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# Endocannabinoid transport tightly controls 2-arachidonoyl glycerol actions in the hippocampus: effects of low temperature and the transport inhibitor AM404

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## Abstract

The control of endocannabinoid actions on cortical neurons by a putative carrier-mediated uptake is still poorly understood at the level of synaptic transmission. We investigated the effect of an endocannabinoid, 2-arachidonoyl glycerol (2-AG), on inhibitory postsynaptic currents (IPSCs) in hippocampal slices under physiological conditions, and when uptake was altered by a selective blocker or lower temperature. Bath application of 2-AG (20  $\mu$ M) caused a 40% reduction in the amplitude of IPSCs evoked in the perisomatic region of CA1 pyramidal neurons at room temperature; this effect could be blocked by a specific CB<sub>1</sub> receptor antagonist, AM251. By contrast, a smaller (20%) but significant suppression of inhibitory transmission was found by 2-AG at 33–35 °C. This reduced blocking effect at physiological temperature could be brought back to 40% by coapplying the endocannabinoid uptake blocker, AM404 (10 or 20  $\mu$ M) with 2-AG. In parallel experiments, we measured [<sup>3</sup>H]2-AG uptake at different temperatures in primary cultures prepared from cortical neurons. These data confirmed a striking inhibition of [<sup>3</sup>H]2-AG uptake at room temperature compared with values observed at 37 °C. Uptake could be significantly modified by anandamide, 2-AG and AM404, suggesting a common transporter for the two endocannabinoids. These findings together demonstrate the presence of an effective endocannabinoid uptake in cortical neurons, which could considerably alter the spatial and temporal constraints of endocannabinoid signalling at physiological temperature, and which may critically change the interpretation of findings at room temperature.

## Introduction

The brain endocannabinoid system consists of signal molecules (endocannabinoids), enzymes for their synthesis and degradation, specific cell-surface receptors and a putative transport system (see for review Freund *et al.*, 2003). Most studies have investigated the functional roles of this signalling system in normal and pathological neuronal activity using plant-derived or synthetic cannabinoid agonists such as delta-9-tetrahydrocannabinol or WIN55,212-2. The main advantage of these compounds is that they are resistant to deactivation, whereas endocannabinoid substances are rapidly eliminated by uptake and intracellular hydrolysis (Freund *et al.*, 2003). Two endocannabinoids have been extensively characterized – anandamide and 2-arachidonoyl glycerol (2-AG). Both activate CB<sub>1</sub> cannabinoid receptors (Devane *et al.*, 1992; Sugiura *et al.*, 1995) and are taken up by neurons and astrocytes (Beltramo *et al.*, 1997; Beltramo & Piomelli, 2000; Hillard & Jarrarian, 2000; Bisogno *et al.*, 2001) *en route* to intracellular degradation by distinct serine hydrolase enzymes: anandamide is broken down by fatty acid amide hydrolase (FAAH), and 2-AG by a monoglyceride lipase (MGL) (Cravatt *et al.*, 1996; Dinh *et al.*, 2002).

In the hippocampus, the endocannabinoid system is thought to play a role in important signalling events such as depolarization-induced

suppression of inhibition (DSI; Ohno-Shosaku *et al.*, 2001; Wilson & Nicoll, 2001) or regulation of neuronal excitability via long-term depression of inhibition (I-LTD; Chevaleyre & Castillo, 2003). In these events, the endocannabinoids may act as retrograde messengers, suppressing GABA release and inhibitory postsynaptic currents by engaging CB<sub>1</sub> receptors present on axon terminals of cholecystokinin-containing GABAergic interneurons (Katona *et al.*, 1999; Hájos *et al.*, 2000).

The clearance of endocannabinoids via uptake and/or degradation is a critical factor in determining the spatial and temporal constraints of their actions. The time-course of DSI is considerably shorter at physiological temperature than at room temperature (Kreitzer & Regehr, 2001), which may reflect the temperature dependence of uptake (Vizi, 1998). By contrast, the induction of I-LTD requires the presence of 2-AG for at least 5–10 min, which may be possible if uptake is slowed by subphysiological temperature. Indeed, the I-LTD experiments of Chevaleyre & Castillo (2003) were carried out at 25 °C. For a better prediction of the significance of endocannabinoid-mediated phenomena under physiological conditions, the temperature dependence of 2-AG uptake and action on GABAergic currents should be investigated.

In the present report, we have used freshly dissected slices of rat hippocampus to investigate the effects of ambient temperature and endocannabinoid transport blockade on the ability of 2-AG to inhibit GABAergic transmission. We have selected 2-AG for these experiments because of its higher abundance in the hippocampus relative to

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anandamide (Stella *et al.*, 1997) and its possible roles in I-LTD (Chevaleyre & Castillo, 2003). Moreover, we have used primary cultures of rat cortical neurons to examine how ambient temperature affects [<sup>3</sup>H]2-AG transport.

## Materials and methods

Experiments were carried out in accordance with the guidelines of the institutional ethical codex and the Hungarian Act of Animal Care and Experimentation (1998. XXVIII. section 243/1998), which is in full agreement with the regulation of animal experiments in the European Union. All efforts were made to minimize the number of animals used.

### Electrophysiology

Male Wistar rats (16–22 days old) were deeply anaesthetized with isoflurane and decapitated. After opening the skull, the brain was quickly removed and immersed in ice-cold (~4 °C) modified artificial cerebrospinal fluid (ACSF), which contained (in mM): 252 sucrose, 2.5 KCl, 26 NaHCO<sub>3</sub>, 2 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub> and 10 glucose. Horizontal slices of the hippocampus (350–400 μm in thickness) were prepared using a Leica VT1000S Vibratome. The slices were then incubated in ACSF containing 126 mM NaCl instead of sucrose at room temperature for at least 1 h before recordings. Whole-cell patch-clamp recordings were obtained at 22–25 °C or at 33–35 °C from CA1 pyramidal cells visualized by infrared DIC videomicroscopy (Zeiss Axioscope, Germany) using a submerged type chamber. The extracellular solution had a composition of (in mM): 126 NaCl, 2.5 KCl, 26 NaHCO<sub>3</sub>, 2 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub> and 10 glucose, and the intrapipette solution contained (in mM): 80 CsCl, 60 Cs-gluconate, 3 NaCl, 1 MgCl<sub>2</sub>, 10 HEPES, 2 Mg-ATP and 5 QX-314 (pH 7.2–7.3 adjusted with CsOH; osmolarity 275–290 mOsm). Patch electrodes were pulled from borosilicate glass capillaries with an inner filament (1.5 mm O.D.; 1.1 mm I.D., Sutter Instruments Co., CA, USA, or 1.5 mm O.D.; 1.12 mm I.D., Hilgenberg, Germany) using a Sutter P-87 puller, and had resistances of 3–6 MΩ when filled with the intrapipette solution. Access resistances (between 4 and 15 MΩ, compensated 70–75%) were frequently monitored and remained constant (±20%) during the period of analysis. Signals were recorded with an Axopatch 200B amplifier or a Multiclamp 700A (Axon Instruments, CA, USA), filtered at 2 kHz, digitized at 6–10 kHz (National Instruments LabPC+ or PCI-6024E A/D board, Austin, TX, USA) and analysed off-line with the EVAN program (courtesy of Professor I. Mody, UCLA, CA, USA). Student's paired *t*-test and Mann–Whitney *U*-test were used to compare changes in the mean amplitude after drug application; a value of *P* < 0.05 was considered significant. Data are presented as mean ± SEM.

The drugs were perfused until the maximal effect was seen. The time needed for the maximal inhibition (at least 3–5 min) correlated with the depth of the recorded cells. In some cases, we applied 2-AG for 7–15 min at 33–35 °C. The longer application did not cause further change in the amplitude compared with the effect seen after 5 min, suggesting that this time period was enough at a flow rate of 3.5–4.0 mL/min to equilibrate the drug effect. To quantify the drug effects, control IPSC amplitudes in a 2–3 min time window were compared with those measured after 5 min drug application for the same period of time.

### Reagents for electrophysiological recordings

2-AG (26.4 mM stock solution in acetonitrile) was purchased from Sigma or Cayman Chemical. AM251 and AM404 [100 mM stock solution in dimethylsulphoxide (DMSO) or 50 mM in ethanol, respectively] were obtained from Tocris (UK). AM374 and URB597 were

dissolved in DMSO. Solvents on their own had no effect on postsynaptic currents (*n* = 8). The perfusion of the drugs did not change the holding current during the recordings. Kynurenic acid was purchased from Sigma.

### Uptake experiments

We prepared primary cultures of rat cortical neurons from 18-day-old Wistar rat embryos in 24-well plates, as described by Stella & Piomelli (2001). We incubated the neurons in Krebs'–Tris buffer containing [<sup>3</sup>H]2-AG (American Radiolabelled Chemicals, 200 Ci/mol; 0.45 nM, brought to 30 nM with nonradioactive 2-AG) for 2–20 min at 37, 22 and 4 °C, rinsed them three times with 0.5 mL Krebs'–Tris buffer containing fatty acid-free bovine serum albumin (BSA, 0.1%), and extracted internalized tracer with 0.5 mL sodium hydroxide (0.1 M). We measured radioactivity by liquid scintillation counting.

## Results

### Temperature-dependence of the suppression of monosynaptically evoked inhibitory postsynaptic currents by 2-AG

We investigated the action of 2-AG on inhibitory neurotransmission in the hippocampal slice preparation. Inhibitory postsynaptic currents (IPSCs) recorded in CA1 pyramidal cells were evoked in the presence of an ionotropic glutamate receptor blocker, kynurenic acid (2–3 mM), by stimulating GABAergic fibres terminating in the perisomatic region (Hájos *et al.*, 2000). First we tested the effect of 2-AG on GABAergic inhibition at room temperature (22–25 °C), at which transporter activity for various transmitters is known to be considerably reduced or even blocked (Vizi, 1998). Bath application of 2-AG (20 μM) significantly reduced the amplitude of evoked IPSCs (eIPSCs) ( $58.9 \pm 6.5\%$ , *n* = 7, *P* < 0.001), an effect that was reversed upon washout (Fig. 1A and D). Pretreatment with or concomitant application of the CB<sub>1</sub> receptor antagonist AM251 (2 μM) abolished the effect of 2-AG ( $98.5 \pm 4.8\%$ , *n* = 3, *P* > 0.05; Fig. 1B and D). Next, we examined the action of 2-AG on inhibitory synaptic transmission at 33–35 °C. Application of 20 μM 2-AG produced a substantially smaller, albeit significant decrease of eIPSCs ( $78.9 \pm 2.7\%$ , *n* = 9, *P* < 0.01; Fig. 1C and D), which contrasted with the change observed at room temperature (Mann–Whitney *U*-test, *P* < 0.02).

### The endocannabinoid uptake inhibitor, AM404, enhances the suppression of evoked IPSCs by 2-AG at physiological temperature

In further investigations, we examined the effect of the endocannabinoid transport inhibitor AM404 on the action of 2-AG at 33–35 °C. In these experiments, 2-AG was applied at 10 μM, which reduced eIPSC amplitude ( $78.1 \pm 2.5\%$ , *n* = 12, *P* < 0.001; Fig. 2A and D) to a similar extent as at 20 μM (Mann–Whitney *U*-test, *P* > 0.05; Fig. 1D). When 2-AG (10 μM) application was either followed by a washing in of 2-AG together with AM404 (10 or 20 μM; Fig. 2C), or this mixture was directly applied (Fig. 2B), the inhibition of eIPSC amplitude was markedly enhanced at physiological temperature ( $63.1 \pm 3.9\%$ , *n* = 7, *P* < 0.001;  $61.1 \pm 4.7\%$ , *n* = 12, *P* < 0.01, respectively), an effect that could be fully reversed by AM251 ( $102.3 \pm 4.6\%$  of control, *n* = 3, *P* > 0.05). Irrespective of the application method, 10 or 20 μM AM404 enhanced the effect of 2-AG to a similar degree (10 μM,  $62.1 \pm 5.4\%$ ; 20 μM,  $62.8 \pm 4.5\%$ ; Mann–Whitney *U*-test, *P* > 0.05; Fig. 2D). Application of AM404 alone had no significant impact on the amplitude of eIPSC (10 μM,  $94.8 \pm 5\%$ ; 20 μM,  $102.5 \pm 8.1\%$ ; *P* > 0.05; Fig. 2D). To exclude the possibility that the enhancement of the action of 2-AG with AM404 is not due to its impact on FAAH

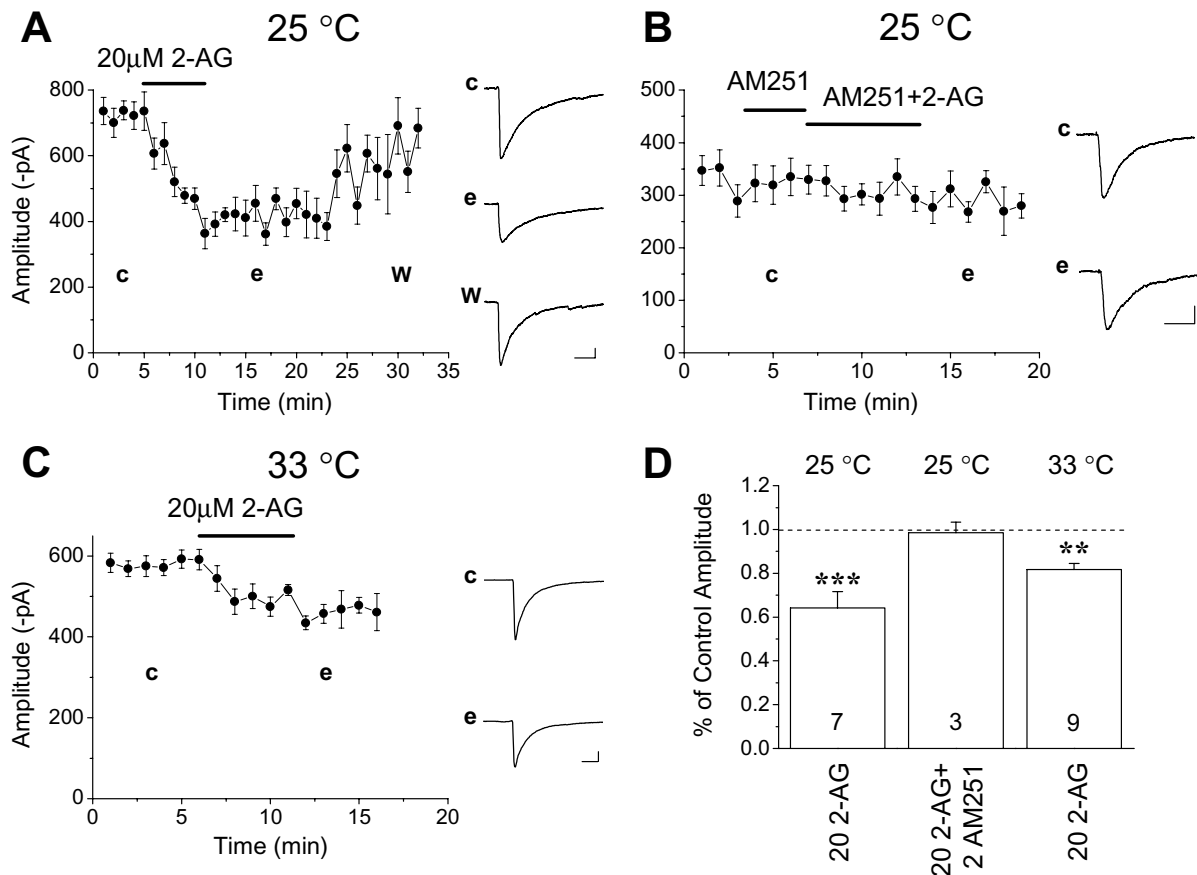


FIG. 1. The efficacy of 2-arachidonoyl glycerol (2-AG), an endocannabinoid ligand, in suppressing evoked inhibitory postsynaptic currents (eIPSCs) in the hippocampus is temperature-dependent. Whole-cell patch-clamp recordings were obtained from CA1 pyramidal cells using a CsCl-based intrapipette solution. IPSCs recorded at a holding potential of  $-60$  mV appear as inward currents. (A) Bath application of 2-AG ( $20 \mu\text{M}$ ) for 5–7 min markedly reduced the amplitude of IPSCs evoked at room temperature, a reduction that could be reversed by washout. (B) Pretreatment with the CB<sub>1</sub> cannabinoid receptor agonist, AM251 ( $2 \mu\text{M}$ ), and its coapplication with 2-AG ( $20 \mu\text{M}$ ) prevented reduction of eIPSC amplitude. (C) At  $33^\circ\text{C}$ , 2-AG application caused only a modest, but significant reduction in the amplitude of eIPSCs. All data points on the plots represent a mean  $\pm$  SEM of six consecutive events evoked by focal electrical stimulation with 0.1 Hz at the border of strata pyramidale and radiatum. Insets are averaged records of six consecutive events taken at the indicated time points. The stimulus artefacts were removed from the traces. Scale bars = 100 pA and 25 ms for A and B, respectively, and 5 ms for C. (D) Summary plot of the effects of 2-AG at different temperatures and its coapplication with the CB<sub>1</sub> receptor antagonist (AM251) on eIPSC amplitudes. All data are normalized to the control values and presented as percentages. The numbers of experiments performed under different conditions are indicated within the columns. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ .

activity, we coapplied 2-AG ( $10 \mu\text{M}$ ) with a specific FAAH inhibitor, URB597 ( $100\text{--}200 \text{ nM}$ ). The perfusion of this mixture caused a similar reduction in IPSC amplitude ( $76.2 \pm 1.6\%$ ,  $n = 4$ ,  $P < 0.05$ ) as with 2-AG application alone, indicating that the enhancement produced by AM404 is most likely mediated by inhibition of the transporter rather than reducing FAAH activity. The IPSC amplitude was not affected by URB597 application alone ( $96.7 \pm 2.2\%$  of control,  $n = 4$ ,  $P > 0.05$ ). In summary, these results demonstrate that there is no significant difference in the 2-AG-induced reduction of eIPSC amplitude at physiological temperature and at room temperature, if at the former AM404 is applied together with 2-AG (Mann–Whitney  $U$ -test,  $P > 0.05$ ). These data imply that 2-AG uptake is considerably reduced at room temperature in slice preparations.

#### 2-AG uptake by cortical neurons in culture is highly temperature-dependent

Human astrocytoma cells accumulate exogenous [ $^3\text{H}$ ]2-AG through a saturable and temperature-dependent process (Beltramo & Piomelli, 2000). The fact that this process is inhibited by either nonradioactive 2-AG or anandamide, as well as by the anandamide transport inhibitor

AM404, suggests that astrocytoma cells may accumulate both endocannabinoids through a common carrier-mediated mechanism. To determine whether neurons internalize 2-AG, we incubated primary cultures of rat cortical neurons in Krebs' buffer containing [ $^3\text{H}$ ]2-AG ( $30 \text{ nM}$ ). After incubation, which lasted 2–20 min, we rinsed the cultures with buffer containing fatty-acid-free BSA, to eliminate residual tracer, and measured radioactivity in the cell extracts. The results of these experiments indicate that cortical neurons rapidly accumulate [ $^3\text{H}$ ]2-AG in a strikingly temperature-dependent manner (Fig. 3A). Next, we incubated the neurons for 4 min in Krebs' buffer containing [ $^3\text{H}$ ]2-AG ( $30 \text{ nM}$ ) plus a large excess of nonradioactive 2-AG or anandamide ( $10$  or  $50 \mu\text{M}$ ). Both compounds prevented [ $^3\text{H}$ ]2-AG internalization in a concentration-dependent manner (Fig. 3B). Moreover, AM404 ( $10 \mu\text{M}$ ) significantly reduced [ $^3\text{H}$ ]2-AG accumulation (Fig. 3B). This effect cannot be attributed to inhibition of FAAH activity by AM404, because two potent FAAH inhibitors, AM374 and URB597, had no effect on [ $^3\text{H}$ ]2-AG accumulation (AM374, 112%; URB597, 99% of control;  $n = 8$ ). These findings indicate that rat brain neurons may internalize 2-AG via a carrier-mediated process similar to that previously described for

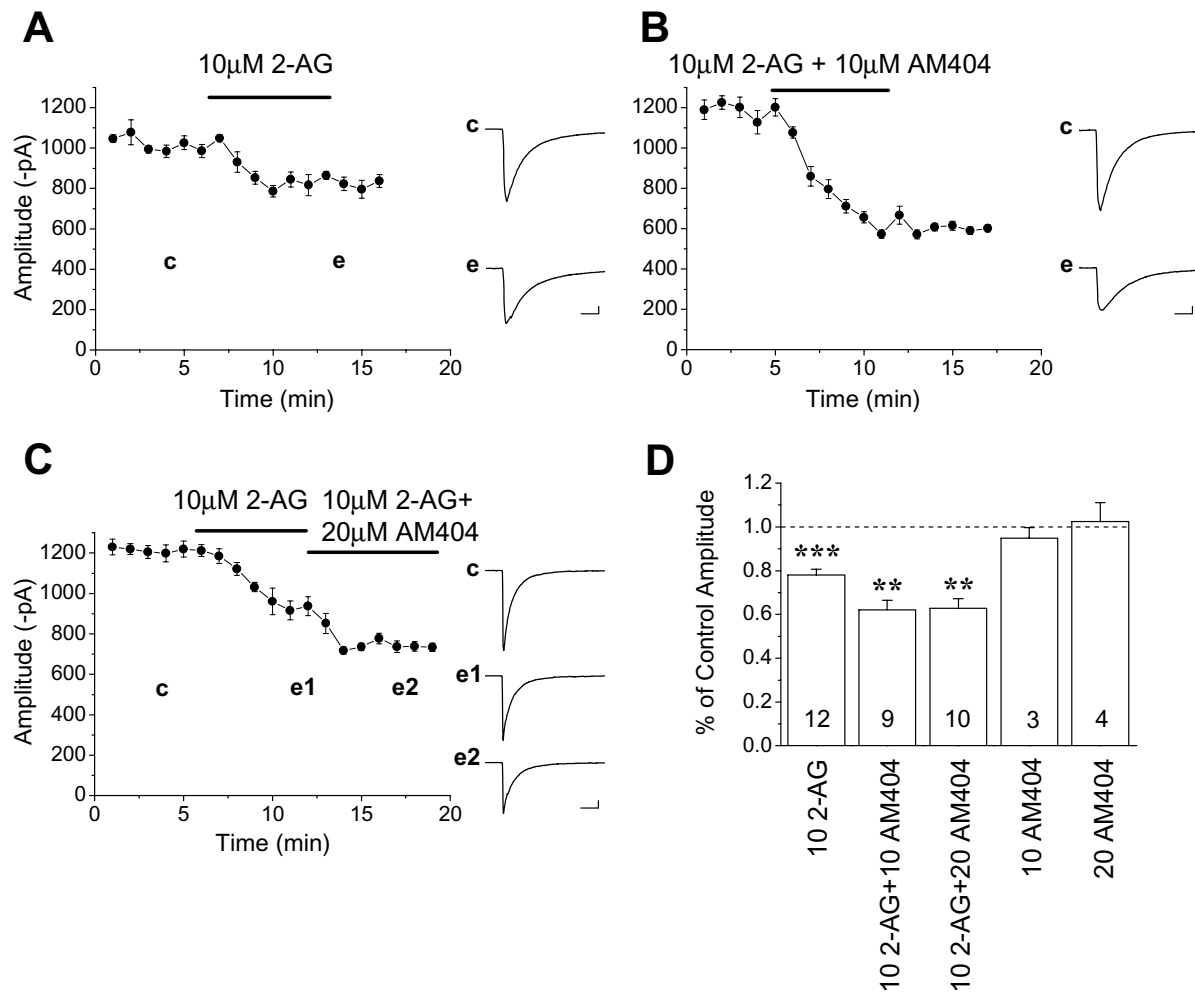


FIG. 2. The endocannabinoid uptake blocker, AM404, enhances the effect of 2-arachidonoyl glycerol (2-AG) on inhibitory postsynaptic currents (IPSCs) recorded at 33–35 °C. (A) Bath application of 10  $\mu$ M 2-AG caused a similar, modest ( $\sim$ 20%) reduction in the amplitude of evoked IPSCs as was noted after treatment with 20  $\mu$ M 2-AG. (B) Coapplication of 2-AG (10  $\mu$ M) with 10  $\mu$ M AM404 resulted in a larger ( $\sim$ 50%) suppression of eIPSC amplitude. (C) Application of the 2-AG/AM404 mix after 2-AG treatment alone further reduced the amplitudes of evoked IPSCs. (D) Summary plot of the effects of 2-AG and AM404 at 33–35 °C on eIPSC amplitudes. Presentation of data is the same as for Fig. 1. Scale bar = 100 pA and 10 ms. \*\*\* $P$  < 0.001, \*\* $P$  < 0.01.

astrocytoma cells (Beltramo & Piomelli, 2000), and that this uptake is highly temperature-dependent.

## Discussion

The 2-AG-mediated suppression of inhibitory synaptic transmission in the hippocampus is probably due to the activation of CB<sub>1</sub> cannabinoid receptors. Our previous observations showed that CB<sub>1</sub> receptors are selectively present on axon terminals and preterminal axon segments of a subset of GABAergic interneurons expressing cholecystokinin, and their activation reduces GABA release as well as inhibitory postsynaptic currents (Katona *et al.*, 1999; Hájos *et al.*, 2000). In addition, our electrophysiological recordings provided direct evidence that cannabinoid actions on GABAergic IPSCs are entirely mediated by CB<sub>1</sub> receptors, because cannabinoid ligands have no effect on GABAergic currents in CB<sub>1</sub> knockout mice (Hájos *et al.*, 2000).

The temperature dependence of [<sup>3</sup>H]2-AG transport has been demonstrated in astrocytoma and other cells (Beltramo & Piomelli, 2000; Bisogno *et al.*, 2001). Here we provide the first measurements of

[<sup>3</sup>H]2-AG uptake in primary neuronal cultures. Our results indicate that [<sup>3</sup>H]2-AG uptake is almost abolished at room temperature, providing a likely explanation for our electrophysiological results. AM404, an uptake blocker without any direct effect on CB<sub>1</sub> receptors, inhibited [<sup>3</sup>H]2-AG uptake at low micromolar concentrations and enhanced the 2-AG-mediated reduction of hippocampal IPSCs at physiological temperature. This enhancement of IPSC reduction at 33 °C was similar to those observed at room temperature. Although the putative endocannabinoid transporter(s) have not yet been identified, uptake studies *in vitro* have provided evidence for the presence of endocannabinoid transport in several brain regions including the hippocampus (Giuffrida *et al.*, 2001). Together, our physiological and biochemical data indicate that the effect of 2-AG is profoundly reduced by its uptake at physiological, but not at ambient temperature. A similar finding has been reported for anandamide in the midbrain (Vaughan *et al.*, 2000).

As suggested by biochemical studies, endocannabinoid uptake can be saturated (Bisogno *et al.*, 2001). Therefore, the question arises as to why we did not see a larger reduction in IPSC amplitude after longer (7–15 min) applications of 2-AG at physiological temperature, as

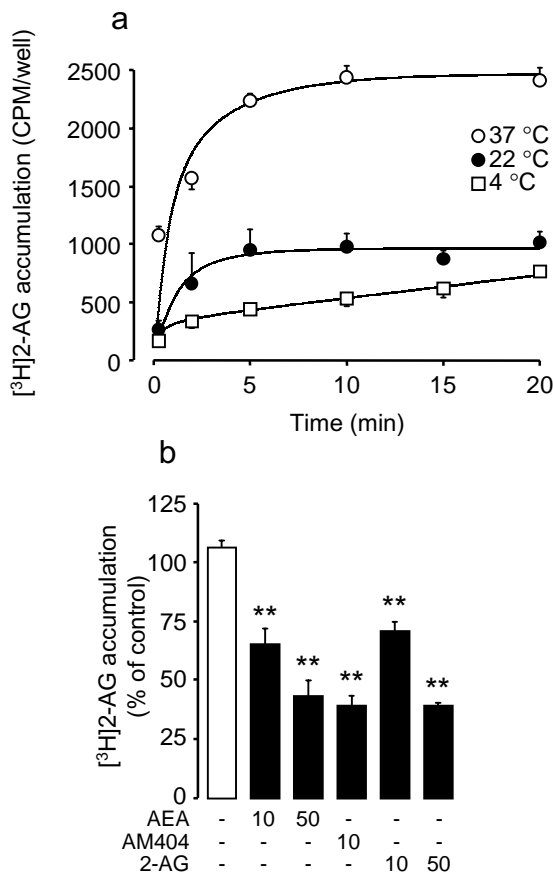


FIG. 3. [ $^3\text{H}$ ]2-AG accumulation in rat cortical neurons. (A) Time-course of [ $^3\text{H}$ ]2-AG accumulation at 37 °C, 22 °C and 4 °C. (B) Effects of various agents on [ $^3\text{H}$ ]2-AG accumulation. AEA, anandamide. \*\* $P < 0.01$ ;  $n = 4$ .

could be predicted from the saturation of uptake. One possibility might be that after uptake 2-AG is metabolized intracellularly, which may allow a constant siphoning of 2-AG from the extracellular space (Beltramo & Piomelli, 2000). Kinetic analyses of anandamide internalization have led to the suggestion that uptake in neuroblastoma and astrocytoma cell lines requires FAAH activity and that no anandamide transporter exists in these cells (Glaser *et al.*, 2003). These findings cannot be generalized to the brain, however, because pharmacological inhibition of FAAH activity in neurons or astrocytes has no effect on anandamide transport (Beltramo *et al.*, 1997; Kathuria *et al.*, 2003).

Most studies on the short- (DSI) or long-term (I-LTD) effects of endocannabinoids at inhibitory synapses have been performed at room temperature (Wilson & Nicoll, 2001; Chevaleyre & Castillo, 2003), at which, according to the present results, endocannabinoid uptake is greatly reduced. This might explain why AM404 increases the magnitude and time course of DSI at 33 °C (Trettel & Levine, 2003) but not at 22 °C (Wilson & Nicoll, 2001). Considering these results, the time window for DSI or for I-LTD at inhibitory synapses in the central nervous system should be narrower than suggested by the experiments performed at room temperature (Wilson & Nicoll, 2001; Chevaleyre & Castillo, 2003). Data by Kreitzer & Regehr (2001) and our present findings imply that this time period, when the CB<sub>1</sub>-expressing subset of inhibitory afferents is temporarily silenced by endocannabinoids, is likely to be in the range of 1–2 *s in vivo*. This time might still be long enough to allow short- or long-term modifications to take place at certain excitatory inputs (Carlson *et al.*, 2002), but may also ensure specificity in space and time.

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## Abbreviations

2-AG, 2-arachidonoyl glycerol; CB<sub>1</sub>, cannabinoid receptor type 1; DSI, depolarization-induced suppression of inhibition; FAAH, fatty acid amide hydrolase; I-LTD, long-term depression of inhibition; IPSC, inhibitory postsynaptic current; MGL, monoglyceride lipase.

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