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Direct inhibition of T-type calcium channels by the endogenous cannabinoid anandamide

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Low-voltage-activated or T-type Ca^{2+} channels (T-channels) are widely expressed, especially in the central nervous system where they contribute to pacemaker activities and are involved in the pathogenesis of epilepsy. Proper elucidation of their cellular functions has been hampered by the lack of selective pharmacology as well as the absence of generic endogenous regulations. We report here that both cloned (α_{1G}, α_{1H} and α_{1I} subunits) and native T-channels are blocked by the endogenous cannabinoid, anandamide. Anandamide, known to exert its physiological effects through cannabinoid receptors, inhibits T-currents independently from the activation of CB1/CB2 receptors, G-proteins, phospholipases and protein kinase pathways. Anandamide appears to be the first endogenous ligand acting directly on T-channels at submicromolar concentrations. Block of anandamide membrane transport by AM404 prevents T-current inhibition, suggesting that anandamide acts intracellularly. Anandamide preferentially binds and stabilizes T-channels in the inactivated state and is responsible for a significant decrease of T-currents associated with neuronal firing activities. Our data demonstrate that anandamide inhibition of T-channels can regulate neuronal excitability and account for CB receptor-independent effects of this signaling molecule.

Keywords: anandamide/cannabinoid receptor/NG 108-15 cells/pharmacology/T-type calcium channel

Introduction

Voltage-dependent Ca^{2+} channels are divided into three families: the L-type channels (or Ca_V1), the neuronal N-, P/Q- and R-type channels (or Ca_V2) and the T-type channels (or Ca_V3) (Ertel *et al*., 2000). T-type Ca^{2+} channel (T-channel) hallmarks are low-voltage-activated Ca^{2+} currents, fast inactivation and slow deactivation kinetics, low unitary conductance and strong steady-state inactivation at physiological resting potential (Carbone and Lux, 1984; Armstrong and Matteson, 1985; Nilius *et al*., 1985; Nowycky *et al*., 1985; Bean and McDonough, 1998). Three genes encoding the T-channel pore subunits were identified recently and designated α_{1G} ($Ca_V3.1$), α_{1H} ($Ca_V3.2$) and α_{1I} ($Ca_V3.3$) (Cribbs *et al*., 1998; Perez-Reyes *et al*., 1998; Klugbauer *et al*., 1999; Lee *et al*., 1999; Monteil *et al*., 2000a,b). T-currents generated by the α_{1I} subunit display slow kinetics that differ markedly from the α_{1G} and α_{1H} currents, which share the typical signature of native T-currents (Lee *et al*., 1999; Monteil *et al*., 2000b). Northern blot analyses have shown that the α_{1G}, α_{1H} and α_{1I} mRNAs are expressed in various tissues, including heart and central nervous system (CNS) (Cribbs *et al*., 1998; Lee *et al*., 1999; Monteil *et al*., 2000a,b). In the CNS, T-channels generate low threshold spikes and participate in spontaneous firing (Llinas and Yarom, 1981; Llinas and Jahnsen, 1982; Huguenard, 1996). T-channels are also involved in cardiac pacemaker activity (Hagiwara *et al*., 1988; Lei *et al*., 1998), aldosterone secretion (Rossier *et al*., 1996) and fertilization (Arnoult *et al*., 1996). Unfortunately, the lack of selective blockers and endogenous ligands targeting T-channels has considerably hampered the elucidation of their functional roles (Huguenard, 1996).

A recent report describing that arachidonic acid (AA) inhibits α_{1H} currents (Zhang *et al*., 2000) suggested to us that anandamide (*N*-arachidonoyl-ethanolamine) (Devane *et al*., 1992) was a potential modulator of T-channel function. Anandamide and 2-arachidonyl glycerol (2-AG) are endogenous ligands of the cannabinoid (CB) receptors, which belong to the G-protein-coupled receptor superfamily. To date, two CB receptors have been cloned, the CB1 receptor expressed primarily in the brain (Matsuda *et al*., 1990) and the CB2 receptor expressed in the immune system (Munro *et al*., 1993). CB1 receptor activation inhibits adenylyl cyclase, increases mitogen-activated protein kinase activities, modulates several potassium channel conductances and inhibits N- and P/Q-type Ca^{2+} channels (Mackie and Hille, 1992; Bouaboula *et al*., 1995; Mackie *et al*., 1995; Twitchell *et al*., 1997; Schweitzer, 2000). Anandamide preferentially binds to the CB1 receptor and mimicks most effects of the major psychoactive component of marijuana, Δ^9-tetrahydrocannabinol (Δ^9-THC), such as enhancement of sensory perception, alteration in cognition, sedation, catalepsy, analgesia and hypothermia (Di Marzo *et al*., 1998; Mechoulam *et al*., 1998; Ameri, 1999). Nevertheless, several physiological roles for anandamide, including modulation of neuronal excitability (Venance *et al*., 1995; Tognetto *et al*., 2001), pain (Adams *et al*., 1998; Vivian *et al*., 1998) and cardiovascular functions (Lake *et al*., 1997; Jarai *et al*., 1999; Zygmunt *et al*., 1999) are independent of the CB1 receptor activation. Indeed, anandamide can directly activate VR-1 vanilloid receptors (Zygmunt *et al*., 1999), inhibit Kv1.2 and TASK-1 potassium channels (Poling *et al*., 1996; Maingret *et al*., 2001) or act on as yet unidentified targets. In the present study, we provide clear evidence that submicromolar concentrations of anandamide directly block T-type calcium channels, which

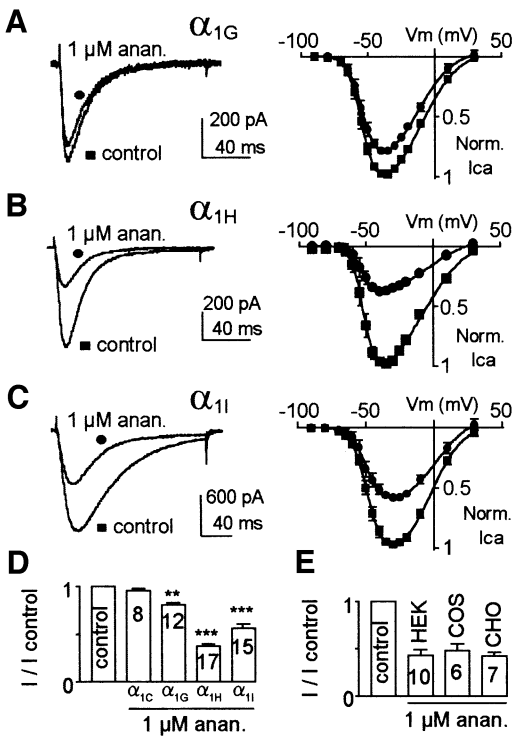

Fig. 1. Anandamide inhibits cloned T-channels. (A–C) Effect of 1 μM anandamide (circle) on Ca^{2+} currents elicited by a –30 mV test pulse (left panel) and current–voltage curves (right panel) for α_{1G} (**A**), α_{1H} (**B**) and α_{1I} subunits (**C**). The holding potential (HP) was –80 mV. (**D**) Effect of 1 μM anandamide on the α_{1G}, α_{1H}, α_{1I} and α_{1C} currents. Anandamide blocked T-currents but not L-type (α_{1C}) currents. α_{1C} currents were elicited by a +20 mV test pulse applied from an HP of –80 mV. (**E**) Anandamide (1 μM) produces a similar block of Ba^{2+} (5 mM) currents when α_{1H} is expressed in HEK 293, CHO or COS cells. For statistical analysis, Student's *t*-tests were used with $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. The number of cells analysed is indicated on the histogram.

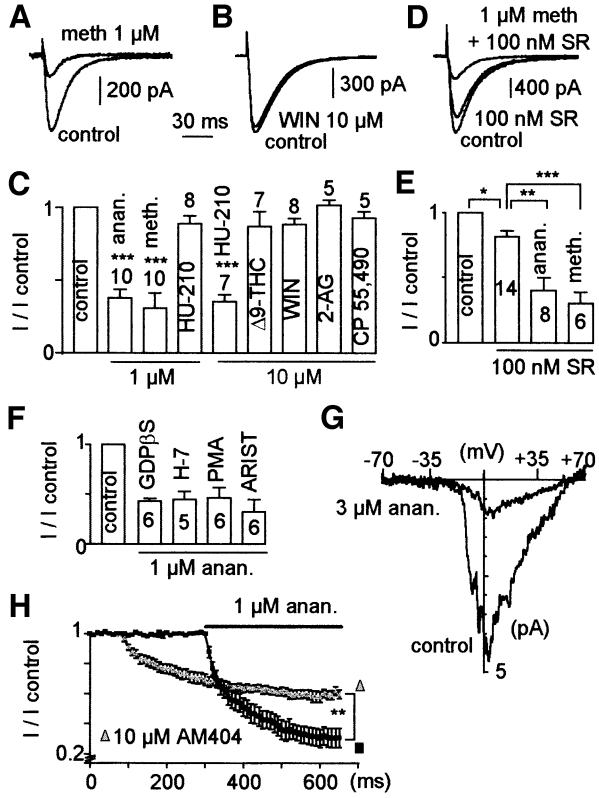

Fig. 2. Anandamide directly inhibits α_{1H} currents independently of G-protein-coupled receptors. (**A**) Methanandamide (meth;1 μM) inhibited α_{1H} currents while 10 μM WIN 55,212-2 (WIN) did not (**B**). (**C**) Effects of various agonists of the CB1/CB2 receptors on α_{1H} current amplitude. Concentrations used are indicated on the histogram. (**D** and **E**) SR141716A (SR; 100 nM), a CB1 antagonist, did not prevent the α_{1H} current inhibition induced by either anandamide (anan) or methanandamide. (**F**) Block of T-current by anandamide in the presence of 1 mM GDPβS in the intracellular medium or following 16 h incubation in the culture medium of 50 μM H7, 100 nM PMA or 250 μM aristolochic acid (ARIST). (**G**) Effect of 3 μM anandamide on α_{1H} currents in inside-out patch recording. The holding potential was –70 mV and currents are stimulated with voltage ramp protocols of 90 ms duration, from –70 to +70 mV. Note that the positive shift for the I–V curve is due to the use of 110 mM barium. (**H**) Effect of 10 μM AM404 on the time course and amplitude of the anandamide block.

therefore represent a novel molecular target for this endocannabinoid.

Results

Micromolar concentrations of anandamide inhibited the three human cloned T-channels α_{1G} ($Ca_V3.1$), α_{1H} ($Ca_V3.2$) and α_{1I} ($Ca_V3.3$) expressed in HEK 293 cells (Figure 1). The α_{1H} and α_{1I} currents were blocked strongly by 1 μM anandamide by ~65 and 45% (I/I control: 0.37 ± 0.02, $n = 17$ and 0.56 ± 0.04, $n = 15$, respectively), while α_{1G} currents were inhibited by 20% at this concentration (I/I control: 0.81 ± 0.02, $n = 12$). These results were observed at every potential (Figure 1A–C) using either extracellular Ca^{2+} (2 mM) or Ba^{2+} (5 mM; $n = 10$). This current inhibition was selective to T-channels since no effect was observed on α_{1C} currents (Figure 1D), even when increasing the anandamide concentration to 10 μM ($n = 5$, not shown). A similar anandamide inhibition of α_{1H} currents was observed in HEK 293, COS and CHO cells (Figure 1E), indicating further that the reported effects were not restricted to HEK 293 cells.

The ability of various cannabinoids to inhibit α_{1H} currents was then investigated (Figure 2A–E). Similarly to anandamide, the non-hydrolysable analogue methanandamide (1 μM) decreased α_{1H} currents by ~70% (I/I control: 0.31 ± 0.09, $n = 10$), indicating that anandamide hydrolysis was not involved in the described effects (Figure 2A and C). In contrast, neither of the synthetic CB1/CB2 receptor agonists WIN 55,212-2 (WIN; Figure 2B) and CP 55,490 decreased α_{1H} currents (Figure 2C). Similarly, neither the endocannabinoid 2-AG nor Δ^9-THC affected α_{1H} currents (Figure 2C). The CB1/CB2 agonist HU-210 had no significant effect on α_{1H} currents at 1 μM (Figure 2C) but inhibited 65% of the current at 10 μM (I/I control: 0.35 ± 0.05, $n = 7$). We next tested the ability of the CB1 receptor antagonist SR141716A to prevent inhibition of α_{1H} currents by anandamide. Although 100 nM SR141716A produced a mild inhibition (~20%) of α_{1H} currents by itself (I/I control: 0.81 ± 0.04, $n = 14$), it failed to prevent current inhibition by anandamide (51 ± 8%, $n = 8$) and methanandamide (64 ± 7%, $n = 6$; Figure 2D and E). An important block of α_{1H} currents was observed using 1 μM SR141716A (70 ± 6%, $n = 6$; not shown).

Activation of CB receptors is known to activate Gi/Go proteins and to regulate various phospholipases (i.e. PLA_2, PLC and PLD) and phosphorylation pathways including protein kinases A and C (PKA and PKC). Application through the patch pipette of 1 mM of GDPβS, an inhibitor of G-protein activity, or cell incubation with 50 μM H7, an inhibitor of cyclic nucleotide-dependent protein kinases, did not prevent the inhibition of α_{1H} currents by anandamide (Figure 2F). Similar results were obtained with cells incubated in the presence of 100 nM phorbol 12-myristate 13-acetate (PMA), which affects PLC/PKC but also PLD pathways in HEK-293 cells (reviewed in Exton, 1999), or in the presence of 250 μM aristolochic acid, an inhibitor of PLA_2 (Figure 2F). More importantly, inhibition of α_{1H} currents was observed in excised inside-out patches of HEK 293 cells superfused in the presence of 3 μM anandamide (I/I control: 0.15 ± 0.07, n = 6; Figure 2G), suggesting further that anandamide binds directly to T-channels. In addition, experiments in the presence of AM404, which inhibits the membrane transport of anandamide, were performed. Although extracellular application of AM404 (10 μM) produced a mild inhibition (~30%) of α_{1H} currents by itself (I/I control: 0.69 ± 0.03, n = 5; Figure 2H), it prevented current inhibition by anandamide (I/I control: 0.6 ± 0.03, n = 5), suggesting that anandamide possibly acts at an intracellular site. Overall, these results demonstrate that neither CB receptors, G-protein pathways nor protein kinases and phospholipases are involved in the inhibition of α_{1H} currents by anandamide and strongly suggest that anandamide directly inhibits T-channels by acting at an intracellular site.

Anandamide block of native T-channels was examined using the neuroblastoma NG108-15 cell line, which expresses well characterized T-channels (Randall and Tsien, 1997) and most of the high-voltage-activated (HVA) channels (Lukyanetz, 1998). Mackie and Hille (1992) demonstrated that in this cell line, activation of CB1 receptors by WIN leads to a pertussis toxin-dependent block of N-currents without any effect on T-currents. As for cloned T-channels, methanandamide but not WIN decreased native T-currents in the NG108-15 cell line (Figure 3A and B). In contrast, both compounds significantly inhibited HVA currents (Figure 3C–F) by acting on CB1 receptors (Mackie and Hille, 1992). The percentages of block by 1 μM methanandamide were 65 ± 6% (n = 12) on T-currents and 31 ± 14% (n = 8) on HVA currents (Figure 3E), while those mediated by 1 μM WIN were 5 ± 9% (n = 11) on T-currents and 43 ± 15% (n = 11) on HVA currents (Figure 3F). The extent of block by methanandamide, as well as its time course (not shown) is similar for cloned and native T-channels. Similar results were obtained using 1 μM anandamide (n = 5, not shown).

Inhibition of Ca^{2+} currents generated by the three cloned T-channels was concentration dependent (Figure 4). Using a holding potential (HP) of –80 mV, submicromolar concentration of anandamide as low as 10 nM significantly decreased α_{1H} and α_{1I} currents, while concentrations higher than 100 nM were required to inhibit α_{1G} currents significantly (Figure 4A and C). Dose–response curves indicated IC_{50} values of 330 ± 66 nM, n = 8 for α_{1H}, 1.10 ± 0.05 μM, n = 8 for α_{1I} and 4.15 ± 0.03 μM, n = 8 for α_{1G}; and Hill slope factors were 0.60 ± 0.05, n = 8 for

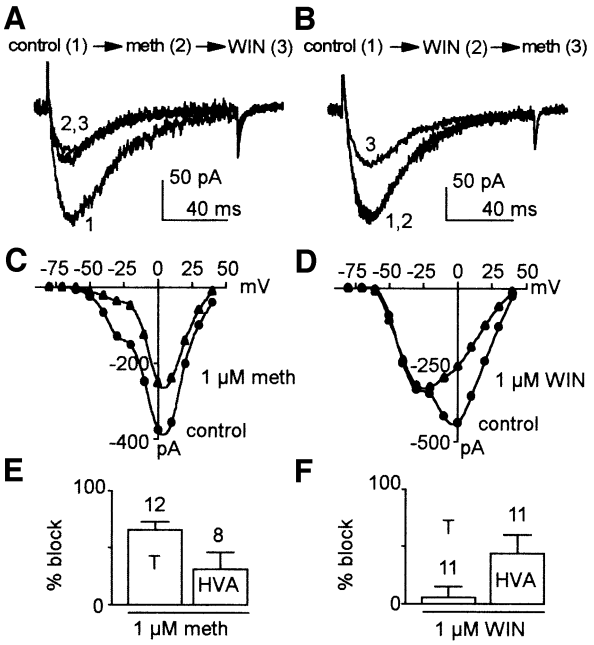

Fig. 3. Methanandamide blocks native T and HVA currents of NG108-15 cells while WIN only affects HVA currents. (**A**) Effects on T-currents of 1 μM methanandamide applied first (2) followed by 1 μM WIN (3). (**B**) Effects on T currents of 1 μM WIN applied first (2) and 1 μM methanandamide applied after (3). (**C** and **D**) Corresponding current–voltage relationships (I–V curves) of the calcium currents presented in (A) and (B), respectively. Drugs were applied at HP –80 mV while I–V curves were performed just after at HP –110 mV in order to record maximal T-currents. (**E** and **F**) Percentage block of T- and HVA currents by 1 μM methanandamide (E) and by 1 μM WIN (F). To avoid contamination of T currents by HVA currents (and reciprocally), the peak of T-currents was measured at –30 mV while HVA currents were measured 100 ms after the beginning of the test pulse at +10 mV. In all experiments, HP was –80 mV and 10 mM Ba^{2+} was used as charge carrier.

α_{1H}, 0.68 ± 0.05, n = 8 for α_{1I} and 0.98 ± 0.02, n = 8 for α_{1G} (Figure 4C). The time course of the α_{1H} current block by increasing concentrations of anandamide was fast and reached steady state in <1 min (Figure 4B). Current inhibition by anandamide was partially reversible. Recovery was ~15% of the total current after 10 min of washout of the drug (n = 5, not shown). Anandamide had little effect on membrane capacitance (7 ± 14%, n = 8, not shown), indicating that its effects could not be explained by simple membrane-disrupting mechanisms.

Closer inspection of T-currents revealed that anandamide also affects their kinetics (Figure 5). Anandamide accelerated inactivation kinetics of the three cloned T-channels in a concentration-dependent way. This effect was most pronounced for α_{1I} currents (significant at 100 nM; Figure 5A), while faster kinetics for α_{1G} and α_{1H} currents were observed at higher concentrations (1 and 10 μM, respectively; Figure 5B and C). Anandamide-induced acceleration of kinetics occurred at all voltages, especially in the negative range of potentials (not shown). Although 1 μM anandamide produced strong effects on inactivation kinetics, it also significantly accelerated the activation kinetics of α_{1G} and α_{1I} currents for potentials lower than –10 mV (P <0.01). Activation kinetics were faster by 1.4- (n = 9), 1.1- (n = 6) and 2-fold (n = 8) at –40 mV for α_{1G}, α_{1H} and α_{1I}, respectively (not shown).

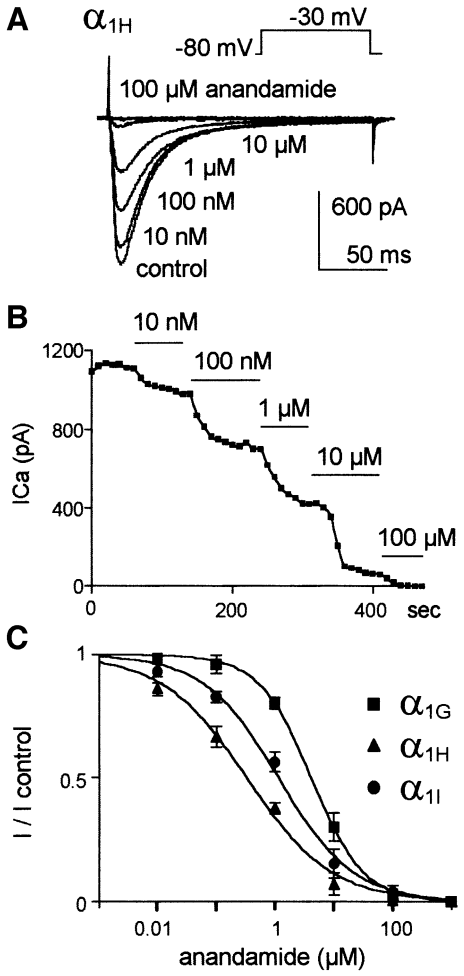

Fig. 4. Submicromolar concentrations of anandamide inhibit α_{1H} and α_{1I} currents but not α_{1G} currents. (**A**) Effects of increasing concentrations of anandamide on calcium currents generated by the α_{1H} subunit. (**B**) Time course of the inhibition of α_{1H} currents by increasing concentrations of anandamide. (**C**) Dose–response curves of anandamide for the three human cloned T-channels. The curves were fitted with a sigmoidal relationship where I/Icontrol = $1/(1 + 10^{[(\text{LogEC50} - \log[\text{anandamide}]) \times \text{Hillslope}]})$.

It is interesting to note that the α_{1I} currents, which display the slowest kinetics, are the most markedly modulated by anandamide. As a consequence, the sustained component of α_{1I} current measured 200 ms after the beginning of the test pulse was abolished in the presence of 1 µM anandamide (Figure 5A). Anandamide-induced acceleration of the inactivation kinetics suggested that it could also modulate steady-state inactivation of T-channels. In order to test this hypothesis, steady-state inactivation protocols were performed in the presence of anandamide concentrations that approximately blocked 50% of T-currents at –80 mV [the half-potential value ($V_{0.5}$) of steady-state inactivation]. Anandamide shifted the steady-state inactivation curves (h_∞) of T-channels (Figure 6A and B) towards more negative potentials (–12.2 ± 2.5 mV, n = 8 for α_{1G}, –10.2 ± 2.2 mV, n = 8 for α_{1H}, and –12.1 ± 2.1 mV, n = 8 for α_{1I}). No shift was observed for steady-state activation curves (m_∞). Consequently, the window current generated by the overlapping steady-state activation and inactivation curves was decreased markedly

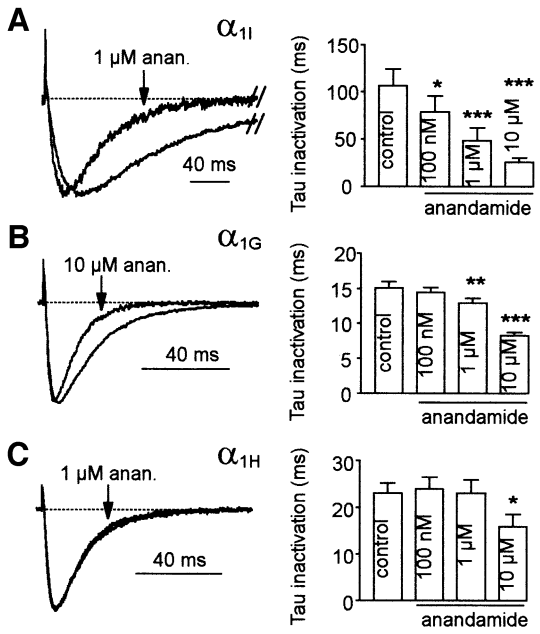

Fig. 5. Anandamide accelerates inactivation kinetics of T-currents. (A–C) Normalized currents in the presence or absence of anandamide (left panel) and effects of increasing concentration of anandamide on inactivation kinetics (right panel) for the α_{1G} (**A**, n = 5), α_{1H} (**B**, n = 6) and α_{1I} subunit (**C**, n = 5). Currents were elicited by a –30 mV test pulse from an HP of –80 mV.

(Figure 6A). Such a negative shift of the steady-state inactivation curve would suggest that anandamide binds and stabilizes T-channels in the inactivated state. A direct method to evaluate anandamide's binding to inactivated T-channels is to measure the current inhibition at various HPs. We therefore examined the inhibition of α_{1H} currents by 1 µM anandamide for HPs ranging from –110 to –70 mV (Figure 6C). Expectedly, anandamide strongly blocked T-currents by 85 ± 8% (n = 7) at –70 mV, while at –110 mV anandamide modestly reduced α_{1H} currents by 8 ± 3% (n = 7).

In order to appreciate better the physiological impact of anandamide's block of T-currents, we performed voltage clamp experiments using a thalamo-cortical relay cell firing activity as waveform. The α_{1I} currents strongly participate in the Ca^{2+} entry during sustained neuronal activities because of their slow inactivation kinetics (Figure 7). Anandamide decreased the amplitude and accelerated the decay of α_{1I} currents in a concentration-dependent manner (Figure 7). Remarkably, 100 nM anandamide completely abolished the α_{1I} current remaining after the seventh spike. To quantify these effects, the integral of the current was measured for the various anandamide concentrations (Figure 7, inset). Since 100 nM anandamide blocked ~50% of the Ca^{2+} influx, these data clearly indicated that anandamide blocks T-channels more efficiently during neuronal activities than during conventional square step stimulations (IC_{50} ~1 µM, Figure 4).

Discussion

This study demonstrates that submicromolar concentrations of the endocannabinoid anandamide blocks cloned α_{1H} and α_{1I} channels (and to a lesser extent α_{1G} channels)

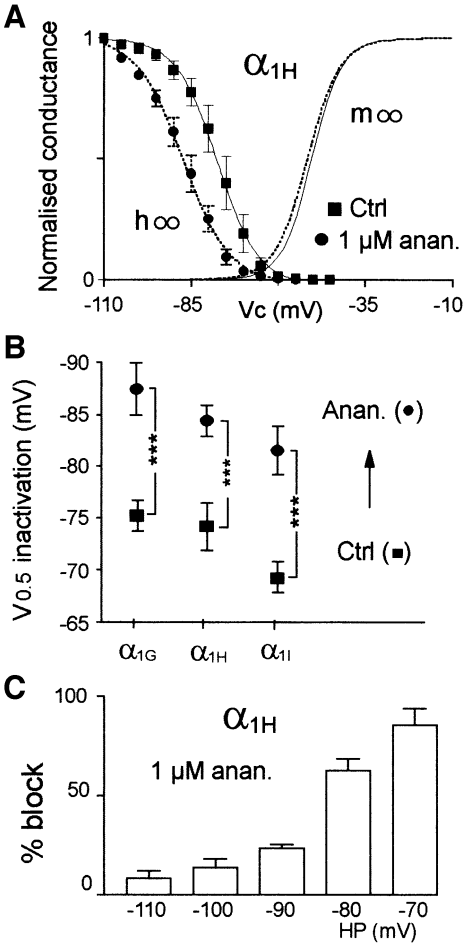

Fig. 6. Effect of anandamide on T-currents is state dependent. (**A**) Anandamide shifts the steady-state inactivation curve (circle) without a corresponding effect on the activation curve (dotted line) of the α_{1H} subunit. Steady-state activation (normalized conductance m_∞) was deduced from the I–V curves presented in Figure 1 ($V_{0.5}$ = –50.3 ± 1.7 mV, k = 5.4 ± 0.4 mV and $V_{0.5}$ = –52.6 ± 1.5 mV, k =0 5.6 ± 0.5 mV (n = 17) in the absence and presence of 1 µM anandamide, respectively). In order to visualize window currents better, corresponding symbols were omitted. Steady-state inactivation curves were obtained by stepping the membrane potential to –30 mV after holding the membrane for 10 s at potentials ranging from –110 to –45 mV. The normalized peak current amplitude (h_∞) was plotted as a function of the HP. (**B**) Effects of 1 µM anandamide (circle) on the $V_{0.5}$ of inactivation for α_{1H} and α_{1I} subunits and of 10 µM anandamide on the $V_{0.5}$ of inactivation for the α_{1G} subunit. No significant difference in the slope factor was observed (**C**) Block of α_{1H} currents by anandamide was dependent on the HP (n = 7 in each condition).

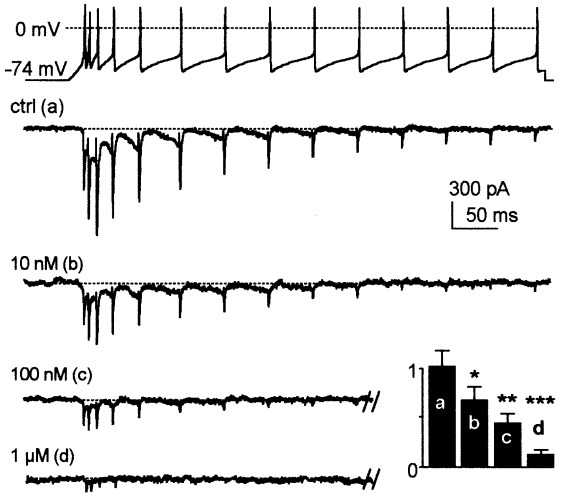

Fig. 7. Anandamide blocks α_{1I} currents during thalamic relay cell-like activities. A firing activity typical of those of thalamic relay neurons was used as waveform (upper panel), and the resulting α_{1I} currents are presented in the absence [ctrl (a)] and presence of increasing concentrations of anandamide [10 nM (b), 100 nM (c) and 1 µM (d)]. The normalized integral of total current (arbitrary units) is shown as an inset (n = 6).

as well as native T-channels. The block is independent of CB receptors, G-proteins, PLA_2, PLC, PLD, cyclic nucleotide-dependent protein kinases and PKC. The block is observed in excised inside-out patch configuration, indicating that inhibition of T-currents by anandamide is probably direct. In addition, block of anandamide membrane transport by AM404 (Beltramo *et al.*, 1997) suggests an intracellular action of anandamide. The pharmacological profile of the endocannabinoid block of T-channels is specific for anandamide and further confirms that this molecule acts directly on the T-channels. First, the classical CB1/CB2 agonists WIN and CP 55,490 are inactive and the CB1 receptor antagonist SR141716A does not prevent inhibition of T-currents by anandamide. Secondly, other natural CB1/CB2 agonists, including the endocannabinoid 2-AG and Δ^9-THC, also fail to affect T-currents. Finally, the non-hydrolysable analogue methanandamide also inhibits T-currents, demonstrating that anandamide hydrolysis to AA is not involved in the inhibition of T-channels. The reported effects are therefore distinct from the recently described inhibition of α_{1H} currents by AA (Zhang *et al.*, 2000). Similarly to cloned channels, native T-channels are blocked potently by anandamide and methanadamide. The NG108-15 cell line, which displays typical T-currents but also N-, P/Q-, R- and L-type currents (Randall and Tsien, 1997; Lukyanetz, 1998), was used previously by Mackie and Hille (1992) to demonstrate that activation of CB1 receptors by WIN leads to the inhibition of N-currents, without affecting T-currents. Using this cell line, we demonstrate that anandamide, but not WIN, is a potent blocker of native T-currents, confirming that this inhibition is independent of CB receptors.

Previous studies have described that other signaling molecules modulate native T-currents. Nociceptin, an endogenous ligand of the opioid receptor ORL_1, mediates inhibition of T-currents from dorsal root ganglion neurons independently of G-proteins (Abdulla and Smith, 1997). This effect is prevented by the κ_3 antagonist, nalbzoh, indicating that nociceptin inhibition of T-currents involves receptor activation. Similarly, the 5-hydroxytryptamine inhibition of T-currents in sensory neurons of *Xenopus* larvae is mediated by a functional domain of the receptor that is distinct from that which couples to G-proteins (Sun and Dale, 1999). In contrast, modulation of T-channels in adrenal glomerulosa cells by angiotensin AII and dopamine D1 receptors occurs through G-protein receptor-coupled pathways (McCarthy *et al.*, 1993; Drolet *et al.*, 1997). Overall, anandamide appears to be one of the first endogenous ligands ever described capable of directly modulating T-channels. The IC_{50} values for T-current

inhibition (330 nM for α_{1H} channels) are equivalent to those obtained for the CB1 receptor activation (Felder *et al*., 1995), suggesting that T-channels could be one of the principal targets of anandamide. Direct effects of anandamide on ionic channels have already been reported. Indeed, anandamide inhibits Kv1.2 potassium channels independently of CB1 receptor activation with an IC_{50} of 2.7 μM (Poling *et al*., 1996), and directly activates vallinoid VR1 receptors at even higher concentrations (Zygmunt *et al*., 1999; Smart *et al*., 2000). In addition, it was shown recently that submicromolar concentrations of anandamide directly inhibit the potassium channel TASK-1, which underlies the background current IKso (Maingret *et al*., 2001). Altogether, besides its classical action on CB receptors, anandamide can act directly on several ionic channels, including T-channels, to produce its powerful pharmacological and physiological effects.

Inhibition of T-currents by anandamide is highly dependent on the inactivation state of the channel. Indeed, the block is negligible for HPs lower than –100 mV and reaches ~85% at –70 mV, an HP at which T-channels are strongly inactivated. In addition, anandamide induces an approximately –10 mV shift of the steady-state inactivation curves of each cloned T-channel, but not of the activation curves. This results in a net decrease of the window current component of T-channels. Overall, these data strongly indicate that anandamide binds and stabilizes T-channels in the inactivated state. Binding to the inactivated T-channels is an important feature of the anandamide block, since T-channels are strongly inactivated at physiological resting potentials. This property could also contribute to tissue selectivity of the anandamide effects. For example, dihydropyridines that bind preferentially to the inactivated state of L-type Ca^{2+} channels are useful as anti-hypertensive drugs by acting on smooth muscle while having little effect on the heart (Triggle, 1992). Another interesting feature of the block of T-currents by anandamide is the acceleration of current kinetics. T-currents generated by the α_{1I} subunit inactivate slowly (Lee *et al*., 1999; Monteil *et al*., 2000b), leading during long test pulses to a sustained current component that is abolished by anandamide. Acceleration of current decay was observed before the decrease in the current amplitude (not shown), suggesting that anandamide could act as an open channel blocker (Bezprozvanny and Tsien, 1995). In that respect, the appearance of a faster inactivation would be due to an increase in the open to blocked transition, which would also explain apparent faster activation kinetics. A similar state-dependent block was described recently for other T-channel blockers such as the antiepileptic drugs methylphenylsuccinimide and ethosuximide (Gomora and Perez-Reyes, 2001). Speed up of current decay is also observed for α_{1G} and α_{1H} subunits but at higher anandamide concentrations, possibly due to their faster inactivation kinetics, as reported for the mibefradil block of HVA Ca^{2+} channels (Bezprozvanny and Tsien, 1995).

By stimulating cells overexpressing α_{1I} channels with thalamic firing activities, we demonstrate that anandamide block of T-currents is ~10 times more potent during neuronal firing activity (IC_{50} ~100 nM for α_{1I} channels). This suggests that anandamide could be an important physiological regulator of T-channels. What could be the physiological relevance of the blockade of T-currents by anandamide? First, the fact than anandamide preferentially binds and stabilizes T-channels in the inactivated state suggests that it could significantly decrease T-currents induced by low-threshold spike (LTS). LTS results from deinactivation of T-channels after inhibitory synaptic input, leading to a Ca^{2+}-dependent depolarization, which activates sodium channels (Huguenard, 1996). This phenomenon is well known in the thalamus where T-channels are thought to mediate action potentials and to control the frequency and time course of repetitive firing (McCormick and Huguenard, 1992). In the thalamus, T-channels are involved in slow wave sleep (Steriade *et al*., 1993) and in the pathogenesis of epilepsy (Tsakiridou *et al*., 1995). It is important to note that a high level of anandamide was detected in the thalamus (Felder *et al*., 1996) while CB1 receptors are expressed at low levels (Herkenham *et al*., 1991). It would be of great interest to test on these neurons whether anandamide could modify their activities via non-CB receptor mechanisms. Inhibition of T-channels could also participate in the antinociceptive effects of anandamide. On one hand, the α_{1H} subunit is highly expressed in dorsal root ganglion neurons (Talley *et al*., 1999) and neuropathic pain alters T-current properties (Hogan *et al*., 2000). On the other hand, anandamide-induced antinociception is not fully prevented by the CB1 receptor antagonist SR141716A (Adams *et al*., 1998; Smith *et al*., 1998; Vivian *et al*., 1998). In addition, the antinociceptive response attributed to tonic activation of CB1 receptors (Chapman, 1999) could be related to the SR141716A block of T-currents described here. Another important property of the anandamide block is the abolition of the window current component of T-channels. The window current occurs near the resting potential of many excitable cells. It contributes to the control of basal levels of Ca^{2+} (Chemin *et al*., 2000) and potentially participates in cell proliferation and differentiation processes (Bijlenga *et al*., 2000). The window current is also suspected to participate in the control of the cardiac pacemaker activity (Lei *et al*., 1998). In the heart, high levels of anandamide but few CB receptors were detected (Felder *et al*., 1996; Herkenham *et al*., 1991). It will therefore be important to determine whether direct inhibition of pacemaker T-channels by anandamide or SR141716A also participates in the bradycardic effect of these compounds (Lake *et al*., 1997). Altogether, anandamide inhibition of T-channels represents a novel non-CB receptor mechanism that can contribute to a wide variety of anandamide's effects. This strong regulation of T-channels should therefore be taken into account in further studies investigating the physiological roles of anandamide.

Materials and methods

Cell culture and transfection protocols

HEK-293, COS-7, CHO and NG 108-15 cells were cultured using standard techniques. Transfection was performed as previously described (Chemin *et al*., 2001) with a DNA mix containing 10% of a GFP plasmid and 90% of either of the pBK-CMV plasmid constructs that code for human α_{1Ga}, α_{1Ia} and α_{1H} T-channel isoforms (Cribbs *et al*., 1998; Monteil *et al*., 2000b) or a 1:1:1 ratio of α_{1C}, β_{1b} and $\alpha_2\delta$1b subunits (Tomlinson *et al*., 1993). When indicated, 50 μM of the kinase inhibitor 1-(5-isoquinolinesulfonyl)-2-methyl-piperazine (H7, Sigma), 100 nM

PMA (Sigma) or 250 μM aristolochic acid (Sigma) were added in the culture medium 16 h prior to the electrophysiological recordings. For NG 108-15 experiments, cells between 10 and 17 passages were plated at low confluence (~0%) and differentiation was induced 5–10 days before recordings by decreasing the serum content in the medium to 1% and by adding 1 mM cAMP (Sigma).

Electrophysiology

Whole-cell currents were recorded at room temperature as previously described (Chemin *et al*., 2001). Extracellular solution contained (in mM): 2 $CaCl_2$, 160 TEACl and 10 HEPES (pH to 7.4 with TEAOH). Pipettes have a resistance of 1–3 MΩ when filled with a solution containing (in mM): 110 CsCl, 10 EGTA, 10 HEPES, 3 Mg-ATP and 0.6 GTP (pH to 7.2 with CsOH). When indicated, 0.6 mM GTP was substituted with 1 mM GDPβS (Sigma). For inside-out patch experiments, a sylgard-coated pipette has a resistance of 8–13 MΩ when filled with a solution containing (in mM): 100 $BaCl_2$, 10 HEPES (pH to 7.2 with TEAOH). Membrane potential was reduced towards 0 mV by bathing the cells in solution containing (in mM): 140 K gluconate, 10 EGTA, 10 glucose, 1 $MgCl_2$, 10 HEPES (pH to 7.3 with KOH). The sampling frequency for acquisition was 10 kHz and data were filtered at 2 kHz. For action potential clamp studies, a thalamo-cortical relay cell firing activity generated by the NEURON model (Hines and Carnevale, 1997) was used in the configuration of a three-compartment model of burst behaviour (Destexhe *et al*., 1998) available at Yale University database (http://senselab.med.yale.edu/senselab/neurondb). With this model, a firing activity produced by a 0.3 nA current injection in the soma during 700 ms was converted into a pCLAMP stimulation file. In this case, leak and capacitive currents were subtracted using a P/–5 procedure. Electrophysiological analysis was performed as previously described (Chemin *et al*., 2001). Student's *t*-tests were used to compare the different values, and were considered significant at $P < 0.05$. Results are presented as the mean ± SEM, and *n* is the number of cells used.

Pharmacological agents

Anandamide solution (10 mg/ml) and its vehicle were obtained from Tocris. WIN 55,212-2, CP 55,490 and HU-210 (Tocris) were dissolved at 50 mM in dimethylsulfoxide (DMSO) and AM404 (Tocris) was dissolved at 50 mM in ethanol. 2-AG, R(+)-methanandamide (RBI) and Δ^9-THC (Sigma) were dissolved at 20 mM in ethanol. SR141716A (10 mM in DMSO) was a gift from Sanofi Recherche (Montpellier, France). Dilutions were prepared daily from these stocks. Solvents were included in all control solutions. Drugs were applied to cells by a gravity-driven home-made perfusion device, controlled by solenoid valves.

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