Chemical Determinants Involved in Anandamide-induced Inhibition of T-type Calcium Channels*

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Anandamide, originally described as an endocannabinoid, is the main representative molecule of a new class of signaling lipids including endocannabinoids and N-acyl-related molecules, eicosanoids, and fatty acids. Bioactive lipids regulate neuronal excitability by acting on G-protein-coupled receptors (such as CB1) but also directly modulate various ion conductances including voltage-activated T-type calcium channels (T-channels). However, little is known about the properties and the specificity of this new class of molecules on their various targets. In this study, we have investigated the chemical determinants involved in anandamide-induced inhibition of the three cloned T-channels: CaV3.1, CaV3.2, and CaV3.3. We show that both the hydroxyl group and the alkyl chain of anandamide are key determinants of its effects on T-currents. As follows, T-currents are also inhibited by fatty acids. Inhibition of the three CaV3 currents by anandamide and arachidonic acid does not involve enzymatic metabolism and occurs in cell-free inside-out patches. Inhibition of T-currents by fatty acids and N-acyl ethanolamides depends on the degree of unsaturation but not on the alkyl chain length and consequently is not restricted to eicosanoids. Inhibition increases for polyunsaturated fatty acids comprising 18–22 carbons when cis-double bonds are close to the carbonyl group. Therefore the major natural (food-supplied) and mammalian endogenous fatty acids including γ-linolenic acid, mead acid, and arachidonic acid as well as the fully polyunsaturated ω3-fatty acids that are enriched in fish oil eicosapentaenoic and docosahexaenoic acids are potent inhibitors of T-currents, which possibly contribute to their physiological functions.

Voltage-dependent calcium channels comprise three families: the L-type channels (CaV1 family), the neuronal N-, P/Q-, and R-type channels (CaV2 family) (CaV2.1 and CaV2.2 subunits), and the T-type channels (CaV3 family). The electrophysiological features of T-type Ca2+ channels (T-channels) are low voltage-activated Ca2+ currents, low unitary conductance, fast inactivation and slow deactivation kinetics, and strong steady-state inactivation at physiological resting potentials (1, 2). Three T-channel subunits have been identified: CaV3.1 (or α1C), CaV3.2 (or α1H), and CaV3.3 (or α1I) (2). In mammalian expression systems, the CaV3.1 and CaV3.2 currents share typical properties of native T-channels, while CaV3.3 currents display unusually slow inactivation kinetics, which are characteristic of specific neurons (1–3). These three subunits are also subject to alternative splicing, which influences their electrophysiological properties (4–6).

In the nervous system, T-channels generate low threshold spikes and participate in spontaneous firing (1, 2). They are involved in slow wave sleep (7), in absence epilepsy (8, 9), and in the pain perception (10–12). In heart cells, T-channels are involved in cardiac pacemaker (13, 14) and cardiac hypertrophy (15). In nonexcitable cells, they participate in hormone secretion (16) and fertilization (17). They are also suspected to induce cellular differentiation of many cell types (18–21). However, the lack of specific blockers and endogenous ligands of the T-channels has precluded the elucidation of their functions.

We recently identified that anandamide, an endogenous signaling molecule that modulates neuronal excitability (22, 23), is a direct blocker of T-type calcium channels (24). Anandamide, primarily described as an endocannabinoid acting on CB1 receptors (25), also modulates various ion conductances (26, 27). Anandamide (N-arachidonoyl ethanolamide, AEA)2 belongs to a new major class of small lipid messengers, including endocannabinoids and N-acyl-related molecules, eicosanoids, and fatty acids (22, 28–32). These bio-active lipids are involved in a multitude of physiological and pathophysiological events, including neuronal excitability (22, 23, 31, 33), sleep (34), epilepsy and neuroprotection (34–37), inflammation and pain (28, 29, 32, 38, 39), as well as cardiovascular modulation (40–42), fertilization, and cell cycle progression (38, 43, 44). Despite the fact that both small lipid messengers and T-channels display similar physiological functions (but with opposite effects), little is known about the properties and the specificity of this new class of molecules on the three CaV3 channels. In the present study, we provide the first detailed analysis of the chemical determinants involved in anandamide-induced inhibition of CaV3 T-channels.

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1The abbreviations used are: AEA, N-arachidonoyl ethanolamide (anandamide); AA, arachidonic acid; EA, ethanalamine; ACEA, arachidonyl 2’-chloroethylamide; ANMA, arachidonic acid N-methylamide; EPA, eicosapentaenoic acid; ETYA, cis-5,8,11,14-eicosatetraynoic acid; FAAH, fatty acid amide hydrolase; DHA, cis-4,7,10,13,16,19-docosahexaenoic acid.

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MATERIALS AND METHODS

Cell Culture and Transfection Protocols—TsA-201 cells were cultivated in Dulbecco’s modified Eagle’s medium supplemented with glutamax and 10% fetal bovine serum (Invitrogen). Transfection was performed using jet-PEI (QBiogen) according to the manufacturer protocol (4 μl of jet-PEI for 1.5 μl of DNA) with a DNA mix containing 0.5% of a green fluorescent protein plasmid and 99.5% of either of the pcDNA3 plasmid constructs that code for human CaV3.1a, CaV3.2, and CaV3.3 T-channel isoforms. Two days after, cells were then dissociated with trypsin, and electrophysiological recordings were performed during the next 2 days.

Electrophysiological Recordings—Macroscopic currents were recorded at room temperature (−24 °C) by the whole-cell patch clamp technique using an Axopatch 200B amplifier (Axon Instruments). Extracellular solution contained (in mM): 135 NaCl, 20 tetraethylammonium chloride, 2 CaCl₂, 1 MgCl₂, and 10 HEPES (pH adjusted to 7.4 with KOH, ~330 mosM). Borosilicate glass pipettes have a typical resistance of 1.5–2.5 MΩ when filled with an internal solution containing (in mM): 145 KCl, 5 EGTA, 1 MgCl₂, and 10 HEPES (pH adjusted to 7.4 with KOH, ~310 mosS). Recordings were filtered at 2 kHz. Data were analyzed as described previously (24) using pCLAMP9 (Axon Instruments) and GraphPad Prism (GraphPad Inc.) software. One-way analysis of variance combined with a Student-Newman-Keuls post-test were used to compare the different values and were considered significant at p < 0.05. Results are presented as the mean ± S.E., and n is the number of cells used.

Chemical Reagents—Fatty acids, N-acyl ethanolamides, as well as other compounds (from Sigma and Cayman Chemical) were dissolved in ethanol at a concentration of 10–100 mM. Stock solutions were briefly sonicated, aliquoted, sealed under argon, and kept at −80 °C. These aliquots were dissolved daily in the extracellular solution, which was sonicated on ice for 5 min before use. Control experiments were carried out using the solvent alone. Drugs were applied by a gravity-driven homemade perfusion device (mostly using Teflon tubing), controlled by solenoid valves.

RESULTS

Anandamide (AEA) inhibits the three cloned T-channels expressed in tsA-201 cells, as illustrated for CaV3.3 (Fig. 1A).
Modulation of T-channels by Fatty Acids

![Diagram showing the inhibition of CaV3 currents by fatty acids](image)

The inhibition is potent (~90% for a concentration of 10 μM, Fig. 1B), occurs in the minute range (maximal after a 2–3-min perfusion), and is partially relieved by perfusion of a bovine serum albumin solution (3 mg/ml) (data not shown).

We first looked for the principal chemical motifs involved in anandamide inhibition. As schematized in Fig. 1A, we identified that both the hydroxyl group of the anandamide head (indicated in blue in Fig. 1A) as well as its alkyl chain (indicated in green in Fig. 1A) are crucial for anandamide effects. Indeed, arachidonic acid (cis-5,8,11,14-eicosatetraenoic acid, AA), which contains a carboxy group and the same alkyl chain as anandamide, mimics anandamide effects. As observed with anandamide, the inhibition of the three cloned T-channels by arachidonic acid occurs in the minute range (maximal after a 2–3-min perfusion) and is partially relieved by perfusion of a bovine serum albumin solution (data not shown). As previously described for lower anandamide concentrations (24), arachidonic acid (10 μM) preferentially inhibits CaV3.2 currents (~66%) compared with CaV3.3 (~53%) and CaV3.1 (~47%) currents (Fig. 1B, p < 0.05). However, the arachidonic acid inhibition is less potent than those induced by anandamide on the three CaV3 currents (Fig. 1B, p < 0.001) indicating that although the ethanolamide group of anandamide does not affect per se T-currents (as assessed with ethanolamine (EA) and acetyl ethanolamide (acetyl-EA) (Fig. 1A)), its presence potently increases the T-current inhibition. Furthermore, arachidonic acid methyl ester (AA-methyl ester), which lacks both the hydroxyl and the amide group of anandamide, has no significant effect on the three CaV3 currents (Fig. 1A and B). It should be noted that the hydroxyl group of AEA seems very important to confer strong T-current inhibition since arachidonoyl 2′-chloroethylamide (ACEA), which contained a chloride instead a hydroxyl group, poorly inhibits the three CaV3 currents (Fig. 1A and B). Indeed, anandamide derivatives possessing the amide linkage (indicated in red in Fig. 1A) but lacking the hydroxyl group (as arachidonamide, arachidonic acid N-methylamide (ANMA), and ACEA) inhibit the three T-currents (~30%) but weakly compared with arachidonic acid (~55%) and anandamide derivatives (~95%) that possess both the hydroxyl and the amide groups (as AEA and R1- and R2-methanandamide) (Fig. 1B, p < 0.001). It is important to note that ANMA and ACEA are potent cannabinoid receptor CB1 agonists. In addition, the carbonyl group of AEA seems less crucial since arachidonoyl alcohol is as potent as AA to inhibit the three CaV3 currents (Fig. 1A and B). It should be noted that both R1- and R2-methanandamide produce maximal inhibition of the three CaV3 currents (Fig. 1A and B).

We next investigated whether the presence of double bonds on the alkyl chain is implicated in T-current inhibition. The fully saturated arachidonic acid (20:0, see schematic representation in Fig. 2A) has no significant effect on T-currents (Fig. 2A). By contrast, the inhibition appears with cis-11,14-eicosadienoic acid (20:2), which inhibits ~36% of CaV3.2 currents, ~21% of CaV3.3 currents, and ~17% of CaV3.1 currents (Fig. 2B). Remarkably, inhibition of the three CaV3 currents increases with the degree of unsaturation (p < 0.001, r² > 0.97) and is maximal for the fully unsaturated eicosapentaenoic acid (20:5, EPA), which inhibits ~79% of CaV3.2 currents, ~64% of CaV3.3 currents, and ~52% of CaV3.1 currents (Fig. 2B). As previously observed for arachidonic acid, polyunsaturated eicosanoids preferentially inhibit CaV3.2 currents compared with CaV3.3 and CaV3.1 currents (Fig. 2B, p < 0.05 for cis-11,14-eicosadienoic acid (20:2), dihomo-γ-linolenic acid (cis-8,11,14-eicosatrienoic acid, 20:3), and EPA (20:5)). It should also be noted that CaV3.2 currents (but not CaV3.3 and CaV3.1 currents) are also inhibited by gondoic acid (20:1, cis-11-eicosenoic acid), although very modestly (~15% inhibition, p < 0.05, Fig. 2B).

We next explored the effects of the most abundant natural fatty acids (which mainly include 16–22 carbons) and analyzed the results with respect to both the chain length and the degree of unsaturation (Fig. 3). It should be noted that most natural fatty acids with less than 18 carbons are saturated or monounsaturated whereas fatty acids with at least 18 carbons can be polyunsaturated. As observed with eicosanoids (20 carbons), no inhibition of the three CaV3 currents is found with saturated fatty acids including palmitic acid (16:0, n = 5–9), stearic acid (18:0, n = 5), and behenic acid (22:0, n = 14–15). Similar results were obtained with short chain fatty acids including lauric acid (12:0, n = 4–5) and myristic acid (14:0, n = 3–4) (data not shown).
significantly inhibit the three CaV3 currents (Fig. 3, data not shown). By contrast, polyunsaturated fatty acids such as arachidonic acid (18:2, n-6), stearidonic acid (18:4, cis-9,12,15-octadecatetraenoic acid, 18:4), EPA (18:5, n-3), DPA (22:5), and DHA (22:6, n-3) strongly inhibit the three CaV3 currents (percent of inhibition: 70% for CaV3.1 currents, 68% for CaV3.2 currents, and 57% for CaV3.3 currents). The fully unsaturated 18-carbon fatty acid, 20:3, inhibits 28% to 30% of the current (CaV3.1 currents, data not shown). As observed with eicosanoids, inhibition increases with the number of cis-double bonds (p < 0.001, r^2 = 0.96, Fig. 3B). Indeed, the fully unsaturated 18-carbon fatty acid, stearidonic acid (18:4, cis-9,12,15-octadecatetraenoic acid, 18:4), strongly inhibits the three CaV3 currents (percent of inhibition: 70 ± 4%, n = 10, for CaV3.2 currents; 68 ± 3%, n = 23, for CaV3.3 currents; and 57 ± 3%, n = 14, for CaV3.1 currents) as well as DHA (22:6, cis-4,7,10,13,16,19-docosahexaenoic acid, 22:6), the fully unsaturated 22-carbon fatty acid (percent of inhibition: 75 ± 3%, n = 17, for CaV3.2 currents; 65 ± 2%, n = 18, for CaV3.3 currents; and 59 ± 5%, n = 16, for CaV3.1 currents). Similar results were obtained with a nonnatural polyunsaturated fatty acid containing an odd number of carbons (19:4, cis-6,9,12,15-eicosatetraenoic acid, n = 3) on each CaV3 currents, data not shown). As observed with eicosanoids, 18 and 22 carbon polyunsaturated fatty acids preferentially inhibit CaV3.2 currents compared with CaV3.3 and CaV3.1 currents (percent of inhibition: 75% for all fatty acids, n = 6–23). Notably, inhibition of the CaV3 currents does not depend on the chain length (Fig. 3B, inset, p > 0.05) but depends on the number of double bonds (Fig. 3B).
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Similar results were obtained with N-acyl ethanolamides (Fig. 4). Indeed, N-acyl ethanolamides with saturated alkyl chain have no significant effect on the three CaV3 currents, as assessed with palmitoyl ethanolamide (16:0 EA, PEA), stearoyl ethanolamide (18:0 EA, SEA), arachidoyl ethanolamide (20:0 EA), and docosanoyl ethanolamide (22:0 EA) (Fig. 4, A–D). As observed with fatty acids, inhibition of T-currents by N-acyl ethanolamides increases with the degree of unsaturation. This is illustrated by linoleoyl ethanolamide (18:2 EA) that produces a mild inhibition of the three CaV3 currents (~50–60% inhibition), whereas docosahexaenoyl ethanolamide (22:6 EA, DHEA) produces a maximal inhibition (~95%) similar to that obtained with AEA (Fig. 4, A–D).

We further investigated whether the position of the double bonds in the alkyl chain is implicated in T-current inhibition (Fig. 5). For the three CaV3 currents, we observed that inhibition increases when double bonds are proximal to the carboxyl group of polyunsaturated fatty acids (see Fig. 5A). Indeed, ω3 eicosatrienoic acid (20:3 cis-11,14,17) modestly inhibits T-currents (percent of inhibition: 29 ± 4%, n = 7, for CaV3.2 currents; 20 ± 3%, n = 10, for CaV3.3 currents; and 12 ± 1%, n = 10, for CaV3.1 currents), while inhibition is increased for ω6 eicosatrienoic acid (20:3 cis-8,11,14-dihomo-γ-linolenic acid) (percent of inhibition: 50 ± 4%, n = 10, for CaV3.2 currents; 39 ± 2%, n = 11, for CaV3.3 currents; and 29 ± 2%, n = 11, for CaV3.1 currents; 20:3 ω3 versus 20:3 ω6; p < 0.001 for each CaV3 current) and is maximal with ω9 eicosatrienoic acid (20:3 cis-5,8,11-meade acid) (percent of inhibition: 70 ± 3%, n = 9, for CaV3.2 currents; 60 ± 2%, n = 10, for CaV3.3 currents; and 46 ± 2%, n = 13, for CaV3.1 currents; 20:3 ω6 versus 20:3 ω9; p < 0.001 for each CaV3 current). Similar results were obtained for α-linolenic and γ-linolenic acid (18:3 ω3 cis-9,12,15- and 18:3 ω6 cis-6,9,12-octadecatrienoic acid, respectively) for the three CaV3 currents (18:3 ω3 versus 18:3 ω6: p < 0.01, Fig. 5, A and D). Also, alteration of cis-double bonds in trans-configuration or in cis-triple bonds, decreases the polysaturated fatty acid effects (p < 0.001), as assessed with linolenelaidic acid (18:3, trans-9,12,15-octadecatrienoic acid) and ETYA (20:4, cis-5,8,11,14-eicosatetraynoic acid). It is important to note that 3 cis-double bonds closer to the carboxyl group is sufficient to produce maximal inhibition since 18:3 ω6 and 20:3 ω9 fatty acids are equally potent that the fully polysaturated 18:4, 20:5, and 22:6 ω3 fatty acids (see Fig. 3) on the three CaV3 currents (~73% inhibition of CaV3.3 currents, p > 0.05 for all these fatty acids; ~61% inhibition of CaV3.3 currents, p > 0.05; and ~52% inhibition of CaV3.1 currents, p > 0.05).

Similar to that described for anandamide (24), arachidonic acid has pronounced effect on T-channel inactivation (Fig. 6). Indeed, arachidonic acid accelerates inactivation kinetics of T-currents as illustrated for CaV3.3 (Fig. 6A). Inactivation kinetics (τ) are ~2 times faster in the presence of arachidonic acid for the three CaV3 currents (p < 0.01; n = 8–14). In addition, arachidonic acid induces a hyperpolarizing shift of the steady-state inactivation curve of T-currents (~12 mV, p < 0.01 for the three CaV3 currents; n = 8–12; Fig. 6B) without a corresponding effect on the activation curve (Fig. 6, C–F). Therefore, in the presence of arachidonic acid, no current is recorded for a resting potential of ~70 mV, whereas ~50% of the current remained in control condition (Fig. 6B). Also, the window current, resulting from the overlap of the activation and inactivation curve, is almost completely abolished in the presence of arachidonic acid (Fig. 6, D–F).

Because the fatty acids and the N-acyl ethanolamides that potently inhibit T-currents contained at least a cis-1,4-pentadiene unit (–C=–C–C=–C–) and therefore are potentially metabolized in mammalian cells by lipooxygenase, cyclooxygenase, and cytochrome P450 epoxygenase and ω-hydrolase pathways (see schematic signaling cascade in Fig. 7A), we investigated whether these latter enzymes could be implicated in AA and AEA induce T-current inhibition (Fig. 7A).
Specific inhibition of cyclooxygenase with indomethacin (10 μM) as well as of lipoxygenase with 5,8,11-eicosatriynoic acid (10 μM) does not alter the T-current inhibition by AA and AEA (Fig. 7A). Similar findings were obtained using 17-oc-tadecynoic acid, which inhibits P450 epoxygenase and -hydroxylase enzymes (Fig. 7A). Furthermore, ETYA (10 μM), which acts as a nonspecific blocker of all AA-metabolizing enzymes, does not suppress T-current inhibition by either AA or AEA (Fig. 7A). Finally, AEA hydrolysis into AA by fatty acid amide hydrolase (FAAH) is not involved in T-current inhibition induced by AEA, as assessed with phenylmethanesulfonyl fluoride (100 μM, Fig. 7A). Because inhibition of T-currents could involve another mechanism (as G-protein receptor and protein kinase activation), we performed cell-free inside-out patches in the presence of AA and AEA (Fig. 7B) using an “intracellular” medium lacking both GTP and ATP. In this configuration, AA potently inhibits the three Caᵥ₃ currents (~50% inhibition of Caᵥ₃.1 and Caᵥ₃.2 currents and ~70% inhibition of Caᵥ₃.3 currents). Similar results are obtained with AEA, which inhibits ~85% of the three Caᵥ₃ currents (Fig. 7B).

**DISCUSSION**

In this study, we have identified the important chemical determinants of anandamide involved in the inhibition of Caᵥ₃ T-currents. We show that both the alkyl chain of anandamide and the hydroxyl present in its head group are primarily responsible for T-current inhibition. Indeed, anandamide derivatives lacking the hydroxyl group are weak inhibitors of T-currents (30%), as assessed with arachidonamide, arachidonic acid N-methyl amide, and arachidonyl 2-chloroethylamide. In addition, the amide linkage also contributes to increase anandamide effects since arachidonic acid (that contains a carboxyl group and the same alkyl chain as anandamide but not the amide linkage) potently inhibits T-currents (60%) but to a lesser extent than anandamide (~90%). Thus, arachidonic acid methyl ester, which lacks both the reactive hydroxyl group and the amide linkage, has no effect on T-currents. In addition, it should be noted that arachidonic alcohol has the same potency as arachidonic acid (that contains a carboxyl group and the same alkyl chain as anandamide) to inhibit T-currents, suggesting that the carbonyl group is not crucial for anandamide effects. Overall, these results suggest that anandamide and fatty acids could act on T-channels primarily using their –OH group, presumably via hydrogen bonds. However, acetyl ethanolamide and ethanolamine, which both lack the alkyl chain of anandamide, have no effect on T-currents, indicating that the fatty acid moiety of anandamide is necessary for the inhibition. Indeed, the inhibition of T-currents mainly depends on the degree of unsaturation of the alkyl chain but not on the chain length and increases...
the carboxyl group. By contrast, fatty acids in trans-configuration or containing cis-triple-bonds (as linolenelaidic acid and ETYA, respectively) have no effect. Therefore, the major natural and endogenous fatty acids, including γ-linolenic acid (18:3 ω6), mead acid (20:3 ω9), and arachidonic acid (20:4 ω6), as well as the fully polyunsaturated ω3 fatty acids stearidonic acid (18:4), EPA (20:5), and DHA (22:6) are potent inhibitors of T-currents (see Fig. 3). Similar results were obtained with the N-acyl related ethanolamides (see Fig. 4).

As observed in our study on T-channels, most of the other voltage-dependent anandamide-sensitive ionic channels are also modulated by fatty acids (reviewed in Ref. 27), including voltage-activated sodium channels (45–48), KV potassium channels (49, 50), and L-type calcium channels (51–53). By contrast, TRPV1 and TASK-1 channels, which are not voltage-sensitive, are modulated by anandamide but weakly by arachidonic acid (54–57). In addition, anandamide and arachidonic acid activate (but not inhibit) voltage-insensitive BK potassium channels (58–60). Also, by contrast with BK channel activation (58), inhibition of voltage-activated channels (including T-channels) occurs with unsaturated but not with the following saturated fatty acids: myristic (14:0), palmitic (16:0), and stearic acid (18:0) (45–47, 49, 51, 52). However, it is important to note that inhibition of T-channels by fatty acids displays specific features when compared with other voltage-activated anandamide-sensitive channels. T-channels are insensitive to long chain mono-unsaturated fatty acids such as palmitoleic (16:1), oleic (18:1) acids, which potently inhibit L-type calcium channels (52). Furthermore, contrary to T-channel inhibition, the inhibition of L-type calcium channels does not increase with polyunsaturated fatty acids (51, 52). In addition, the inhibition of sodium channels by polyunsaturated fatty acids comprising 18–22 carbons is different from the T-channel inhibition since it does not increase with the number of double bonds and therefore linoleic acid

| A | B |
|---|---|
| CaV3.3 currents elicted by incremental test pulse depolarizations (from −80 mV to +50 mV, 10-mV increments) of 450-ms duration applied every 5 s from a holding potential of −100 mV. Inactivation kinetics (τ inact) at −50 mV and −30 mV in the presence and in the absence of AA are presented as an inset. Inactivation τ values were obtained by monoeponential fits. B, effects of AA on steady-state inactivation of CaV3.3 currents. Currents were elicited by a −30 mV test pulse (450-ms duration) applied from holding potentials ranged from −110 mV to −50 mV (10-s duration, 5-mV increments). C, effects of AA on current-voltage (I-V) curves of CaV3.3 currents. I-V curves were obtained from experiments illustrated in A, D, AA shifts the steady-state inactivation (h∞) curve of CaV3.3 currents toward negative potentials without the corresponding effect on the steady-state activation (m∞) curve. The steady-state inactivation (h∞) curve was obtained from experiments illustrated in B, while the steady-state activation (m∞) curve were deduced from the fit of the I-V curves presented in C. For clarity, the corresponding symbol of the m∞ curve was omitted. E and F, AA shifts the steady-state inactivation (h∞) curve of CaV3.3, 3,1 (E) and CaV3.2 currents (F) toward negative potentials without the corresponding effect on the steady-state activation (m∞) curves. Steady-state activation (m∞) and inactivation (h∞) curves of CaV3.1 and CaV3.2 currents were deduced from the same experiments as in A and B except that the test pulses were of 200-ms duration.

with the number of cis-double bonds. Therefore the inhibition of T-currents is not restricted to eicosanoids (20 carbons) and also occurs with fatty acids and N-acyl ethanolamides containing 18–22 carbons. Furthermore, inhibition is also increased for unsaturated fatty acids when cis-double bonds are close to

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produces the same inhibitory effects than the fully polyunsaturated DHA (22:6) and EPA (22:5) (45, 46). In contrast, inhibition of Kv channels occurs with polyunsaturated fatty acids containing four or more double bonds (as arachidonic acid and DHA) but not with linoleic (18:2) and linolenic fatty acid (18:3) (49), which weakly inhibit T-channels. Also, contrary to T-channels, inhibition of Kv channels is maximal with ETYA (49), which contains four triple bonds. It should also be noted that TRPV1 is fully activated by N-acyl ethanolamides possessing at least one double bond and therefore linoleoyl ethanolamide, which weakly inhibits T-channels, is a full activator of TRPV1 (61). Overall, our structure-activity study of fatty acids on T-channels reveals unique features among anandamide-sensitive channels.

We also have demonstrated that the position of the double bonds in fatty acids is important for T-current inhibition. Inhibition is increased for unsaturated fatty acids when cis-double bonds are proximal to the carboxyl group. Therefore natural and endogenous fatty acids containing three double bonds near to the carboxyl group, such as γ-linolenic acid (18:3 cis-6,9,12-octadecatrienoic acid) and mead acid (20:3 cis-5,8,11-eicosatrienoic acid), produce maximal inhibition equivalent to those obtained with the fully polyunsaturated fatty acids, including stearidonic acid (18:4), EPA (20:5), and DHA (22:6). This observation suggests that the fully polyunsaturated ω3 fatty acids stearidonic acid, EPA, and DHA produce their effects not because they contain cis-double bonds in the ω3 position but rather because they are fully polyunsaturated and therefore contain double bonds near to the carboxyl group. It should be noted that the importance of the position of the double bonds in ω3 fatty acids has not been systematically tested on ionic currents, and most studies have concluded that stearidonic acid, EPA, and DHA produce maximal effect because they are ω3 fatty acids, providing molecular explanation for their beneficial physiological effects, as in neuroprotection and cardioprotection (31, 34–36, 40, 42). Furthermore, contrary to ω3 and ω6 fatty acids, which come from diet (from green plants, algae, and especially from fish oil) and are not endogenously produced in mammals, bio-active lipids acting on T-channels are synthesized in mammalian cells (which possess the unsaturase
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enzymes allowing double bond insertions near the carboxyl group, i.e. after the @ position) and therefore are likely to operate as signal transduction molecules.

Fatty acids and N-acyl ethanolamides inhibiting T-channels possess at least a cis-1,4-pentadiene unit (\(-C-H-C-C-C-H\)) and therefore are potentially metabolized in mammalian cells by several enzymes (see schematic signaling pathways for anandamide and arachidonic acid in Fig. 7A). We demonstrated with different enzyme inhibitors that the inhibition of the three T-channels by anandamide and arachidonic acid does not involve lipoxygenase, cyclooxygenase, as well as cytochrome P450 pathways. Furthermore, anandamide hydrolysis into arachidonic acid by FAAH is not involved in anandamide effects since T-current inhibition is resistant to phenylmethanesulfonyl fluoride and also occurs with R1- and R2-methanamide, which are resistant to FAAH hydrolysis. We also demonstrated that anandamide and arachidonic acid inhibit the three Ca\(3\) T-channels in cell-free inside-out patches, suggesting that both anandamide and arachidonic acid exert their action by binding directly to T-channels or by acting on their near lipid environment. For instance, it has been reported that fatty acids possibly modulate BK and TREK-1 potassium channels by acting directly on the membrane, as these channels are sensitive to membrane stretch (57–59). It should be noted, however, that in these two later cases, fatty acids and membrane stretch induce activation rather than inhibition of the channels.

We have tested the membrane hypothesis by mimicking the membrane effects of arachidonic acid with unrelated molecules trinitrophenol and dinitriphenol that also are anionic amphipathic molecules (62, 63). These molecules, like arachidonic acid, have been previously shown to cause membrane creation and opening of the mechano-sensitive TRENK-1 channels (57, 62, 63). We found that these compounds had very weak effects on T-currents (~5% increase) even at a concentration of 400 \(\mu M\) (data not shown). Furthermore, a hypo-osmotic solution (~150 mosm), which also mimics arachidonic acid creation effects on cell membrane and TRENK-1 channels (57), induced a slight increase of T-currents (~20%, data not shown) but no inhibition. In addition, by contrast with our data on T-channel inhibition, membrane effects of fatty acids mainly depend on the chain length and the degree of unsaturation rather than the position of double bonds. Although we cannot completely exclude a membrane effect of anandamide and fatty acids on T-channels, our results are overall in favor of a direct binding of these molecules to T-channels.

We have previously shown that anandamide accelerates the inactivation kinetics and shifts the steady-state inactivation curve of the three Ca\(3\) currents toward more negative potentials (~10 mV) (24). We demonstrated in this study that arachidonic acid induces similar changes in T-current properties, confirming a common molecular mechanism (64–66). Also, similar effects were obtained with polyunsaturated fatty acids on inactivation properties of other anandamide-sensitive voltage-dependent channels such as sodium channels (45–47), \(K_{\text{v}}\) potassium channels (49, 50), and L-type calcium channels (51, 52), indicating that the mechanism of inhibition by anandamide and fatty acids might be conserved among voltage-dependent channels.

CONCLUSIONS

Polyunsaturated fatty acids and N-acyl ethanolamides are signaling molecules implicated in a multitude of physiological and pathophysiological events, including cardiac and neuronal excitability (31, 33, 40, 42), cardioprotection and neuroprotection from ischemic and epileptic episodes (31, 34–36, 40), inflammation, and pain (32, 39). Considering the role of T-channels in cardiac and neuronal pacemaker (1, 2, 13, 14), cardiac hypertrophy (15), neuroprotection, and absence epilepsy (8, 9, 67) as well as in pain perception (10–12), it is attractive to suggest that T-channel inhibition may contribute to polyunsaturated fatty acid and N-acyl ethanolamide effects. In addition, it is important to note that both anandamide and arachidonic acid also act as intracellular second messengers (68, 69) and therefore could potentially participate in the intracellular signal transduction cascades by which neurotransmitters inhibit T-currents (2, 70). It will be of great interest to investigate whether endogenously produced anandamide and arachidonic acid can modulate T-currents as recently demonstrated for TRPV1 receptors (69).

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