Multiple Mechanisms of Endocannabinoid Response Initiation in Hippocampus

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Edwards, David A., Jimok Kim, and Bradley E. Alger. Multiple mechanisms of endocannabinoid response initiation in hippocampus. J Neurophysiol 95: 67–75, 2006. First published October 5, 2005; doi:10.1152/jn.00813.2005. Endocannabinoids (eCBs) act as retrograde messengers at inhibitory synapses of the hippocampal CA1 region. Current models place eCB synthesis in the postsynaptic pyramidal cell and the site of eCB action at cannabinoid receptors located on presynaptic interneuron terminals. Four responses at the CA1-interneuron synapse are attributed to eCBs: depolarization-induced suppression of inhibition (DSI), G-protein-coupled receptor-mediated enhancement of DSI (∆DSI), persistent suppression of evoked inhibitory postsynaptic currents (eIPSCs), and finally, mGlur-dependent long-term depression (iLTD). It has been proposed that all are mediated by the eCB, 2-arachidonoyl glycerol, yet there is evidence that DSI does not arise from the same underlying biochemical processes as the other responses. In view of the increasing importance of eCB effects in the brain, it will be essential to understand the mechanisms by which eCB effects are produced. Our results reveal new differences in the biochemical pathways by which the eCB-dependent responses are initiated. Both U73122, a phospholipase C antagonist, and RHC-80267, a diacylglycerol (DAG) lipase antagonist, prevented eCB-dependent iLTD induction by 3,5-dihydroxyphenylglycine (DHPG). However, mACHR activation does not cause eCB-dependent iLTD. Neither enzyme inhibitor affects DSI, and persistent eCB-dependent eIPSC suppression induced by either mGlur or mACHRs is unaffected by U73122. On the other hand, inhibition of DAG lipase prevents persistent eCB-dependent eIPSC suppression triggered by mACHRs. The results show that the biochemical pathways for the various eCB-dependent responses differ and might therefore be independently manipulated.

INTRODUCTION

The active component of marijuana, Δ9-tetrahydrocannabinol (THC), has many effects on the brain: it alters cognition, induces euphoria and relaxation, produces hypothermia, and prevents nausea (Ameri 1999). These actions are the result of THC binding to a cannabinoid receptor (CB1) in brain regions that subserve these functions. In the CNS, there exist natural ligands to CB1, the endocannabinoids (eCBs). Anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) are the two predominant eCBs discovered so far (Devane et al. 1992; Mechoulam et al. 1996; Sugiura et al. 1995). In the hippocampus 2-AG is presently thought to be the predominant eCB (Kim and Alger 2004; Makara et al. 2005; Stella et al. 1997), however, this has not yet been definitively established, and a role for AEA or other messengers cannot be entirely ruled out. CB1 is found in high concentrations in the hippocampus, an area of the brain involved in learning and memory. In the hippocampal CA1 region, CB1 is found in highest concentration on the axon terminals of a subset of inhibitory interneurons, the CCK-containing cells that synapse onto the pyramidal cells (Freund et al. 2003). Regulation of inhibitory synaptic inputs onto pyramidal cells can significantly affect CA1 excitability as well as plasticity at excitatory synapses. eCBs are thought to be produced and released from the postsynaptic CA1 pyramidal cells and then to suppress GABA release from CB1-expressing presynaptic interneuron terminals (Ohno-Shosaku et al. 2001; Wilson and Nicoll 2001; Wilson et al. 2001). By binding to CB1, eCBs can suppress inhibition, and therefore play an important role in regulating synaptic plasticity of both inhibitory (Chevaleyre and Castillo 2003) and excitatory (Carlson et al. 2002; Chevaleyre and Castillo 2004) synapses onto CA1 pyramidal cells.

A great deal is known about the molecular basis of the eCB system (Piomelli 2003 for review). ECB-mediated responses are produced after two distinct kinds of cellular stimulation: a strong increase in [Ca2+]i, and activation of certain G-protein-coupled receptors, including group I mGluRs (Maejima et al. 2001; Varma et al. 2001), mAChRs (Kim et al. 2002, Ohno-Shosaku et al. 2003), and D2 receptors (Giuffrida et al. 1999). At the interneuron synapses in CA1, four different eCB-mediated effects can be observed after different stimuli. First, a postsynaptic rise in [Ca2+]i causes a transient, eCB-dependent reduction of GABA release. This is called depolarization-induced suppression of inhibition (DSI). Second, low-to-moderate activation of postsynaptic mAChRs or group I mGluRs enhances DSI (∆DSI). Third, activation of mACHRs or mGlurRs with a higher concentration of agonist causes a persistent, relatively Ca2+-insensitive, eCB-dependent suppression of evoked inhibitory postsynaptic currents (eIPSCs). Fourth, prolonged activation of group I mGluRs produces a long-term depression of GABA release (iLTD) that is dependent on CB1 activation for induction but not maintenance.

These four eCB-mediated responses enable the CA1 pyramidal cell to regulate inhibitory synaptic inputs under a variety of stimulus conditions. The responses are distinguished by differences in their induction mechanisms and their durations. They are all thought to reflect eCB actions, and all may be mediated by 2-AG, and yet there is evidence that they may not all be mediated by the same underlying processes. Differences in these responses could reflect differences in the eCB induction pathways or other factors. For example, both M1/M3...
mAChRs and group I mGluRs are G-protein-coupled receptors (GPCR) that are linked to the phosphatidyl inositol/phospholipase C (PI/PLC) pathway, and the production of inositol trisphosphate (IP3) and diaacylglycerol (DAG). The synthesis of 2-AG can proceed from the conversion of phosphatidyl inositol to DAG by PLC and from DAG to 2-AG by DAG lipase (Di Marzo et al. 1998). Both mGluR- and mAChR-activated, persistent eCB responses are absent in mutant mice genetically engineered to lack PLCβ1 (Hashimotodani et al. 2005). Nevertheless, the mAChR and the mGluR pathways for inducing eCB responses might not be exactly the same (e.g., Kim et al. 2002). In addition, DSI is unaffected by pharmacological inhibition of PLC or DAG lipase (Chevaleyre and Castillo 2003) and is intact in the PLCβ1−/− mouse. Prolonged activation of mGluR-induced eCB release can lead to iLTD (Chevaleyre and Castillo 2003); however, it is not clear if induction of iLTD is exclusively due to CB1 activation. Because of the numerous roles they serve, it will be important to understand the various pathways involved in eCB-dependent responses. We have therefore investigated these and other questions involved in eCB actions in rat hippocampal CA1 pyramidal cells.

Our findings suggest that multiple pathways may be involved in producing eCB-dependent responses. In addition to the difference between the DSI pathway and the GPCR-mediated responses, we find that the mGluR and mAChR-dependent eCB pathways are biochemically distinct. The data also suggest that, like some forms of LTD (Ronesi et al. 2004; Sjostrom et al. 2003), hippocampal mGluR-dependent iLTD may require the participation of additional factors that work in conjunction with eCBs as eCB-dependent activation of CB1 appears necessary but not sufficient for iLTD induction.

This work is contained in the Ph.D. theses of J. Kim and D. A. Edwards.

**METHODS**

**Preparation of slices**

After deeply sedating and decapitating 4- to 6-wk-old male Sprague-Dawley rats, we removed the hippocampi and cut them into 400-μm-thick slices in ice-cold bath solution [see following text, excluding NBQX (Tocris, Ballwin, MO), and 2-amino-5-phosphonopentanoic acid (AP5; Tocris)] using a vibratome (Technical Products International, St. Louis, MO). The protocols were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. The slices were maintained at room temperature for >1 h in an interface holding chamber filled with humid 95% O2-5% CO2.

**Electrophysiology**

Whole cell voltage-clamp recordings of CA1 pyramidal cells were carried out at 30–31°C. Electrode resistances in the bath were 3–6 MΩ, and series resistances of <30 MΩ were accepted. If the series resistance, when checked by −1- or −2-mV steps, changed significantly (~15%), we discarded the data. At the holding potential of −70 mV, monosynaptic eIPSCs were elicited by 100-μs extracellular stimuli delivered with concentric bipolar stimulating electrodes placed in s. pyramidal or s. radiatum between CA3 and CA1. In Fig. 1E and F, stratum radiatum was also stimulated alternately, 1.5 s apart from s. pyramidal stimulation, every 6 s. Data were collected using an Axopatch 1C amplifier (Axon Instruments, Union City, CA), filtered at 2 kHz and digitized at 5 kHz using a Digidata 1200 and Clampex 8 software (Axon Instruments).

The pipette solution contained (in mM) 88 CsCl, 50 CsCl2, 1 MgCl2, 2 Mg-ATP, 0.3 tri-GTP, 0.2 Cs2-BAPTA, 10 HEPES, and 5 QX-314 (pH = 7.20 with CsOH, and 295 mosM with sucrose). The bath solution contained (in mM) 120 NaCl, 3 KCl, 25 NaHCO3, 1 NaH2PO4, 2.5 CaCl2, 2 MgSO4, 20 glucose, 0.01 NBQX and 0.05 dl-AP5 (or 0.02 d-AP5; 300 mosM). The bath solution was oxygenated with 95% O2-5% CO2 and flowed through the recording chamber at a rate of ~1 ml/min. In Figs. 1F, 2B, 3, A and B, and 5B–D, the slices were preincubated with AM251 (Tocris), RHC-80267 (Biomol, Plymouth Meeting, PA), U73122 (Tocris), or THL (Sigma) for >1 h, and the corresponding drug was present in the bath solution throughout recording. The final concentration of DMSO, a solvent, was 0.1% (vol/vol) for RHC-80267, U73122, THL, and 0.01% for AM251. All other chemicals were purchased from Sigma (St. Louis, MO).

**Data analysis**

To measure DSI, we evoked eIPSCs at 4-s intervals and depolarized the postsynaptic cell to 0 nV for 1 s at 89- or 120-seintervals. When DSI was not measured, eIPSCs were evoked every 6 s. DSI was calculated as follows: DSI (%) = 100 × [mean amplitude of 4 eIPSCs after depolarization/mean amplitude of 5 eIPSCs before depolarization]. Values of two to three DSI trials were averaged to obtain a mean DSI in a given condition. Changes in DSI (∆DSI) were calculated as ∆DSI = test DSI − control DSI. Evoked IPSC suppression was calculated as follows: 100 × [mean amplitude of 15 eIPSCs after drug treatment/mean amplitude of 15 eIPSCs before]. iLTD was determined to be present if eIPSCs were significantly suppressed after a 20-min washout of the agonist. Statistical tests were done in Excel XP (Microsoft, Redmond, WA), and the P value for significance was <0.05. Paired t-test were used, unless otherwise stated. For multiple comparisons, we used ANOVA followed by multiple t-test or Holm-Sidak tests.

**RESULTS**

We first replicated the four known eCB-mediated effects on monosynaptic eIPSCs in rat hippocampal CA1 pyramidal cells: DSI, ∆DSI, eIPSC suppression by postsynaptic GPCR activation, and mGluR-dependent iLTD (Fig. 1, see METHODS for measurement details). After obtaining a stable whole cell voltage-clamp recording of a CA1 cell, eIPSCs were elicited by stimulation in the s. radiatum every 4 s. A 1-s depolarization of the pyramidal cell was used to produce a small degree of DSI (IPSCs were transiently suppressed to 87.3 ± 2% of control, n = 8, e.g., Fig. 1A, left). The same depolarization following bath application of low concentrations, 2.5 (Fig. 1A, middle) or 5 μM, of the group I mGluR selective agonist 3,5-dihydroxyphenylglycine (DHPG) increased the inhibition of eIPSCs by an additional 16.5 ± 5% of control at 2.5 μM DHPG (n = 4) and an additional 23.4 ± 2% of control at 5.0 μM DHPG (n = 4) (∆DSI, Fig. 1B). 2.5 μM DHPG did not significantly alter the baseline eIPSC amplitude (Fig. 1C, 95 ± 5%, n = 4, P > 0.1).

Concentrations of DHPG ≥5 μM decreased the baseline amplitude of the eIPSCs (cf. Desai et al. 1994; Jouveneau et al. 1995; Morishita et al. 1998) (68 ± 14%, n = 3, and 43 ± 3%, n = 4, and 4, for 5.0 and 50 μM DHPG, respectively, P < 0.05).

At 50 μM, DHPG reduced the eIPSCs enough to occlude DSI (∆DSI, 0.57 ± 5.09%, n = 4, P > 0.1). If the application of 50 μM DHPG was continued for 10 min, iLTD was induced (e.g.,
Fig. 1. Endocannabinoid (eCB)-mediated effects on monosynaptic evoked inhibitory postsynaptic currents (eIPSCs) in hippocampal CA1 pyramidal cells. eIPSCs were elicited by stimulating in stratum radiatum every 4 s, and a 1-s depolarization was used to produce depolarization-induced suppression of inhibition (DSI). A: representative trace from the same cell showing minimal DSI, left, G-protein-coupled receptor-mediated enhancement of DSI (ΔDSI) caused by 2.5 μM 3,5-dihydroxyphenylglycine (DHPG) (group I mGluR agonist), middle, and suppression of eIPSCs caused by 50 μM DHPG, right. B: group data showing ΔDSI (DSI in the presence of DHPG minus control DSI). DHPG enhanced DSI at 2.5 and 5.0 μM but occluded DSI at 50 μM (n = 4 for each, respectively; *P < 0.05; t-test). C: group data showing eIPSCs suppressed at DHPG ≥5 μM (n = 3, 4 for 5.0 and 50 μM DHPG, respectively; *P < 0.05; t-test); 2.5 μM DHPG (n = 4) did not significantly suppress eIPSCs (P > 0.1; t-test). D: representative trace showing that 50 μM DHPG for 10 min causes iLTD. E: mGluR-dependent long-term depression (iLTD) can be produced by DHPG (50 μM) applied for 10 min (n = 4; *P < 0.05; t-test). In 3 of 4 cells, 5 μM MPEP (6-methyl-2-(phenylethynyl)-pyridine; mGluR5 antagonist) and 100 μM LY367385 ((+)-2-methyl-4-carboxyphenylglycine mGluR1 antagonist) were applied during wash-out of DHPG. S. pyramidal (●) and s. radiatum (○) were stimulated alternately. Traces are from 1 cell and averaged over 1–2 min at the indicated time points. F: slices were preincubated for >1 h with 3 μM AM251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; CB1 antagonist), which was also present in the bath. DHPG (50 μM) had little effect on eIPSCs (P > 0.05). S. pyramidal and s. radiatum were stimulated alternately, and traces are from 1 cell. 2, 1-s depolarization steps.

Fig. 1D). That is, the higher concentration of DHPG caused persistent suppression of eIPSCs for as long as it was applied (≥20 min), and the eIPSCs were suppressed to 48 ± 5% of control for eIPSCs evoked in s. radiatum and to 36 ± 9% of control for eIPSCs evoked in s. pyramidal at 10 min (n = 4). Activation of mGluRs was halted by washout of DHPG from the chamber (n = 4) and, in three of the cells, by the simultaneous addition of the group I mGluR antagonists, (+)-2-methyl-4-carboxyphenylglycine (LY367385) (100 μM; mGluR1) and 6-methyl-2-(phenylethynyl)-pyridine (MPEP) (5 μM; mGluR5). In all cases, eIPSCs recovered from prolonged DHPG treatment only partially, remaining significantly depressed 20 min after termination of DHPG application, confirming the report of Chevaleyre and Castillo (2003). eIPSCs at 20 min after beginning DHPG removal were 74 ± 7% of control for s.-radiatum-evoked IPSCs and 70 ± 5% of control for s.-pyramidale-evoked IPSCs (n = 4; Fig. 1E). Previous reports have shown that group I mGluR activation suppresses eIPSCs through an eCB-dependent action (Maejima et al. 2001; Varma et al. 2001). Indeed we found that in slices pretreated with the CB1 antagonist, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251; 3 μM) for >1 h, 10-min application of 50 μM DHPG had little effect on eIPSCs (P > 0.05). s. pyramidal and s. radiatum were stimulated alternately, and traces are from 1 cell.
The question arises whether CB1 activation is sufficient for iLTD induction or is only necessary for it. Other factors might also be involved (cf. Sjostrom et al. 2003; Ronesi et al. 2004). DSI is mediated by eCBs (Ohno-Shosaku et al. 2001; Wilson andNicoll 2001), and so to test the hypothesis that CB1 activation is sufficient for iLTD induction, we continuously delivered 2-s depolarizing steps to the pyramidal cell at 12-s intervals for 10 min (e.g., Fig. 2A). Because recovery from DSI is not complete within 12 s (e.g., Lenz and Alger 1999), this procedure effectively activated CB1 continuously for 10 min, as the depression during the tetanus could be blocked by AM251 (Fig. 2B). During the repetitive DSI, the envelope of the eIPSCs was reduced to levels comparable to mGluR activation with 50 μM DHPG (cf. Fig. 1E). Nevertheless, despite persistent CB1 activation, repetitive DSI failed to induce iLTD; shortly after cessation of the DSI-inducing voltage steps, the eIPSCs recovered completely to control levels.

Evoked IPSC amplitudes during repetitive DSI differed significantly from control or recovery phases (group data in Fig. 2C, n = 5, ANOVA, P < 0.01), but eIPSCs during control and recovery phases did not differ (ANOVA followed by Holm-Sidak test, P > 0.1), i.e., iLTD was not induced.

Because CB1 activation alone was insufficient to cause iLTD, it appeared that some other effect of mGluR activation might be required for iLTD induction. mGluRs are coupled to G proteins and activate PLC and DAG lipase pathways, and these pathways are probably not activated during DSI (e.g., Hashimotodani et al. 2005). Chevaleyre and Castillo (2003) showed that induction of iLTD by synaptic stimulation can be blocked by 1,6-bis(cyclohexyloiminocarboxylamino)hexane (RHC-80267; DAG lipase inhibitor) or 1-6-(((17β)-3-methoxyestra-1,3,5(10)-trien-17-y1amino)hexyl)-1H-pyrrole-2,5-dione (U73122; PLC inhibitor) and was occluded by a 10-min application of DHPG. Although Hashimotodani et al. (2005) were unable to use these compounds in dissociated culture because of adverse side effects, in our preparation, we observed no changes in eIPSCs caused by acute application of either U73122 or RHC-80267 (n = 6, data not shown). We now show that in slices treated with RHC-80267 (70 μM), the eIPSC amplitude completely recovered to its control level 5–10 min after termination of DHPG bath application (n = 4; Fig. 3A, 101 ± 6% of pre-DHPG). Similarly, in slices treated with 6 μM U73122, eIPSCs were 99 ± 5% (n = 4; Fig. 3B) of their control levels 5–10 min after DHPG termination. The differences from the degree of eIPSC suppression during iLTD are significant (cf. Fig. 1E; P < 0.05, t-test after ANOVA). Hence iLTD induction by persistent mGluR activation depended on the PLC/DAG lipase pathway.

![Image](http://jn.physiology.org/)

**FIG. 2.** Continuous cannabinoid receptor (CB1) activation by multiple DSI trials does not induce iLTD. After 10 min of stable baseline eIPSC measurement, 2-s depolarizing steps were delivered to the pyramidal cell at 12-s intervals for 10 min. A and B: representative traces showing multiple DSI trials activate CB1 essentially continuously for 10 min but fail to induce iLTD. AM251 (3 μM) pretreatment for 1 h prevented DSI. C: group data showing that at 20 min after multiple DSI trials, eIPSCs were not significantly suppressed (n = 5, P > 0.1).

**FIG. 3.** mGluRs are tightly linked to phospholipase C (PLC) and diacylglycerol (DAG) lipase for iLTD induction but not for eCB release. Slices were pretreated with either RHC-80267 (70 μM, n = 4; DAG lipase inhibitor) or U73122 (6 μM, n = 4; PLC inhibitor) for ≥1 h and were present in the bath during the experiment. A and B: representative cells showing either treatment prevented iLTD by 50 μM DHPG. Neither drug blocked the suppression of eIPSCs by DHPG. The traces on the right are from the cell shown and are means of eIPSCs obtained over 1–2 min during the times labeled. C: group data showing the suppression of eIPSCs with either RHC-80267 (n = 7) or U73122 (n = 5) pretreatment was not significantly different from the suppression seen in vehicle-treated controls (DMSO, n = 6; P > 0.1, ANOVA). D: group data showing both treatments prevented ILTD induction by DHPG compared with control (same cells as in C; *P < 0.05, t-test after ANOVA). MPEP (5 μM; mGluR5 antagonist) and 100 μM LY367385 (mGluR1 antagonist) were applied during wash-out of DHPG in 3 experiments treated with RHC-80267, 2 with U73122, and in 3 vehicle treated slices.
The expectation was that inhibitors of DAG lipase and PLC would prevent iLTD because they inhibit eCB synthesis. However, we found that RHC-80267 and U73122 failed to prevent the eCB-dependent reduction of eIPSCs by DHPG. In vehicle-treated slices, DHPG suppressed eIPSCs to 42 ± 8% of control (n = 6). In slices treated with RHC-80267 50 μM DHPG suppressed eIPSCs to 54 ± 9% of control (n = 7; Fig. 3C). Similarly, in slices treated with U73122, 50 μM DHPG suppressed eIPSCs to 37 ± 10% of control (n = 5; Fig. 3C). Neither of these values was significantly different from the eIPSC suppression in vehicle control (P > 0.1, ANOVA). Yet because both extracellular RHC-80267 and U73122 prevented iLTD (Fig. 3D), the drugs were clearly active in our hands. These results suggest that the eCB-mediated responses produced by mGluR-activation are not tightly coupled to the PLC/DAG lipase pathway. They also suggest that inhibition of DAG lipase and PLC alter iLTD through some mechanism other than the synthesis of eCBs.

Pretreatment with bath application of RHC-80267 or U73122 prevented iLTD, but whether pre- or postsynaptic mGluR-linked second-messenger systems were blocked could not be determined with bath application. We therefore loaded each drug individually into patch pipettes and repeated the experiments. Interestingly, iLTD was not blocked by either RHC-80267 (63.74 ± 7.61%, n = 4; Fig. 4, A and D) or U73122 (50.35 ± 2.15%, n = 4; Fig. 4, B and D) applied inside the pyramidal cell (iLTD vehicle was 49.67 ± 8%, n = 4; Fig. 4D, P > 0.1, ANOVA). Moreover, the degree of eIPSC suppression caused by DHPG in control conditions (39.39 ± 6%, n = 4) was not affected by intracellular application of either RHC-80267 (40.84 ± 7.12%, n = 4) or U73122 (50.25 ± 6.62%, n = 4; Fig. 4C, P > 0.1, ANOVA). Controls for the efficacy of intracellular application are provided in the following text. These results suggest that DAG might induce iLTD by actions not confined to the postsynaptic cell and are consistent with a lack of tight coupling between mGluR-activation and PLC and DAG lipase in the production of eCB responses.

Like mGluRs, mACHRs are also G-protein coupled and linked to PLC and DAG lipase pathways. Activation of mACHRs by carbachol (CCh) causes DASS and at ≤ 1 μM suppresses eIPSCs exclusively by an eCB-dependent mechanism (Kim et al. 2002). We asked whether mACHrs and mGluRs induce eCB responses through the same process. First we tried to induce iLTD by bath-applying CCh. We observed that a 10 (n = 2)- or 20 (n = 3)-min application of 1 μM CCh suppressed eIPSCs to the same degree that 50 μM DHPG did (Fig. 5A). The eIPSCs were reduced to 47 ± 9% of control after 10 min of CCh application (n = 5). CCh was then washed out while the mACHR antagonist 1 μM atropine was simultaneously applied. This procedure reversed the eIPSC amplitude completely, i.e., CCh failed to induce iLTD even when applied for 20 min, 10 min longer than required for mGluR activation to induce iLTD. At 7 min after atropine application, the eIPSC amplitude was 100 ± 9% of control (n = 5). Atropine alone does not affect eIPSCs or DASS (e.g., Kim et al. 2002; Pitler and Alger 1994) and seemed unlikely to affect iLTD. Nevertheless, in two additional experiments, we applied CCh for 10 min and washed it off in the absence of atropine. No iLTD was observed (data not shown). Evoked IPSC suppression by 1 μM CCh or 50 μM DHPG is mediated entirely via CB1 (Kim et al. 2002 and Fig. 1F, respectively), therefore eIPSC suppression reflects the degree of the eCB-mediated response. The eIPSC suppression caused by CCh and DHPG was statistically indistinguishable (P > 0.1, t-test). Thus the difference between the ability of mGluR and mACHr to induce iLTD cannot obviously be attributed to differences in the degree of CB1 activation. Moreover, in three cells, CCh was applied for twice as long as DHPG, and yet no iLTD was produced. Finally, to ensure that activation of the mACHr and mGluR systems were as similar as possible, we bath-applied 50 μM CCh. Although the persistent suppression of eIPSCs by 50 μM concentration of CCh was markedly greater than the suppression produced by 1 μM CCh, owing largely to eCB-independent actions (see Kim et al. 2002), eIPSCs recovered fully to baseline when 50 μM CCh was removed and atropine applied (n = 4; Fig. 5A, D). The data also argue that other factors besides CB1 activation are required to induce iLTD.
DAG lipase inhibitor (e.g., Bisogno et al. 2003) at 10 

tested tetrahydrolipstatin, THL, a second, chemically unrelated

responses obtained over 1–2 min. 

THL-pretreated slices, 1

C

without affecting the DHPG response. In

found that it also prevented CCh from persistently reducing

ANOVA; n

5

80267 prevented CCh from persistently reducing eIPSCs (Fig.

1

mGluR-activated system.

mAChR-induced eCB effects. Indeed, we found that RHC-

DAG-lipase nor PLC is required for DSI or modulation of DSI

DHPG might saturate the eCB-synthesis mechanism and thereby obscure an effect of RHC-80267. We therefore repeated the test using 5 

M HPG suppressed eIPSCs to 63 ±

9% of pre-DHPG values (n = 5; Fig. 4E), which is not significantly different from the effect of this dose of DHPG in vehicle conditions, 68 ±

% (n = 3; Fig. 1C; P > 0.1, t-test).

Consequently, even with milder activation of mGluRs, RHC-80267 still did not prevent activation of the eCB system by DHPG. In summary, the results show that mACHR-dependent eCB responses depend on DAG lipase activation and that the mGluR and mACHR-dependent systems differ in this fundamental respect. Somewhat unexpectedly, in view of the positive effect of the DAG lipase inhibitor, the PLC inhibitor did not affect mACHR-activated eCB responses. In slices treated with 6 

mM tetrahydrolipstatin (THL) for >1 h, 1 

M CCh did not reduce the amplitude of the basal eIPSCs, although it still enhanced DSI. In slices treated with 10 

M tetrahydrolipstatin (THL) for >1 h, 1 

M CCh did not reduce the amplitude of the eIPSCs, although DSI was enhanced. In slices treated with 6 

mM U73122 for >1 h, 1 

M CCh reduced eIPSCs to 60 ±

% of control (n = 4; Fig. 5D), which was not different from the CCh-induced depression in vehicle-treated slices (46 ±

%, n = 5; P > 0.1, t-test after ANOVA). Group data are shown in Fig. 5E.

The degree of eIPSC suppression during DSI was to 69 ±

% of control (n = 5) and was not altered by RHC-80267 (63 ±

%: n = 8) or U73122 (69 ±

%: n = 4; e.g., Fig. 5, B and D; group data not shown; P > 0.1, ANOVA) as reported by Chevaleyre and Castillo (2003). Interestingly, we found that 

DSI induced by CCh (Kim et al. 2002) was also unaffected by RHC-80267 or U73122. 

DSI induced by 1 

M CCh was 18 ±

% (n = 5) in vehicle, 17 ±

% (n = 8) in RHC-80267 and 18 ±

% (n = 4) in U73122 (e.g., Fig. 5, B and D; group data not shown; P > 0.1, ANOVA). Therefore neither DAG-lipase nor PLC is required for DSI or modulation of DSI by CCh.

Because RHC-80267 was bath-applied in the previous experiments, it was not clear where it acted in inhibiting CCh effects. To determine if RHC-80267 acted on the pyramidal cell, we loaded it into the cells for 25–40 min through the recording pipette only. In these cells, the capability of 1 

M CCh to reduce eIPSCs was significantly diminished. In control cells, 1 

M CCh reduced eIPSCs to 58 ±

% of pre-CCh levels (n = 5; e.g., Fig. 6, A and C), whereas in RHC-80267-filled cells, the mean reduction was only to 82 ±

% of control (n = 5; Fig. 6, B and C). Thus it appears that DAG lipase activation in the pyramidal cell is required for eCB responses induced by mACHR.

Our data revealed two distinctions between mGluR- and mACHR-mediated eCB signaling pathways: apparently different enzymatic requirements for eCB production and different ability to induce iLTD. Because prolonged activation of CB1 is not sufficient for iLTD induction and because PLC and DAG lipase are required for iLTD, we postulate an additional factor, probably induced at some site outside the pyramidal cell, may be required for iLTD (Fig. 7). The significance of these observations is considered in the following text.

**DISCUSSION**

Our results suggest that differences among the four eCB-mediated actions that occur at inhibitory inputs to CA1 pyramidal cells reflect in part differences in the biochemical pathways involved from the initiating stimulus to eCB release. DSI

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**FIG. 5.** The eCB system stimulated by mACHR activation differs from the mGluR-activated system. A: eCBs produced by the mACHR activation did not induce ILTD. CCh (1 

mM; mACHR agonist) was applied for 10 min (n = 2) or 20 min (n = 3). Five cells were pooled because the recoveries of eIPSCs from suppression were similar. CCh was washed out with application of 1 

mM atropine (mACHR antagonist). The traces are from 1 cell and are means of responses obtained over 1–2 min. B: in slices treated with 70 

mM RHC-80267 for >1 h, 1 

M CCh did not reduce the amplitude of the basal eIPSCs, although it still enhanced DSI. C: in slices treated with 10 

mM tetrahydrolipstatin (THL) for >1 h, 1 

M CCh did not reduce the amplitude of the eIPSCs, although DSI was enhanced. D: in slices treated with 6 

mM U73122 for >1 h, 1 

M CCh reduced eIPSCs and enhanced DSI. E: group data showing the reduction of basal eIPSC by 1 

M CCh (*, P < 0.001, t-test with vehicle after ANOVA; n = 5, 8, 6, and 4 for vehicle, RHC-80267, THL, and U73122, respectively).
is clearly distinct from the GPCR-mediated responses, and mAChR- and mGluR-initiated eCB responses are affected differently by an inhibitor of DAG lipase. iLTD is dependent on CB1 activation, but CB1 activation is apparently not sufficient for iLTD induction.

Neither the conventional model of eCB synthesis and release nor our model (see following text) can obviously account for DSI, which although thought to be mediated by 2-AG (e.g., Kim and Alger 2004) is not blocked by pharmacological inhibitors of PLC or DAG lipase (Chevaleyre and Castillo 2003) and is present in PLCβ1−/− mice (Hashimotodani et al. 2005). DSI is very calcium-sensitive (Lenz and Alger 1999; Llano et al. 1991; Pitler and Alger 1990; Wilson and Nicoll 2001). In this sense also, DSI can be distinguished from the GPCR-dependent forms of eCB response, which have a very much reduced level of sensitivity to calcium (Kim et al. 2002; Maejima et al. 2001) and require the co-activation of the GPCR (Hashimotodani et al. 2005). Our observations therefore confirm that the DSI and GPCR-dependent eCB mechanisms are not the same.

The activation of mGluRs (Maejima et al. 2001; Varma et al. 2001) and mAChRs (Kim et al. 2002) initiate the same kinds of eCB-dependent responses: both types of GPCR enhance DSI when stimulated with low concentrations of agonist, and in addition, both directly suppress eIPSCs by persistently triggering eCB responses at high agonist concentrations. Biochemically, both GPCRs are linked to the PI/PLC system, and their abilities to liberate eCBs are ultimately dependent on the activation of PLC/DAG lipase in the hippocampus (Hashimotodani et al. 2005). Nevertheless we find that the eCB-dependent responses initiated by mAChR and mGluR are not identical. Only stimulation of mGluRs was capable of inducing iLTD; even prolonged stimulation of mAChRs did not do so. On the other hand, only the persistent induction of the eCB-mediated eIPSC suppression triggered by mAChRs was sensitive to the DAG lipase inhibitors, RHC-80267 and THL. We had previously suggested that the pathways might differ (Kim et al. 2002), but that suggestion had been based on subtle differences between their efficacy in initiating eCB synthesis and their apparent ability to occlude DSI. The present data thus confirm and extend that suggestion.

The major eCB in the hippocampus is thought to be 2-AG, and a wealth of neurochemical evidence shows that 2-AG is chiefly produced by the PLC/DAG lipase pathway (Stella et al. 1997), although alternative pathways exist (Bisogno et al. 1999; Sugira et al. 2002). The ability of mAChR or mGluR agonists to stimulate eCB responses is lost in the PLCβ1−/− mouse (Hashimotodani et al. 2005). It was expected that pharmacological inhibition of PLC and DAG lipase would be similarly effective in antagonizing these GPCR-dependent responses. Accordingly, we were surprised to find that this is not the case: the PLC inhibitor was ineffective in blocking either GPCR-activated eIPSC suppression or ΔDSI. The DAG lipase inhibitor, whether applied extra- or intracellularly, was very effective in antagonizing the mAChR-induced eCB responses but ineffective against mGluR responses. Lack of efficacy of the drugs cannot account for their inability to alter mGluR-mediated eCB responses because RHC-80267 did affect the mAChR responses, and both RHC-80267 and U73122 prevented iLTD. We emphasize that we used the same concentrations of the same drugs previously demonstrated to prevent
RHC-80267 can act as an mAChR antagonist in a cerebral cortical membrane preparation (Sekar et al. 1990), hence its ability to antagonize the effect of CCh could conceivably be related to receptor antagonism. However, we found that THL, a chemically unrelated DAG lipase inhibitor that has not been reported to have antagonist effects at mAChRs, also blocked the CCh- but not the DHPG-induced persistent eIPSC suppression. Our data demonstrate that RHC-80267 cannot be a general mAChR antagonist in hippocampal slices because CCh continued to enhance DSI an mAChR-mediated action (Kim et al. 2002) in its presence (Fig. 5B). In addition, when applied internally to the pyramidal cell, RHC-80267 continued to block the CCh effect on eIPSCs (Fig. 6B). Clearly more work must be done to identify the mechanism of action of RHC-80267 and THL on the mAChR-induced eCB responses, but blocking DAG lipase is the parsimonious explanation for their effects at present.

The data show that there are differences between the mGluR- and mAChR-induced eCB responses. Conceivably, the 2-AG synthetic enzymes could be differentially coupled to the two GPCRs, and sensitivity of the enzymes to the pharmacological antagonists could somehow be related to the strength or immediacy of the coupling. It could also be proposed that the two receptors initiate the synthesis of different eCBs, but this seems unlikely in view of the numerous similarities between them (Hashimotodani et al. 2005; Kim et al. 2002; Ohno-Shosaku et al. 2003).

As a novel alternative explanation that can reconcile all of the reported data, we propose that the PLC/DAG lipase pathway is the main pathway for de novo synthesis but not for release, of 2-AG. Rather, release is triggered “on-demand” from preformed pools of 2-AG or DAG. It is well known that 2-AG participates in numerous cellular processes, many of which are not associated with eCB signaling (Bisogno et al. 1997; Sugiuira et al. 2002), and unstimulated basal levels of 2-AG within membranes are readily measurable (Stella et al. 1997). Hence 2-AG production must occur independently of eCB responses, and as a corollary, 2-AG synthesis is not invariably coupled to its release. Under our model, the measured increases in 2-AG synthesis would participate in the refilling of the eCB signaling pools of 2-AG or its precursors, or perhaps to the generation of 2-AG for other cellular roles, but not be inextricably linked to eCB-release. Deficiency of PLCβ1 would prevent GPCR-dependent eCB signaling by depleting the pools of 2-AG and DAG that ultimately become available for signaling. It is well established that different GPCRs on the same cell can generate different responses, probably by virtue of their coupling to the G proteins in different cellular microdomains (Delmas et al. 2004). In this vein, we propose that in hippocampal pyramidal cells, mAChRs could initiate eCB responses by triggering DAG lipase activation and the production of 2-AG from extant DAG, whereas the mGluR pathway would initiate 2-AG release from an existing stock of 2-AG. The details of 2-AG release are not known for any system, and hence the mechanism by which GPCRs actually cause the release cannot presently be specified. It is interesting that intracellular infusion of the anandamide transport blocker can prevent release of AEA or 2-AG from cells (Roness et al. 2004) and eCB-mediated iLTD, suggesting that efflux of eCBs might come about by reversal of the transporter. It is possible that GPCR-dependent eCB release bypasses initial enzymatic steps and involves stimulation of the transporter. In summary, although speculative, our model of the relationship of eCB signaling to eCB synthesis can explain both previously published findings, as well as the data we present here. Testing it will require further work.


