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# Cross-Modulation and Molecular Interaction at the Ca<sub>v</sub>3.3 Protein between the Endogenous Lipids and the T-Type Calcium Channel Antagonist TTA-A2.

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## ABSTRACT

T-type calcium channels (T/Ca<sub>v</sub>3-channels) are implicated in various physiologic and pathophysiologic processes such as epilepsy, sleep disorders, hypertension, and cancer. T-channels are the target of endogenous signaling lipids including the endocannabinoid anandamide, the ω3-fatty acids, and the lipoamino-acids. However, the precise molecular mechanism by which these molecules inhibit T-current is unknown. In this study, we provided a detailed electrophysiologic and pharmacologic analysis indicating that the effects of the major *N*-acyl derivatives on the Ca<sub>v</sub>3.3 current share many similarities with those of TTA-A2 [(*R*)-2-(4-cyclopropylphenyl)-*N*-(1-(5-(2,2,2-trifluoroethoxy)pyridin-2-yl)ethyl)acetamide], a synthetic T-channel inhibitor. Using radioactive binding assays with the TTA-A2 derivative [<sup>3</sup>H]TTA-A1 [(*R*)-2-(4-(*tert*-butyl)phenyl)-*N*-(1-(5-methoxypyridin-2-yl)ethyl)acetamide], we demonstrated that polyunsaturated lipids, which inhibit the Ca<sub>v</sub>3.3 current, as NAGly (*N*-arachidonoyl glycine), NASer (*N*-arachidonoyl-L-serine), anandamide, NADA (*N*-arachidonoyl dopamine), NATau

(*N*-arachidonoyl taurine), and NA-5HT (*N*-arachidonoyl serotonin), all displaced [<sup>3</sup>H]TTA-A1 binding to membranes prepared from cells expressing Ca<sub>v</sub>3.3, with *K*<sub>i</sub> in a micromolar or submicromolar range. In contrast, lipids with a saturated alkyl chain, as *N*-arachidoyl glycine and *N*-arachidoyl ethanolamine, which did not inhibit the Ca<sub>v</sub>3.3 current, had no effect on [<sup>3</sup>H]TTA-A1 binding. Accordingly, bio-active lipids occluded TTA-A2 effect on Ca<sub>v</sub>3.3 current. In addition, TTA-Q4 [(*S*)-4-(6-chloro-4-cyclopropyl-3-(2,2-difluoroethyl)-2-oxo-1,2,3,4-tetrahydroquinazolin-4-yl)benzotrile], a positive allosteric modulator of [<sup>3</sup>H]TTA-A1 binding and TTA-A2 functional inhibition, acted in a synergistic manner to increase lipid-induced inhibition of the Ca<sub>v</sub>3.3 current. Overall, our results demonstrate a common molecular mechanism for the synthetic T-channel inhibitors and the endogenous lipids, and indicate that TTA-A2 and TTA-Q4 could be important pharmacologic tools to dissect the involvement of T-current in the physiologic effects of endogenous lipids.

## Introduction

Low-voltage-activated (T-type/Ca<sub>v</sub>3) calcium channels are a subclass of voltage-dependent calcium channels allowing calcium entry near the resting potential of most cells (Perez-Reyes, 2003). T-channels are implicated in many physiologic processes as diverse as neuronal firing (Perez-Reyes, 2003; Cain and Snutch, 2010), slow wave sleep (Lee and Shin, 2007), hormone secretion (Weiss and Zamponi, 2013), the cell cycle

(Lory et al., 2006), heart rhythm (Ono and Iijima, 2010), and vasodilatation (Kuo et al., 2011). They emerge as important pharmacologic targets in several diseases such as epilepsy, insomnia, neuropathic pain, cancer, and hypertension (McGivern, 2006; Todorovic and Jevtovic-Todorovic, 2013).

Various synthetic T-channel blockers have been described in the past few years (McGivern, 2006; Lory and Chemin, 2007; Giordanetto et al., 2011), including TTA-A2 [(*R*)-2-(4-cyclopropylphenyl)-*N*-(1-(5-(2,2,2-trifluoroethoxy)pyridin-2-yl)ethyl)acetamide], a potent and specific inhibitor of T-current (Uebele et al., 2009a,b; Kraus et al., 2010; Reger et al., 2011). In vivo studies have demonstrated that TTA-A2 reduces

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**ABBREVIATIONS:** BAPTA, 1,2-bis(o-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; CP-55940, (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; FAAH, fatty acid amide hydrolase; 18:2 Gly, *N*-linoleoyl glycine; 20:0 Gly, *N*-arachidoyl glycine; 22:6 Gly, *N*-docosahexaenoyl glycine; HEK, human embryonic kidney cells; HP, holding potential; NAAla, *N*-arachidonoyl alanine; NADA, *N*-arachidonoyl dopamine; NAEA, *N*-arachidonoyl ethanolamine (anandamide); NAGABA, *N*-arachidonoyl-gaba-butyric acid; NAGABA-OH, *N*-arachidonoyl-3-OH-gaba-butyric acid; NAGly, *N*-arachidonoyl glycine; NA-5HT, *N*-arachidonoyl serotonin; NASer, *N*-arachidonoyl-L-serine; NATau, *N*-arachidonoyl taurine; PMSF, phenylmethylsulfonyl fluoride; T-channel, T-type calcium channel; TTA-A1, (*R*)-2-(4-(*tert*-butyl)phenyl)-*N*-(1-(5-methoxypyridin-2-yl)ethyl)acetamide; TTA-A2, (*R*)-2-(4-cyclopropylphenyl)-*N*-(1-(5-(2,2,2-trifluoroethoxy)pyridin-2-yl)ethyl)acetamide; TTA-Q4, (*S*)-4-(6-chloro-4-cyclopropyl-3-(2,2-difluoroethyl)-2-oxo-1,2,3,4-tetrahydroquinazolin-4-yl)benzotrile.

absence epilepsy seizures (Uebele et al., 2009b; Reger et al., 2011), pain perception (Francois et al., 2013), nicotine self-administration (Uslaner et al., 2010), and weight gain (Uebele et al., 2009a), and it ameliorates the sleep quality (Uebele et al., 2009a; Kraus et al., 2010; Reger et al., 2011) and displays antipsychotic properties (Uslaner et al., 2012).

T-channels are also inhibited by several endogenous signaling lipids. These molecules include arachidonic acid,  $\omega$ 3-fatty acids, endocannabinoids (as anandamide), lipo-amino-acids and lipo-neurotransmitters (Zhang et al., 2000; Chemin et al., 2001, 2007; Talavera et al., 2004; Danthi et al., 2005; Barbara et al., 2009; Ross et al., 2009; Gilmore et al., 2012). These lipids are implicated in multiple physiologic functions and more specifically in pain perception (Bradshaw and Walker, 2005; Burstein, 2008; Basbaum et al., 2009), sleep and epilepsy (Chen and Bazan, 2005), and heart rhythm and vasodilatation (Roman, 2002; Leaf et al., 2003).

Lipid-mediated inhibition of the T-current occurs in excised cell-free membrane patches consistent with the direct effect of lipids on T-channels or via perturbation of their near membrane environment (Chemin et al., 2001, 2007; Talavera et al., 2004; Barbara et al., 2009). In this context, it is interesting to note that a radiolabeled derivative of TTA-A2, [<sup>3</sup>H]TTA-A1 [(*R*)-2-(4-(*tert*-butyl)phenyl)-*N*-(1-(5-methoxypyridin-2-yl)ethyl)acetamide], was shown to bind membranes from cells expressing Ca<sub>v</sub>3.3 with a *K*<sub>d</sub> of 1.8 nM (Uebele et al., 2009b). Moreover a structurally distinct antagonist, TTA-Q4 [(*S*)-4-(6-chloro-4-cyclopropyl-3-(2,2-difluoroethyl)-2-oxo-1,2,3,4-tetrahydroquinazolin-4-yl)benzotrile], increased both [<sup>3</sup>H]TTA-A1 binding and TTA-A2-induced inhibition of the Ca<sub>v</sub>3.3 current by acting on a distinct molecular site of the Ca<sub>v</sub>3.3 protein (Uebele et al., 2009b). In this study, we used these new pharmacologic tools to explore whether bioactive lipids and TTA-A2 share similar mechanisms and molecular determinants at the Ca<sub>v</sub>3.3 protein.

## Materials and Methods

**Cell Culture and Transfection Protocols.** We cultivated tsA-201 (SV40 temperature-sensitive T antigen transformed) cells and a human embryonic kidney (HEK) 293 cell line stably expressing Ca<sub>v</sub>3.3 [a generous gift from Dr. Perez-Reyes (Xie et al., 2007)] in Dulbecco's modified Eagle's medium supplemented with GlutaMax and 10% fetal bovine serum (Invitrogen/Life Technologies, Carlsbad, CA). The tsA-201 cell transfection was performed using jet-PEI (QBiogene, Illkirch, France) with a DNA mix containing 0.5% of a green fluorescent protein plasmid and 99.5% of either of the plasmid constructs that code for human Ca<sub>v</sub>3.1a, Ca<sub>v</sub>3.2, and Ca<sub>v</sub>3.3. Two days after transfection, cells were dissociated with Versene (Invitrogen) and plated at a density of  $\sim 35 \times 10^3$  cells per 35-mm Petri dish for electrophysiologic recordings.

**Electrophysiologic Recordings.** Macroscopic currents were recorded in the whole-cell configuration using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). The extracellular solution contained the following (in mM): 135 NaCl, 20 TEACl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, and 10 HEPES (pH adjusted to 7.25 with KOH  $\sim 330$  mOsm). Borosilicate glass pipettes have a typical resistance of 1.5–2.5 M $\Omega$  when filled with an internal solution containing the following (in mM): 140 CsCl, 10 EGTA, 10 HEPES, 3 Mg-ATP, 0.6 GTPNa, and 3 CaCl<sub>2</sub> (pH adjusted to 7.25 with KOH  $\sim 315$  mOsm). In a subset of experiments, EGTA was substituted with 10 mM BAPTA [1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid]. Recordings were performed at room temperature and were filtered at 2–5 kHz. Data were analyzed using pCLAMP9 (Molecular Devices) and GraphPad Prism (GraphPad

Software, Inc., San Diego, CA). Drugs were applied by a gravity-driven homemade perfusion device, and the control experiments were performed using the solvent alone. Results are presented as the mean  $\pm$  S.E.M., and *n* is the number of cells used. Student's *t* test was used to compare the different values, and *P* < 0.05 was considered statistically significant.

**Binding Experiments.** Membranes were prepared from the HEK-293 cells stably expressing Ca<sub>v</sub>3.3 (Uebele et al., 2009b). The protein concentration was determined using a protein assay (Bio-Rad Laboratories, Hercules, CA). Binding assays were performed at room temperature for 3 hours with 8  $\mu$ g protein, 1 nM [<sup>3</sup>H]TTA-A1, and increasing concentrations of lipids (ranging from 1 nM to 40  $\mu$ M) in 1 ml final volume in Unifilter-96 GF/C plates (Packard, Meriden, CT) coated with 0.3% ethylene imine polymer solution (Sigma-Aldrich, St. Louis, MO). The assay and wash buffer contained 20 mM HEPES, 125 mM NaCl, and 5 mM KCl.

After incubation, the reactions were aspirated and washed with 4°C buffer using a 96-well Filtermate Harvester (PerkinElmer Life and Analytic Sciences, Shelton, CT). The plates were dried before the addition of Microscint-20 (PerkinElmer), and the remaining radioactivity was quantified on a PerkinElmer NXT HTS Top Count. Total and nonspecific binding were determined in the absence and the presence of 100 nM of TTA-A2, respectively.

Data were collected in triplicate. The inhibition constants *K*<sub>i</sub> were calculated with GraphPad Prism using the following equation:

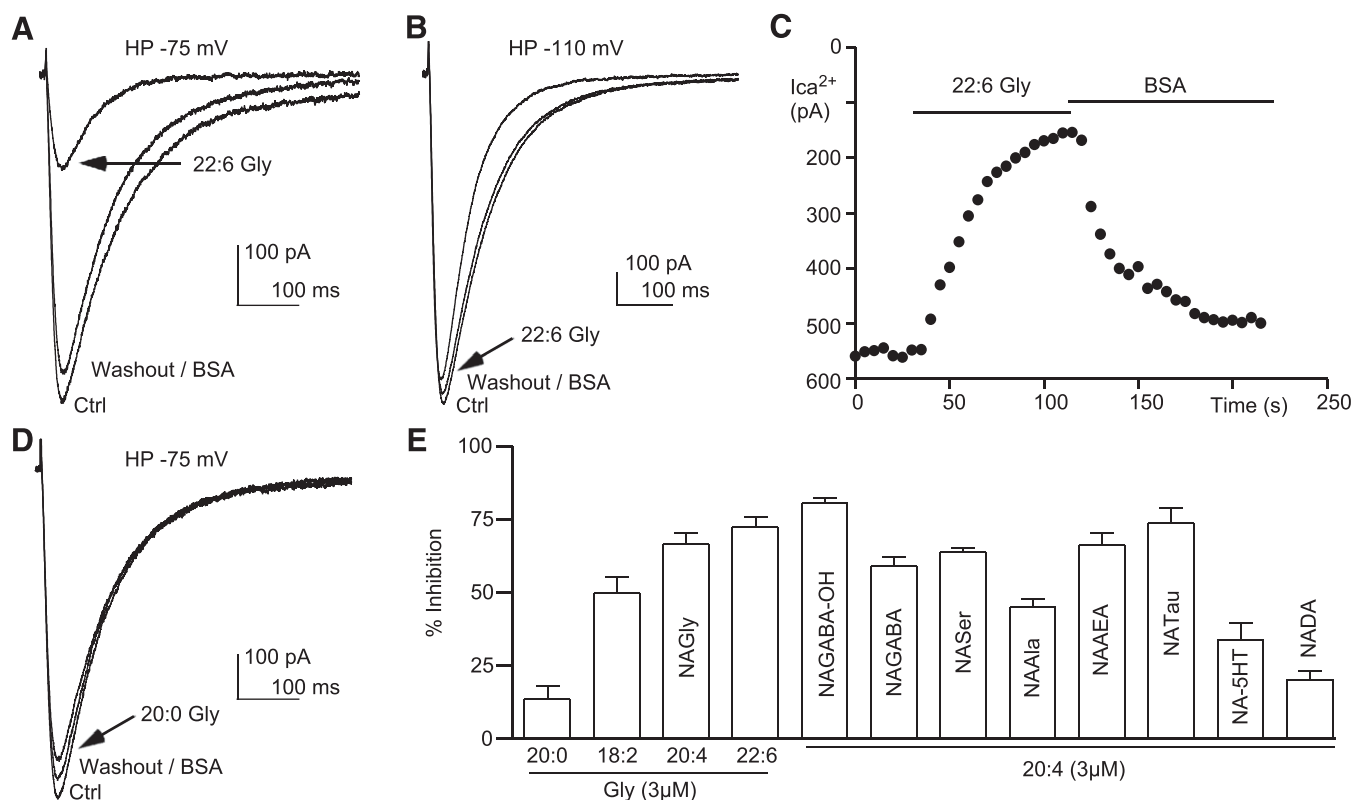
$$K_i = \frac{IC_{50}}{\left(1 + \left(\frac{[{}^3\text{H}]\text{TTA-A1}}{K_d}\right)\right)}$$

where IC<sub>50</sub> was the half maximal inhibitory concentration, [<sup>3</sup>H]TTA-A1 was 1 nM, and *K*<sub>d</sub> was 1.8 nM, as previously described elsewhere (Uebele et al., 2009b).

**Chemical Reagents.** [<sup>3</sup>H]TTA-A1 (56–65 mCi/mmol), TTA-A2, and TTA-Q4 were synthesized at Merck (West Point, PA), as previously described elsewhere (Uebele et al., 2009b), dissolved in dimethylsulfoxide at 10 mM, aliquoted, and kept at –20°C. Lipids were obtained from Cayman Chemicals (Ann Arbor, MI) and were dissolved in ethanol purged with argon at a concentration of 10 mM. NA-5HT (*N*-arachidonoyl serotonin) and NADA (*N*-arachidonoyl dopamine) were also obtained from Tocris Bioscience (Bristol, UK) and Enzo Life Sciences (Farmingdale, NY), and the results were similar using lipids from different suppliers. Stock lipid solutions were briefly sonicated, aliquoted, sealed under argon, and kept at –80°C. These aliquots were dissolved daily in the extracellular solution. Control experiments were performed using the solvent alone.

## Results

**Inhibition of Ca<sub>v</sub>3.3 Currents by Polyunsaturated Amino Acids.** We have characterized the effects of lipo-amino-acids on the Ca<sub>v</sub>3.3 current because among lipids inhibiting T-currents, only this class showed a higher T-type specificity with no effect on cannabinoid receptors and transient receptor potential vanilloid-1 (Huang et al., 2001; Bradshaw and Walker, 2005; Barbara et al., 2009). We found that the Ca<sub>v</sub>3.3 current was strongly inhibited by 3  $\mu$ M *N*-docosahexaenoyl glycine (22:6 Gly; chain length 22 carbons, 6 double bonds) at a holding potential (HP) of –75 mV (Fig. 1A) but not at HP –110 mV (Fig. 1B). The inhibition occurred in the minute range and was relieved by application of 3 mg/ml of bovine serum albumin (Barbara et al., 2009; Gilmore et al., 2012) (Fig. 1C). The extent of inhibition increased gradually with the number of double bonds in *N*-acyl glycine (Fig. 1E). Saturated *N*-arachidoyl glycine (20:0 Gly) produced weak inhibition ( $\sim 13\%$ , *n* = 5; Fig. 1, D and E); *N*-linoleoyl glycine



**Fig. 1.** Inhibition of Ca<sub>v</sub>3.3 currents by endogenous lipids. (A and B) Inhibition of Ca<sub>v</sub>3.3 currents by 3  $\mu$ M 22:6 Gly and subsequent washout with a solution containing 3 mg/ml bovine serum albumin (BSA). Ca<sub>v</sub>3.3 currents were elicited by a 450-millisecond depolarization to  $-30$  mV from a HP of  $-75$  mV (A) or  $-110$  mV (B) at a frequency of 0.2 Hz. (C) Time course of the decrease in Ca<sub>v</sub>3.3 current amplitude during 22:6 Gly application at HP  $-75$  mV and subsequent washout with the BSA solution. Same cell as in A. (D) Inhibition of Ca<sub>v</sub>3.3 currents by 20:0 Gly at HP  $-75$  mV and subsequent washout with a solution containing 3 mg/ml BSA. (E) Summary of the inhibitory effect on Ca<sub>v</sub>3.3 current at HP  $-75$  mV of 20:0 Gly, 18:2 Gly, NAGly, 22:6 Gly, NAGABA-OH, NAGABA, NASer, NAAla, NAEA, NATau, NA-5HT, and NADA. Ctrl, control.

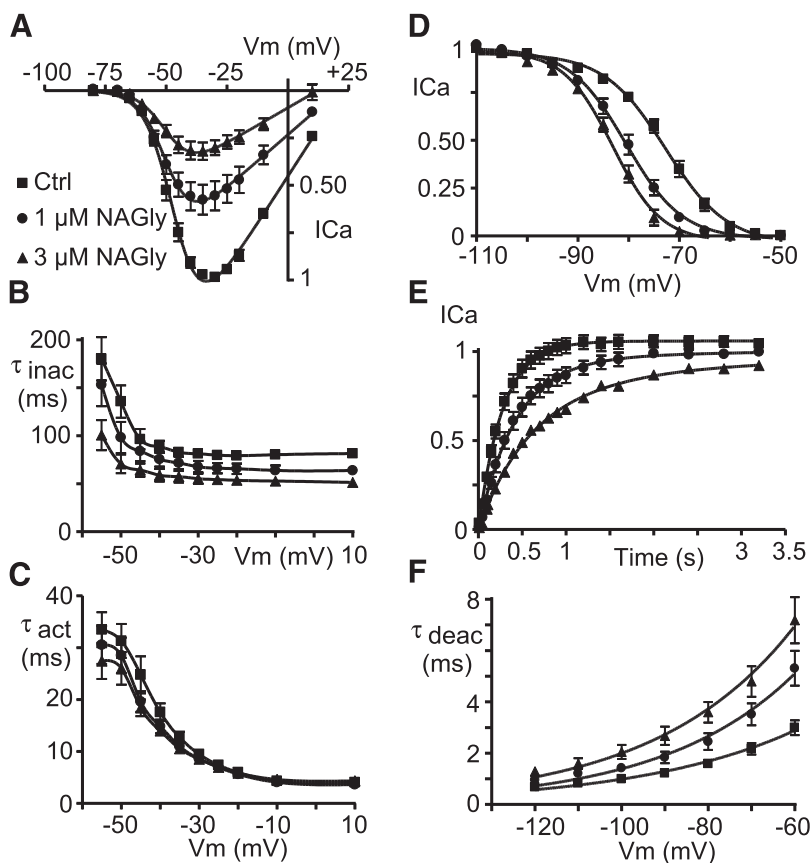
(18:2 Gly) produced  $\sim 50\%$  inhibition ( $n = 7$ ; Fig. 1E;  $P \leq 0.001$  compared with 20:0 Gly); *N*-arachidonoyl glycine (20:4, NAGly) produced  $\sim 67\%$  inhibition ( $n = 11$ ; Fig. 1E;  $P \leq 0.05$  compared with 18:2 Gly); and the fully polyunsaturated 22:6 glycine produced  $\sim 74\%$  inhibition ( $n = 12$ ; Fig. 1E; not statistically significant compared with 20:4 Gly). Interestingly, the increase in the number of double bonds in fatty acids augmented the membrane fluidity while restricting the conformational freedom of the molecule, possibly leading to the adequate conformation to inhibit the T-current.

The Ca<sub>v</sub>3.3 current was also inhibited by several *N*-arachidonoyl amino acids, including *N*-arachidonoyl-3-OH- $\gamma$ -butyric acid (NAGABA-OH,  $\sim 81\%$  inhibition,  $n = 22$ ), *N*-arachidonoyl- $\gamma$ -butyric acid (NAGABA,  $\sim 59\%$  inhibition,  $n = 24$ ), *N*-arachidonoyl-L-serine (NASer,  $\sim 64\%$  inhibition,  $n = 22$ ), and *N*-arachidonoyl alanine (NAAla,  $\sim 45\%$  inhibition,  $n = 23$ ; Fig. 1E). In addition, the lipo-neurotransmitters *N*-arachidonoyl taurine (NATau), NA-5HT, and *N*-arachidonoyl dopamine (NADA) inhibited the Ca<sub>v</sub>3.3 current by  $\sim 74\%$ ,  $\sim 34\%$ , and  $\sim 20\%$ , respectively ( $n \geq 8$ ; Fig. 1E). Similar findings were obtained with NADA and NA-5HT using an intracellular medium containing 10 mM BAPTA ( $n = 5$ ) (Ross et al., 2009; Gilmore et al., 2012) as well as on Ca<sub>v</sub>3.1 and Ca<sub>v</sub>3.2 current (data not shown). Application of 3  $\mu$ M NADA induced  $\sim 18\%$  inhibition of the Ca<sub>v</sub>3.1 current ( $n = 5$ ) and  $\sim 27\%$  inhibition of the Ca<sub>v</sub>3.2 current ( $n = 6$ ). Similarly, application of 3  $\mu$ M NA-5HT induced  $\sim 44\%$  inhibition of the Ca<sub>v</sub>3.1 current ( $n = 16$ ) and  $\sim 50\%$  inhibition of the Ca<sub>v</sub>3.2 current ( $n = 14$ ). As

previously described elsewhere (Chemin et al., 2001), in these experiments, 3  $\mu$ M NAEA [*N*-arachidonoyl ethanolamine (anandamide)] strongly inhibited the three Ca<sub>v</sub>3 currents by  $\sim 74\%$ – $80\%$  ( $n \geq 8$ ; Fig. 1E).

**NAGly Effect on Ca<sub>v</sub>3.3 Biophysical Properties.** NAGly inhibited the Ca<sub>v</sub>3.3 current at every potential, with inhibition of the peak current at  $-35$  mV by  $\sim 40\%$  with 1  $\mu$ M NAGly and by  $\sim 70\%$  with 3  $\mu$ M NAGly (Fig. 2A). NAGly had a weak and not statistically significant effect on the Ca<sub>v</sub>3.3 current–voltage relationship, as the  $V_{0.5}$  values were  $-45.7 \pm 1.3$  mV ( $n = 8$ ) in control conditions,  $-49.6 \pm 1.4$  mV ( $n = 8$ ) during 1  $\mu$ M NAGly application, and  $-48.6 \pm 0.8$  mV ( $n = 5$ ) during 3  $\mu$ M NAGly application ( $P > 0.05$ ). We also observed that 3  $\mu$ M NAGly accelerated the inactivation rate of the Ca<sub>v</sub>3.3 current at every tested potential ( $P < 0.05$ ,  $n = 5$ ; Fig. 2B) without a significant corresponding effect on the activation rate (Fig. 2C). Furthermore, NAGly induced a  $\sim 10$  mV negative shift in the steady-state inactivation properties of Ca<sub>v</sub>3.3 (Fig. 2D). The  $V_{0.5}$  values were  $-73.1 \pm 0.9$  mV ( $n = 8$ ) in control conditions,  $-80.6 \pm 1.07$  mV ( $P < 0.001$ ,  $n = 8$ ) during 1  $\mu$ M NAGly application, and  $-83.2 \pm 0.8$  mV ( $P < 0.001$ ,  $n = 7$ ) during 3  $\mu$ M NAGly application. Application of 3  $\mu$ M NAGly also decreased the slope factor of the steady-state inactivation curve of Ca<sub>v</sub>3.3 from  $5.2 \pm 0.2$  in control conditions to  $4.3 \pm 0.2$  ( $P < 0.05$ ).

We next investigated the effect of NAGly on the recovery from inactivation of the Ca<sub>v</sub>3.3 current (Fig. 2E). The recovery from inactivation of the Ca<sub>v</sub>3.3 current was well



**Fig. 2.** Effects of NAGly on biophysical properties of Ca<sub>v</sub>3.3 currents. (A) Current-voltage (I-V) curves of Ca<sub>v</sub>3.3 current in the absence or presence of 1 or 3 μM NAGly. Currents were elicited by increasing depolarizations (−80 to +10 mV) from a HP of −80 mV at a frequency of 0.2 Hz. (B–C) Effects of 1 and 3 μM NAGly on inactivation (τ<sub>inac</sub>; B) and activation (τ<sub>act</sub>; C) kinetics of Ca<sub>v</sub>3.3 currents. (D) Steady-state inactivation curves of Ca<sub>v</sub>3.3 currents in the absence or presence of 1 or 3 μM NAGly. Currents were recorded at −30 mV from HPs ranging from −110 to −50 mV (5-second duration). (E) Recovery from inactivation of Ca<sub>v</sub>3.3 current in the absence or presence of 1 or 3 μM NAGly. Recovery from inactivation was measured using two −30 mV depolarizations lasting 450 milliseconds, which were applied from a HP of −80 mV of increasing duration. (F) Effects of 1 or 3 μM NAGly on deactivation kinetics of Ca<sub>v</sub>3.3 currents (τ<sub>deac</sub>). Currents were elicited by a 28-millisecond depolarization at −30 mV, and deactivation kinetics were measured at repolarization potentials ranging from −130 to −60 mV. Ctrl, control.

fit by a monoexponential revealing that NAGly strongly increased the  $\tau$  of recovery from  $298 \pm 20$  milliseconds ( $n = 8$ ) in control conditions to  $453 \pm 22$  milliseconds ( $P < 0.001$ ,  $n = 8$ ) during 1 μM NAGly application and to  $771 \pm 43$  milliseconds ( $P < 0.001$ ,  $n = 8$ ) during 3 μM NAGly application. Finally, we found that both 1 and 3 μM NAGly slowed the deactivation of the Ca<sub>v</sub>3.3 current at repolarization potentials ranging from −120 to −60 mV ( $P < 0.05$ ,  $n = 8$ ; Fig. 2F).

**Pharmacologic Interaction of Endogenous Lipids and TTA-A2 on the Ca<sub>v</sub>3.3 Channel.** Recently, TTA-A2, a potent and specific synthetic inhibitor of T-current, was described (Uebele et al., 2009a,b; Kraus et al., 2010; Reger et al., 2011). As observed with endogenous lipids, TTA-A2 inhibited the Ca<sub>v</sub>3 current at physiologic HP but not at very negative potentials (i.e., −110 mV) (Kraus et al., 2010; Francois et al., 2013). Furthermore, as observed with endogenous lipids, TTA-A2 induced a negative shift in the steady-state inactivation properties of Ca<sub>v</sub>3 current and slowed their recovery from inactivation (Kraus et al., 2010; Francois et al., 2013). It should be noted that several other structurally unrelated T-channel inhibitors, including mibefradil, flunarizine, and pimoizide, which also exhibit similar state-dependent inhibition of T-currents (Martin et al., 2000; Santi et al., 2002), were shown to interact with [<sup>3</sup>H]TTA-A1 binding to membranes containing Ca<sub>v</sub>3.3 (Uebele et al., 2009b).

Therefore, we investigated whether endogenous lipids and TTA-A2 could share a common inhibitory mechanism on Ca<sub>v</sub>3.3. To this purpose, the effect of NAEA (which strongly inhibited Ca<sub>v</sub>3.3 current) and NADA or NA-5HT (which mildly inhibited Ca<sub>v</sub>3.3 current) were compared in the presence and the absence

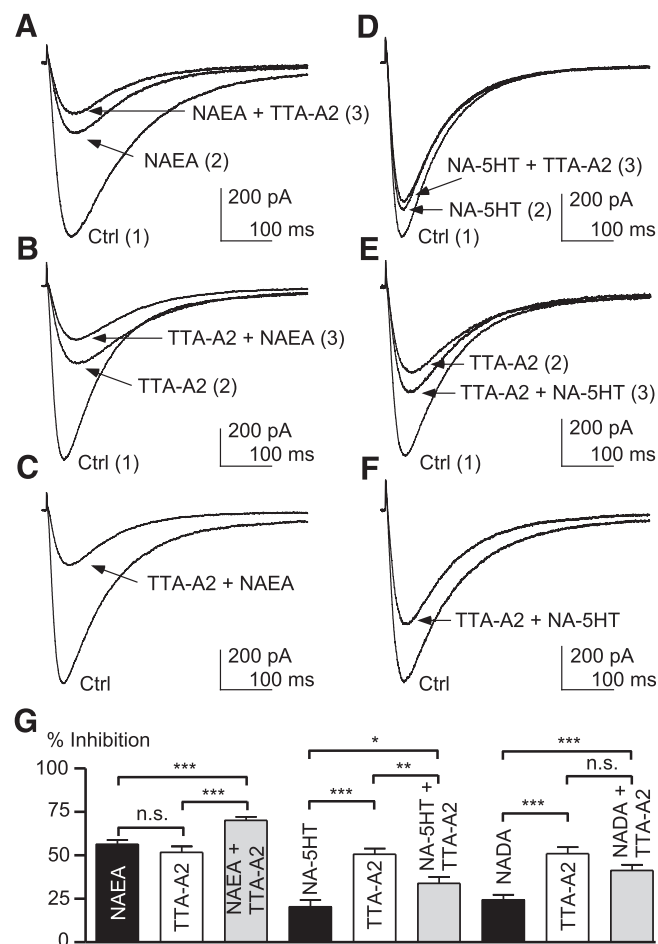
of TTA-A2 (Fig. 3). We found that 300 nM NAEA alone induced  $56\% \pm 2\%$  inhibition of the Ca<sub>v</sub>3.3 current and accelerated the inactivation kinetic ( $\tau$ ) by  $32.5\% \pm 2.6\%$  ( $P < 0.05$ ,  $n = 8$ ; Fig. 3A), as previously described elsewhere (Chemin et al., 2001). Similarly, 3 nM TTA-A2 alone induced  $52\% \pm 3\%$  inhibition of the Ca<sub>v</sub>3.3 current but slowed the inactivation kinetic ( $\tau$ ) by  $34.1\% \pm 5.4\%$  ( $P < 0.05$ ,  $n = 7$ ; Fig. 3B).

Interestingly, when NAEA and TTA-A2 were applied simultaneously, the Ca<sub>v</sub>3.3 current was inhibited by  $70\% \pm 2\%$  ( $n = 10$ ; Fig. 3C), indicating that the effect of NAEA and TTA-A2 are only partially additive (Fig. 3G). In this latter case, the inactivation kinetic ( $\tau$ ) was accelerated by  $21.7\% \pm 4.9\%$  ( $P < 0.05$ ,  $n = 10$ ; Fig. 3C). The results were not different ( $P > 0.05$ ) when both molecules were applied after NAEA application ( $n = 5$ ; Fig. 3A) or TTA-A2 application ( $n = 5$ ; Fig. 3B).

These results were further confirmed using the mild inhibitors NADA and NA-5HT. Indeed, when 3 μM NA-5HT, which inhibited the Ca<sub>v</sub>3.3 current by  $20\% \pm 4\%$  ( $n = 16$ ; Fig. 3D), was applied with TTA-A2, the resulting inhibition was only  $33\% \pm 4\%$  ( $n = 19$ ; Fig. 3F), demonstrating no additive effects. This was clearly evidenced when NA-5HT and TTA-A2 were applied together after TTA-A2 treatment (Fig. 3E). In this case, the inhibition induced by both compounds was less than those obtained with TTA-A2 alone ( $P < 0.01$ ,  $n = 11$ ; Fig. 3, E and G). Similar findings were obtained with 3 μM NADA ( $n = 15$ ; Fig. 3G).

Overall, these results demonstrated a pharmacologic interaction between endogenous lipids and TTA-A2 and suggested that both molecules could share the same molecular site on the Ca<sub>v</sub>3.3 protein.

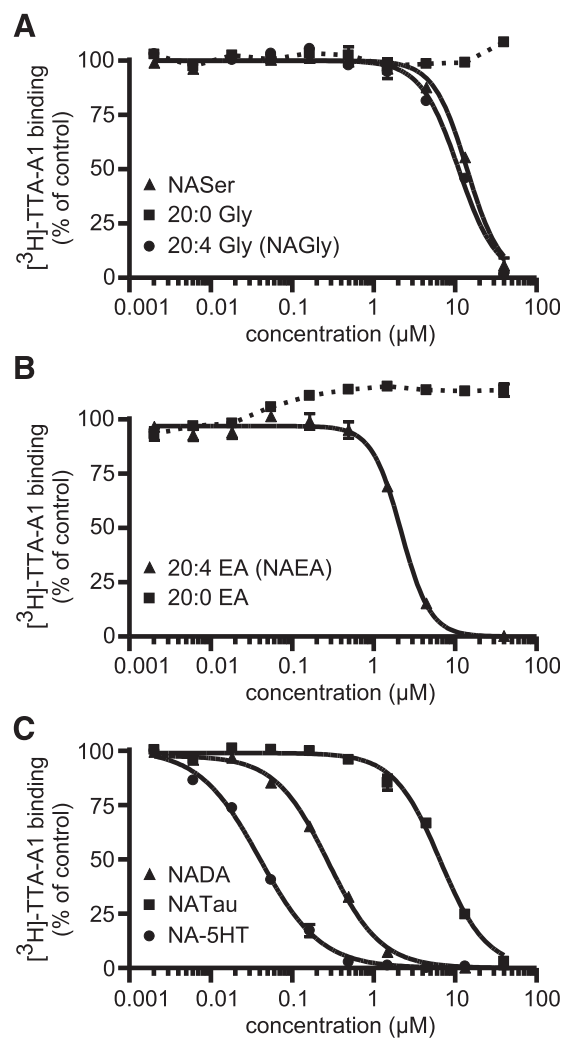




**Fig. 3.** Inhibition of  $Ca_v3.3$  currents by combined application of endogenous lipids and TTA-A2. (A) Effect of a 300 nM NAEA solution (2) followed by the application of both 300 nM NAEA and 3 nM TTA-A2 (3), compared with the control solution (1). (B) Effect of a 3 nM TTA-A2 solution followed by the application of both 300 nM NAEA and 3 nM TTA-A2. (C) Effect of a solution containing both 300 nM NAEA and 3 nM TTA-A2. (D–F) Similar experiments with 3  $\mu$ M NA-5HT and 3 nM TTA-A2. (G) Summary of the average effects of NAEA, NADA, and NA-5HT alone or combined with 3 nM TTA-A2. Currents were elicited at  $-30$  mV from a HP of  $-75$  mV at a frequency of 0.2 Hz. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Ctrl, control; n.s., not statistically significant.

**N-Acyl Derivatives That Inhibited  $Ca_v3.3$  Current Displaced [ $^3$ H]TTA-A1 Binding.** Because lipids and TTA-A2 possibly act at the same molecular site, we investigated whether bioactive lipids that inhibit  $Ca_v3.3$  current could displace [ $^3$ H]TTA-A1 (a radiolabeled derivative of TTA-A2) binding to membranes containing  $Ca_v3.3$ , as demonstrated for several state-dependent T-channel antagonists (Uebele et al., 2009b). We found that *N*-arachidonoyl amino-acids NAser and NAGly, which inhibited  $Ca_v3.3$  current (Fig. 1D), displaced [ $^3$ H]TTA-A1 binding in a concentration-dependent manner (Fig. 4A). The  $K_i$  for NAser was  $9.02 \pm 0.06 \mu$ M and  $7.12 \pm 0.31 \mu$ M for NAGly whereas the Hill slope number ( $n_{Hill}$ ) was 2.1 and 1.8 for NAser and NAGly, respectively.

Displacement of [ $^3$ H]TTA-A1 binding was not observed with the saturated arachidoyl glycine at concentrations up to 40  $\mu$ M (20:0; Fig. 4A). Similarly, the endocannabinoid anandamide (20:4 EA) displaced [ $^3$ H]TTA-A1 binding with a  $K_i$  of  $1.35 \pm 0.04 \mu$ M and a  $n_{Hill}$  of 2.4, but not the saturated form, *N*-arachidoyl ethanolamine (Fig. 4B). In addition, the



**Fig. 4.** Displacement of [ $^3$ H]TTA-A1 binding by (A) *N*-acyl amino acids, (B) *N*-acyl ethanolamines, and (C) *N*-acyl neurotransmitters. Lipids are NAser, NAGly, 20:0 Gly, NAEA (20:4), *N*-arachidoyl ethanolamine (20:0 EA), NADA (20:4), NA-5HT (20:4), and NATau (20:4).

*N*-arachidonoyl neurotransmitters NATau, *N*-arachidonoyl dopamine (NADA) and NA-5HT, also displaced [ $^3$ H]TTA-A1 binding (Fig. 4C). The  $K_i$  was  $4.13 \pm 0.11 \mu$ M for NATau,  $0.170 \pm 0.004 \mu$ M for NADA and  $0.026 \pm 0.001 \mu$ M for NA-5HT whereas the  $n_{Hill}$  was 1.5, 1.3, and 1.1 for NATau, NADA, and NA-5HT, respectively.

**TTA-Q4, a Positive Allosteric Modulator of [ $^3$ H]TTA-A1 Binding, Increased Lipid-Induced Inhibition of  $Ca_v3.3$  Current.** The structurally distinct T-type antagonist TTA-Q4 was shown to increase [ $^3$ H]TTA-A1 binding on  $Ca_v3.3$  expressing membranes as well as TTA-A2-induced  $Ca_v3.3$  current inhibition (Uebele et al., 2009b). It was demonstrated that TTA-Q4 increased TTA-A2-mediated  $Ca_v3.3$  current inhibition by a synergistic mechanism (Uebele et al., 2009b). Therefore, we investigated whether TTA-Q4 had a similar effect on lipid-induced  $Ca_v3.3$  current inhibition.

We found that 20 nM TTA-Q4 alone induced  $22\% \pm 4\%$  inhibition of  $Ca_v3.3$  current ( $n = 8$ ; Fig. 5A), 100 nM anandamide alone induced  $18\% \pm 5\%$  inhibition ( $n = 5$ ; Fig. 5B), but application of both compounds induced  $72\% \pm 4\%$  inhibition ( $n = 7$ ; Fig. 5, C and D), which is much greater than

the anticipated sum of ~40% expected for additive effects (as indicated by an arrow in Fig. 5I). We also found that 300 nM NA-5HT induced a negligible effect on Ca<sub>v</sub>3.3 current (8% ± 5% inhibition, *n* = 5; Fig. 5E) whereas when NA-5HT was applied with TTA-Q4, the inhibition of Ca<sub>v</sub>3.3 current was

52% ± 2% (*n* = 5; Fig. 5, G–I). Similar results were obtained using 3 μM NADA and 20 nM TTA-Q4, as application of both compounds induced 72% ± 2% inhibition of Ca<sub>v</sub>3.3 current (*n* = 11; Fig. 5I). Overall, these results indicated that TTA-Q4 and polyunsaturated lipids modulated Ca<sub>v</sub>3.3 current in a synergistic manner.

## Discussion

In this study, we demonstrated that lipid effects on Ca<sub>v</sub>3.3 biophysical properties share many features with those induced by TTA-A2, especially regarding the gating properties. Importantly, inhibition of Ca<sub>v</sub>3.3 current by both lipids and TTA-A2 occurs only at depolarized resting potentials at which Ca<sub>v</sub>3.3 channels are partly inactivated. We also documented that endogenous lipids and TTA-A2 share similar molecular mechanisms.

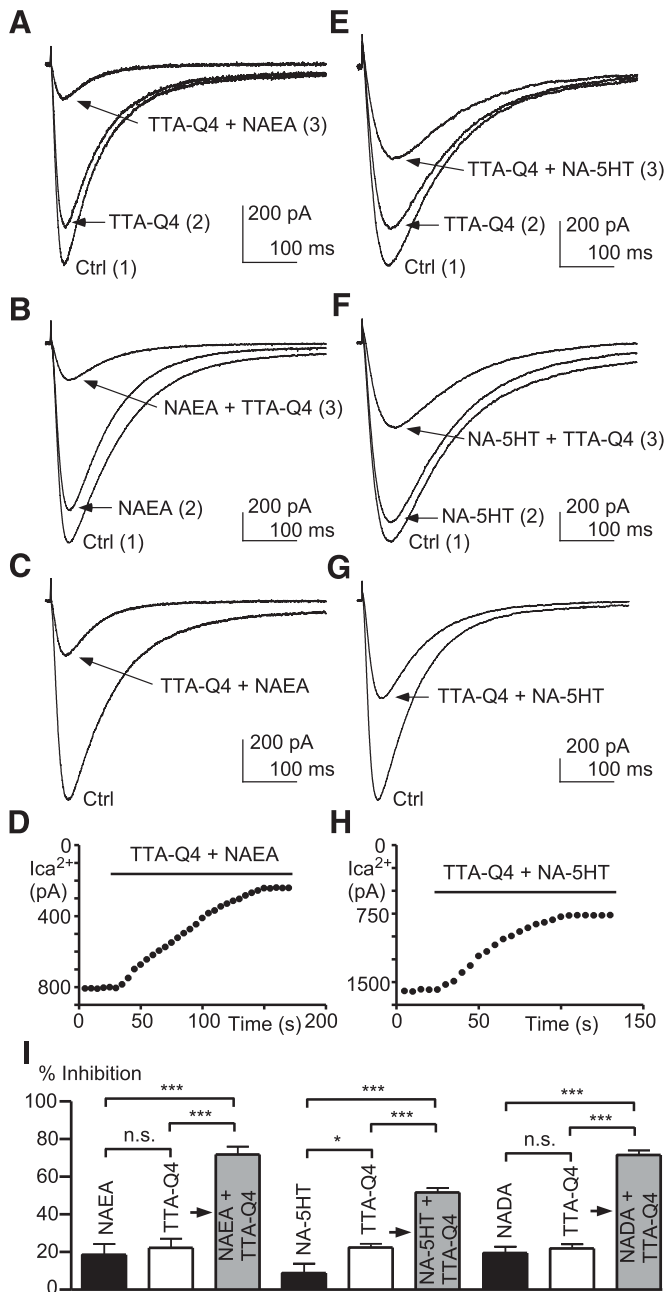
First, we found that TTA-A2 effects on Ca<sub>v</sub>3.3 current were weakly additive with those produced by lipids or were even decreased when both lipids and TTA-A2 were applied together. Second, using [<sup>3</sup>H]TTA-A1, a radioactive derivative of TTA-A2 that specifically binds membranes expressing Ca<sub>v</sub>3.3 (Uebele et al., 2009b), we found that endogenous lipids inhibiting Ca<sub>v</sub>3.3 all displaced [<sup>3</sup>H]TTA-A1 binding, with *K<sub>i</sub>* in the micromolar range. Third, using TTA-Q4, which increased [<sup>3</sup>H]TTA-A1 binding on Ca<sub>v</sub>3.3 expressing membranes as well as TTA-A2-induced Ca<sub>v</sub>3.3 current inhibition (Uebele et al., 2009b), we demonstrated a synergistic mechanism between this molecule and lipids for Ca<sub>v</sub>3.3 current inhibition.

Overall, our results indicate a common molecular mechanism between the synthetic inhibitor TTA-A2 and the endogenous lipids, and suggest that lipids inhibiting the T-current could act directly on the Ca<sub>v</sub>3.3 protein at a site overlapping that of TTA-A2. However, we cannot exclude that lipids could displace [<sup>3</sup>H]TTA-A1 binding by acting at the membrane rather than on the Ca<sub>v</sub>3.3 protein. Importantly, no [<sup>3</sup>H]TTA-A1 binding was observed in membranes from HEK-293 cells that did not express Ca<sub>v</sub>3.3 (Uebele et al., 2009b).

We have shown that *N*-arachidonoyl derivatives containing a glycine, a serine, an alanine, a γ-butyric acid, a 3-OH-γ-butyric acid, and a taurine inhibited the Ca<sub>v</sub>3.3 current. Inhibition did not occur with the saturated *N*-arachidonoyl glycine (20:0 Gly) and increased with the number of double bonds, leading to maximal effect on Ca<sub>v</sub>3.3 current with the fully polyunsaturated ω3 22:6 Gly. Similar findings were obtained for Ca<sub>v</sub>3.1 and Ca<sub>v</sub>3.2 currents (not shown), as previously observed with fatty acids and *N*-acyl ethanolamines (Chemin et al., 2007).

In contrast with previous studies (Ross et al., 2009; Gilmore et al., 2012), we found that NA-5HT and NADA were weak inhibitors of the Ca<sub>v</sub>3.3 currents (as well as Ca<sub>v</sub>3.1 and Ca<sub>v</sub>3.2 currents). Similar results were obtained using a stable HEK-293 cell line expressing Ca<sub>v</sub>3.3 current or using an intracellular medium containing 10 mM BAPTA (Ross et al., 2009; Gilmore et al., 2012). We do not have yet any satisfactory explanation for this discrepancy because the Ca<sub>v</sub>3 subunits and the lipids used here are identical and from the same companies as those previously used (Ross et al., 2009; Gilmore et al., 2012).

We also demonstrated that the inhibition occurred only at depolarized resting potentials, indicating that lipids preferentially affect T-channels in the inactivated state or in intermediate



**Fig. 5.** Inhibition of Ca<sub>v</sub>3.3 currents by combined application of endogenous lipids and TTA-Q4. (A) Effect of a 20 nM TTA-Q4 solution followed by the application of both 100 nM NAEA and 20 nM TTA-Q4. (B) Effect of a 100 nM NAEA solution followed by the application of both 100 nM NAEA and 20 nM TTA-Q4. (C) Effect of a solution containing both 100 nM NAEA and 20 nM TTA-Q4. (D) Time course of the decrease in Ca<sub>v</sub>3.3 current amplitude during application of 100 nM NAEA and 20 nM TTA-Q4. Same cell as in C. (E–H) Similar experiments with 20 nM TTA-Q4 and 300 nM NA-5HT. (I) Summary of the effects of NAEA, NADA, and NA-5HT alone or combined with 20 nM TTA-Q4. The arrows indicate the anticipated sum of the effects of lipids plus TTA-Q4. Currents were elicited at –30 mV from a HP of –75 mV at a frequency of 0.2 Hz. \**P* < 0.05; \*\*\**P* < 0.001. Ctrl, control; n.s., not statistically significant.

closed states (Talavera et al., 2004). Furthermore, NAGly induced a hyperpolarized shift in the  $Ca_v3.3$  steady-state inactivation properties, leading to current inhibition at physiologic resting potentials. In addition, we found that NAGly slowed the recovery from inactivation of the  $Ca_v3.3$  current, a property that was not investigated before on recombinant channels. These effects on inactivation properties were reminiscent of those induced by TTA-A2, which induced similar effects on steady-state inactivation and recovery from inactivation (Kraus et al., 2010; Francois et al., 2013).

NAGly also induced a hyperpolarized shift in the steady-state inactivation of  $Ca_v3.1$  and  $Ca_v3.2$  currents and slowed their recovery from inactivation confirming a common biophysical mechanism (data not shown). However, NAGly had specific effects on the  $Ca_v3.3$  current kinetics. NAGly accelerated the inactivation kinetics of  $Ca_v3.3$  current at every tested potential without the corresponding effect on  $Ca_v3.1$  and  $Ca_v3.2$  currents (data not shown). In the same way, NAGly induced a deceleration of the  $Ca_v3.3$  deactivation kinetic, which was not observed on  $Ca_v3.1$  or  $Ca_v3.2$  currents (data not shown) and is not yet documented for TTA-A2.

We found that TTA-A2-induced inhibition of  $Ca_v3.3$  current was not fully additive with those produced by anandamide when both compounds were applied together. Moreover, the presence of NADA or NA-5HT decreased the TTA-A2 effect, probably because they did not produce an important inhibition per se, which suggests that they could share with TTA-A2 a common site on  $Ca_v3.3$ . This was confirmed using [ $^3H$ ]TTA-A1, a radioactive derivative of TTA-A2 that specifically binds membranes expressing  $Ca_v3.3$  (Uebele et al., 2009b). Indeed, we demonstrated that polyunsaturated lipids NAGly, NASer, NAEA, NADA, NA-5HT, and NATau all displaced the [ $^3H$ ]TTA-A1 binding, with  $K_i$  in a micromolar or submicromolar range whereas saturated lipids (which did not inhibit  $Ca_v3.3$  current) had no effect. The affinity ( $K_i$  in  $\mu M$ ) obtained with polyunsaturated lipids was NA-5HT (0.02) < NADA (0.17) < NAEA (1.35) < NATau (4.13) < NAGly (7.12) < NASer (9.02). These findings are in good agreement with our electrophysiologic studies on  $Ca_v3.3$  current inhibition.

Interestingly, we also found that NADA and NA-5HT, which had a mild effect on  $Ca_v3.3$  current, strongly bound to  $Ca_v3.3$ -expressing membranes. Accordingly, the presence of NADA or NA-5HT prevented TTA-A2 inhibitory effects in electrophysiologic experiments. We had previously shown that the inhibitory effects of lipids on the T-current depend on both the amide and the hydroxyl groups (Chemin et al., 2007). In this context, the aromatic heterocyclic rings of NADA and NA-5HT, which increase the distance between the amide and the hydroxyl groups and are also hydrophobic, could impair interaction with key amino acids in the  $Ca_v3.3$  protein and therefore their inhibitory effect without decreasing their binding to membranes expressing  $Ca_v3.3$ . This suggests that the polyunsaturated alkyl chain of lipids would be mostly important for their binding whereas the amide and hydroxyl groups would mediate current inhibition. Accordingly, both NADA and NA-5HT strongly occluded the TTA-A2 effect but weakly inhibited the  $Ca_v3.3$  current whereas saturated lipids (which did not produce inhibition) provided no consistent displacement.

We also found that the Hill slope number ( $n_{Hill}$ ) in these binding assays was high (ranging from 1.5 to 2.4) for NATau,

NAGly, NASer, and NAEA, especially for NAEA ( $n_{Hill} = 2.4$ ), whereas the  $n_{Hill}$  for NA-5HT and NADA was more typical of competitive displacement curves ( $n_{Hill}$  between 1.1 and 1.3). These results might indicate lipid degradation, because in these binding experiments we did not use a fatty acid amide hydrolase [FAAH, the main enzyme metabolizing NAEA and others NArachidonoyl-conjugates (Huang et al., 2001; Saghatelian et al., 2004, 2006)] inhibitor and this enzyme is known to be very active even in crude membrane extracts (Deutsch and Chin, 1993; Childers et al., 1994; Pinto et al., 1994). For instance, displacement of the cannabinoid receptor 1 agonist [ $^3H$ ]CP-55940 (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol] by NAEA indicated a  $K_i$  of 2  $\mu M$  in the absence of the FAAH inhibitor phenylmethylsulfonyl fluoride (PMSF) whereas the  $K_i$  was 12 nM in the presence of PMSF (Pinto et al., 1994), suggesting NAEA degradation. Interestingly, the same investigators showed that the slope of the displacement curve was particularly steep ( $n_{Hill} > 2$ ) in the absence of PMSF whereas in the presence of PMSF the  $n_{Hill}$  was near a value of 1 (Pinto et al., 1994). It is also interesting that NA-5HT and NADA are inhibitors of FAAH and particularly NA-5HT (Bisogno et al., 1998, 2000) and this FAAH inhibition could account for the strong NA-5HT potency in the binding assay. Moreover, it is important to note that in these membrane extracts the transmembrane resting potential was likely lost, and the  $Ca_v3.3$  channels might be in a complete inactivated state that was not achieved in electrophysiologic experiments, which could also explain the potency of NA-5HT and NADA in these binding assays.

Because the structurally distinct antagonist TTA-Q4 was shown to be a positive allosteric modulator of TTA-A2, increasing [ $^3H$ ]TTA-A1 binding on  $Ca_v3.3$ -expressing membranes as well as TTA-A2 induced  $Ca_v3.3$  current inhibition (Uebele et al., 2009b), we have investigated whether it could also promote lipid-induced  $Ca_v3.3$  current inhibition. We found that TTA-Q4 potentiated the NAEA effect on  $Ca_v3.3$  current, indicating that TTA-Q4 and anandamide could inhibit  $Ca_v3.3$  current in a synergistic manner. Moreover, in the presence of TTA-Q4 we revealed important NADA and NA-5HT inhibitory effects, as previously described elsewhere (Ross et al., 2009; Gilmore et al., 2012). Interestingly, the binding and the potency of anandamide at cannabinoid receptor 1 and transient receptor potential vanilloid-1 receptors is increased by palmitoyl ethanolamine and oleoyl ethanolamine, which act as an allosteric modulator of these receptors in a phenomenon called the "entourage" effect (Ben-Shabat et al., 1998; De Petrocellis et al., 2001; Ho et al., 2008).

It has been demonstrated that TTA-A2 has important pharmacologic effects, including the reduction of absence epilepsy seizures (Uebele et al., 2009b; Reger et al., 2011) and of pain perception (Francois et al., 2013). In addition, TTA-A2 affects sleep/wake patterns (Uebele et al., 2009a; Kraus et al., 2010; Reger et al., 2011) and displays antipsychotic properties (Uslaner et al., 2012). Similarly, bioactive lipids inhibiting T-currents have been implicated in several functions, including pain perception (Bradshaw and Walker, 2005; Burstein, 2008; Basbaum et al., 2009), sleep, and epilepsy (Chen and Bazan, 2005). The analogy between in vivo effects of TTA-A2 and lipids suggests, in the light of our results, that many physiologic effects of endogenous lipids are supported by T-current inhibition. Furthermore, TTA-A2 and TTA-Q4 could be important



pharmacologic tools to dissect the involvement of T-current in the physiologic effects of endogenous lipids.

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#### Authorship Contributions

*Participated in research design:* Uebele, Lory, Chemin.

*Conducted experiments:* Cazade, Nuss, Bidaud, Chemin.

*Contributed new reagents or analytic tools:* Renger, Uebele.

*Performed data analysis:* Uebele, Chemin.

*Wrote or contributed to the writing of the manuscript:* Uebele, Lory, Chemin.

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