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Anandamide transport inhibitor AM404 and structurally related compounds inhibit synaptic transmission between rat hippocampal neurons in culture independent of cannabinoid CB_1 receptors

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Abstract

N-(hydroxyphenyl)-arachidonamide (AM404) is an inhibitor of endocannabinoid transport. We examined the effects of AM404 on glutamatergic synaptic transmission using network-driven increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]$ spikes) as an assay. At a concentration of 1 μM AM404 inhibited $[Ca^{2+}]_i$ spiking by 73 ± 8%. The cannabinoid CB_1 receptor antagonist *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride (SR141716A), the vanilloid VR_1 receptor antagonist capsazepine (CPZ), and treatment with pertussis toxin failed to block AM404-mediated inhibition. AM404 (3 μM) inhibited action-potential-evoked Ca^{2+} influx by 58 ± 3% but failed to affect calcium influx evoked by depolarization with 30 mM K^+, suggesting that the inhibition of electrically evoked $[Ca^{2+}]_i$ increases and that $[Ca^{2+}]_i$ spiking was due to inhibition of Na^+ channels. Palmitoylethanolamide (PMEA), capsaicin (CAP) and (5Z,8Z,11Z,14Z)-*N*-(4-hydroxy-2-methylphenyl)-5,8,11,14-eicosatetraenamide (VDM11), compounds structurally similar to AM404, inhibited $[Ca^{2+}]_i$ spiking by 34 ± 10%, 42 ± 18% and 67 ± 12%, respectively. Thus, AM404 and related compounds inhibit depolarization-induced Ca^{2+} influx independent of cannabinoid receptors, suggesting caution when using these agents as pharmacological probes to study synaptic transmission.

Keywords: AM404; Excitatory synaptic transmission; *N*-acetyl ethanolamide; CB_1 receptor; Cannabinoid; Hippocampus

1. Introduction

Cannabinoids act on inhibitory G-protein-coupled receptors primarily located on neurons (CB_1) and cells of the immune system (CB_2; Howlett, 1998). Agonist binding to cannabinoid CB_1 receptors results in activation of K^+ channels and members of the mitogen-activated protein kinase family and in inhibition of adenylyl cyclase and voltage-gated Ca^{2+} channels (Porter and Felder, 2001). Several endogenous ligands for these receptors have been identified including arachidonoyl ethanolamide (anandamide), 2-arachidonoyl glycerol (2-AG) and noladin ether (Fowler, 2003). Endocannabinoids are thought to modulate neuronal activity via depolarization-induced suppression of excitatory (DSE; Kreitzer and Regehr, 2001) or inhibitory neurotransmission (DSI; Wilson and Nicoll, 2001). This phenomenon is initiated by a depolarization-induced elevation in the postsynaptic intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) resulting in the production of endocannabinoids, which diffuse in a retrograde manner to act on presynaptic cannabinoid CB_1 receptors to inhibit neurotransmitter release. Activation of metabotropic glutamate receptors and muscarinic acetylcholine receptors also produce endocannabinoids (Kim et al., 2002; Maejima et al., 2001). The endogenous cannabinoid system modulates synaptic plasticity, neurodegeneration, pain, inflammation and addiction (Lichtman et al., 2002; Shen and Thayer, 1998b; Sinor et al., 2000; Walker and Huang, 2002; Wilson and Nicoll, 2001).

Endocannabinoid signaling is postulated to be terminated by carrier-mediated reuptake, and catabolism of the ligand by fatty acid amino hydrolase and monoglyceride lipase (Dinh et al., 2002; Freund et al., 2003). A specific transporter for endocannabinoids has yet to be identified although the

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reuptake process has been characterized as rapid, temperature-dependent and saturable, characteristics of carrier mediated transport (Beltramo et al., 1997). *N*-(hydroxyphenyl)-arachidonamide (AM404) inhibits endocannabinoid transport across cell membranes (Beltramo et al., 1997) and thus potentiates the cannabinoid CB_1 receptor-mediated effects of endocannabinoids (Beltramo et al., 2000; Calignano et al., 1997).

There are several examples of AM404 acting at sites other than the endocannabinoid system. AM404 activates the vanilloid VR_1 receptor (Zygmunt et al., 2000), increases Ca^{2+} in Madin Darby Canine Kidney cells (Chen et al., 2001), inhibits proliferation of C6 glioma cells (Jonsson et al., 2003) and inhibits Na^+ channels (Nicholson et al., 2003).

We examined the effect of AM404 on synaptic transmission between cultured hippocampal neurons. AM404 inhibited synaptically driven $[Ca^{2+}]_i$ spiking, but not via the cannabinoid CB_1 receptor. AM404 and structurally similar compounds attenuated synaptic transmission by inhibiting action-potential-mediated Ca^{2+} influx, most likely by acting on voltage-gated Na^+ channels.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle media (DMEM) and sera were purchased from Gibco-BRL (Grand Island, NY, USA). Indo-1 and Indo-5F were obtained from Molecular Probes (Eugene, OR, USA). AM404, (5Z,8Z,11Z,14Z)-*N*-(4-Hydroxy-2-methylphenyl)-5,8,11,14-eicosatetraenamide (VDM11) and capsazepine (CPZ) were purchased from Tocris (Ellisville, MO). All other reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture

Rat hippocampal neurons were grown in primary culture as described previously (Wang et al., 1994), with minor modifications. Fetuses were removed on embryonic day 17 from maternal rats anesthetized with CO_2 and sacrificed by decapitation, according to a protocol approved by the University of Minnesota Institutional Animal Care and Use Committee. Hippocampi were dissected and placed in Ca^{+2}- and Mg^{+2}-free HEPES-buffered Hanks salt solution (HHSS), pH 7.45. HHSS was composed of the following: 20 mM HEPES, 137 mM NaCl, 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 5.0 mM KCl, 0.4 mM KH_2PO_4, 0.6 mM Na_2HPO_4, 3.0 mM $NaHCO_3$ and 5.6 mM glucose. Cells were dissociated by titration through a 5-ml pipette and a flame-narrowed Pasteur pipette. Cells were pelleted and resuspended in DMEM without glutamine, supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/ml and 100 mg/ml, respectively). Dissociated cells were then plated at a density of 25,000–50,000 cells/well onto 25-mm round cover glasses that had been coated with poly-D-lysine (0.1 mg/ml) and were washed with H_2O. Neurons were grown in a humidified atmosphere of 10% CO_2 and 90% air (pH, 7.4) at 37 °C, and fed every 7 days by exchange of 30% of the media with DMEM supplemented with 10% horse serum and penicillin/streptomycin. Cells used in these experiments were cultured without mitotic inhibitors for a minimum of 12 days.

2.3. $[Ca^{+2}]_i$ measurement

$[Ca^{+2}]_i$ was determined using a previously described dual-emission microfluorimeter (Werth and Thayer, 1994) to monitor indo-1 (Grynkiewicz et al., 1985). Cells were loaded with 2 μM indo-1 acetoxymethyl ester or 2 μM indo-5F acetoxymethyl ester for 45 min at 37 °C in HHSS containing 0.5% bovine serum albumin (BSA). Loaded cells were placed in a flow-through chamber (Thayer et al., 1988), and experiments were performed at room temperature (22 °C). The chamber was mounted on an inverted microscope, and cells were superfused with HHSS containing 10 μM glycine at a rate of 1–2 ml/min for 15 min before starting an experiment. Bovine serum albumin at a concentration of 0.1% was present in all solutions to prevent adherence of lipophilic compounds to the solution delivery system. Superfusion solutions were selected with a multiport valve coupled to several reservoirs. Depolarization-induced Ca^{2+} influx was elicited by changing the K^+ concentration in the superfusion solution from 5 to 30 mM, with K^+ substituted for Na^+ reciprocally. Action potentials were evoked by electric field stimulation as described previously (Piser et al., 1994). Lipophilic compounds were dissolved in dimethylsulfoxide (DMSO) and were diluted at least 1000-fold in HHSS.

For excitation of indo-1 and indo-5F, light from a 75-W Xe arc lamp was passed through a 350/10-nm band-pass filter (Omega Optical; Brattleboro, VT), reflected from a dichroic mirror (380 nm) and focused with a 70× phase–contrast oil immersion objective (Leitz, numerical aperture 1.15). Emitted light was reflected sequentially from dichroic mirrors (440 and 516 nm), through band-pass filters (405/20 and 495/20 nm, respectively), to photomultiplier tubes operating in photon-counting mode (Thorn EMI, Fairfield, NJ). Cells were illuminated with transmitted light (580-nm-long pass) and visualized with a video camera placed after the second emission dichroic. Recordings were defined spatially with a rectangular diaphragm. The 5-V photomultiplier output was integrated by passing the signal through an eight-pole Bessel filter at 2.5 Hz. This signal was input into two channels of an analog-to-digital converter (Indec Systems; Sunnyvale, CA) sampling at 1 Hz. After completion of each experiment, cells were wiped from the microscope field using a cotton-tipped applicator, and background light levels were determined (approximately

5% of cell counts). Autofluorescence from cells that had not been loaded with dye was not detectable.

After subtracting background, the 405/495 fluorescence ratio (R) was converted to $[Ca^{+2}]_i$ by the equation $[Ca^{+2}]_i = K_d\beta(R - R_{min})/(R_{max} - R)$. The K_d used for indo-1 was 250 nM, the K_d used for indo-5F was 470 nM and β was the emitted fluorescence at 495 nm, in the absence and presence calcium. R_{min}, R_{max} and β were determined in ionomycin-permeabilized cells in Ca^{+2}-free (1 mM EGTA) and 5 mM Ca^{+2} buffers. R_{min}, R_{max} and β were, respectively, 1.29, 14.1 and 3.70 for indo-1 and 0.8, 8.6 and 3.94 for indo-5F.

2.4. Data analysis

The frequency of $[Ca^{+2}]_i$ spiking was calculated from data collected during a 10-min window starting 5 min after changing the bath to 0.1 mM $[Mg^{+2}]_o$ for control ($F_{control}$) and from a 5-min window starting 5 min after drug application for drug-treated (F). Percent inhibition (I) was calculated by the formula $I=[(F_{control} - 2F)/(F_{control})] \times 100$. Data are presented as mean ± S.E. and significance was determined by unpaired Student's t-test. $P < 0.05$ was accepted as the level of significance.

3. Results

3.1. AM404 inhibits synaptic transmission

The effects of the anandamide transport inhibitor AM404 on excitatory neurotransmission were studied using network-driven $[Ca^{2+}]_i$ spikes as an index of glutamatergic synaptic transmission. Indo-1-based microfluorimetry was used to measure $[Ca^{2+}]_i$ spikes evoked by reducing the extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$) from 1 to 0.1 mM in the solution bathing rat hippocampal neurons (Werth et al., 1996). The frequency of $[Ca^{2+}]$ spiking accurately reflects the intensity of synaptic transmission as confirmed by evoked excitatory synaptic currents (McLeod et al., 1998).

AM404 inhibits endocannabinoid transport across cell membranes (Beltramo et al., 1997). Application of 1 μM AM404 inhibited $[Ca^{2+}]_i$ spiking by 73 ± 8% (Fig. 1A). However, coapplication of the cannabinoid CB_1 receptor antagonist *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride (SR141716A; 300 nM) did not block AM404-mediated inhibition of $[Ca^{2+}]_i$ spiking (Fig. 1B,C), indicating that the effect of AM404 was not mediated via the cannabinoid CB_1 receptor. This concentration of SR141716A blocked completely WIN55,212-2 [((*R*)-(+)-[2,3-dihydro-5-methyl-3-[4-morpholinylmethyl]-pyrrolo[1,2,3-de]1,4-benzoxazin-6-yl](1-naphthyl) methanone mesylate)]-mediated inhibition of $[Ca^{2+}]_i$ spiking (Shen and Thayer, 1998a). AM404 inhibited synaptic

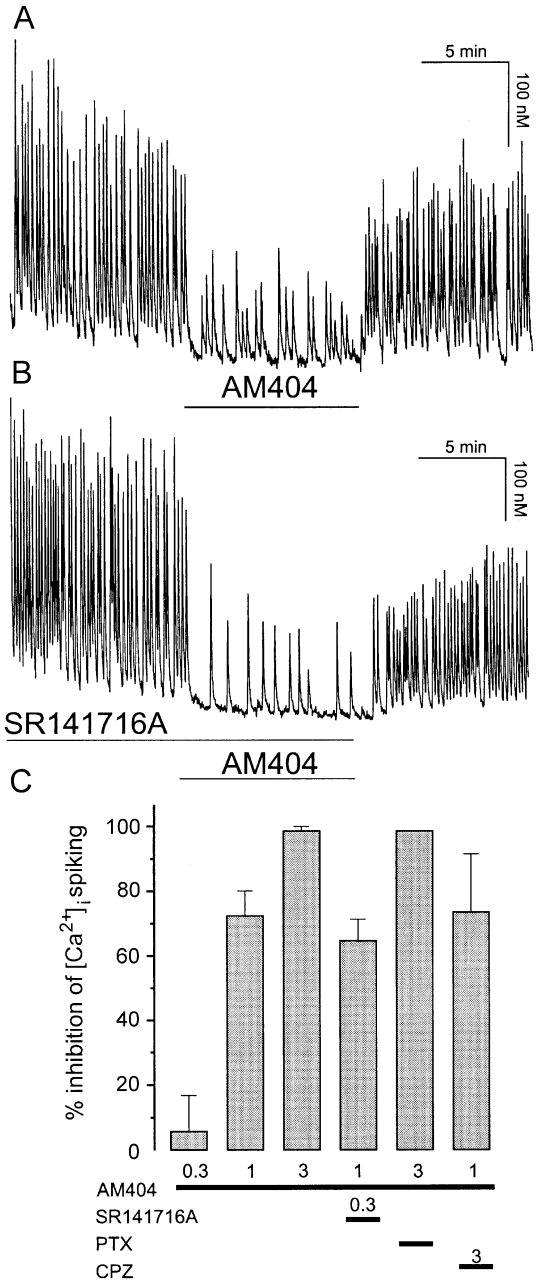

Fig. 1. AM404 inhibits synaptic transmission. $[Ca^{2+}]_i$ spiking induced by 0.1 mM $[Mg^{2+}]_o$ was recorded from single-hippocampal neurons as described under Materials and methods. Drugs were applied by superfusion at the times indicated by the horizontal bars. (A) Representative trace shows inhibition of $[Ca^{2+}]_i$ spiking when superfusion solution contained 1 μM AM404 for 10 min. (B) Pretreatment with SR141716A (0.3 μM) for 10 min failed to block the effects of 1 μM AM404. (C) Bar graph summarizes inhibition of the frequency of $[Ca^{2+}]_i$ spiking by superfusion of AM404 (shown in μM) in the presence of the indicated agents. AM404-mediated inhibition of $[Ca^{2+}]_i$ spiking was not blocked by superfusion of the cannabinoid CB_1 receptor antagonist SR141716A (0.3 μM, $p = 0.46$), the vanilloid VR_1 receptor antagonist capsazepine (CPZ; 3 μM) or by treatment with pertussis toxin (500 ng/ml), applied 24 h prior to recording. Data plotted are mean ± S.E. of 3–6 experiments.

transmission in a concentration-dependent manner (Fig. 1C), with a maximally effective concentration of 3 μM, producing 99 ± 1% inhibition of $[Ca^{2+}]_i$ spiking. Vehicle (0.1% DMSO) did not significantly affect $[Ca^{2+}]_i$ spiking frequency ($n = 4$).

Cannabimimetic compounds have been reported to act at sites on central neurons in addition to cannabinoid CB_1 receptors (Chemin et al., 2001; Nicholson et al., 2003; Poling et al., 1996; Zygmunt et al., 2000). To determine whether inhibitory G-proteins were involved, hippocampal cultures were treated with pertussis toxin (500 ng/ml) for 18 to 24 h. In pertussis toxin-treated neurons, AM404 (3 μM) inhibited $[Ca^{2+}]_i$ spiking by 100% thus eliminating the involvement of G_i-coupled receptors (Fig. 1C). This same treatment completely blocked the cannabinoid CB_1 receptor-mediated inhibition of $[Ca^{2+}]_i$ spiking produced by 100 nM WIN55,212-2 ($n = 7$). AM404 is an agonist at the vanilloid VR_1 receptor (Zygmunt et al., 2000). Coapplication of the vanilloid VR_1 receptor antagonist capsazepine (3 μM) also failed to block AM404-mediated inhibition of $[Ca^{2+}]_i$ spiking (Fig. 1C). These results show that, in addition to acting independent of cannabinoid CB_1 receptors, the inhibition of synaptic transmission by AM404 was not mediated by any inhibitory G-protein-coupled receptor or the vanilloid VR_1 receptor.

3.2. AM404 action at sodium channels

Synaptic transmission requires Ca^{2+} influx for neurotransmitter release (Augustine, 2001). We next examined the effects of AM404 on the $[Ca^{2+}]_i$ increase evoked by electrical field stimulation to determine whether the drug interfered directly with excitation–secretion coupling. Electric field stimulation was applied by passing current between two platinum electrodes placed on either side of the recording chamber. Electrically evoked Ca^{2+} influx requires functioning sodium channels as it is tetrodotoxin-sensitive (Piser et al., 1994). AM404(3μM) inhibited Ca^{2+} influx by 58 ± 3% (Fig. 2A). The inhibition of action-potential-evoked Ca^{2+} influx was dependent on the concentration of AM404 (Fig. 2B). These data suggest that AM404 inhibits the Ca^{2+} influx required to trigger neurotransmitter release. A recent report by Nicholson et al. (2003) suggested that anandamide analogs inhibited voltage-operated sodium channels. To determine whether Na^+ or Ca^{2+} channels were the site of AM404 action in this study, we investigated the effects of AM404 on depolarization-induced Ca^{2+} influx using the low-affinity $[Ca^{2+}]_i$-sensitive dye, indo-5F. Superfusion of HHSS containing 30 mM K^+ and 300 nM tetrodotoxin evoked Ca^{2+} influx independent of voltage-operated Na^+ channels (Fig. 2C). AM404 (3 μM) did not significantly affect 30-mM K^+-induced Ca^{2+} influx (Fig. 2D). The inhibition of action-potential-induced $[Ca^{2+}]_i$ responses and the failure to affect responses evoked by direct activation of voltage-gated Ca^{2+} channels suggest that AM404 inhibits voltage-operated Na^+ channels. Thus,

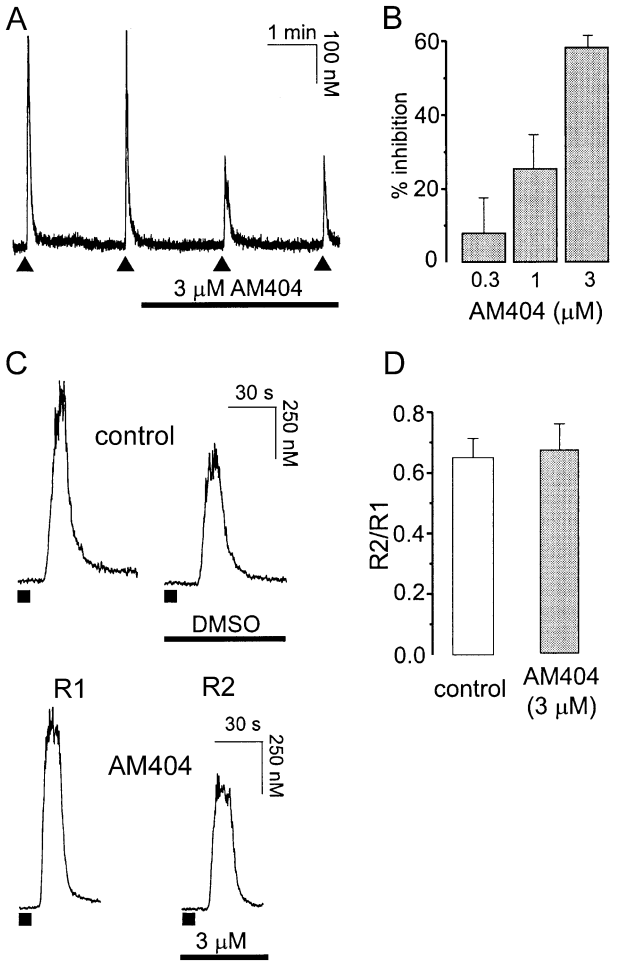

Fig. 2. AM404 inhibits Ca^{2+} influx via Na^+ channels. Increases in $[Ca^{2+}]_i$ were recorded from single-hippocampal neurons following a train of action potentials elicited by field stimulation or 30 mM $[K^+]_o$ as described under Materials and methods. Drugs were applied by superfusion at the times indicated by the horizontal bars. (A) Representative trace shows inhibition of electrically evoked (8 Hz, 3 s; ▲) Ca^{2+} influx when superfusion solution contained 3 μM AM404. (B) Bar graph summarizes the concentration-dependent inhibition of electrically evoked Ca^{2+} influx by AM404. (C) Representative traces show depolarization-induced (30 mM K^+, 30 s; ■) $[Ca^{2+}]_i$ increases in the presence of tetrodotoxin (300 nM). Twenty minutes following an initial control response (R1), a second depolarization-induced response (R2) was elicited in the presence of either vehicle or 3 μM AM404. (D) Bar graph summarizes the ratio of the amplitude of the second response (R2) to the first response (R1). Experiments in the presence of DMSO vehicle or AM404 were not significantly different. Data plotted are mean ± S.E. of 3–5 experiments.

AM404 blocks the depolarization required to activate voltage-gated Ca^{2+} channels and to trigger neurotransmitter release.

3.3. Structurally similar N-arachidonyl compounds inhibit neurotransmission

Lipid amide compounds have been shown to affect cell signaling through cannabinoid and noncannabinoid-mediated mechanisms (Berdyshev et al., 2001; Hansen et al.,

2002). We examined the effects of compounds structurally related to AM404 on $[Ca^{2+}]_i$ spiking (Fig. 3). Palmitoylethanolamide (PMEA) may interact with cannabinoid receptors at high concentrations (Calignano et al., 1998; Franklin et al., 2003; Griffin et al., 2000), and at a concentration of 3 μM, inhibited $[Ca^{2+}]_i$ spiking by 34 ± 9%. The vanilloid VR_1 receptor agonist capsaicin (CAP; 3 μM) inhibited $[Ca^{2+}]_i$ spiking 42 ± 18%. However, capsaicin inhibition of $[Ca^{2+}]_i$ spiking was not sensitive to the vanilloid VR_1 receptor antagonist capsazepine ($n = 4$). VDM11 is an anandamide transport inhibitor (De Petrocellis et al., 2000), and at a concentration of 3 μM, inhibited $[Ca^{2+}]_i$ spiking by 67 ± 12%. Arachidonic acid did not affect $[Ca^{2+}]_i$ spiking. These data show that lipid amides structurally similar to AM404 also inhibit synaptic transmission.

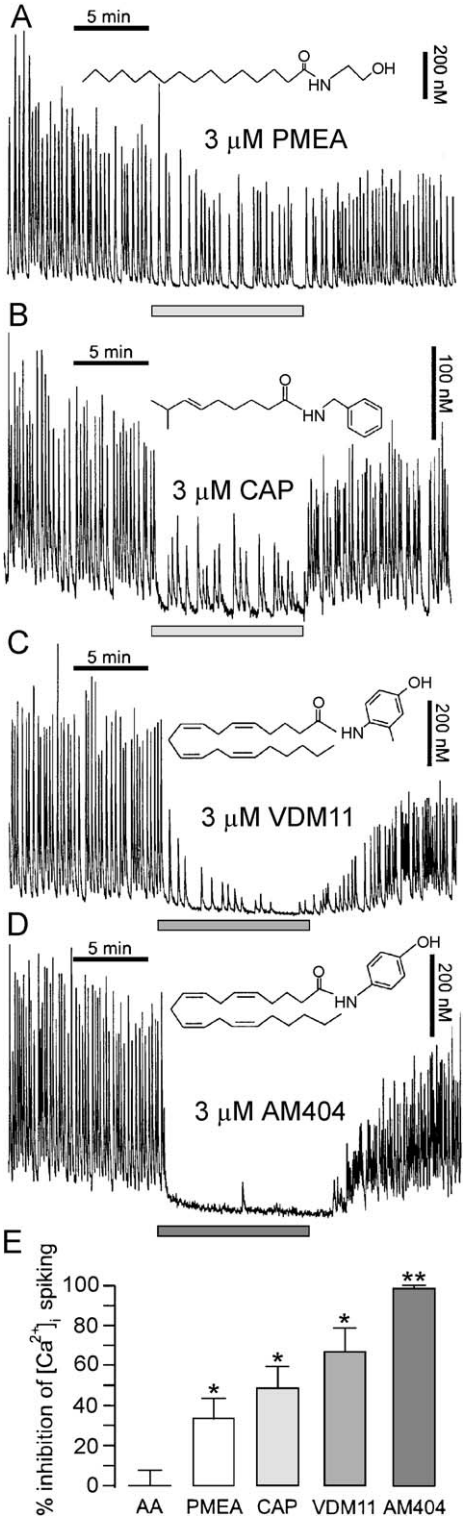

Fig. 3. Anandamide analogs inhibit synaptic transmission. 0.1 mM $[Mg^{2+}]_o$-induced $[Ca^{2+}]_i$ spiking was recorded from single-hippocampal neurons as described under Materials and methods. Drugs were applied by superfusion for 10 min at the times indicated by the horizontal bars. Representative traces show inhibition of $[Ca^{2+}]_i$ spiking by superfusion with 3 μM palmitoylethanolamide (PMEA; A), 3 μM capsaicin (CAP; B), 3 μM VDM11 (C) or 3 μM AM404 (D). (E) Bar graph summarizes inhibition of $[Ca^{2+}]_i$ spiking by anandamide analogs. At a concentration of 3 μM, $[Ca^{2+}]_i$ spiking was inhibited 34 ± 9% by PMEA, 42 ± 18% by capsaicin, 67 ± 12% by VDM11 and 99 ± 1% by AM404. Data plotted are mean ± S.E. of 3–6 experiments. $*P < 0.05$ and $**P < 0.001$ inhibition significantly greater than zero.

4. Discussion

The endocannabinoid transport inhibitor AM404 and structural analogs inhibited synaptic transmission between cultured hippocampal neurons. The effects of AM404 were not mediated by cannabinoid CB_1 receptors, vanilloid VR_1 receptors or inhibitory G-proteins but instead via inhibition of Na^+ channels. These results suggest that AM404 and structurally related compounds may have effects that mimic cannabinoid CB_1 receptor activation through a distinctly different mechanism. Thus, when using AM404 to modulate endocannabinoid signaling, the results should be validated by demonstrating block with a cannabinoid receptor antagonist.

AM404 inhibited $[Ca^{2+}]_i$ spiking in a concentration-dependent manner, with essentially complete inhibition achieved by a concentration of 3 μM. The observed inhibition was insensitive to the cannabinoid CB_1 receptor antagonist SR141716A. This result is in contrast to previous reports in which the actions of AM404 were blocked by SR141716A and thus attributed to cannabinoid CB_1 receptor activation secondary to reduced endocannabinoid reuptake (Beltramo et al., 2000; Calignano et al., 1997). AM404 inhibits endocannabinoid transport with an IC_{50} of 1 μM in neurons (Beltramo et al., 1997) and is used to study endocannabinoid signaling at concentrations in the 10 μM range (Huang et al., 2003; Steffens et al., 2003). Thus, the noncannabinoid-receptor-mediated effects shown here could complicate the interpretation of some studies of endocannabinoid signaling. The inhibition of $[Ca^{2+}]_i$ spiking by AM404 was not prevented by treatment with pertussis toxin ruling out the possible contribution of a third cannabinoid receptor subtype (Hajos et al., 2001) or the possibility of a novel *N*-acyl fatty acid-sensitive receptor coupled to inhib-

itory G-proteins. AM404 activates vanilloid VR_1 receptors (Zygmunt et al., 2000), although the inhibition of Ca^{+2} spiking described here was unaffected by the vanilloid VR_1 receptor antagonist capsazepine. It is possible that AM404 acted indirectly by elevating endocannabinoid levels at some noncannabinoid receptor site of action. However, the $[Ca^{2+}]_i$ spiking assay used here is inhibited by endocannabinoids via a cannabinoid CB_1 receptor-dependent mechanism (Shen et al., 1996), suggesting that if endocannabinoid levels were elevated by AM404, a cannabinoid CB_1 receptor antagonist would have blocked the response. We conclude that AM404 inhibited synaptic transmission by a previously unreported mechanism.

Direct action of cannabimimetics on ion channels has been previously described (Chemin et al., 2001; Shen and Thayer, 1998a). In this study, we found that AM404 inhibited electrically evoked Ca^{2+} influx in a concentration-dependent manner. At a concentration of 3 μM, AM404 inhibited electrically evoked Ca^{2+} influx by $58 \pm 3\%$, whereas the same concentration resulted in $99 \pm 1\%$ inhibition of synaptic transmission in the $[Ca^{2+}]_i$ spiking assay. A partial reduction of Ca^{2+} influx is sufficient for complete block of synaptic transmission due to a power function greater than 1, relating $[Ca^{2+}]_i$ to neurotransmitter release (Dodge and Rahamimoff, 1967; Mintz et al., 1995; Shen and Thayer, 1998a). AM404 did not affect the 30 mM K^+-induced $[Ca^{2+}]_i$ increase in the presence of the Na^+ channel blocker tetrodotoxin. This result suggests that the observed inhibition of electrically evoked Ca^{2+} responses and inhibition of $[Ca^{2+}]_i$ spiking are due to AM404-blocking Na^+ channels and the depolarization required for Ca^{2+} influx in these models. This finding is in good agreement with a report by Nicholson et al. (2003) in which AM404 inhibited depolarization and neurotransmitter release from synaptosomes induced by the Na^+ channel activator veratridine (Nicholson et al., 2003). In that study, AM404 inhibited veratridine-induced depolarization with an IC_{50} of 9.3 μM and demonstrated maximal effect at 50 μM. In the same study, AM404 inhibited release of L-glutamate with an IC_{50} of 1.6 μM and γ-aminobutyric acid release with an IC_{50} of 3.3 μM. Although higher concentrations of AM404 were used to inhibit depolarization, the reported effects of AM404 on neurotransmitter release are in good agreement with the low micromolar potency for AM404 inhibition of $[Ca^{2+}]_i$ spiking described here.

Compounds structurally similar to anandamide, but without activity at cannabinoid CB_1 receptors, inhibited synaptic transmission. Evidence that anandamide and its analogs modulate ion channels independent of cannabinoid CB_1 receptors or vanilloid VR_1 receptors is growing. Anandamide (Chemin et al., 2001), capsaicin (Fischer et al., 2001; Sim et al., 2001) and the anandamide–capsaicin hybrid, *N*-arachidonoyl-vanillyl-amine (arvanil) (Lo et al., 2003) all inhibit voltage-gated Ca^{2+} channels. Anandamide was also reported to directly inhibit Shaker-related K^+ channels (Poling et al., 1996) and recently, Na^+ channels (Nicholson et al., 2003). Previous work from our lab reported that high concentrations of the synthetic cannabinoid WIN55,212-2 directly inhibits Ca^{2+} channels (Shen and Thayer, 1998a). The antiproliferative effects of anandamide and its analogs appear to result from a mechanism independent of cannabinoid CB_1 receptor activation (Berdyshev et al., 2001; Hansen et al., 2002; Jonsson et al., 2003). In addition, palmitoylethanolamide was reported to have analgesic properties not attributed to the activation of cannabinoid CB_1 receptors (Jaggar et al., 1998). In summary, our results present further evidence that anandamide analogs have significant effects on neuronal function independent of cannabinoid receptor activation, yet mimic the receptor-mediated inhibition of synaptic transmission.

Inhibitors of carrier-mediated transport of endocannabinoids have been used as pharmacological tools to investigate endogenous cannabinoid action. A cautious approach should be taken in interpreting results from such studies as evidence for noncannabinoid-receptor-mediated action of these compounds grows.

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References

Augustine, G.J., 2001. How does calcium trigger neurotransmitter release? Curr. Opin. Neurobiol. 11, 320–326.

Beltramo, M., Stella, N., Calignano, A., Lin, S.Y., Makriyannis, A., Piomelli, D., 1997. Functional role of high-affinity anandamide transport, as revealed by selective inhibition. Science 277, 1094–1097.

Beltramo, M., de Fonseca, F.R., Navarro, M., Calignano, A., Gorriti, M.A., Grammatikopoulos, G., Sadile, A.G., Giuffrida, A., Piomelli, D., 2000. Reversal of dopamine D(2) receptor responses by an anandamide transport inhibitor. J. Neurosci. 20, 3401–3407.

Berdyshev, E.V., Schmid, P.C., Krebsbach, R.J., Hillard, C.J., Huang, C., Chen, N., Dong, Z., Schmid, H.H., 2001. Cannabinoid-receptor-independent cell signaling by *N*-acylethanolamines. Biochem. J. 360, 67–75.

Calignano, A., Larana, G., Beltramo, M., Makriyannis, A., Piomelli, D., 1997. Potentiation of anandamide hypotension by the transport inhibitor, AM404. Eur. J. Pharmacol. 337, R1–R2.

Calignano, A., La Rana, G., Giuffrida, A., Piomelli, D., 1998. Control of pain initiation by endogenous cannabinoids. Nature 394, 277–281.

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

Chen, W.C., Huang, J.K., Cheng, J.S., Tsai, J.C.R., Chiang, A.J., Chou, K.J., Liu, C.P., Jan, C.R., 2001. AM-404 elevates renal intracellular Ca^{2+}, questioning its selectivity as a pharmacological tool for investigating the anandamide transporter. J. Pharmacol. Toxicol. Methods 45, 195–198.

De Petrocellis, L., Bisogno, T., Davis, J.B., Pertwee, R.G., Di Marzo, V., 2000. Overlap between the ligand recognition properties of the anandamide transporter and the VR_1 vanilloid receptor: inhibitors of anandamide uptake with negligible capsaicin-like activity. FEBS Lett. 483, 52–56.

Dinh, T.P., Carpenter, D., Leslie, F.M., Freund, T.F., Katona, I., Sensi, S.L., Kathuria, S., Piomelli, D., 2002. Brain monoglyceride lipase participating in endocannabinoid inactivation. Proc. Natl. Acad. Sci. U. S. A. 99, 10819–10824.

Dodge, F.A., Rahamimoff, R., 1967. Co-operative action of calcium ions in transmitter release at the neuromuscular junction. J. Physiol. 193, 419–432.

Fischer, B.S., Qin, D.M., Kim, K., McDonald, T.V., 2001. Capsaicin inhibits Jurkat T-cell activation by blocking calcium entry current I-CRAC. J. Pharmacol. Exp. Ther. 299, 238–246.

Fowler, C.J., 2003. Plant-derived, synthetic and endogenous cannabinoids as neuroprotective agents—Non-psychoactive cannabinoids, 'entourage' compounds and inhibitors of *N*-acyl ethanolamine breakdown as therapeutic strategies to avoid psychotropic effects. Brain Res. Rev. 41, 26–43.

Franklin, A., Parmentier-Batteur, S., Walter, L., Greenberg, D.A., Stella, N., 2003. Palmitoylethanolamide increases after focal cerebral ischemia and potentiates microglial cell motility. J. Neurosci. 23, 7767–7775.

Freund, T.F., Katona, I., Piomelli, D., 2003. Role of endogenous cannabinoids in synaptic signaling. Physiol. Rev. 83, 1017–1066.

Griffin, G., Tao, Q., Abood, M.E., 2000. Cloning and pharmacological characterization of the rat CB_2 cannabinoid receptor. J. Pharmacol. Exp. Ther. 292, 886–894.

Grynkiewicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

Hajos, N., Ledent, C., Freund, T.F., 2001. Novel cannabinoid-sensitive receptor mediates inhibition of glutamatergic synaptic transmission in the hippocampus. Neuroscience 106, 1–4.

Hansen, H.S., Moesgaard, B., Petersen, G., Hansen, H.H., 2002. Putative neuroprotective actions of *N*-acyl-ethanolamines. Pharmacol. Ther. 95, 119–126.

Howlett, A.C., 1998. The CB_1 cannabinoid receptor in the brain. Neurobiol. Dis. 5, 405–416.

Huang, Y.-C., Wang, S.-J., Chiou, L.-C., Gean, P.-W., 2003. Mediation of amphetamine-induced long-term depression of synaptic transmission by CB_1 cannabinoid receptors in the rat amygdala. J. Neurosci. 23, 10311–10320.

Jaggar, S.I., Hasnie, F.S., Sellaturay, S., Rice, A.S., 1998. The anti-hyperalgesic actions of the cannabinoid anandamide and the putative CB_2 receptor agonist palmitoylethanolamide in visceral and somatic inflammatory pain. Pain 76, 189–199.

Jonsson, K.O., Andersson, A., Jacobsson, S.O., Vandevoorde, S., Lambert, D.M., Fowler, C.J., 2003. AM404 and VDM 11 non-specifically inhibit C6 glioma cell proliferation at concentrations used to block the cellular accumulation of the endocannabinoid anandamide. Arch. Toxicol. 77, 201–207.

Kim, J., Isokawa, M., Ledent, C., Alger, B.E., 2002. Activation of muscarinic acetylcholine receptors enhances the release of endogenous cannabinoids in the hippocampus. J. Neurosci. 22, 10182–10191.

Kreitzer, A.C., Regehr, W.G., 2001. Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto Purkinje cells. Neuron 29, 717–727.

Lichtman, A.H., Varvel, S.A., Martin, B.R., 2002. Endocannabinoids in cognition and dependence. Prostaglandins, Leukot. Essent. Fat. Acids 66, 269–285.

Lo, Y.K., Chiang, H.T., Wu, S.N., 2003. Effect of arvanil (*N*-arachidonoyl-vanillyl-amine), a nonpungent anandamide–capsaicin hybrid, on ion currents in NG108-15 neuronal cells. Biochem. Pharmacol. 65, 581–591.

Maejima, T., Hashimoto, K., Yoshida, T., Aiba, A., Kano, M., 2001. Presynaptic inhibition caused by retrograde signal from metabotropic glutamate to cannabinoid receptors. Neuron 31, 463–475.

McLeod Jr., J.R., Shen, M., Kim, D.J., Thayer, S.A., 1998. Neurotoxicity mediated by aberrant patterns of synaptic activity between rat hippocampal neurons in culture. J. Neurophysiol. 80, 2688–2698.

Mintz, I.M., Sabatini, B.L., Regehr, W.G., 1995. Calcium control of transmitter release at a cerebellar synapse. Neuron 15, 675–688.

Nicholson, R.A., Liao, C., Zheng, J., David, L.S., Coyne, L., Errington, A.C., Singh, G., Lees, G., 2003. Sodium channel inhibition by anandamide and synthetic cannabimimetics in brain. Brain Res. 978, 194–204.

Piser, T.M., Lampe, R.A., Keith, R.A., Thayer, S.A., 1994. ω-Grammotoxin blocks action-potential-induced Ca^{2+} influx and whole-cell Ca^{2+} current in rat dorsal root ganglion neurons. Pfluger's Arch. Eur. J. Physiol. 426, 214–220.

Poling, J.S., Rogawski, M.A., Salem, N., Vicini, S., 1996. Anandamide, an endogenous cannabinoid, inhibits shaker-related voltage-gated K^+ channels. Neuropharmacology 35, 983–991.

Porter, A.C., Felder, C.C., 2001. The endocannabinoid nervous system: unique opportunities for therapeutic intervention. Pharmacol. Ther. 90, 45–60.

Shen, M., Thayer, S.A., 1998a. The cannabinoid agonist Win55,212-2 inhibits calcium channels by receptor-mediated and direct pathways in cultured rat hippocampal neurons. Brain Res. 783, 77–84.

Shen, M., Thayer, S.A., 1998b. Cannabinoid receptor agonists protect cultured rat hippocampal neurons from excitotoxicity. Mol. Pharmacol. 54, 459–462.

Shen, M., Piser, T.M., Seybold, V.S., Thayer, S.A., 1996. Cannabinoid receptor agonists inhibit glutamatergic synaptic transmission in rat hippocampal cultures. J. Neurosci. 16, 4322–4334.

Sim, J.H., Kim, Y.C., Kim, S.J., Lee, S.J., Suh, S.H., Jun, J.Y., So, I., Kim, K.W., 2001. Capsaicin inhibits the voltage-operated calcium channels intracellularly in the antral circular myocytes of guinea-pig stomach. Life Sci. 68, 2347–2360.

Sinor, A.D., Irvin, S.M., Greenberg, D.A., 2000. Endocannabinoids protect cerebral cortical neurons from in vitro ischemia in rats. Neurosci. Lett. 278, 157–160.

Steffens, M., Szabo, B., Klar, M., Rominger, A., Zentner, J., Feuerstein, T.J., 2003. Modulation of electrically evoked acetylcholine release through cannabinoid CB_1 receptors: evidence for an endocannabinoid tone in the human neocortex. Neuroscience 120, 455–465.

Thayer, S.A., Sturek, M., Miller, R.J., 1988. Measurement of neuronal Ca^{2+} transients using simultaneous microfluorimetry and electrophysiology. Pfluger's Arch. Eur. J. Pharmacol. 412, 216–223.

Walker, J.M., Huang, S.M., 2002. Endocannabinoids in pain modulation. Prostaglandins Leukot. Essent. Fat. Acids 66, 235–242.

Wang, G.J., Randall, R.D., Thayer, S.A., 1994. Glutamate-induced intracellular acidification of cultured hippocampal neurons demonstrates altered energy metabolism resulting from Ca^{2+} loads. J. Neurophysiol. 72, 2563–2569.

Werth, J.L., Thayer, S.A., 1994. Mitochondria buffer physiological calcium loads in cultured rat dorsal root ganglion neurons. J. Neurosci. 14, 348–356.

Werth, J.L., Usachev, Y.M., Thayer, S.A., 1996. Modulation of calcium efflux from cultured rat dorsal root ganglion neurons. J. Neurosci. 16, 1008–1015.

Wilson, R.I., Nicoll, R.A., 2001. Endogenous cannabinoids mediate retrograde signaling at hippocampal synapses. Nature 410, 588–592.

Zygmunt, P.M., Chuang, H.H., Movahed, P., Julius, D., Hogestatt, E.D., 2000. The anandamide transport inhibitor AM404 activates vanilloid receptors. Eur. J. Pharmacol. 396, 39–42.