British Journal of Pharmacology (2009), 156, 740–750

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

Inhibition of human recombinant T-type calcium channels by the endocannabinoid *N*-arachidonoyl dopamine

Hamish R Ross[1], Andrew J Gilmore[1] and Mark Connor[1,2]

[1]*Pain Management Research Institute, Kolling Institute, University of Sydney at Royal North Hospital, St Leonards, NSW, Australia, and* [2]*Brain and Mind Research Institute, University of Sydney, Camperdown, NSW, Australia*

Background and purpose: *N*-arachidonoyl dopamine (NADA) has complex effects on nociception mediated via cannabinoid CB_1 receptors and the transient receptor potential vanilloid receptor 1 (TRPV1). Anandamide, the prototypic CB_1/TRPV1 agonist, also inhibits T-type voltage-gated calcium channel currents (I_{Ca}). These channels are expressed by many excitable cells, including neurons involved in pain detection and processing. We sought to determine whether NADA and the prototypic arachidonoyl amino acid, *N*-arachidonoyl glycine (NAGly) modulate T-type I_{Ca}
Experimental approach: Human recombinant T-type I_{Ca} (Ca_V3 channels) expressed in HEK 293 cells and native mouse T-type I_{Ca} were examined using standard whole-cell voltage clamp electrophysiology techniques.
Key results: *N*-arachidonoyl dopamine completely inhibited Ca_V3 channels with a rank order of potency *(p*EC_{50}*)* of $Ca_V3.3$ (6.45) $\geq$ $Ca_V3.1$ (6.29) > $Ca_V3.2$ (5.95). NAGly (10 $\mu mol \cdot L^{-1}$) inhibited Ca_V3 I_{Ca} by approximately 50% or less. The effects of NADA and NAGly were voltage- but not use-dependent, and both compounds produced significant hyperpolarizing shifts in Ca_V3 channel steady-state inactivation relationships. By contrast with anandamide, NADA and NAGly had modest effects on Ca_V3 channel kinetics. Both NAGly and NADA inhibited native T-type I_{Ca} in mouse sensory neurons.
Conclusions and implications: *N*-arachidonoyl dopamine and NAGly increase the steady-state inactivation of Ca_V3 channels, reducing the number of channels available to open during depolarization. These effects occur at NADA concentrations at or below to those affecting CB_1 and TRPV1 receptors. Together with anandamide, the arachidonoyl neurotransmitter amides, NADA and NAGly, represent a new family of endogenous T-type I_{Ca} modulators.
British Journal of Pharmacology (2009) **156**, 740–750; doi:10.1111/j.1476-5381.2008.00072.x; published online 18 February 2009

Keywords: T-type calcium channel; NADA; anandamide; *N*-arachidonoyl glycine; nociception; endocannabinoid; endovanilloid; acyl amino acid; arachidonoyl amino acid

Abbreviations: FAAH, fatty acid amide hydrolase; I_{Ca}, voltage-gated calcium channel current; NADA, *N*-arachidonoyl dopamine; NAGly, *N*-arachidonoyl glycine; TRPV1, transient receptor potential vanilloid receptor 1

Introduction

Endogenous compounds consisting of arachidonic acid conjugated with amino acids or neurotransmitter amines are a large class of molecules found in many tissues including brain and spinal cord (Huang *et al.*, 2001; 2002; Milman *et al.*, 2006; Saghatelian *et al.*, 2006; Rimmerman *et al.*, 2008). While details of their synthesis and metabolism are only beginning to emerge, several of these compounds have been shown to have profound effects on nociception (Burstein *et al.*, 2000; Huang *et al.*, 2001; 2002), inflammation (Burstein *et al.*, 2007) and vascular function (O'Sullivan *et al.*, 2004; Milman *et al.*, 2006) following systemic administration or incubation with tissue *in vitro*. The molecular targets of these compounds are also beginning to be defined, and in some respects they resemble those of the endogenous cannabinoid agonist, anandamide (AEA) (*N*-arachidonoyl ethanolamide). Thus, *N*-arachidonoyl dopamine (NADA) is an agonist at cannabinoid CB_1 receptors (Bisogno *et al.*, 2000), transient receptor potential vanilloid receptor 1 (TRPV1) receptors (Huang *et al.*, 2002) and a weak inhibitor of a major AEA hydrolysing enzyme, fatty acid amide hydrolase (FAAH, Bisogno *et al.*, 2000). Intriguingly, the *N*-arachidonoyl amide most closely related to AEA, *N*-arachidonoyl glycine (NAGly), has very low affinity for CB_1 receptors (Sheskin *et al.*, 1997), modest inhibitory potency at FAAH (Huang *et al.*, 2001) and negligible

Correspondence: Mark Connor, Brain & Mind Research Institute, University of Sydney MO2G, 100 Mallet Street, Camperdown, NSW 2050, Australia. E-mail: markc@med.usyd.edu.au
Received 4 September 2008; revised 30 September 2008; accepted 23 October 2008

activity at TRPV1 receptors (De Petrocellis *et al.*, 2000). It is, however, the prototypic agonist for the orphan G protein coupled receptor, GPR 18 (Kohno *et al.*, 2006), and an agonist of another orphan receptor, GPR 92 (Oh *et al.*, 2008).

The mechanisms through which NADA and NAGly modulate nociception remain incompletely understood. NADA has pro- and anti-nociceptive effects, depending on the route and site of administration (Bisogno *et al.*, 2000; Huang *et al.*, 2002). In different experiments, both pro- and anti-nociceptive effects have been explained on the basis of TRPV1 activation (Sagar *et al.*, 2004; Huang and Walker, 2006), while some anti-nociceptive actions involve CB_1 receptors (Sagar *et al.*, 2004). There is also evidence for CB_1/TRPV1-independent actions of NADA in sensory neurons (Price *et al.*, 2004). The antinociceptive effects of NAGly are not sensitive to CB_1 antagonists (Succar *et al.*, 2007; Vuong *et al.*, 2008), indicating that its main mechanism of action is unlikely to be elevation of endocannabinoids following inhibition of FAAH. Recent studies have described selective NAGly inhibition of the glycine transporter GlyT2A and complex effects on glycine receptors (Wiles *et al.*, 2006; Yang *et al.*, 2008), both of which could conceivably contribute to anti-nociception. As part of a search for possible sites of action relevant to the anti-nociceptive effects of NADA and NAGly, we examined the effects of these compounds on T-type calcium channels, low-voltage-activated channels that have an important role in sensory processing and are an emerging target for analgesics (Shin *et al.*, 2008). We report that NADA and NAGly robustly inhibit recombinant human and native mouse T-type calcium channels. The effects are broadly similar to those previously reported for AEA (Chemin *et al.*, 2001; 2007), but quite distinct from the effects of NADA and NAGly on high-voltage-activated, N-type calcium channels (Guo *et al.*, 2008).

Methods

Cell culture

HEK 293 cells stably transfected with plasmids containing cDNA for the human $Ca_V3.1$, $Ca_V3.2$ or $Ca_V3.3$ (Cribbs *et al.*, 1998; 2000; Gomora *et al.*, 2002, Ross *et al.*, 2008) were cultivated in Dulbecco's modified Eagle's Medium supplemented with 100 U penicillin, 100 μg streptomycin, 10% fetal bovine serum or donor bovine serum and 1 $mg \cdot mL^{-1}$ G418 (Invitrogen, Mt. Waverly, Australia).

Isolation of sensory neurons

All animal procedures were approved by the Royal North Hospital Animal Care and Ethics Committee. Male C57Bl6 mice at least 8 weeks old were anaesthetized with isofluorane, decapitated and the trigeminal ganglia removed. Adult mouse trigeminal ganglion neurons were isolated as described in Ross *et al.* (2008). Briefly, ganglia were placed in a modified HEPES-buffered saline (mHBS) containing (in mM): 130 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 10 $MgCl_2$. 10 HEPES, 10 glucose (pH to 7.3 with NaOH, osmolarity 330 ± 5 mosmol). The ganglia were cut into pieces with iridectomy scissors and incubated in mHBS containing 20 $U \cdot mL^{-1}$ papain for 25 min at 37°C. The reaction was stopped with mHBS containing 1 $mg \cdot mL^{-1}$ BSA and 1 $mg \cdot mL^{-1}$ trypsin inhibitor (Type II-O). The tissue was then washed with mHBS and cells released by gentle trituration through fire-polished Pasteur pipettes. Cells were plated onto tissue culture dished and used within 8 h of isolation.

Electrophysiology

Voltage-gated calcium channel currents (I_{Ca}) in HEK 293 cells were recorded in the whole-cell configuration of the patch-clamp method (Hamill *et al.*, 1981) at room temperature, unless otherwise noted (Ross *et al.*, 2008). Dishes were perfused with HBS containing (in $mmol \cdot L^{-1}$): 140 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose (pH to 7.3, osmolarity = 330 ± 5 mosmol). For recording $Ca_V3.1$ and 3.2 currents, cells were bathed in an external solution containing (in mM): 140 tetraethylammonium chloride, 2.5 CsCl, 10 HEPES, 10 glucose, 1 $MgCl_2$, 5 $CaCl_2$ (pH to 7.3, osmolarity = 330 ± 5 mosmol). For recording $Ca_V3.3$ currents, 5 $mmol \cdot L^{-1}$ $CaCl_2$ was replaced by 5 $mmol \cdot L^{-1}$ $BaCl_2$ in the external solution (see Ross *et al.*, 2008). Recordings were made with fire-polished borosilicate glass pipettes with resistance ranging from 2 to 3 MΩ. For recording $Ca_V3.1$ and 3.2 currents, the internal solution contained (in $mmol \cdot L^{-1}$): 130 CsCl, 10 HEPES, 2 $CaCl_2$, 10 EGTA, 5 MgATP (pH to 7.3, osmolarity = 285 ± 5 mosmol). For recording of $Ca_V3.3$ currents, 10 $mmol \cdot L^{-1}$ EGTA was replaced by 10 $mmol \cdot L^{-1}$ BAPTA, and the concentration of MgATP was reduced to 1 $mmol \cdot L^{-1}$. Recordings were made with a HEKA EPC 10 amplifier with Patchmaster acquisition software (HEKA Elektronik, Lambrecht/Pfalz, Germany), an Axopatch 1D amplifier using pClamp 5 software (Molecular Devices, Sunnyvale, CA, USA) and an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) using AxoGraph X software (http://axographx.com/). Data were sampled at 5–20 kHz, filtered at 2 kHz and recorded on a hard disk for later analysis. Series resistance ranged from three to 10 MΩ, and was compensated by at least 80% in all experiments. Leak subtraction using a *P* over 4 protocol (with 10 mV test steps) was used for some experiments where cells were being stepped to a single potential, but it was not employed for experiments where more complex waveforms were applied to the cells (e.g. inactivation). Uncompensated leak in these latter experiments did not exceed –30 pA at –106 mV, and cells with a leak current of greater than –30 pA were not used for any experiment. Cells were exposed to drugs via flow pipes positioned approximately 200 μm from the cell.

Recordings from trigeminal ganglion sensory neurons were made as outlined in Borgland *et al.* (2001). The solutions were the same as those outlined above, except that T-type I_{Ca} were recorded in an external TEA solution containing 2.5 $mmol \cdot L^{-1}$ Ca^{2+} and 1 $mmol \cdot L^{-1}$ Mg^{2+}. Other membrane currents were recorded using HBS as the external solution. Recordings were made from small to medium sized cells (<25 μm diameter) which were identified as Type 1 or Type 2 cells from their I_{Ca} signatures derived from a current–voltage protocol, as outlined in Borgland *et al.* (2001). Type 1 neurons express little or no T-type I_{Ca} (measured at a test potential of –40 mV) and typically express TRPV1 and μ-opioid receptors. Type 2 neurons have significant I_{Ca} at a test potential of –40 mV, do not express TRPV1 and are not sensitive to μ-opioid agonists.

Effects of drug vehicle (ethanol) Drugs were kept in concentrated stock solutions in ethanol and stored at –20°C. Daily dilutions from these stocks were made; the final ethanol concentration in all solutions was 0.1%. Ethanol at this concentration did not significantly affect the properties of the Ca_V3 channels. After 10 min recording in control conditions or ethanol (0.1%), the V_{50} for activation for the Ca_V3 channels was not different: $Ca_V3.1$: 43 ± 1 mV, 43 ± 1 mV; $Ca_V3.2$: 40 ± 2 mV, 39 ± 2 mV; $Ca_V3.3$: –37 ± 1 mV, –35 ± 2 mV (n = 5 to 9 cells for each condition). The amplitudes of the currents elicited by stepping from –106 mV to –26 mV were also similar after 10 min in control conditions and 10 min in ethanol (0.1%): $Ca_V3.1$: –642 ± 115 pA, –742 ± 150 pA; $Ca_V3.2$: –331 ± 74 pA, –258 ± 77 pA; $Ca_V3.3$: –1.82 ± 0.4 nA, –2.02 ± 0.4 nA (n = 5 to 9 cells for each condition). Acute application of ethanol (0.1%) inhibited $Ca_V3.1$ channels activated by stepping from –86 mV to –26 mV by 1 ± 1% (n = 12).

Data analysis

Concentration–response (I_{Ca}) curves were generated by fitting data to a sigmoidal dose–response function in GraphPad Prism 4. Steady-state activation curves were generated from current–voltage relationships, while steady-state inactivation curves were generated by measuring the peak current from a 50 ms step to –26 mV following a series of 5 s steps ranging from potentials of –126 mV to –46 mV. Reported potentials are corrected for a junction potential of –6 mV. Activation curves were generated by fitting data to a Boltzmann sigmoidal function $Y = 1/(1 + e^{((V0.5-Vm)/Slope)})$. Inactivation curves were generated by fitting data to a Boltzmann sigmoidal function $Y = 1 - 1/(1 + e^{((V0.5-Vm)/Slope)})$.

Statistical significance for comparing the $V_{0.5}$ values of activation and inactivation was determined using unpaired *t*-tests comparing values of $V_{0.5}$ calculated for individual experiments. In order to compare the changes in the time constant of inactivation and deactivation, two-way ANOVA was used with a Bonferroni post-test to compare values at different potentials.

Materials *N*-arachidonoyl dopamine, NAGly and AEA were obtained from either Alexis Biochemicals (Lausen, Switzerland), Biomol (Plymouth Meeting, PA, USA) or Cayman Chemical (Ann Arbor, MI, USA). Where possible, the same drug was purchased from several sources. In all cases, results were similar with drugs purchased from different suppliers. Papain was from Worthington, and all other drugs and chemicals were from Sigma Australia.

Drug and molecular target nomenclature conforms to the BJP Guide to Receptors and Channels (Alexander *et al.*, 2008).

Results

N-arachidonoyl glycine is structurally very similar to the endocannabinoid AEA, differing only by having an additional oxygen molecule, that is, having a terminal carboxylic acid instead of alcohol. AEA is a reasonably potent inhibitor of T-type calcium channels (Chemin *et al.*, 2001; 2007), so we examined the effects NAGly on T-type calcium channels in mouse trigeminal sensory neurons (Borgland *et al.*, 2001). Superfusion of NAGly onto Type 2 trigeminal ganglion neurons inhibited the I_{Ca} evoked by a step from –86 mV to –46 mV (Figure 1). The highest concentration of NAGly testable (30 μmol·L^{-1}) inhibited the currents at –46 mV by 80 ± 8% (n = 6), 10 μmol·L^{-1} NAGly inhibited the currents by 52 ± 7%. As the low-voltage-activated calcium currents in native sensory neurons are likely to represent a mixed population of channels, we examined the effects of NAGly and the CB_1/TRPV1 agonist NADA on recombinant human Ca_V3 channels stably expressed in HEK293 cells.

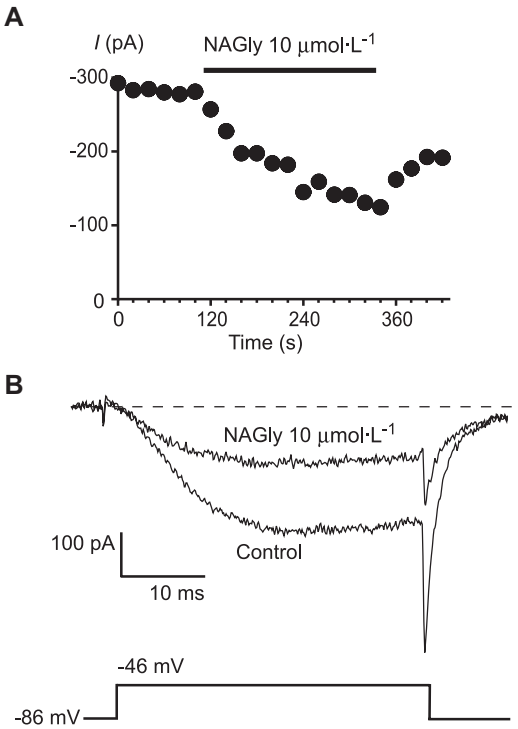

Figure 1 *N*-arachidonoyl glycine (NAGly) inhibits native T-type calcium channels. Whole-cell patch clamp recordings were made from acutely isolated mouse trigeminal ganglion neurons. Currents were evoked by stepping from –86 mV to –46 mV. (A) Time plot of the peak amplitude of the I_{Ca} at –46 mV, illustrating the effects of 10 μmol·L^{-1} NAGly (perfused for the duration of the bars). (B) Example traces from the above cell in the absence and presence of NAGly. The dotted line represents zero current. I_{Ca}, voltage-gated calcium channel current.

N-arachidonoyl glycine and NADA inhibited each of the human Ca_V3 subtypes (Figures 2 and 3). The inhibitory effects of lower concentrations of drug on Ca_V3 channels could be at least partially reversed by washing. At a concentration of 10 μmol·L^{-1} or 30 μmol·L^{-1}, NADA completely inhibited each of the Ca_V3 channels, while NAGly was considerably less effective, with about 50% or less inhibition of each channel at a concentration of 10 μmol·L^{-1} (Figure 4). The potency of NADA and NAGly inhibition of Ca_V3 channels was determined by superfusing single concentrations of drug onto cells repetitively stepped from –86 mV to –26 mV. NADA inhibited $Ca_V3.1$ with pEC_{50} of 6.29 ± 0.03, $Ca_V3.2$ with a pEC_{50} of 5.95 ± 0.02 and $Ca_V3.3$ with a pEC_{50} of 6.45 ± 0.02. The endogenous compound *N*-oleoyl dopamine (18 : 1ω9,

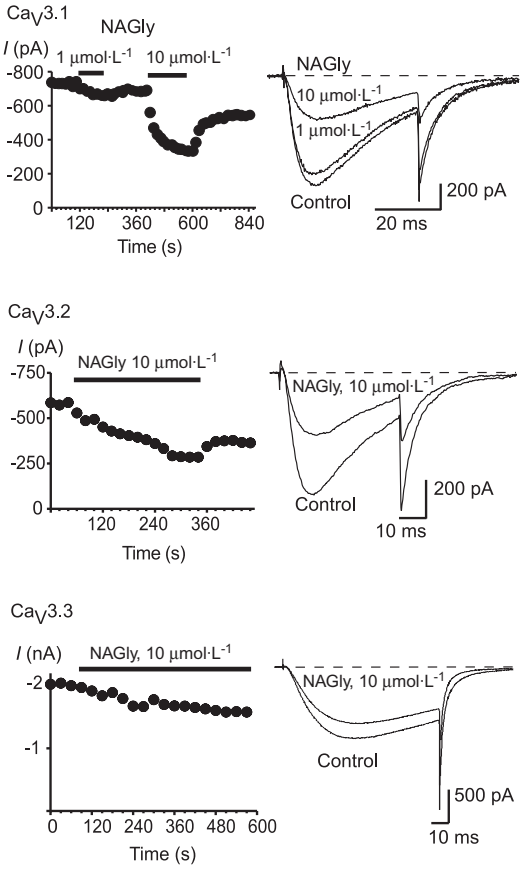

Figure 2 *N*-arachidonoyl glycine (NAGly) inhibits recombinant human T-type calcium channels. Whole-cell patch clamp recordings were made from human Ca$_V$3.1, Ca$_V$3.2 and Ca$_V$3.3 channels stably expressed in HEK 293 cells. Currents were evoked by stepping from –86 mV to –26 mV. The effect of NAGly on each of Ca$_V$3.1, Ca$_V$3.2 and Ca$_V$3.3. are illustrated, with a representative time plot and example traces. Each trace is an example of at least six similar experiments. The dotted line represents zero current.

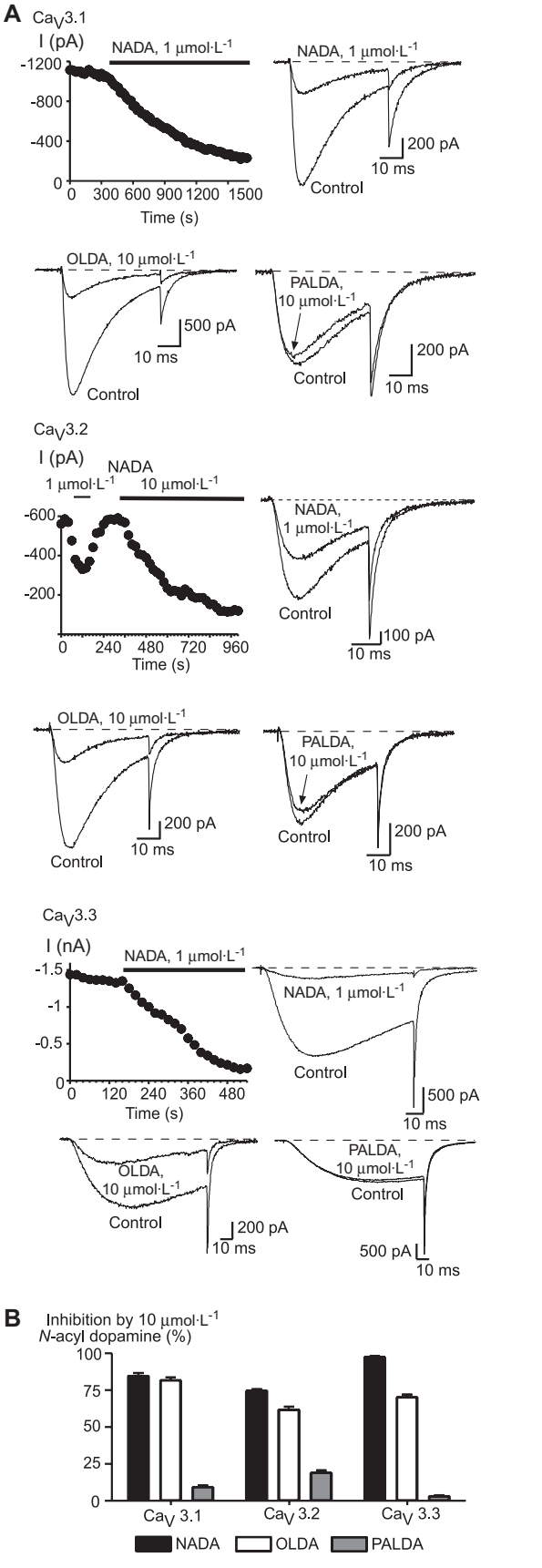

Figure 3 *N*-acyl dopamine compounds inhibit recombinant human T-type calcium channels. Whole-cell patch clamp recordings were made from human Ca$_V$3.1, Ca$_V$3.2 and Ca$_V$3.3 channels stably expressed in HEK 293 cells. Currents were evoked by stepping from –86 mV to –26 mV. (A) The effect of *N*-arachidonoyl dopamine (NADA) *N*-oleoyl dopamine (OLDA) and *N*-palmitoyl dopamine (PALDA) on each of Ca$_V$3.1, Ca$_V$3.2 and Ca$_V$3.3. are illustrated, with a time plot for the effects of NADA. (B) A summary of the effects of 10 μmol·L^{-1} of each of the *N*-acyl dopamines on each of the recombinant human Ca$_V$3 channels. The bars are the mean ± SEM of 6–9 cells for each drug on each channel. The dotted line represents zero current.

10 μmol·L^{-1}, Chu *et al*., 2003) inhibited Ca$_V$3 channels to similar degree as NADA (10 μmol·L^{-1}), but the unsaturated *N*-palmitoyl dopamine (C16, 10 μmol·L^{-1}) was much less effective (Figure 3). NAGly inhibited Ca$_V$3.1 channels with a notional EC$_{50}$ of 16 μmol·L^{-1}, but the highest concentration tested (30 μmol·L^{-1}) did not completely inhibit the channels (Figure 4).

We further examined the interactions of NADA and NAGly on Ca$_V$3.1 by examining whether inhibition of the channel was use-dependent or influenced by the membrane potential at which the cell was voltage clamped during the experiment.

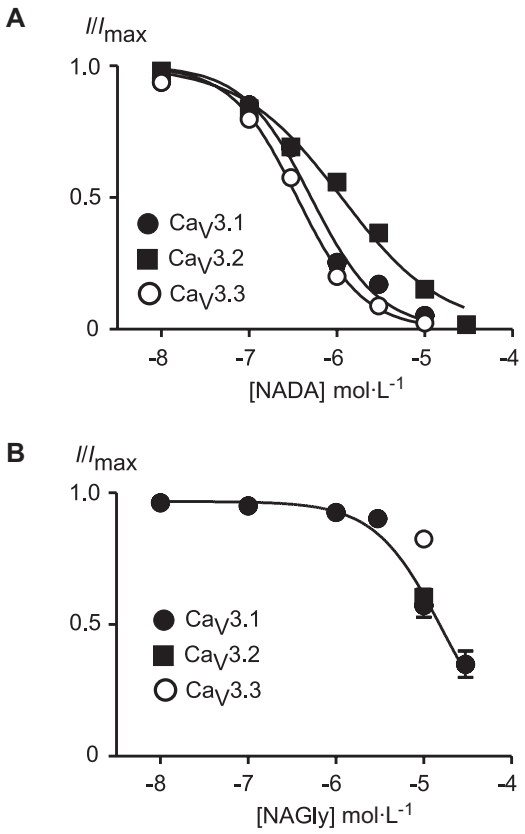

Figure 4 Concentration–response plots for *N*-arachidonoyl dopamine (NADA) and *N*-arachidonoyl glycine (NAGly) inhibition of Ca_V3 channels. Whole-cell patch clamp recordings were made from human $Ca_V3.1$, $Ca_V3.2$ and $Ca_V3.3$ channels stably expressed in HEK 293 cells. Currents were evoked by stepping from –86 mV to –26 mV. A single concentration of drug was superfused over each cell, each point represents the mean ± SEM of at least six cells. (A) NADA inhibits Ca_V3 channels with a rank order of $Ca_V3.3$ (350 nmol·L^{-1}) ≥ $Ca_V3.1$ (500 nmol·L^{-1}) > $Ca_V3.2$ (1.13 µmol·L^{-1}). (B) Concentration-response plot for NAGly inhibition of $Ca_V3.1$. The highest concentration of NAGly tested was 30 µmol·L^{-1}, assuming complete inhibition the notional EC_{50} was 16 µmol·L^{-1}. The inhibition of $Ca_V3.2$ and $Ca_V3.3$ by 10 µmol·L^{-1} NAGly is plotted for comparison.

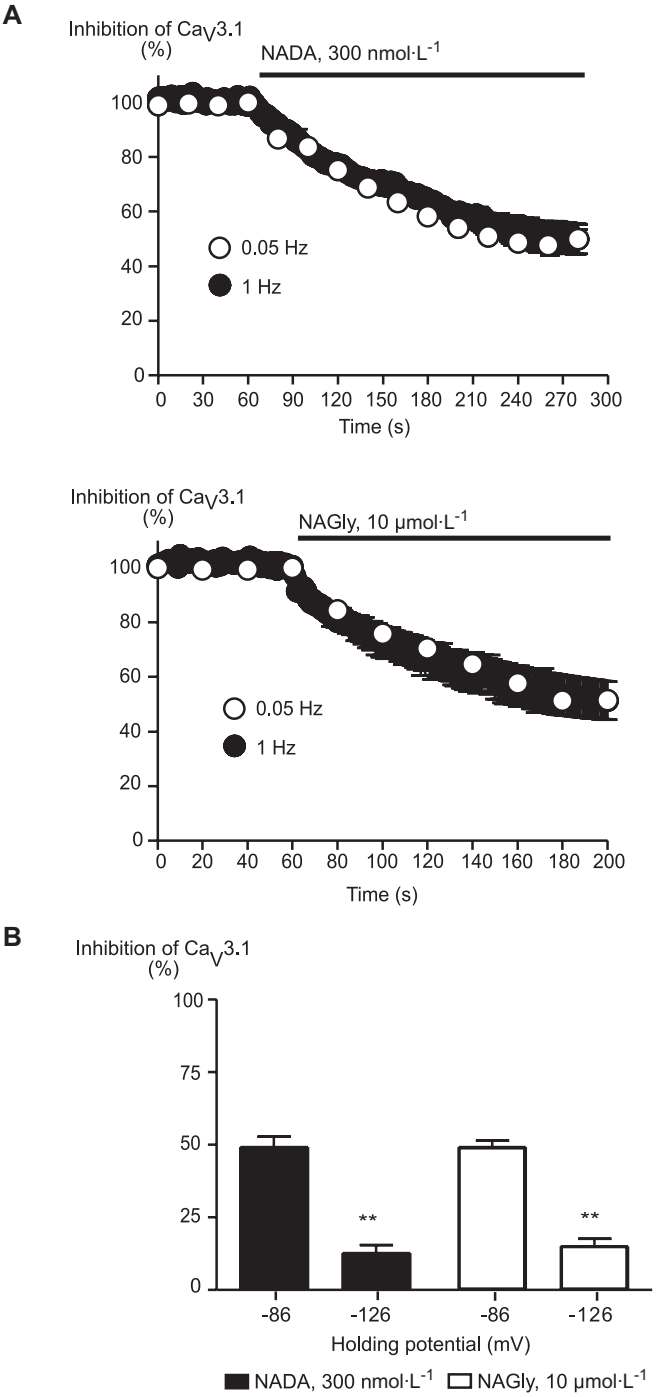

Figure 5 Inhibition of $Ca_V3.1$ by *N*-arachidonoyl dopamine (NADA) and *N*-arachidonoyl glycine (NAGly) was voltage- but not use-dependent. Whole-cell patch clamp recordings were made from human $Ca_V3.1$ channels stably expressed in HEK 293 cells. (B) The time course of inhibition of $Ca_V3.1$ currents by NADA (300 nmol·L^{-1}) and NAGly (10 µmol·L^{-1}) were similar when currents were evoked by stepping from –86 mV to –26 mV at 1 Hz or 0.05 Hz. (B) The inhibition of $Ca_V3.1$ by NADA (300 nmol·L^{-1}) and NAGly (10 µmol·L^{-1}) was significantly less when cells were voltage clamped at –126 mV and stepped to –26 mV than when cells were stepped to –26 mV from a holding potential of –86 mV. **$P < 0.01$ versus –86 mV, Student's *t*-test.

The amount and macroscopic time-course of inhibition of $Ca_V3.1$ were the same for NADA (300 nmol·L^{-1}) and NAGly (10 µmol·L^{-1}) whether the currents were evoked at 1 Hz or 0.05 Hz (Figure 5). However, the amount of inhibition by NADA (300 nmol·L^{-1}) and NAGly (10 µmol·L^{-1}) was strongly influenced by the holding potential of the cell, with significantly greater inhibition at –86 mV than –126 mV (Figire 5). Inclusion of the competitive inhibitor of G protein activation, GDPβS (1.2 mmol·L^{-1}), failed to affect the inhibition of $Ca_V3.1$ by NADA (300 nmol·L^{-1}; 50 ± 5% in GTP vs. 49 ± 6% in GDPβS, $n = 6$ each) or NAGly (10 µmol·L^{-1}; 52 ± 2% in GTP vs. 53 ± 8% in GDPβS, $n = 6$ each) applied 10 min after breaking into the cells.

We compared the effects of NADA (500 nmol·L^{-1}), NAGly (10 µmol·L^{-1}) and AEA (300 nmol·L^{-1}) on $Ca_V3.1$ channel kinetics by comparing the effects of 5 min applications of compound with time-matched controls. These concentrations of drug inhibited the peak current currents by about 50% (Figure 6). Currents were elicited from a holding potential of

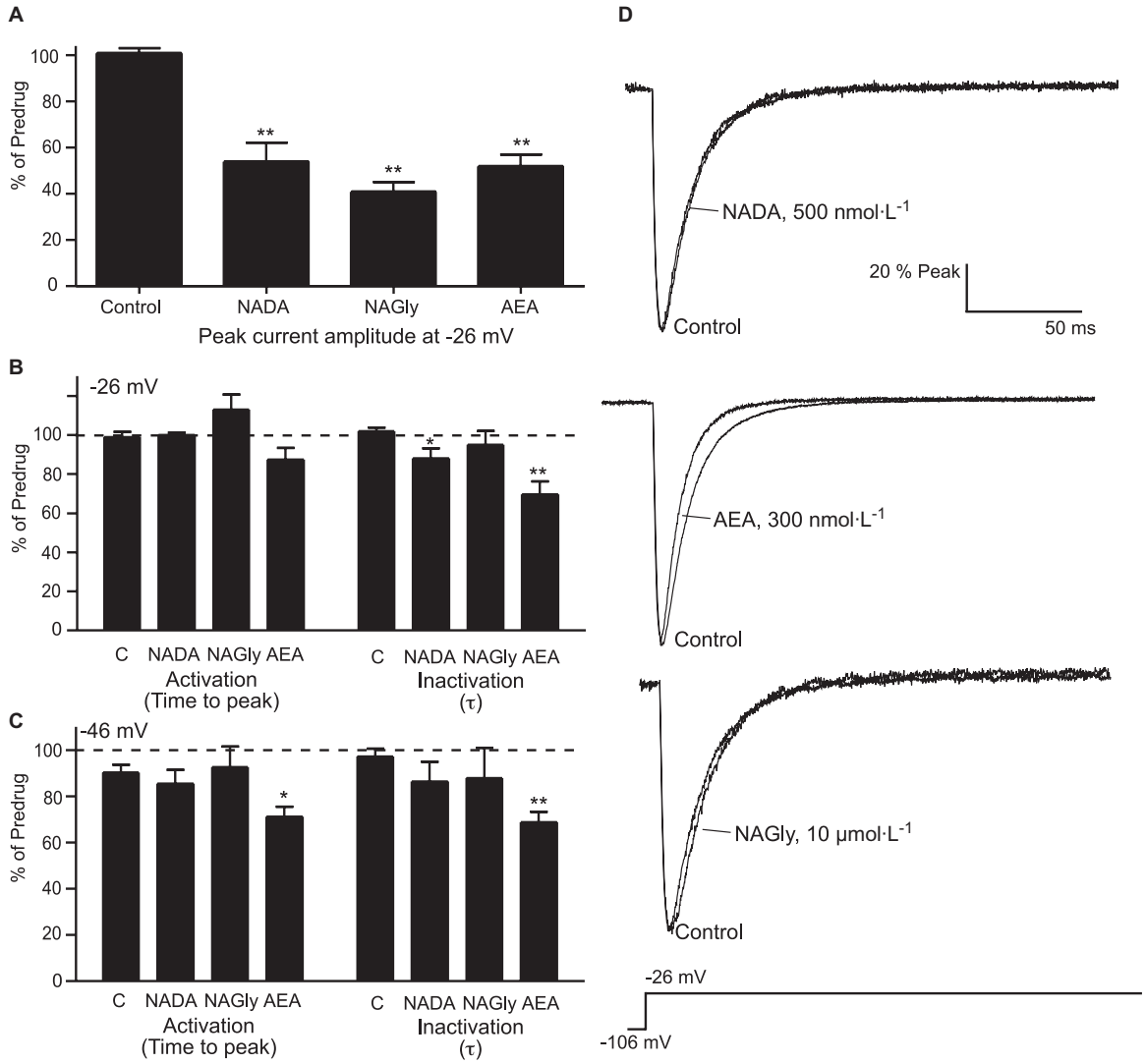

Figure 6 *N*-arachidonoyl dopamine (NADA) and *N*-arachidonoyl glycine (NAGly) do not strongly affect $Ca_V3.1$ channel kinetics. Whole-cell patch clamp recordings were made from human Ca_V3 channels stably expressed in HEK 293 cells. Channel activation from a holding potential of –106 mV was measured 5 min after breaking into the cell and then again after 5 min in NADA (500 nmol·L^{-1}), NAGly (10 µmol·L^{-1}) or anandamide (AEA, 300 nmol·L^{-1}). The graphs illustrate: (A) current amplitude at –26 mV, the time to peak and time constant of inactivation at (B) –26 mV and (C) –46 mV. The values in drug are expressed as a percentage of the values at 5 min, control cells were continuously superfused with vehicle alone. Each bar represents the mean ± SEM of at least six cells. Statistical comparisons were made with control cells recorded on the same day (Student's *t*-test, **P* < 0.05, ***P* < 0.01). Panel (D) illustrates typical currents elicited by a step from –106 mV to –26 mV recorded in control conditions and after 5 min in NADA, AEA and NAGly. Currents have been normalized to the peak inward current to allow ready comparison of inactivation kinetics.

–106 mV and we measured the time to peak and time constant of channel inactivation from an open state. Data from test potentials of –46 mV (approximately 50% of channels active) and –26 mV (all channels open) are illustrated in (Figure 6). NAGly did not affect time to peak or inactivation from an open state at either potential, while NADA modestly accelerated inactivation from an open state at –26 mV ($P < 0.05$, Figure 6). AEA significantly accelerated open-state inactivation kinetics of $Ca_V3.1$ channels at both potentials ($P < 0.01$), and also decreased the time to peak at –46 mV, consistent with the previously reported effects of AEA on $Ca_V3.1$ (Chemin *et al.*, 2001). NADA did not affect time to peak of $Ca_V3.2$ or $Ca_V3.3$ currents evoked from –106 mV. NADA or NAGly did not affect the time to peak or deactivation time constant of currents evoked from a holding potential of –86 to a test potential of –26 mV in any Ca_V3 channel (Table 1).

Effects of NADA and NAGly on channel activation and inactivation

AEA inhibits Ca_V3 channels in part by increasing steady-state inactivation and thus reducing the numbers of channels available to open during a depolarization. We examined whether the inhibition of Ca_V3 channels by NADA or NAGly could be due to effects on channel availability or activation. Activation curves were constructed by stepping cells from –106 mV to potentials between –86 and +59 mV, and then

Table 1 The effects of NADA and NAGly on the kinetics of Ca_V3 channels

Ca_V channel	Time to peak (ms)			Deactivation τ (ms)		
	Control	NADA	NAGly	Control	NADA	NAGly
3.1	7.2 ± 0.3	6.2 ± 0.3	7.3 ± 0.3	3.2 ± 0.1	3.1 ± 0.1	3.2 ± 0.2
3.2	11.2 ± 0.4	11.5 ± 0.6	11.6 ± 0.4	3.1 ± 0.2	3.1 ± 0.2	3.1 ± 0.2
3.3	43 ± 1	44 ± 2	43 ± 2	2.4 ± 0.1	2.4 ± 0.2	2.3 ± 0.1

Cells expressing recombinant Ca_V3 channels were voltage clamped at –86 mV and then stepped to –26 mV. For examples of these experiments see Figures 2 and 3. The time to peak was measured directly and the decay of the current following repolarization of the membrane to –86 mV fit with a single exponential function. The concentration of NADA was 300 nmol·L^{-1} for $Ca_V3.1$ and $Ca_V3.3$ and 1 μmol·L^{-1} for $Ca_V3.2$. 10 μmol·L^{-1} NAGly was used for each channel. There were no differences in time to peak or deactivation for any current with either drug (paired *t*-test vs. predrug values for each cell). $n = 6$–8 for each condition.
NADA, *N*-arachidonoyl dopamine; NAGly, *N*-arachidonoyl glycine.

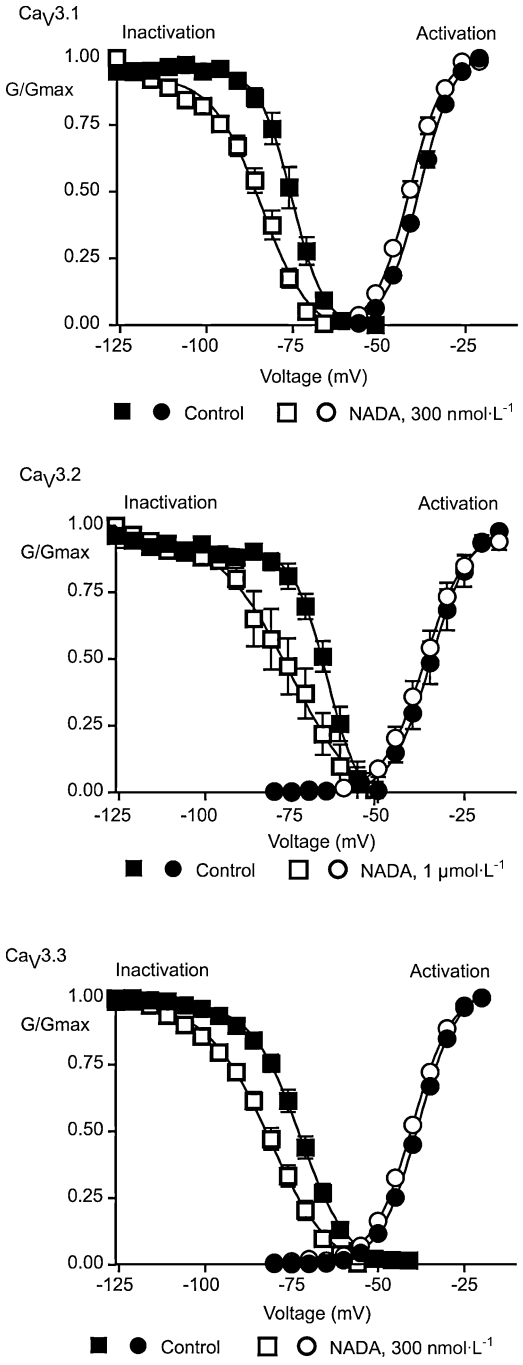

Figure 7 *N*-arachidonoyl dopamine (NADA) affects steady-state inactivation but not activation of Ca_V3 channels. Whole-cell patch clamp recordings were made from human Ca_V3 channels stably expressed in HEK 293 cells, 5 min after breaking into the cell and then again after 5 min in NADA. To measure channel activation, cells were voltage clamped at –106 mV and stepped to potentials above –86 mV in 5 mV increments. To measure steady-state inactivation, cells were voltage clamped for 5 s at potentials between –126 mV and –46 mV, and then stepped to a test potential of 26 mV. The peak current at each test potential is plotted for activation curves, the current at –26 mV following 5 s at the indicated holding potential is plotted for inactivation. Curves are a Boltzmann fit of the data (see Methods). NADA did not affect activation, but produced a significant hyperpolarizing shift in the membrane at which 50% of the channels are inactivated for each Ca_V3 subtype (Table 2).

were repeated after 5 min perfusion of approximately EC_{50} concentrations of NADA (Figure 7), NAGly (Figure 8) and AEA (not shown). In the presence of the compounds there were small (2–3 mV) shifts in the potential at which half the channels were activated, these shifts were not different from those seen with time-matched vehicle controls (Table 2).

Steady-state inactivation was determined by holding cells at –106 mV and then stepping them for 5 s to test potentials between –126 mV and –51 mV before measuring the current following a step to –26 mV. This was repeated after 5 min in approximately EC_{50} concentrations of drug. NADA produced a significant hyperpolarizing shift in the membrane potential at which 50% of the channels were available for activation for all channels examined (Figure 7, Table 2). A similar effect was observed for NAGly (10 μmol·L^{-1}) on $Ca_V3.1$ (Figure 8, Table 2). The shifts in steady-state inactivation in cells exposed to vehicle alone for 5 min were less than 2 mV (Table 2). The increase in steady-state inactivation is likely to make a major contribution to the inhibition of Ca_V3 channel currents by NADA and NAGly.

In small to medium sized mouse trigeminal ganglion neurons, T-type I_{Ca} and capsaicin responses are largely segregated into two different populations of neurons (Borgland *et al.*, 2001; Roberts *et al.*, 2002). Type 1 cells are usually sensitive to capsaicin but do not express T-type I_{Ca} while Type 2 cells express prominent T-type I_{Ca} but are insensitive to capsaicin. Superfusion of NADA (300 nmol·L^{-1}) inhibited the I_{Ca} evoked by stepping Type 2 neurons from –86 mV to –46 mV by 30 ± 7% (Figure 9). NADA alone did not produce an inward current in Type 2 cells. NADA is also an agonist at

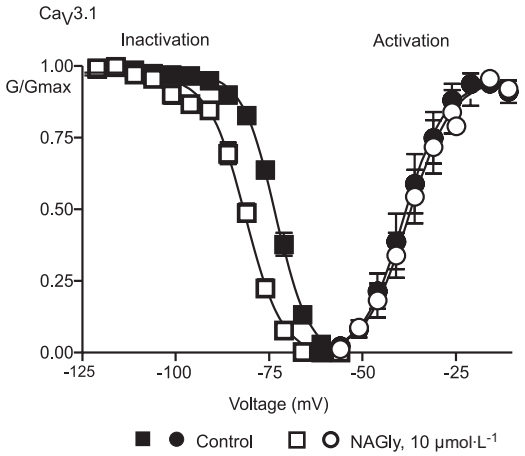

Figure 8 *N*-arachidonoyl glycine (NAGly) affects steady-state inactivation but not activation of $Ca_V3.1$. Whole-cell patch clamp recordings were made from human $Ca_V3.1$ channels stably expressed in HEK 293 cells, 5 min after breaking into the cell and then again after 5 min in NAGly (10 µmol·L^{-1}). To measure channel activation, cells were voltage clamped at –106 mV and stepped to potentials above –86 mV in 5 mV increments. To measure steady-state inactivation, cells were voltage clamped for 5 s at potentials between –126 mV and –46 mV, and then stepped to a test potential of –26 mV. The peak current at each test potential is plotted for the activation curve, the current at –26 mV following 5 s at the indicated holding potential is plotted for inactivation. Curves are a Boltzmann fit of the data (see Methods). NAGly did not affect activation, but produced a significant hyperpolarizing shift in the membrane at which 50% of the $Ca_V3.1$ channels are inactivated (Table 2).

Table 2 The effects of NADA, NAGly and anandamide (AEA) on the parameters of steady-state activation and inactivation of Ca_V3 channels

Drug	*Ca_V channel*	*Channel $V_{0.5}$ (mV)*	
		Activation	*Inactivation*
300 nmol·L^{-1} NADA	3.1	–3 ± 0.3	–11 ± 1**
1 µmol·L^{-1} NADA	3.2	–2.5 ± 2	–10 ± 3**
300 nmol·L^{-1} NADA	3.3	–1 ± 0.3	–10 ± 1**
10 µmol·L^{-1} NAGly	3.1	1 ± 1	–8.5 ± 1**
300 nmol·L^{-1} AEA	3.1	–2.7 ± 0.5	ND
No drug	3.1	–2 ± 1	–2 ± 3
No drug	3.2	1 ± 2	–2 ± 2
No drug	3.3	–2 ± 2	–2 ± 2

Cells expressing recombinant Ca_V3 channels were voltage clamped at –106 mV and then stepped to potentials above –86 mV (activation) or stepped for 5 s to potentials between –126 and –36 mV before stepping to the test potential of –26 mV. The resulting peak currents were fitted to a Boltzmann equation. Changes in the voltage for half activation/inactivation ($V_{0.5}$) of the curves are reported. The "No drug" values represent time-dependent changes under our recording conditions. Curves for NADA and NAGly are illustrated in Figures 7 and 8. NADA, *N*-arachidonoyl dopamine; NAGly, *N*-arachidonoyl glycine; ND, not determined.
*P < 0.05, **P < 0.01 from control.

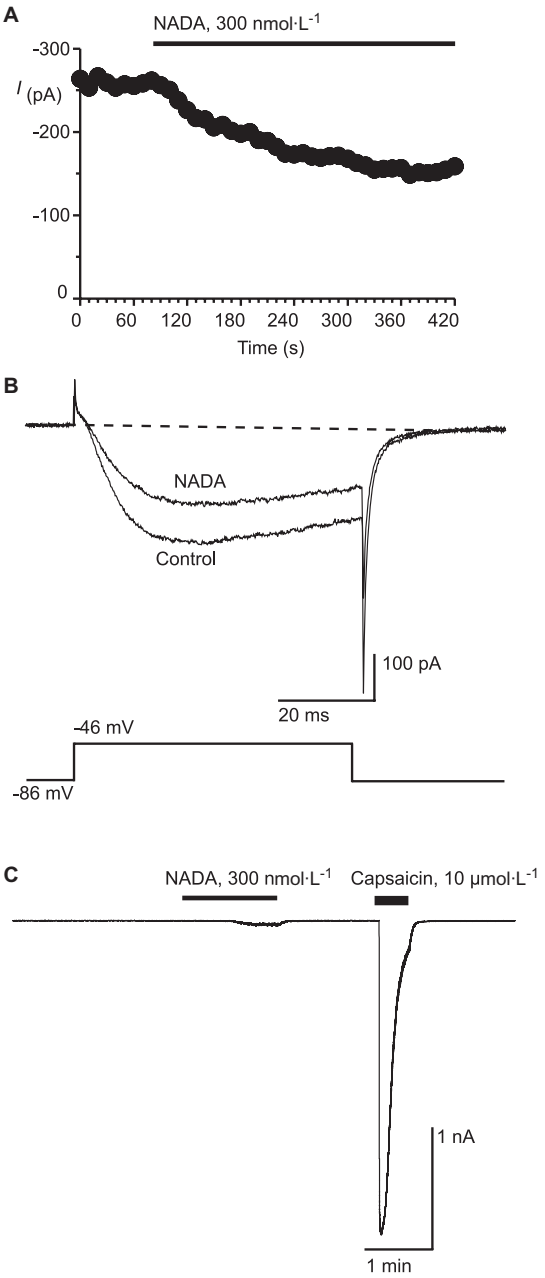

Figure 9 *N*-arachidonoyl dopamine (NADA) inhibits native T-type calcium channels and activates TRPV1. Whole-cell patch clamp recordings were made from acutely isolated mouse trigeminal ganglion neurons. Currents were evoked by stepping from –86 mV to –46 mV. (A) Time plot of the peak amplitude of the I_{Ca} at –46 mV, illustrating the effect of NADA (perfused for the duration of the bar). (B) Typical traces from the same cell as above in (A), in the absence and presence of NADA. (C) Example trace from a Type 1 sensory neuron voltage clamped at –60 mV and superfused with NADA and a maximally effective concentration of the TRPV1 agonist capsaicin. NADA (300 nmol·L^{-1}) produced an inward current of, on average, 10 ± 6% of the capsaicin (10 µmol·L^{-1}) current. The dotted line represents zero current. These traces are not leak subtracted. I_{Ca}, voltage-gated calcium channel current; TRPV1, transient receptor potential vanilloid receptor 1.

TRPV1 receptors, and superfusion of NADA (300 nmol·L^{-1}) produced modest inward currents in capsaicin-sensitive Type 1 cells, but not Type 1 cells insensitive to capsaicin (n = 6) (Figure 9). The NADA currents were 10 ± 6% of the size of the current produced by a subsequent application of high concentration of capsaicin (10 µmol·L^{-1}, mean current 1.5 ± 0.5 nA, n = 7). NAGly (10 µmol·L^{-1}) did not activate currents in capsaicin-sensitive Type 1 cells at room temperature (n = 6) and only produced a small inward current at 33°C in capsaicin-sensitive Type 1 cells (16 ± 8 pA, n = 6).

Discussion

The major finding of this study is that NADA inhibited T-type calcium channels with a similar potency to its agonist actions at native CB_1 or TRPV1 receptors and with a similar or greater potency to the inhibitory effects of the prototypic endocannabinoid AEA at Ca_V3 channels (Chemin *et al.*, 2001). At concentrations of 300 nmol·L^{-1} to 1 μmol·L^{-1}, NADA strongly inhibited native and recombinant T-type calcium channels, while other studies have reported EC_{50} values of approximately 1 μmol·L^{-1} for NADA activation of native TRPV1 (Huang *et al.*, 2002, Price *et al.*, 2004) and CB_1 receptors (Bisogno, *et al.*, 2000). By contrast, NAGly, the prototypic arachidonoyl amino acid and close structural analogue of AEA, was a much weaker inhibitor of T-type calcium channels than AEA or NADA. Interestingly, unlike AEA or NADA, NAGly is also not a CB_1 receptor ligand (Sheskin *et al.*, 1997), has little activity at TRPV1 (De Petrocellis *et al.*, 2000) and does not activate a recently described AEA-stimulated current in mouse trigeminal neurons (Roberts *et al.*, 2008).

T-type calcium channels are involved in a wide range of physiological processes (Perez-Reyes, 2003, Shin *et al.*, 2008), including many that are also modulated by activation of CB or TRPV1 receptors. For several reasons, it is difficult to assess the contribution of NADA modulation of T-type channels to the known effects of NADA administered to animals. First, the role of T-type channels cannot be readily dissected pharmacologically because of the lack of inhibitors selective for T-type channels over other voltage-gated calcium channels (Heady *et al.*, 2001). Conversely, while there are neurobehavioural assays of nociception, sleep and seizure activity sensitive to known T-type channel inhibitors (Perez-Reyes, 2003; Shin *et al.*, 2008), none of these assays are specific assays for T-type channel blockers. Second, many of the drugs used to antagonize the effects of NADA at CB_1 receptors or TRPV1 also inhibit T-channels at pharmacologically relevant concentrations. These include the CB_1 antagonists SR 141716A (Chemin *et al.*, 2001) and AM251 (Ross *et al.*, 2008) and the TRPV1 antagonist capsazepine, (Docherty *et al.*, 1997; Connor, unpublished observations). Thus, while the reversal of a NADA effect by any of these agents provides good evidence for the involvement of the cognate protein, pre-treatment with these ligands will occlude any effects of subsequently administered NADA mediated by actions on T-type channels.

The effects of NADA and NAGly on Ca_V3 channel activity shared some but not all of the hallmarks of AEA modulation of the channel. All three ligands produce strong hyperpolarizing shifts in the membrane potential at which Ca_V3 channels inactivate, and this would have the effect of reducing the number of channels available to open from all but the most negative membrane potentials. This is a common mechanism for modulation of Ca_V3 channels, and is seen with arachidonic acid (Zhang *et al.*, 2000; Talavera *et al.*, 2004; Chemin *et al.*, 2007) and cannabinoid ligands such as Δ^9-tetrahydrocannabinol and cannabidiol (Ross *et al.*, 2008). However, AEA (and arachidonic acid) both have effects on the kinetics of Ca_V3 channels, manifested as an acceleration of channel opening and open-state inactivation, with this latter effect further limiting calcium entry through Ca_V3 channels. Neither NADA nor NAGly strongly affected channel opening or open-state inactivation, which is similar to the effect of cannabidiol on Ca_V3 channels (Ross *et al.*, 2008). NADA inhibition of Ca_V3 channels, particularly $Ca_V3.3$, was more potent than cannabidiol (EC_{50} approximately 800 nmol·L^{-1} for $Ca_V3.1$ and $Ca_V3.2$, 4 μmol·L^{-1} for $Ca_V3.3$) and Δ^9-tetrahydrocannabinol (EC_{50} approximately 1 μmol·L^{-1} for $Ca_V3.1$ and $Ca_V3.2$, 4 μmol·L^{-1} for $Ca_V3.3$) recorded in identical conditions, (Ross *et al.*, 2008) while that of NAGly was similar to that of arachidonic acid (Chemin *et al.*, 2007). The rank order of potency for NADA effects on Ca_V3 channels ($3.3 \geq 3.1 > 3.2$) is also distinct from that reported for AEA ($3.2 > 3.3 > 3.1$) (Chemin *et al.*, 2001) and cannabidiol/Δ^9-tetrahydrocannabinol ($3.1 = 3.2 > 3.3$) (Ross *et al.*, 2008).

It is likely, but not proven, that NADA and NAGly were acting directly on the Ca_V3 channels. NADA has not been reported to be a ligand for any G protein coupled receptor other than the CB_1 receptor and, while HEK 293 cells express mRNA for the NAGly-activated GPR 18 (Kohno *et al.*, 2006, Johnson and Connor, unpublished observations), the inhibition of $Ca_V3.1$ by either compound was not sensitive to the non-specific inhibitor of G protein activation, GDPβS. Receptor- or second messenger-dependent modulation of T-type calcium channels is complex (Perez-Reyes, 2003) but we are not aware of any mechanisms described that produce inhibitory effects on all three isoforms of Ca_V3 channels that are similar to those observed in this study (Hildebrand *et al.*, 2007; Iftinca *et al.*, 2007; Tao *et al.*, 2008).

The site(s) of action for fatty acids and their derivatives modulating Ca_V3 channels is not known and there is limited information about where drugs that modulate Ca_V3 channels could bind to affect channel function. Unsaturated fatty acids seem to be relatively non-selective inhibitors of Ca_V3 channels, and a hyperpolarizing shift in channel inactivation potential is commonly observed with lipid-soluble modulators of Ca_V3 (Heady *et al.*, 2001; Ross *et al.*, 2008) and other voltage-dependent channels (Lundbaek 2008). Amphiphiles which act in the plasma membrane to increase bilayer elasticity produce hyperpolarizing shifts in the inactivation of N-type calcium channels and voltage-gated sodium channels (Lundbaek *et al.*, 1996; 2004), reminiscent of the effects of AEA, NADA and NAGly. The partial reversibility of NADA, NAGly and AEA (Chemin *et al.*, 2001) are also consistent with an interaction mediated through the plasma membrane. However, the effects of AEA on bilayer lipid dynamics have been suggested to be minimal (Tian *et al.*, 2005) and those of NADA and NAGly are unknown. Further evidence inconsistent with a major effect of NADA and NAGly on lipid elasticity, at the concentrations that inhibit Ca_V3 channels, comes from a study showing that NADA (10 μmol·L^{-1}) had no effect on native N-type calcium channels while NAGly (10 μmol·L^{-1}) *enhanced* N-channel activation (Guo *et al.*, 2008). The differences in the absolute potency (up to 30-fold), differences in rank order of potency at Ca_V3 subtypes and the distinct effects on $Ca_V3.1$ kinetics of iso-inhibitory concentrations of AEA, NADA and NAGly suggest that a specific site mediates the effects of these compounds. This site seems to have a sufficiently well defined structure to be sensitive to the nature of the head group of the compounds and we suggest is likely to be part of the Ca_V3 channel itself.

In this study, we have shown that endogenous acyl amides are powerful inhibitors of T-type calcium channels, with potencies similar to that previously reported for their major sites of action, CB_1 receptors and TRPV1 ion channels. T-type calcium channels, CB_1 receptors and TRPV1 are often co-expressed, and thus at concentrations previously shown to modulate peripheral nociception (Chu *et al*., 2003; Sagar *et al*., 2004; Huang and Walker, 2006) and synaptic transmission (Marinelli *et al*., 2007), NADA is also likely to be strongly affecting T-type calcium channels in these tissues. However, confirmation that T-type calcium channel modulation contributes to the physiological or pharmacological effects of NADA and NAGly awaits the development of more selective T-type calcium channel modulators, or studies in Ca_V3 knockout animals.

Acknowledgements

This work was supported by National Health and Medical Research Council of Australia Project 402564 to MC. HR was supported by a University of Sydney postgraduate award and Kolling Institute award, AG was supported by a University of Sydney postgraduate award and Kolling Institute award. HR, AG and MC performed experiments, HR and MC wrote the paper. Thanks to Catherine Morris and Ann Rittenhouse for their helpful discussion of the membrane effects of arachidonic acid and related compounds.

Conflict of interest

None.

References

Alexander SPH, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC), 3rd edition (2008 revision). *Br J Pharmacol* **153** (Suppl. 2): S1–S209.

Bisogno T, Melck D, Bobrov MY, Gretskaya NM, Bezuglov VV, De Petrocellis L *et al.* (2000). N-acyl-dopamines: novel synthetic CB_1 cannabinoid-receptor ligands and inhibitors of anandamide inactivation with cannabimimetic activity *in vitro* and in vivo. *Biochem J* **351**: 817–824.

Borgland SL, Connor M, Christie MJ (2001). Nociceptin inhibits calcium channel currents in a subpopulation of small nociceptive trigeminal ganglion neurons in mouse. *J Physiol* **536**: 35–47.

Burstein SH, Rossetti RG, Yagen B, Zurier RB (2000). Oxidative metabolism of anandamide. *Prostaglandins Other Lipid Mediat* **61**: 29–41.

Burstein SH, Adams JK, Bradshaw HB, Fraioli C, Rossetti RG, Salmonsen RA *et al*. (2007). Potential anti-inflammatory actions of the elmiric (lipoamino) acids. *Bioorg Med Chem* **15**: 3345–3355.

Chemin J, Monteil A, Perez-Reyes E, Nargeot J, Lory P (2001). Direct inhibition of T-type calcium channels by the endogenous cannabinoid anandamide. *EMBO J* **20**: 7033–7040.

Chemin J, Nargeot J, Lory P (2007). Chemical determinants involved in anandamide-induced inhibition of T-type calcium channels. *J Biol Chem* **282**: 2314–2323.

Chu CJ, Huang SM, De Petrocellis L, Bisogno T, Ewing SA, Miller JD *et al*. (2003). *N*-oleoyldopamine, a novel endogenous capsaicin-like lipid that produces hyperalgesia. *J Biol Chem* **278**: 13633–13639.

Cribbs LL, Lee JH, Yang J, Satin J, Zhang Y, Daud A *et al*. (1998). Cloning and characterization of alpha1H from human heart, a member of the T-type Ca^{2+} channel gene family. *Circ Res* **83**: 103–109.

Cribbs LL, Gomora JC, Daud AN, Lee JH, Perez-Reyes E (2000). Molecular cloning and functional expression of $Ca_V3.1c$, a T-type calcium channel from human brain. *FEBS Lett* **466**: 54–58.

De Petrocellis L, Bisogno T, Davis JD, Pertwee RG, Di Marzo V (2000). Overlap between the ligand recognition properties of the anandamide transporter and the VR1 receptor: inhibitors of anandamide uptake with negligible capsaicin-like activity. *FEBS Lett* **483**: 52–56.

Docherty RJ, Yeats JC, Piper AC (1997). Capsazepine block of voltage-activated calcium channels in adult dorsal root ganglion neurones in culture. *Br J Pharmacol* **121**: 1461–1467.

Gomora JC, Murbartian J, Arias JM, Lee J-H, Perez-Reyes E (2002). Cloning and expression of the human T-type channel $Ca_V3.3$: insights into prepulse facilitation. *Biophys J* **83**: 229–241.

Guo J, Williams DJ, Ikeda SR (2008). *N*-arachidonyl serine, a putative endocannabinoid, alters the activation of N-type calcium channels in sympathetic neurons. *J Neurophysiol* **100**: 1147–1151.

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch* **391**: 85–100.

Heady TN, Gomora JC, MacDonald TL, Perez-Reyes E (2001). Molecular pharmacology of T-type calcium channels. *Jpn J Pharmacol* **85**: 339–350.

Hildebrand ME, David LS, Hamid J, Mulatz K, Garcia E, Zamponi GW *et al*. (2007). Selective inhibition of $Ca_V3.3$ T-type calcium channels by $G\alpha_{q/11}$-coupled muscarinic acetylcholine receptors. *J Biol Chem* **282**: 21043–21055.

Huang SM, Walker JM (2006). Enhancement of spontaneous and heat-evoked activity in spinal nociceptive neurons by the endovanilloid/endocannabinoid *N*-arachidonyldopamine (NADA). *J Neurophysiol* **95**: 1207–1212.

Huang SM, Bisogno T, Petros TJ, Chang S-Y, Zavitsanos PA, Zipkin RE *et al*. (2001). Identification of a new class of molecules, the arachidonyl amino acids, and characterization of one member that inhibits pain. *J Biol Chem* **276**: 42639–42634.

Huang SM, Bisogno T, Trevisani M, Al-Hayani A, de Petrocellis L, Fezza F *et al*. (2002). An endogenous capsaicin-like substance with high potency at recombinant and native vanilloid receptors. *Proc Natl Acad Sci USA* **99**: 8400–8405.

Iftinca M, Hamid J, Chen L, Varela D, Tadayonnejad R, Altier C *et al*. (2007). Regulation of T-type calcium channels by Rho-associated kinase. *Nat Neurosci* **10**: 854–860.

Kohno M, Hasegawa H, Inoue A, Muraoka M, Miyazaki T, Oka K *et al*. (2006). Identification of *N*-arachidonylglycine as the endogenous ligand for orphan G-protein-coupled receptor GPR18. *Biochem Biophys Res Commun* **347**: 827–832.

Lundbaek JA (2008). Lipid bilayer-mediated regulation of ion channel function by amphiphilic drugs. *J Gen Physiol* **131**: 421–429.

Lundbaek JA, Birn P, Girshman J, Hansen AJ, Andersen OS (1996). Membrane stiffness and channel function. *Biochemistry* **35**: 3825–3830.

Lundbaek JA, Birn P, Hansen AJ, Sogaard R, Nielsen C, Girshman J *et al*. (2004). Regulation of sodium channel function by bilayer elasticity: the importance of hydrophobic coupling. Effects of micelle-forming amphiphiles and cholesterol. *J Gen Physiol* **123**: 599–621.

Marinelli S, Di Marzo V, Florenzano F, Fezza F, Viscomi MT, van der Stelt M *et al*. (2007). *N*-arachidonoyl-dopamine tune synaptic transmission onto dopaminergic neurons by activating both

cannabinoid and vanilloid receptors. *Neuropsychopharmacology* **32**: 298–308.

Milman G, Maor Y, Abu-Lafi S, Horowitz M, Gallily R, Baktai S *et al*. (2006). N-arachidonyl L-serine, an endocannabinoid-like brain constituent with vasodilatory properties. *Proc Natl Acad Sci USA* **103**: 2428–2433.

O'Sullivan SE, Kendall DA, Randall MD (2004). Characterisation of the vasorelaxant properties of the novel endocannabinoid *N*-arachidonoyl-dopamine (NADA). *Br J Pharmacol* **141**: 803–812.

Oh DY, Yoon JM, Moon MJ, Hwang J-I, Choe H, Lee JE *et al*. (2008). Identification of farnesyl pyrophosphate and *N*-arachidonylglycine as endogenous ligands for GPR92. *J Biol Chem* **283**: 21054–21064.

Perez-Reyes E (2003). Molecular physiology of low-voltage-activated T-type calcium channels. *Physiol Rev* **83**: 117–161.

Price TJ, Patwardhan A, Akopian AN, Hargreaves KM, Flores CM (2004). Modulation of trigeminal sensory neurons activity by the dual cannabinoid-vanilloid agonists anandamide, N-arachidonoyl-dopamine and arachidonyl-2-chloroethylamide. *Br J Pharmacol* **141**: 1118–1130.

Rimmerman N, Bradshaw HB, Hughes HV, Chen JS-C, Hu SS-J, McHugh D *et al*. (2008). *N*-Palmitoyl glycine, a novel endogenous lipid that acts as a modulator of calcium influx and nitric oxide production in sensory neurons. *Mol Pharmacol* **74**: 213–224.

Roberts LA, Christie MJ, Connor M (2002). Anandamide is a partial agonist at native vanilloid receptors in acutely isolated mouse trigeminal sensory neurons. *Br J Pharmacol* **137**: 421–428.

Roberts LA, Ross HR, Connor M (2008). Methanandamide activation of a novel current in mouse trigeminal ganglion sensory neurons in vitro. *Neuropharmacology* **54**: 172–180.

Ross HR, Napier I, Connor M (2008). Inhibition of recombinant human T-type calcium channels by Δ^9-Tetrahydrocannabinol and cannabidiol. *J Biol Chem* **283**: 16124–16134.

Sagar DR, Smith PA, Millns PJ, Smart D, Kendall DA, Chapman V (2004). TRPV1 and CB_1 receptor-mediated effects of the endovanilloid/endocannabinoid *N*-arachidonoyl-dopamine on primary afferent fibre and spinal cord neuronal responses in the rat. *Eur J Neurosci* **20**: 175–184.

Saghatelian A, McKinney MK, Bandell M, Patapoutian A, Cravatt BF (2006). A FAAH-regulated class of *N*-acyl taurines that activates TRP ion channels. *Biochemistry* **45**: 9007–9015.

Sheskin T, Hanus L, Slager J, Vogel Z, Mechoulam R (1997). Structural requirements for binding of anandamide-type compounds to the rat brain cannabinoid receptor. *J Med Chem* **40**: 659–667.

Shin H-S, Cheong E-J, Choi S, Lee J, Na HS (2008). T-type calcium channels as therapeutic targets in the nervous system. *Curr Opin Pharmacol* **8**: 33–41.

Succar R, Mitchell VA, Vaughan CW (2007). Actions of *N*-arachidonyl-glycine in a rat inflammatory pain model. *Mol Pain* **3**: 24.

Talavera K, Staes M, Janssens A, Droogmans G, Nilius B (2004). Mechanism of arachidonic acid modulation of the T-type calcium channel α_{1g}. *J Gen Physiol* **124**: 225–238.

Tao J, Hildebrand ME, Liao P, Liang MC, Tan G, Li S *et al*. (2008). Activation of corticotrophin-releasing factor receptor 1 selectively inhibits $Ca_V3.2$ T-type calcium channels. *Mol Pharmacol* **73**: 1596–1609.

Tian X, Guo J, Yao F, Yang D-P, Makriyannis A (2005). The conformation, location and dynamic properties of the endocannabinoid ligand anandamide in a membrane bilayer. *J Biol Chem* **280**: 29788–29795.

Vuong LAQ, Mitchell VA, Vaughan CW (2008). Actions of *N*-arachidonyl-glycine in a rat neuropathic pain model. *Neuropharmacology* **54**: 189–193.

Wiles AL, Pearlman RJ, Rosvall M, Aubrey K, Vandenberg RJ (2006). *N*-arachidonyl glycine inhibits the glycine transporter, GLYT2a. *J Neurochem* **99**: 781–786.

Yang Z, Aubrey KR, Alroy I, Harvey RJ, Vandenberg RJ, Lynch JW (2008). Subunit-specific modulation of glycine receptors by cannabinoids and *N*-arachidonyl-glycine. *Biochem Pharmacol* **76**: 1014–1023.

Zhang Y, Cribbs L, Satin J (2000). Arachidonic acid modulation of α1H, a cloned human T-type calcium channel. *Am J Physiol Heart Circ Physiol* **278**: H184–H193.