Cross modulation and molecular interaction at the Ca,3.3 protein between the endogenous lipids and the T-type calcium channel antagonist TTA-A2.


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Lipids and TTA-A2 interaction at the Ca_{3.3} protein

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T-channel: T-type calcium channel; NAGly: N-arachidonoyl glycine; NAGABA-OH: N-arachidonoyl-3-OH-gaba-butyric acid; NAGABA: N-arachidonoyl-gaba-butyric acid; NASer: N-arachidonoyl-L-serine; NAAAla: N-arachidonoyl alanine; NATau: N-arachidonoyl taurine; NA-5HT: N-arachidonoyl serotonin; NADA: N-arachidonoyl dopamine; NAEA: N-arachidonoyl ethanolamine (anandamide); 22:6 Gly: N-docosahexaenoyl glycine; 20:0 Gly: N-arachidoyl glycine; 18:2 Gly: N-linoleoyl glycine; 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)benzonitrile.
Abstract.

T-type calcium channels (T/Ca\(_{3}\) channels) are implicated in various physiological and patho-physiological processes such as epilepsy, sleep disorders, hypertension and cancer. T-channels are the target of endogenous signaling lipids including the endocannabinoid anandamide, the \(\omega\)3-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 electrophysiological and pharmacological analysis indicating that the effects of the major \(N\)-acyl derivatives on the Ca\(_{3.3}\) current share many similarities with those of TTA-A2, a synthetic T-channel inhibitor. Using radioactive binding assays with the TTA-A2 derivative \(^3\text{H}\)-TTA-A1, we demonstrated that poly-unsaturated lipids which inhibit the Ca\(_{3.3}\) current, as \(N\)AGly, \(N\)ASer, anandamide, NADA, NATau and NA-5HT, all displaced \(^3\text{H}\)-TTA-A1 binding to membranes prepared from cells expressing Ca\(_{3.3}\), with \(K_i\) in a micromolar or sub-micromolar range. In contrast, lipids with a saturated alkyl chain, as \(N\)-arachidoyl glycine and \(N\)-arachidoyl ethanolamine, which did not inhibit the Ca\(_{3.3}\) current, had no effect on \(^3\text{H}\)-TTA-A1 binding. Accordingly, bio-active lipids occluded TTA-A2 effect on Ca\(_{3.3}\) current. In addition, TTA-Q4, a positive allosteric modulator of \(^3\text{H}\)-TTA-A1 binding and TTA-A2 functional inhibition, acted in a synergistic manner to increase lipid-induced inhibition of the Ca\(_{3.3}\) 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 pharmacological tools to dissect the involvement of T-current in the physiological effects of endogenous lipids.
Introduction.

Low-voltage-activated (T-type / Ca_{v3}) 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 physiological processes as diverse as neuronal firing (Cain and Snutch, 2010; Perez-Reyes, 2003), slow wave sleep (Lee and Shin, 2007), hormone secretion (Weiss and Zamponi, 2012), cell cycle (Lory et al., 2006), heart rhythm (Ono and Iijima, 2010) and vasodilatation (Kuo et al., 2011). They emerge as important pharmacological 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 (Giordanetto et al., 2011; Lory and Chemin, 2007; McGivern, 2006), including TTA-A2, a potent and specific inhibitor of T-current (Kraus et al., 2010; Reger et al., 2011; Uebele et al., 2009a; Uebele et al., 2009b). In-vivo studies demonstrated that TTA-A2 reduces absence epilepsy seizures (Reger et al., 2011; Uebele et al., 2009b), pain perception (Francois et al., 2013), nicotine self administration (Uslaner et al., 2010), weight gain (Uebele et al., 2009a) whereas it ameliorates the sleep quality (Kraus et al., 2010; Reger et al., 2011; Uebele et al., 2009a) and displays anti-psychotic properties (Uslaner et al., 2012).

T-channels are also inhibited by several endogenous signaling lipids. These molecules include arachidonic acid, ω3-fatty acids, endocannabinoids (as anandamide), lipoamino-acids and lipo-neurotransmitters (Barbara et al., 2009; Chemin et al., 2001; Chemin et al., 2007; Danthi et al., 2005; Gilmore et al., 2012; Ross et al., 2009; Talavera et al., 2004; Zhang et al., 2000). These lipids are implicated in multiple physiological functions and more specifically in pain perception (Basbaum et al., 2009; Bradshaw and Walker, 2005; Burstein, 2008), sleep and epilepsy (Chen and Bazan, 2005), heart rhythm and vasodilatation (Leaf et al., 2003; Roman, 2002).
Lipid-mediated inhibition of T-current occurred in excised cell-free membrane patches consistent with direct effect of lipids on T-channels or via perturbation of their near membrane environment (Barbara et al., 2009; Chemin et al., 2001; Chemin et al., 2007; Talavera et al., 2004). In this context, it is interesting to note that a radiolabeled derivative of TTA-A2, $[^3]$H-TTA-A1, was shown to bind membranes from cells expressing Ca$_{v}$3.3 with a Kd of 1.8 nM (Uebele et al., 2009b). Moreover a structurally distinct antagonist, TTA-Q4, increased both $[^3]$H-TTA-A1 binding and TTA-A2-induced inhibition of the Ca$_{v}$3.3 current, by acting on a distinct molecular site of the Ca$_{v}$3.3 protein (Uebele et al., 2009b). In this study, we used these new pharmacological tools to explore whether bio-active lipids and TTA-A2 share similar mechanisms and molecular determinants at the Ca$_{v}$3.3 protein.
Materials and Methods.

Cell culture and transfection protocols.

tsA-201 cells and a HEK-293 cell line stably expressing Ca\textsubscript{3.3} (a generous gift from Dr. Perez-Reyes (Xie et al., 2007)) were cultivated in DMEM supplemented with GlutaMax and 10\% fetal bovine serum (Invitrogen). tsA-201 cell transfection was performed using jet-PEI (QBiogen) with a DNA mix containing 0.5\% of a GFP plasmid and 99.5\% of either of the plasmid constructs that code for human Ca\textsubscript{3.1a}, Ca\textsubscript{3.2}, and Ca\textsubscript{3.3}. Two days after transfection, cells were dissociated with Versene (Invitrogen) and plated at a density of ~35 x 10\textsuperscript{3} cells per 35 mm Petri dish for electrophysiological recordings.

Electrophysiological recordings.

Macroscopic currents were recorded in the whole cell configuration using an Axopatch 200B amplifier (Molecular Devices). The extracellular solution contained the following (in mM): 135 NaCl, 20 TEACl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, and 10 HEPES (pH adjusted to 7.25 with KOH, ~330 mOsm). Borosilicate glass pipettes have a typical resistance of 1.5–2.5 MΩ 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\textsubscript{2} (pH adjusted to 7.25 with KOH, ~315 mOsm). In a subset of experiments, EGTA was substituted with 10 mM BAPTA. 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. Drugs were applied by a gravity-driven homemade perfusion device and control experiments were performed using the solvent alone. Results are presented as the mean ± SEM, and \( n \) is the number of cells used. Student’\( t \) test were used to compare the different values, which were considered significant at \( p<0.05 \).

Binding Experiments.
Membranes were prepared from the HEK 293 cells stably expressing Ca_{3.3} (Uebele et al., 2009b). Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Binding assays were performed at room temperature for 3 h with 8 µg protein, 1 nM [^{3}H]-TTA-A1 and increasing concentrations of lipids (ranging from 1 nM to 40 µM) in 1 ml final volume in Packard Unifilter-96, GF/C plates (Packard, Meriden, CT, USA) coated with 0.3% ethylene imine polymer solution (Sigma-Aldrich). 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 Perkin-Elmer 96-well Filtermate Harvester. The plates were dried before the addition of Microscint-20 (Perkin-Elmer Life and Analytic Sciences, Shelton, CT, USA) and the remaining radioactivity was quantified on a Perkin-Elmer NXT HTS Top Count. Total and non-specific binding were determined in the absence and the presence of 100 nM of TTA-A2, respectively. Data were collected in triplicate. The inhibition constants $K_i$ were calculated with GraphPad Prism using the following equation: $K_i = IC_{50} / (1+([^{3}H]-TTA-A1)/Kd))$ where $IC_{50}$ was the half maximal inhibitory concentration, $[^{3}H]-TTA-A1$ was 1 nM and Kd was 1.8 nM, as previously described (Uebele et al., 2009b).

Chemical reagents.

$[^{3}H]$-TTA-A1 (56–65 mCi/mmol), TTA-A2 and TTA-Q4 were synthesized at Merck (West Point, PA), as previously described (Uebele et al., 2009b), dissolved in DMSO at 10 mM, aliquoted and kept at -20°C. Lipids were obtained from Cayman Chemical and were dissolved in ethanol purged with argon at a concentration of 10 mM. NA-5HT and NADA were also obtained from Tocris Bioscience and Enzo Life Sciences, 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,3.3 currents by poly-unsaturated amino-acids.

We have characterized the effects of lipoamino-acids on the Ca,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 TRPV1 (Barbara et al., 2009; Bradshaw and Walker, 2005; Huang et al., 2001). We found that the Ca,3.3 current was strongly inhibited by 3 µM N-docosahexaenoyl glycine (chain length 22 carbons, 6 doubles bonds, 22:6 Gly) 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 bovine serum albumin (Barbara et al., 2009; Gilmore et al., 2012)(BSA, Fig. 1C). The extent of inhibition increased gradually with the number of double bonds in the N-acyl glycine (Fig. 1E). The saturated N-arachidoyl glycine (20:0 Gly) produced weak inhibition (~13 %, n=5, Fig. 1D-E), N-linoleoyl glycine (18:2 Gly) produced ~50 % inhibition (n=7, Fig. 1E, p≤0.001 compared to 20:0 Gly), N-arachidonoyl glycine (20:4, NAGly) produced ~67 % inhibition (n=11, Fig. 1E, p≤0.05 compared to 18:2 Gly) and the fully poly-unsaturated 22:6 glycine produced ~74 % inhibition (n=12, Fig. 1E, non-significant compared to 20:4 Gly). Interestingly, the increase in the number of double bonds in fatty acids augments the membrane fluidity whereas it restricts the conformational freedom of the molecule possibly leading to the adequate conformation to inhibit T-current. The Ca,3.3 current was also inhibited by several N-arachidonoyl amino-acids including N-arachidonoyl-3-OH-γ-butyric acid (NAGABA-OH, ~81 % inhibition (n=22)), N-arachidonoyl-γ-butyric acid (NAGABA, ~59 % inhibition (n=24)), N-arachidonoyl-L-serine (NASer, ~64 % inhibition (n=22)) and N-arachidonoyl alanine (NAAAla, ~45 % inhibition (n=23, Fig. 1E)). In addition, the lipo-neurotransmitters N-arachidonoyl taurine (NATau), N-arachidonoyl serotonin (NA-5HT) and N-arachidonoyl dopamine (NADA) inhibited the Ca,3.3 current by ~74 %, ~34 % and ~20 %, respectively (Fig. 1E,
n≥8). Similar findings were obtained with NADA and NA-5HT using an intracellular medium containing 10 mM BAPTA (n=5) (Gilmore et al., 2012; Ross et al., 2009) as well as on Ca\(_{v}\)3.1 and Ca\(_{v}\)3.2 current (data not shown). Application of 3 µM NADA induced ~18 % inhibition of the Ca\(_{v}\)3.1 current (n=5) and ~27 % inhibition of the Ca\(_{v}\)3.2 current (n=6). Similarly, application of 3 µM NA-5HT induced ~44 % inhibition of the Ca\(_{v}\)3.1 current (n=16) and ~50 % inhibition of the Ca\(_{v}\)3.2 current (n=14). As previously described (Chemin et al., 2001), in these experiments, 3 µM NAEA strongly inhibited the three Ca\(_{v}\)3 currents by ~74-80 % (n≥8, Fig. 1E).

**NAGly effect on Ca\(_{v}\)3.3 biophysical properties.**

NAGly inhibited the Ca\(_{v}\)3.3 current at every potential with inhibition of the peak current at -35 mV by ~40 % with 1 µM NAGly and by ~70 % with 3 µM NAGly (Fig. 2A). NAGly had weak and not significant effect on the Ca\(_{v}\)3.3 current-voltage relationship since the \(V_{0.5}\) values were -45.7 ± 1.3 mV (n=8) in control condition, -49.6 ± 1.4 mV (n=8) during 1 µM NAGly application and -48.6 ± 0.8 mV (n=5) during 3 µM NAGly application (\(p>0.05\)). We also observed that 3 µM NAGly accelerated the inactivation rate of Ca\(_{v}\)3.3 current at every tested potential (\(p<0.05\), n=5, Fig. 2B) without significant corresponding effect on the activation rate (Fig. 2C). Furthermore, NAGly induced a ~10 mV negative shift in the steady-state inactivation properties of Ca\(_{v}\)3.3 (Fig. 2D). The \(V_{0.5}\) values were -73.1 ± 0.9 mV (n=8) in control condition, -80.6 ± 1.07 mV (\(p<0.001\), n=8) during 1 µM NAGly application and -83.2 ± 0.8 mV (\(p<0.001\), n=7) during 3 µM NAGly application. Application of 3 µM NAGly also decreased the slope factor of the steady-state inactivation curve of Ca\(_{v}\)3.3 from 5.2 ± 0.2 in control condition to 4.3 ± 0.2 (\(p<0.05\)). We next investigated the effect of NAGly on the recovery from inactivation of Ca\(_{v}\)3.3 current (Fig. 2E). The recovery from inactivation of Ca\(_{v}\)3.3 current was well fit by a mono-exponential revealing that NAGly strongly increased
the \( \tau \) of recovery from \( 298 \pm 20 \) ms (\( n=8 \)) in control condition to \( 453 \pm 22 \) ms (\( p<0.001, n=8 \)) during \( 1 \) \( \mu \)M NAGly application and to \( 771 \pm 43 \) ms (\( p<0.001, n=8 \)) during \( 3 \) \( \mu \)M NAGly application. Finally, we found that both \( 1 \) \( \mu \)M and \( 3 \) \( \mu \)M NAGly slowed the deactivation of the \( \text{Ca}_{3.3} \) current at repolarization potentials ranging from -120 to -60 mV (\( p<0.05, n=8, \text{Fig. 2F} \)).

**Pharmacological interaction of endogenous lipids and TTA-A2 on the \( \text{Ca}_{3.3} \) channel.**

Recently, TTA-A2, a potent and specific synthetic inhibitor of T-current was described (Kraus et al., 2010; Reger et al., 2011; Uebele et al., 2009a; Uebele et al., 2009b). As observed with endogenous lipids, TTA-A2 inhibited the \( \text{Ca}_{3} \) current at physiological HP but not at very negative potentials (i.e. -110 mV) (Francois et al., 2013; Kraus et al., 2010). Furthermore, as observed with endogenous lipids, TTA-A2 induced a negative shift in the steady-state inactivation properties of \( \text{Ca}_{3} \) current and slowed their recovery from inactivation (Francois et al., 2013; Kraus et al., 2010). It should be noted that several other structurally-unrelated T-channel inhibitors, including mibefradil, flunarizine and pimozide, which also exhibit similar state-dependent inhibition of T-currents (Martin et al., 2000; Santi et al., 2002), were shown to interact with \( [\text{H}] \)-TTA-A1 binding to membranes containing \( \text{Ca}_{3.3} \) (Uebele et al., 2009b). Therefore we investigated whether endogenous lipids and TTA-A2 could share a common inhibitory mechanism on \( \text{Ca}_{3.3} \). To this purpose, the effect of NAEA (which strongly inhibited \( \text{Ca}_{3.3} \) current) and NADA or NA-5HT (which mildly inhibited \( \text{Ca}_{3.3} \) 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 \( \text{Ca}_{3.3} \) current and accelerated the inactivation kinetic (\( \tau \)) by \( 32.5 \pm 2.6 \% \) (\( p<0.05, n=8, \text{Fig. 3A} \)), as previously described (Chemin et al., 2001). Similarly, 3 nM TTA-A2 alone induced \( 52 \pm 3 \% \) inhibition of the \( \text{Ca}_{3.3} \) current but slowed the inactivation kinetic (\( \tau \)) by \( 34.1 \pm 5.4 \% \) (\( p<0.05, n=7; \)}
Interestingly, when NAEA and TTA-A2 were applied simultaneously, the Ca\(_{v}\)3.3 current was inhibited by 70 ± 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 ± 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\(_{v}\)3.3 current by 20 ± 4 % (n=16; Fig. 3D), was applied with TTA-A2, the resulting inhibition was only 33 ± 4 % (n=19; Fig. 3F) demonstrating no additive effects. This was clearly evidenced when NA-5HT and TTA-A2 where 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. 3E and Fig. 3G). Similar findings were obtained with 3 µM NADA (n=15, Fig. 3G). Overall, these results demonstrated a pharmacological interaction between endogenous lipids and TTA-A2 and suggested that both molecules could share the same molecular site on the Ca\(_{v}\)3.3 protein.

**N-acyl derivatives that inhibited Ca\(_{v}\)3.3 current displaced \[^{3}\text{H}]-\text{TTA-A1} binding.**

Because lipids and TTA-A2 possibly act at the same molecular site, we investigated whether bio-active lipids that inhibit Ca\(_{v}\)3.3 current could displace \[^{3}\text{H}]-\text{TTA-A1} (a radiolabeled derivative of TTA-A2) binding to membranes containing Ca\(_{v}\)3.3, 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\(_{v}\)3.3 current (Fig. 1D), displaced \[^{3}\text{H}]-\text{TTA-A1} binding in a concentration–dependent manner (Fig. 4A). The \(K_i\) for NASer was 9.02 ± 0.06 µM and 7.12 ± 0.31 µM for NAGly whereas the Hill slope number (\(n_{Hill}\)) was 2.1 and 1.8 for NASer and NAGly, respectively. Displacement of \[^{3}\text{H}]-
TTA-A1 binding was not observed with the saturated arachidoyl glycine at concentrations up to 40 µM (20:0, Fig. 4A). Similarly, the endocannabinoid anandamide (20:4 EA) displaced $[^3H]$-TTA-A1 binding with a $K_i$ of $1.35 \pm 0.04$ µM and a $n_H$ill of 2.4, but not the saturated form, N-arachidoyl ethanolamine (Fig. 4B). In addition, the N-arachidonoyl neurotransmitters NATau, N-arachidonoyl dopamine (NADA) and N-arachidonoyl serotonin (NA-5HT), also displaced $[^3H]$-TTA-A1 binding (Fig. 4C). The $K_i$ was $4.13 \pm 0.11$ µM for NATau, $0.170 \pm 0.004$ µM for NADA and $0.026 \pm 0.001$ µM for NA-5HT whereas the $n_H$ill was 1.5, 1.3 and 1.1 for NATau, NADA and NA-5HT, respectively.

**TTA-Q4, a positive allosteric modulator of $[^3H]$-TTA-A1 binding, increased lipid-induced inhibition of Ca,3.3 current.**

The structurally distinct T-type antagonist, TTA-Q4, was shown to increase $[^3H]$-TTA-A1 binding on Ca,3.3 expressing membranes as well as TTA-A2-induced Ca,3.3 current inhibition (Uebele et al., 2009b). It was demonstrated that TTA-Q4 increased TTA-A2-mediated Ca,3.3 current inhibition by a synergistic mechanism (Uebele et al., 2009b). Therefore, we investigated whether TTA-Q4 had a similar effect on lipid-induced Ca,3.3 current inhibition. We found that 20 nM TTA-Q4 alone induced 22 ± 4 % inhibition of Ca,3.3 current (n=8, Fig. 5A), 100 nM anandamide alone induced 18 ± 5 % inhibition (n=5, Fig. 5B) whereas application of both compounds induced 72 ± 4 % inhibition (n=7, Fig. 5C-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 negligible effect on Ca,3.3 current (8 ± 5 % inhibition, n=5, Fig. 5E) whereas when NA-5HT was applied with TTA-Q4, the inhibition of Ca,3.3 current was 52 ± 2 % (n=5, Fig 5G-I). Similar results were obtained using 3 µM NADA and 20 nM TTA-Q4 since application of both compounds
induced 72 ± 2 % inhibition of Ca_{3.3} current (n=11, Fig. 5I). Overall, these results indicated that TTA-Q4 and poly-unsaturated lipids modulated Ca_{3.3} current in a synergistic manner.
Discussion

In this study we demonstrated that lipid effects on Ca\textsubscript{v}3.3 biophysical properties share many features with those induced by TTA-A2, especially regarding the “gating properties”. Importantly, inhibition of Ca\textsubscript{v}3.3 current by both lipids and TTA-A2 occurs only at depolarized resting potentials at which Ca\textsubscript{v}3.3 channels are partly inactivated. We also documented that endogenous lipids and TTA-A2 share similar molecular mechanisms. Firstly, we found that TTA-A2 effects on Ca\textsubscript{v}3.3 current were weakly additive with those produced by lipids or were even decreased when both lipids and TTA-A2 were applied together. Secondly, using \[^{3}H\]-TTA-A1, a radioactive derivative of TTA-A2, which specifically binds membranes expressing Ca\textsubscript{v}3.3 (Uebele et al., 2009b), we found that endogenous lipids inhibiting Ca\textsubscript{v}3.3, all displaced \[^{3}H\]-TTA-A1 binding with $K_i$ in the micromolar range. Thirdly, using TTA-Q4, which increased \[^{3}H\]-TTA-A1 binding on Ca\textsubscript{v}3.3 expressing membranes as well as TTA-A2-induced Ca\textsubscript{v}3.3 current inhibition (Uebele et al., 2009b), we demonstrated a synergistic mechanism between this molecule and lipids for Ca\textsubscript{v}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\textsubscript{v}3.3 protein at a site overlapping that of TTA-A2. However, we cannot exclude that lipids could displace \[^{3}H\]-TTA-A1 binding by acting at the membrane rather than on the Ca\textsubscript{v}3.3 protein. Importantly, no \[^{3}H\]-TTA-A1 binding was observed in membrane from HEK-293 cells that did not express Ca\textsubscript{v}3.3 (Uebele et al., 2009b).

We have shown that $N$-arachidonoyl derivatives containing a glycine, a serine, an alanine, a $\gamma$-butyric acid, a 3-OH-$\gamma$-butyric acid and a taurine inhibited the Ca\textsubscript{v}3.3 current. Inhibition did not occur with the saturated $N$-arachidoyl glycine (20:0) and increased with the number of double bonds leading to maximal effect on Ca\textsubscript{v}3.3 current with the fully polyunsaturated $\omega3$ $N$-docosahexaenoyl glycine (22:6 gly). Similar findings were obtained for
Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 currents (not shown), as previously observed with fatty acids and N-acyl ethanolamines (Chemin et al., 2007). In contrast with previous studies (Gilmore et al., 2012; Ross et al., 2009), we found that NA-5HT and NADA were weak inhibitors of the Ca\textsubscript{v}3.3 currents (as well as Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 currents). Similar results were obtained using a stable HEK-293 cell line expressing Ca\textsubscript{v}3.3 current or using an intracellular medium containing 10 mM BAPTA (Gilmore et al., 2012; Ross et al., 2009). We do not have yet any satisfactory explanation for this discrepancy because the Ca\textsubscript{v}3 subunits and the lipids used here are identical and from the same companies as those previously used (Gilmore et al., 2012; Ross et al., 2009). 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 closed states (Talavera et al., 2004). Furthermore, NAGly induced a hyperpolarized shift in the Ca\textsubscript{v}3.3 steady-state inactivation properties leading to current inhibition at physiological resting potentials. In addition, we found that NAGly slowed the recovery from inactivation of Ca\textsubscript{v}3.3 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 (Francois et al., 2013; Kraus et al., 2010). NAGly also induced a hyperpolarized shift in the steady-state inactivation of Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 currents and slowed their recovery from inactivation confirming a common biophysical mechanism (data not shown). However, NAGly had specific effects on the Ca\textsubscript{v}3.3 current kinetics. NAGly accelerated the inactivation kinetics of Ca\textsubscript{v}3.3 current at every tested potential without the corresponding effect on Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 currents (data not shown). In the same way, NAGly induced a deceleration of the Ca\textsubscript{v}3.3 deactivation kinetic, which was not observed on Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 currents (data not shown) and is not yet documented for TTA-A2.
We found that TTA-A2 induced inhibition of Ca\textsubscript{v}3.3 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 \textit{per-se}, suggesting that they could share with TTA-A2 a common site on Ca\textsubscript{v}3.3. This was confirmed using [\textsuperscript{3}H]-TTA-A1, a radioactive derivative of TTA-A2, which specifically binds membranes expressing Ca\textsubscript{v}3.3 (Uebele et al., 2009b). Indeed, we demonstrated that poly-unsaturated lipids NAGly, NASer, NAEA, NADA, NA-5HT and NATau, all displaced the [\textsuperscript{3}H]-TTA-A1 binding with K\textsubscript{i} in a micromolar or sub-micromolar range whereas saturated lipids (which did not inhibit Ca\textsubscript{v}3.3 current) had no effect. The affinity (K\textsubscript{i} in µM) obtained with poly-unsaturated lipids were 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 electrophysiological studies on Ca\textsubscript{v}3.3 current inhibition. Interestingly, we also found that NADA and NA-5HT, which had mild effect on Ca\textsubscript{v}3.3 current, strongly bound to Ca\textsubscript{v}3.3 expressing membranes. Accordingly, the presence of NADA or NA-5HT prevented TTA-A2 inhibitory effects in electrophysiological experiments.

We have previously shown that inhibitory effects of lipids on 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\textsubscript{v}3.3 protein and therefore their inhibitory effect, without decreasing their binding to membranes expressing Ca\textsubscript{v}3.3. This suggests that the poly-unsaturated 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 TTA-A2 effect but weakly inhibited Ca\textsubscript{v}3.3 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 were high (ranged 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 were 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., 2006; Saghatelian et al., 2004)) inhibitor and this enzyme is known to be very active even in crude membrane extracts (Childers et al., 1994; Deutsch and Chin, 1993; Pinto et al., 1994). For instance, displacement of the cannabinoid receptor CB1 agonist [3H]-CP-55940 by NAEA, indicated K_i of 2 µM in the absence of the FAAH inhibitor PMSF whereas K_i was 12 nM in the presence of PMSF (Pinto et al., 1994), suggesting NAEA degradation. Interestingly, the same authors 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). Interestingly, NA-5HT and NADA are inhibitors of FAAH and particularly NA-5HT (Bisogno et al., 2000; Bisogno et al., 1998) and this FAAH inhibition could account for the strong NA-5HT potency in the binding assay. In addition, it is important to note that in these membrane extracts, the trans-membrane resting potential was likely lost, and the Ca_{v}3.3 channels might be in a complete inactivated state that was not achieved in electrophysiological experiments, and this 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_{v}3.3 expressing membranes as well as TTA-A2 induced Ca_{v}3.3 current inhibition (Uebele et al., 2009b), we have investigated whether it could also promote lipid-induced Ca_{v}3.3 current inhibition. We found that TTA-Q4 potentiated NAEA effect on Ca_{v}3.3 current, indicating that TTA-Q4 and anandamide could inhibit Ca_{v}3.3 current in a synergistic manner. Moreover, in the presence of
TTA-Q4 we revealed important NADA and NA-5HT inhibitory effects, as previously described (Gilmore et al., 2012; Ross et al., 2009). Interestingly, the binding and the potency of anandamide at CB1 and TRPV1 receptors is increased by palmitoyl ethanolamine and oleoyl ethanolamine, which acts as an allosteric modulator of these receptors, a phenomenon called the “entourage” effect (Ben-Shabat et al., 1998; De Petrocellis et al., 2001; Ho et al., 2008).

It was demonstrated that TTA-A2 had important pharmacological effects including the reduction of absence epilepsy seizures (Reger et al., 2011; Uebele et al., 2009b) and of pain perception (Francois et al., 2013). In addition, TTA-A2 affected sleep/wake patterns (Kraus et al., 2010; Reger et al., 2011; Uebele et al., 2009a) and displayed anti-psychotic properties (Uslaner et al., 2012). Similarly, bio-active lipids inhibiting T-currents have been implicated in several functions, including pain perception (Basbaum et al., 2009; Bradshaw and Walker, 2005; Burstein, 2008), 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 physiological effects of endogenous lipids are supported by T-current inhibition. Furthermore, TTA-A2 and TTA-Q4 could be important pharmacological tools to dissect the involvement of T-current in the physiological effects of endogenous lipids.
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Authorship Contributions

Participated in research design: Uebele, Lory and Chemin

Conducted experiments: Cazade, Nuss, Bidaud and Chemin

Contributed new reagents or analytic tools: Renger and Uebele

Performed data analysis: Uebele and Chemin

Wrote or contributed to the writing of the manuscript: Uebele, Lory and Chemin
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Footnotes

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Figure Legends.

Figure 1.
Inhibition of Ca\(_{\text{v}}\)3.3 currents by endogenous lipids. (A-B) Inhibition of Ca\(_{\text{v}}\)3.3 currents by 3 µM \(N\)-docosahexaenoyl glycine (22:6 Gly) and subsequent washout with a solution containing 3 mg/ml BSA. Ca\(_{\text{v}}\)3.3 currents were elicited by a 450 ms depolarization to -30 mV from a holding potential (HP) of -75 mV (A) or -110 mV (B) at a frequency of 0.2 Hz. (C) Time course of the decrease in Ca\(_{\text{v}}\)3.3 current amplitude during 22:6 Gly application at HP -75 mV and subsequent washout with the BSA solution. Same cell than in (A). (D) Inhibition of Ca\(_{\text{v}}\)3.3 currents by \(N\)-arachidoyl glycine (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\(_{\text{v}}\)3.3 current at HP -75 mV of 20:0 Gly, 18:2 Gly (\(N\)-linoleoyl glycine), 20:4 Gly (\(N\)-arachidonoyl glycine, \(NAGly\)), 22:6 Gly, \(N\)-arachidonoyl-3-OH-\(\gamma\)-butyric acid (\(NAGABA-OH\)), \(N\)-arachidonoyl-\(\gamma\)-butyric acid (\(NAGABA\)), \(N\)-arachidonoyl-L-serine (\(NASer\)), \(N\)-arachidonoyl alanine (\(NAAla\)), \(N\)-arachidonoyl ethanolamine (anandamide, \(NAEA\)), \(N\)-arachidonoyl taurine (\(NATau\)), \(N\)-arachidonoyl serotonin (\(NA-5HT\)) and \(N\)-arachidonoyl dopamine (\(NADA\)).

Figure 2.
Effects of \(NAGly\) on biophysical properties of Ca\(_{\text{v}}\)3.3 currents. (A) Current-voltage (I-V) curves of Ca\(_{\text{v}}\)3.3 current in the absence and in the presence of 1 µM and 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 (\(\tau_{\text{inac}}\), B) and activation (\(\tau_{\text{act}}\), C) kinetics of Ca\(_{\text{v}}\)3.3 currents. (D) Steady-state inactivation curves of Ca\(_{\text{v}}\)3.3 currents in the absence and in the presence of 1 µM and 3 µM \(NAGly\). Currents were recorded at -30 mV from HPs ranging from -110 to -50 mV (5 s duration). (E) Recovery from inactivation of Ca\(_{\text{v}}\)3.3 current in the absence and in the presence of 1 µM and 3 µM \(NAGly\).
Recovery from inactivation was measured using two -30 mV depolarizations lasting 450 ms, which were applied from a HP of -80 mV of increasing duration. (F) Effects of 1 and 3 µM NAGly on deactivation kinetics of Ca\textsubscript{v}3.3 currents (\(\tau\text{deac}\)). Currents were elicited by a 28 ms depolarization at -30 mV and deactivation kinetics were measured at repolarization potentials ranging from -130 to -60 mV.

**Figure 3.**

Inhibition of Ca\textsubscript{v}3.3 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 to 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 µM 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. *, \(p<0.05\); **, \(p<0.01\); ***, \(p<0.001\); n.s., non significant.

**Figure 4.**

Displacement of \(^{3}\text{H}\) TTA-A1 binding by N-acyl amino acids (A), by N-acyl ethanolamines (B) and N-acyl neurotransmitters (C). Lipids are N-arachidonoyl-L-serine (NASer), N-arachidonoyl glycine (NAGly), N-arachidoyl glycine (20:0 Gly), N-arachidonoyl ethanolamine (NAEA, 20:4), N-arachidoyl ethanolamine (20:0 EA), N-arachidonoyl dopamine (NADA, 20:4), N-arachidonoyl serotonin (NA-5HT, 20:4) and N-arachidonoyl taurine (NATau, 20:4).
Figure 5.
Inhibition of Ca,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 a 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 a 20 nM TTA-Q4. (D) Time course of the decrease in Ca,3.3 current amplitude during application of 100 nM NAEA and 20 nM TTA-Q4. Same cell than 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; n.s., non significant.
Figure 1
Figure 2
Figure 3
Figure 4

A

$[^3H]$-TTA-A1 binding (% of control)

colorbar

concentration (µM)

NASer

20:0 Gly

20:4 Gly (NAGly)

B

$[^3H]$-TTA-A1 binding (% of control)

concentration (µM)

20:4 EA (NAEA)

20:0 EA

C

$[^3H]$-TTA-A1 binding (% of control)

concentration (µM)

NADA

NATau

NA-5HT
Figure 5