Endocannabinoid-Mediated Depolarization-Induced Suppression of Inhibition in Hilar Mossy Cells of the Rat Dentate Gyrus

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INTRODUCTION

Depolarization-induced suppression of inhibition (DSI) is a form of short-term synaptic plasticity that is dependent on retrograde transmission mediated by endogenous cannabinoids (ECs). DSI has been observed in a number of brain areas where presynaptic cannabinoid type 1 (CB1) receptors are expressed, including the amygdala, substantia nigra, basal ganglia, neocortex, cerebellum, and hippocampus (Engler et al. 2006; Kreitzer and Regehr 2001a; Llano et al. 1991; Pitler and Alger 1992b; Trettel and Levine 2003; Wilson and Nicoll 2001; Yanovsky et al. 2003; Zhu and Lovinger 2005). In fact, across many areas of the CNS, variations in factors such as the mechanism of induction (and source) of EC release, the duration of EC-dependent signaling, and the location of cannabinoid receptor expression have implicated ECs either directly or indirectly in multiple forms of synaptic plasticity (Chevaleyre and Castillo 2003, 2004; Gerdeyam et al. 2002; Hashimoto and Llano 2005; Kreitzer and Regehr 2001b; Robbe et al. 2002; Safo and Regehr 2005).

Several recent lines of evidence have begun to suggest that ECs might play a similarly important role in modulating synaptic activity in the dentate gyrus, an area that serves as the entry point for the primary afferent projections to the hippocampus from both the medial septum and the entorhinal cortex (Johnston and Amaral 1998). For example, it is clear that both CB1 receptors and the enzymes necessary for degradation of ECs are expressed in the dentate gyrus (Gulyas et al. 2004; Katona et al. 1999, 2000; Romero et al. 2002; Tsou et al. 1998). Further, previous reports have demonstrated that exogenous CB1 agonists can modulate the activity of inhibitory inputs to dentate granule cells (Nakatsuka et al. 2003) and that EC-dependent signaling is enhanced in the dentate after febrile seizures (Chen et al. 2003). Finally, a recent study has provided the first clear evidence that DSI may be induced by experimental excitation of granule cells under normal conditions (Isokawa and Alger 2005).

In the present study we test the hypothesis that endocannabinoid-dependent retrograde signaling can also be initiated by depolarization of mossy cells found in the hilar region of the dentate gyrus. These cells are unique among local circuit neurons in the hippocampus and dentate gyrus in that they are glutamatergic instead of GABAergic (Scharfman 1995). They are also extremely sensitive to both ischemia and excitotoxicity (Freund and Magloczky 1993; Hsu and Buzsaki 1993; Magloczky and Freund 1993), have a strong longitudinal projection (Amaral 1978; Amaral and Witter 1989; Buckmaster and Schwartzkroin 1994; Buckmaster et al. 1992), and have been consistently implicated (through either their loss or dysfunction) in several competing theories on the etiology of temporal lobe epilepsy (Houser 1999; Lothman et al. 1996; Ratzliff et al. 2002, 2004; Santhakumar et al. 2000; Sloviter 1991). Our results indicate that excitation of hilar mossy cells produces a robust inhibition of local GABAergic transmission and suggest a prominent role for EC-dependent retrograde signaling in hilar neurophysiology.

METHODS

Hippocampal slice preparation

Male Sprague–Dawley rats between 18 and 25 days of age were given an intraperitoneal injection of ketamine (80–100 mg/kg) and rapidly decapitated using a small animal guillotine. The brain was quickly removed and horizontal slices were cut at a thickness of 300 μm in ice-cold artificial cerebral spinal fluid (ACSF) using a Pelco Series 3000 Vibratome (Pelco, Redding, CA). Immediately after being cut, slices were placed in a submerged incubator containing ACSF preheated to 30–35°C. They were maintained at this temperature for 30 min and then allowed to equilibrate to room temperature in the same incubator. The ACSF used for both cutting and incubating slices...
contained (in mM): 124 NaCl, 2.5 KCl, 1.2 NaH2PO4, 2.5 MgSO4, 10 d-glucose, 1 CaCl2, and 25.9 NaHCO3, saturated with 95% O2-5% CO2. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida and conformed to animal welfare guidelines issued by the National Institutes of Health.

Whole cell recording

After incubation, slices were transferred to a recording chamber where they were perfused at a rate of 2 ml/min with ACSF that was heated to 30°C and saturated with 95% O2-5% CO2. This ACSF contained (in mM): 126 NaCl, 3 KCl, 1.2 NaH2PO4, 1.5 MgSO4, 10 d-glucose, 2.4 CaCl2, and 25.9 NaHCO3 (pH 7.3). In some cases, 3 µM CCh was added directly to the ACSF. While in the recording chamber, slices were visualized with infrared differential interference contrast microscopy (IR DIC) using an Olympus BX51WI microscope. Whole cell patch-clamp recordings were made using pipettes pulled on a Flaming/Brown electrode puller (Sutter P-97, Sutter Instruments, Novato, CA). Pipette resistance was typically 3–5 MΩ when filled with an internal solution that contained (in mM): 105 Cs-MeSO3, 55 CsCl, 1 MgCl2, 0.2 EGTA, 10 HEPES, 2 MgATP, 0.3 Na2GTP, and 5 QX-314-Cl. This solution was pH adjusted to 7.3 using CsOH and volume adjusted to 300–315 MΩm. For the experiments presented in Fig. 4A, 10 mM 1,2-bis(o-aminophenoxo)ethane-N,N',N',N'-tetraacetic acid (BAPTA) was also present in this solution. On each experimental day sulforhodamine 101 was added to the internal solution (about 63 µM) and cells were then visualized after whole cell recording using fluorescence microscopy. Access resistance was typically between 10 and 40 MΩ and was generally uncompensated. Evoked responses were typically generated at 0.33 Hz using a concentric bipolar stimulator (FHC, Bowdoin, ME) connected to a constant current stimulator isolator (World Precision Instruments, Sarasota, FL). Current intensity varied between 50 and 300 µA. Stimuli lasted 0.1 ms. In some cases when paired recordings were used, an extra stimulus was inserted during the 5-s depolarization so as to not interrupt the regular 3-s interstimulus interval. Voltage-clamp experiments were performed using an Axon Multiclamp 700A or 700B amplifier (Molecular Devices, Sunnyvale, CA). The data were sampled at 20 kHz, filtered at 2 kHz, and recorded on a computer with a Digidata 1322A A/D converter using Clampex version 9 (Molecular Devices, Sunnyvale, CA). All chemicals used in these experiments were obtained from Sigma (St. Louis, MO) except for γ-aminobutyric acid (GABA), (R)(-)()-3,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolol[1,2,3-de]-1,4-benzoazxin-6-yl]-1-naphthalenylmethanone (WIN55,212-2), and N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM-251), which were obtained from Tocris Cookson (Ellisville, MO).

Identification of mossy cells

Hilar mossy cells were identified using a wide range of physiological and anatomical criteria as outlined in a previous publication (Frazier et al. 2003). In brief, the prototypical mossy cell studied here was clearly located in the hilus, appeared larger than other types of hilar cells when visualized under IR DIC, had a whole cell capacitance >200 pF (average of 50 randomly chosen but representative cells was 298.1 ± 8.0 pF), and, importantly, displayed at least some (and usually many) large-amplitude (>100 pA) spontaneous excitatory postsynaptic currents (sEPSCs) when voltage clamped at −70 mV in the absence of glutamate receptor antagonists. Although previous work has indicated that some hilar interneurons may have some of these features, no cell was considered a mossy cell unless it met all of the above criteria and additionally was confirmed to be both multi-polar and spiny (with thorny excrescences being particularly prominent on the proximal dendrites) when examined by fluorescence microscopy.

Data analysis

In experiments involving either spontaneous or miniature inhibitory postsynaptic currents (sIPSCs and mIPSCs, respectively), events were detected using appropriate parameters in MiniAnalysis v. 6.03 (Synaptosoft, Decatur, GA). Event tables were then exported from MiniAnalysis and imported into OriginPro version 7.5 (OriginLab, Northampton, MA) for further analysis and binning using custom software developed in OriginC by CJF. Power analysis for data as presented in Figs. 1 and 2 was conducted in ClampFit version 9. “Theta power” was defined as (absolute power from 4 to 14 Hz ± absolute power from 2 to 50 Hz) × 100, where absolute power is the summation of all spectral bins in the stated range.

In experiments that involved inducing retrograde transmission by depolarization of hilar mossy cells, the baseline period was defined as the eight sweeps or the 24 s immediately before depolarization, and the test period was defined as the two sweeps or the 6 s immediately after depolarization. Recovery was measured from 66 to 90 s after depolarization. The response to depolarization was quantified as the mean response observed during the test period divided by the mean response observed during the baseline period. This value represents the response to one trial or “set” of DSI. In most cases, the response to a minimum of three sets of DSI was averaged to obtain a representative value for each cell. No cell was included for analysis unless at least two complete sets of DSI were obtained. In all cases, 95% confidence intervals were calculated around the baseline mean and the mean during the recovery period as (2.37 × SE of the relevant mean). A cell was considered to have expressed DSI if the mean response during the test period was <95% confidence bands for the sIPSC as observed during both the baseline and recovery periods.

Statistical hypothesis testing was done using standard tools in OriginPro or Excel 2003 (Microsoft, Seattle, WA). The Kolmogorov-Smirnov (K-S) test was implemented in OriginC using a function provided from the Numerical Algorithms Group Library. Error bars in all figures represent the SE.

RESULTS

DSI of sIPSCs in hilar mossy cells

Identified hilar mossy cells (see METHODS) were voltage clamped at −70 mV in whole cell mode using a CsMeSO4-based internal solution that contained ~60 mM chloride. About 3 min after obtaining a whole cell recording, the ionotropic glutamate receptor antagonists 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium (NBQX, 10 µM) and 2-amino-5-phosphonovaleric acid (APV, 40 µM) were bath applied to isolate sIPSCs and 3 µM carbamoylcholine chloride (CCh, a muscarinic acetylcholine receptor (mAChR) agonist) was applied in an attempt to increase sIPSC frequency. Mossy cells could be readily divided into three groups based on the nature of the spontaneous activity that emerged under these experimental conditions.

In one group of cells, application of CCh produced a large, sudden, and stable increase in sIPSC frequency and amplitude (Fig. 1A). In four cells in which this effect was observed, the average sIPSC frequency after application of CCh was 14.5 ± 3.42 Hz, whereas the average amplitude was 71.5 ± 6.44 pA. A power analysis revealed that spontaneous activity in these cells uniformly showed one or more strong peaks in the theta band [e.g., 4–14 Hz, Fig. 1B, average “theta power” (see METHODS) was 50.1 ± 3.38%]. In this population of cells,
Depolarization from -70 to 0 mV for 5 s produced robust DSI that could be observed as a transient decrease in both frequency and amplitude of sIPSCs (Fig. 1, C–E). Bicuculline-sensitive (e.g., GABA<sub>A</sub>-mediated) eIPSCs were generated in the presence of NBQX and APV at a rate of 0.33 Hz using a bipolar stimulator placed in the hilus. In the absence of bath-applied CCh, depolarization of hilar mossy cells from -70 to 0 mV for 5 s (bar) caused a transient reduction in both frequency and amplitude of CCh-induced sIPSCs. Inset: averaged 3–6 sIPSCs recorded during the baseline period (24 s before depolarization), depolarization-induced suppression of inhibition (DSI) period (6 s immediately after depolarization), and recovery period (66–90 s after depolarization), respectively. B: power analysis of the 1 min of data before depolarization reveals several strong peaks in the theta band. These peaks were completely absent during the DSI period (data not shown). Average theta power for this cell during the 1 min preceding depolarization was 50% (see METHODS). C and D: cumulative probability histograms reveal that depolarization of this cell produced a statistically significant decrease in both frequency and amplitude of sIPSCs. Events were collected from the baseline and DSI periods as defined above. Thick lines in each histogram represent the baseline period; thin lines represent the DSI period [P < 0.001 in both cases, Kolmogorov–Smirnov (K-S) test]. E: summary plot indicating the average effect of depolarization on sIPSC frequency and amplitude in 4 cells that displayed an effect of CCh as shown in A. At least 2–3 sets of DSI were averaged for each cell to obtain a representative effect. Every cell tested that exhibited high theta power sIPSCs after application of CCh showed robust DSI of both frequency and amplitude. *P < 0.05, **P < 0.01 on a 2-tailed paired Student’s t-test, n = 4.

Finally, in a third group, cells either failed to show clear increases in sIPSC frequency (beyond 3 Hz) after bath application of 3 µM CCh or showed transient bursts in activity that were not sufficiently sustained to allow for reliable detection of DSI. This group represented the strong majority (roughly 90%) of all mossy cells examined. Consequently, we also examined the ability to detect DSI in hilar mossy cells as a transient reduction in the amplitude of evoked IPSCs (eIPSCs).

**DSI of eIPSCs in hilar mossy cells**

Bicuculline-sensitive (e.g., GABA<sub>A</sub>-mediated) eIPSCs were generated in the presence of NBQX and APV at a rate of 0.33 Hz using a bipolar stimulator placed in the hilus. In the absence of bath-applied CCh, depolarization of hilar mossy cells from -70 to 0 mV for 5 s transiently reduced eIPSC amplitude by 18.9 ± 5.00% (Fig. 3A, n = 6, P < 0.01). Interestingly, when DSI was examined in a separate population of mossy cells exposed to 3 µM CCh, the effect was significantly more robust...
and amplitude of nontheta sIPSCs recorded in the presence of 3 µM CCh. Data in A–D are all from a single representative experiment. A: sample sIPSCs recorded in the presence of 3 µM CCh in a cell where CCh failed to produce large-amplitude high-frequency theta-band sIPSCs as in Fig. 1. Insets: highlight the effect of depolarization from −70 to 0 mV for 5 s (bar). B: power analysis indicating that this cell, as every cell that lacked a clear CCh effect on sIPSCs, failed to show clear peaks in the theta band. C and D: cumulative probability histograms reveal that depolarization of this cell produced a statistically significant decrease in sIPSC frequency (P < 0.001, K-S test), with no change in amplitude (P = 0.87, K-S test). E: summary plot indicating the average effect of depolarization on sIPSC frequency and amplitude in 3 cells that lacked an effect of CCh as shown in A, but still had baseline sIPSC frequency of ≥3 Hz. Three sets of DSI were averaged for each cell to acquire a representative effect. *P < 0.05, on a 2-tailed paired Student’s t-test, n = 3.

DSI in hilar mossy cells is mediated by calcium-dependent release of endogenous cannabinoids that act on presynaptic CB1 receptors

We observed that the magnitude of DSI in hilar mossy cells depends directly on both depolarization duration and on postsynaptic calcium influx. Specifically, in separate groups of cells, we measured DSI of 10.3 ± 1.84% after a 0.1-s depolarization, 21.9 ± 2.91% after a 1-s depolarization, and 30.7 ± 3.8% after a 5-s depolarization (n = 8, 9, and 13, respectively, data not shown). Further, we demonstrated that DSI was largely eliminated in cells that were filled with 10 mM BAPTA by the recording pipette (eIPSC amplitude after depolarization was reduced by only 2.53 ± 2.08%, n = 18; Fig. 4A). We also found that there was no effect of depolarization on the response to rapid local application of exogenous GABA (100 µM by a picospritzer) in four cells that collectively showed DSI of eIPSCs of 20 ± 3.0% (data not shown). Cumulatively, these results suggest that calcium-dependent release of a retrograde messenger is involved in DSI as observed in hilar mossy cells and, consistent with other systems, several lines of experimental evidence implicate endogenous cannabinoids in that role. Specifically, we found that in a group of mossy cells that showed 30 ± 5.0% reduction in eIPSC amplitude after a 5-s depolarization to 0 mV, wash-in of AM-251 (a CB1 receptor antagonist) for ≥20 min produced a statistically significant block of DSI (to 9.0 ± 3%, n = 6, P < 0.01, Fig. 4B). Similarly, incubation of slices in 5 µM AM-251 before whole cell recording completely eliminated DSI (eIPSC amplitude...
after depolarization was 100.76 ± 2.08% of control, \( n = 14 \), Fig. 4C). We also demonstrated that bath application of a cannabinoid receptor agonist (WIN55,212–2) both reduces eIPSC amplitude (by 36.4 ± 7.17%, \( n = 4 \), a value comparable to that transiently produced by a 5-s depolarization) and completely occludes DSI (Fig. 5A).

**Activation of presynaptic CB1 receptors preferentially inhibits calcium-dependent exocytosis**

We next sought to determine whether calcium dependency of exocytosis from GABAergic afferents to hilar mossy cells predicts sensitivity to CB1-mediated inhibition. As a first approach we examined the effect of depolarization-induced release of endogenous cannabinoids on action potential–independent miniature IPSCs recorded in the presence of 1 μM tetrodotoxin (TTX). Because voltage-gated calcium channels rely on the depolarization initiated by TTX-sensitive Na⁺ channels for their activation, these conditions are expected to dramatically reduce calcium-dependent exocytosis. To eliminate false negatives resulting from failures in retrograde transmission, experiments on mIPSCs were completed only in cells that had previously demonstrated robust DSI of eIPSCs under normal conditions. In six of eight cases we found that depolarization of hilar mossy cells (from ~70 to 0 mV for 5 s) had no significant effect on frequency or amplitude of mIPSCs recorded in normal ACSF (frequency: 111.5 ± 12.6% of control, amplitude: 98.7 ± 2.44% of control, \( P > 0.4 \) in both cases; Fig. 6D). In sharp contrast, in eight of 11 cases where mIPSCs were tested in ACSF that contained 15 mM KCl and 5 mM CaCl₂ (conditions designed to rescue calcium-dependent exocytosis) depolarization reduced mIPSC frequency by 32.9 ± 4.6% without affecting mIPSC amplitude (101 ± 1.59% of control, \( P < 0.01 \), \( P > 0.5 \) respectively, Fig. 6D). In fact, in three cells where DSI was examined in all three conditions, we sequentially observed robust DSI of eIPSCs, followed by no DSI of mIPSCs, followed by robust DSI of mIPSCs in high KCl and CaCl₂. One such series of experiments is shown in Fig. 6, A–C.

Although we did observe DSI in two of eight cases in normal media, these cells had notably higher mIPSC frequencies than that of others for these conditions (13.7 ± 3.1 vs. 6.2 ± 2.2 Hz, respectively). Therefore we assessed mIPSC frequency in three cells after a 25-min wash-in of an external solution that contained 0 mM Ca²⁺, 3.9 mM Mg²⁺, and 100 μM BAPTA-AM and found it significantly reduced [e.g., from 7.0 ± 1.4 to 4.1 ± 0.7 Hz, \( P = 0.03 \) (one-tailed), 0.07 (two-tailed); data not shown].

We also tested the ability of bath applied WIN55,212–2 (5 μM) to modulate the frequency of calcium-independent mIPSCs induced by local application of a hypertonic solution (100 mM sucrose; Khvotchev et al. 2000; Rosenmund and Stevens 1996; for previous evidence of calcium independence see Fatt and Katz 1952). Specifically, we found that local application of 100 mM sucrose (2 min × roughly 20 psi) to the surface of the slice just above a patched hilar mossy cell reliably increased mIPSC frequency to nearly 500% of control (e.g., to about 20 Hz) and, further, that this effect was unaltered...
in a separate population of cells that had been pretreated with 10 mM 1,2-bis(o-aminophenoxy)ethane-N,N',N'-tetraacetic acid (BAPTA, delivered by the internal solution), eIPSC amplitude after depolarization was 97.5 ± 2.08% of baseline, n = 18. B: DSI was significantly reduced by bath application of 5 μM N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM-251). Filled circles indicate magnitude of DSI in a group of 6 mossy cells in control conditions (30 ± 5.0%). Open circles indicate amount of DSI observed in the same 6 mossy cells after wash-in of 5 μM AM-251, a CB1 receptor antagonist, for ≥20 min (9.0 ± 3%). This represents a statistically significant reduction in the magnitude of DSI (P < 0.01 on paired Student’s t-test). C: when slices were preincubated in 5 μM AM-251 before whole cell recording, DSI was completely eliminated (average eIPSC amplitude after depolarization was 99.2 ± 1.31% of control, n = 14). D: summary plot indicating the amount of DSI observed in mossy cells filled with 10 mM BAPTA, mossy cells not exposed to AM-251, and mossy cells pretreated with AM-251 (5 μM) before whole cell recording. Insets in A–C: averages of 2–6 individual traces recorded during the times indicated. In B, sample traces for all 3 periods are overlaid both before and after wash-in of AM-251.

Endocannabinoid-mediated signaling in the dentate gyrus is subject to tight spatial constraints

A final question of interest was whether endogenous cannabinoids released as a result of depolarization of one mossy cell could lead to reduction in transmitter release from GABAergic afferents to nearby mossy cells. To address this question we...
performed paired whole cell recordings from 14 mossy cells (seven pairs) in which both cells showed robust DSI on depolarization and in which the cell somas were located within 100 μm of each other (average: 59 ± 9.4 μm, as calculated in three-dimensional space). In that population, we failed to observe any detectable DSI in the nondepolarized cell in 11 of 14 cases, seven pairs, and DSI of spontaneous IPSCs is present, generated as in earlier figures. Filled circles represent normalized averages from 3 sets of DSI. Insets: averages of 2–6 individual traces from the indicated period. B, top traces: representative miniature IPSCs recorded after bath application of 1 μM tetrodotoxin (TTX) as observed both before (Base) and immediately after (No DSI) a 5-s depolarization from −70 to 0 mV. A cumulative probability histogram is presented below the traces to indicate that depolarization did not decrease the frequency of miniature IPSCs (mIPSCs) in this cell. In fact, in this case, a slight, but not significant, increase was observed (P = 0.12, K-S test). C, top traces: representative mIPSCs recorded in the same cell after increasing external KCl to 15 mM and external CaCl2 to 5 mM. Under these conditions, depolarization produced a significant reduction in mIPSC frequency (P = 0.001, K-S test). D: summary plot indicating average affect of depolarization in the conditions described in A–C. Numbers on the bar are n values. *P = 0.03, unpaired Student’s t-test. **P < 0.01, one-sample Student’s t-test (null hypothesis, mean = 0).

DISCUSSION

In the present study we used whole cell recording techniques in acute preparations of the rat dentate gyrus to examine retrograde transmission initiated by depolarization of hilar mossy cells. Our results indicate that depolarization of hilar mossy cells produces calcium-dependent release of endogenous cannabinoids that is facilitated by activation of mAChRs. We further provide specific experiments to indicate endocannabinoids liberated by depolarization of mossy cells activate presynaptic CB1 receptors on traditional GABAergic afferents and that this activation (whether by endogenous or exogenous agonists) preferentially inhibits calcium-dependent exocytosis. We further provide evidence from paired whole cell recordings that indicates EC-mediated retrograde transmission in this system is subject to tight spatial constraints. Overall, our results represent just the second example of EC-mediated retrograde signaling occurring under normal conditions in the dentate gyrus (Isokawa and Alger 2005), the first formal report of DSI at this synapse and, indeed, the first report of endocannabinoids being liberated by depolarization of a local circuit neuron in the hippocampus or dentate gyrus (although see Howard et al., Soc Neurosci Abstr 808.11, 2003). There are several areas in which our results deserve careful comparison to those previously reported in other systems.

The role of muscarinic acetylcholine receptors in DSI

In early experiments on DSI in area CA1 of the hippocampus, CCh was often used because of its ability to increase sIPSC frequency and amplitude (Behrends and ten Bruggencate 1993; Pitler and Alger 1992a,b). DSI of spontaneous

FIG. 6. Depolarization-induced release of endogenous cannabinoids preferentially inhibits calcium-dependent exocytosis. Data presented in A–C were all collected sequentially from a single cell. A: DSI of evoked IPSCs is present, generated as in earlier figures. Filled circles represent normalized averages from 3 sets of DSI.Insets: averages of 2–6 individual traces from the indicated period. B, top traces: representative miniature IPSCs recorded after bath application of 1 μM tetrodotoxin (TTX) as observed both before (Base) and immediately after (No DSI) a 5-s depolarization from −70 to 0 mV. A cumulative probability histogram is presented below the traces to indicate that depolarization did not decrease the frequency of miniature IPSCs (mIPSCs) in this cell. In fact, in this case, a slight, but not significant, increase was observed (P = 0.12, K-S test). C, top traces: representative mIPSCs recorded in the same cell after increasing external KCl to 15 mM and external CaCl2 to 5 mM. Under these conditions, depolarization produced a significant reduction in mIPSC frequency (P = 0.001, K-S test). D: summary plot indicating average affect of depolarization in the conditions described in A–C. Numbers on the bar are n values. *P = 0.03, unpaired Student’s t-test. **P < 0.01, one-sample Student’s t-test (null hypothesis, mean = 0).
IPSCs was almost always absent in cells that lacked this CCh-induced effect and yet DSI of evoked IPSCs was readily apparent, even in the absence of CCh and the presence of atropine (Martin and Alger 1999). Cumulatively, those observations led to the conclusion that activation mAChRs was not necessary for DSI per se, but rather allowed easy detection of DSI by increasing the activity of DSI-sensitive (e.g., CB1 positive) interneurons. Implicit in that conclusion, and strongly supported by immunohistochemical analysis (e.g., Katona et al. 1999; Tsou et al. 1999), was the suggestion that there exists a clear population of GABAergic afferents to CA1 pyramidal cells that are CB1 negative.

In the present study, we report robust DSI of large-amplitude, high-frequency, CCh-induced sIPSCs. These sIPSCs show strong peaks at theta frequencies on spectral analysis and DSI is apparent as a transient reduction in frequency, amplitude, and theta power. However, whereas Martin and Alger (1999) reported sustained large-amplitude CCh-induced sIPSCs in >50% of CA1 pyramidal cells examined, we see similar activity in <10% of mossy cells tested. The mechanism by which CCh increases the amplitude of sIPSCs in these cases is not clear. One possibility is that CCh causes a dramatic increase in quantal content, although a competing explanation is that CCh promotes synchronous release from somatic and perisomatic GABAergic inputs. Although we did not perform experiments aimed directly at this question, both early speculation and more recent experimental work in area CA1 favors the latter hypothesis (Alger et al. 1996; Reich et al. 2005).

In the present study, we also report DSI of smaller-amplitude sIPSCs that lack clear peaks in the power spectrum at theta frequencies. In these cases DSI is apparent as a transient reduction in sIPSC frequency, with no detectable change in sIPSC amplitude. Although it might be argued that this represents a new and nontraditional form of DSI expression, it is in fact exactly what would be predicted from a purely presynaptic effect of ECs on asynchronous release events of low quantal

![Image of graphs and data](http://jn.physiology.org/)

**FIG. 7.** Bath application of WIN55,212–2 preferentially inhibits calcium-dependent exocytosis. A: application of hypertonic solution (100 mM sucrose, applied by a picospritzer to the surface of the slice, roughly 20 psi × 2 min, bars) caused a significant and reproducible increase in mIPSC frequency (App. 1: 506 ± 104%, App. 2: 441 ± 68.7%, n = 5). B: identical experiment was performed on a group of mossy cells pretreated with 5 μM WIN55,212–2 for a minimum of 12 min (App. 1: 572 ± 113%, App. 2: 493 ± 106%, n = 6). C: summary plot indicating that there was no significant difference in the effect of sucrose on mIPSC frequency in the WIN55,212–2 treated group (dark bars) vs. the control group (white bars) on either the 1st or 2nd sucrose application. Effect of sucrose was calculated as the average mIPSC frequency from 1 to 2 min after starting the sucrose application divided by the average mIPSC frequency in the 1 min immediately preceding sucrose application. D: summary plot indicating that bath-applied 5 μM WIN55,212–2 is able to effectively reverse KCl-mediated increases in mIPSC frequency (n = 3 out of approximately 6 tested. Cells were not included in analysis if 10 mM KCl failed to significantly increase mIPSC frequency). E: raw data from a representative experiment recorded in the presence of 10 mM KCl [but before application of 5 μM WIN55,212–2 (left traces) and approximately 25 min after application of WIN55,212–2 (right traces)].
content. In that light, it becomes interesting to note that, in contrast to our results, CCh-insensitive presumably nontheta sIPSCs recorded in CA1 pyramidal cells have generally been reported to be DSI insensitive (Martin and Alger 1999; Martin et al. 2001). Even though the reasons for this apparent difference are not yet entirely clear, immunohistochemical studies have demonstrated that innervation by parvalbumin immunoreactive terminals, although present, is much weaker in mossy cells than in traditional hippocampal principal cells, whereas innervation by cholecystokinin immunoreactive terminals remains robust (Acsady et al. 2000). It should be interesting for future studies to begin to examine the resultant differences in CB1-mediated modulation of network activity.

We further demonstrated that although DSI of evoked IPSCs is apparent in the absence of cholinergic stimulation, the magnitude of this effect is enhanced by bath application of 3 μM CCh in an atropine-sensitive manner. Based on current data we cannot definitively determine whether this effect of CCh on DSI of eIPSCs is mediated presynaptically or postsynaptically. A potential presynaptic mechanism might involve a CCh-mediated increase in the number of DSI-sensitive afferents that are recruited by the stimulus, whereas a postsynaptic mechanism might involve direct mAChR-mediated facilitation of depolarization-induced EC release. Although significant further work will be necessary to distinguish between these possibilities in the current system, significant precedence for the postsynaptic hypothesis is developing based on recent work in CA1 (Hashimotodani et al. 2005; Kim et al. 2002; Ohno-Shosaku et al. 2003).

Presynaptic effects of endocannabinoids

Although it is clear that activation of CB1 receptors is negatively coupled to exocytosis in a number of CNS synapses, the precise mechanism by which this occurs has been a matter of some controversy. Activation of CB1 receptors in cultured hippocampal neurons has been directly linked to Gi/Go-mediated inhibition of N- and P/Q-type calcium channels (Sullivan 1999; Twitchell et al. 1997). This result has been used to suggest that presynaptic CB1 receptors in acute CNS preparations may inhibit transmitter release primarily by reducing action potential–mediated calcium influx. Indeed, consistent with that hypothesis, miniature IPSCs (recorded in the presence of 1 μM TTX) in hippocampal slices have generally been
shown to be insensitive to CB1 activation in normal media, but sensitive to both CB1 agonists and Cd$^{2+}$ (indicating their calcium dependency) after bath application of high KCl (Hajos et al. 2000; Hoffman and Lupica 2000; Pitter and Alger 1994; Wilson and Nicoll 2001). By contrast, endogenous and/or exogenous CB1 agonists have been shown to inhibit presumably calcium-independent miniature synaptic events in several other brain areas including the cerebellum, nucleus accumbens, and spinal cord (Diana et al. 2002; Hoffman and Lupica 2001; Kreitzer and Regehr 2001a; Llano et al. 1991; Morisset and Urban 2001; Robbe et al. 2001). Cumulatively, these data implied that contingent on the specific synapse involved, activation of CB1 receptors may inhibit exocytosis either by reducing calcium influx into the presynaptic terminal and/or by direct modulation of the release machinery. Our own results in the dentate gyrus indicate that depolarization-induced release of endogenous cannabinoids generally fails to reduce mIPSC frequency in normal ACSF and yet reliably does so in external solution that contains 15 mM K$^+$ and 5 mM Ca$^{2+}$. Similarly, we have further demonstrated that WIN55,212–2 fails to reduce robust calcium-independent exocytosis produced by focal application of a hypertonic solution and yet reduces the frequency of mIPSCs recorded in the presence of high extracellular K$^+$. These results are consistent with the conclusion that CB1 receptors on GABAergic afferents to hilar mossy cells preferentially inhibit calcium-dependent exocytosis, but do not directly speak to whether the site of action is at voltage-gated calcium channels and/or downstream of calcium influx.

An important footnote here is that we did observe DSI of mIPSCs in normal ACSF in two of eight cases. One possible explanation for these outliers is that there is an additional mechanism for CB1-mediated inhibition of calcium independent exocytosis. However, we also noted that mIPSC frequency could sometimes be reduced in hilar mossy cells by switching to a Ca$^{2+}$-free external containing 100 $\mu$M BAPTA-AM, suggesting that TTX may not always completely eliminate calcium-dependent exocytosis of GABA. Considered together, these results suggest that the calcium dependency of mIPSCs in hilar mossy cells most likely predicts their sensitivity to inhibition by CB1 activation. In that respect, our conclusions parallel those of Yamasaki et al. (2006), who recently and convincingly demonstrated that the differential sensitivity of mEPSCs versus mIPSCs in cerebellar Purkinje cells to WIN55,212–2 in normal (2 mM) Ca$^{2+}$ can be explained by a previously unnoted differential Ca$^{2+}$ dependency of the miniature events.

**Spatial constraints on endocannabinoid-dependent signaling**

Despite the rapid nature of recent progress, relatively few studies have directly examined spatial constraints on EC-dependent retrograde signaling. In the cerebellum, strong depolarization of Purkinje cells has produced a clear spread of EC-dependent retrograde signaling to both inhibitory and parallel inputs to nondepolarized cells (Kreitzer et al. 2002; Vincent and Marty 1993), and yet more selective induction of EC release has shown exquisite specificity in retrograde transmission, even within the dendritic tree of a single Purkinje cell (Brown et al. 2003). In area CA1 of the hippocampus, Wilson and Nicoll (2001) reported that EC-dependent retrograde signaling can occur over a range of about 20 microns and that the extent of inhibition detectable in nondepolarized cells is strongly correlated with somatic distance from a depolarized cell. Our results in the dentate gyrus indicate that it is generally quite difficult to detect the spread of EC-dependent signaling between mossy cells with somatic distances between 17 and 87 $\mu$m. The fact that all cells in the pairs experiment exhibited robust DSI on depolarization eliminates the possibility of CB1 negative afferents masking the spread of EC-dependent signaling. Although we observed exceptional cases where the spread of EC-dependent signaling clearly affected afferent inputs to a nondepolarized cell, we did not find any clear correlation between this effect and somatic distance and/or magnitude of DSI. In our view this suggests that EC-dependent retrograde signaling initiated by depolarization of mossy cells is likely confined to very small spaces (e.g., $\leq$20 $\mu$m). Although this may be functionally important, experimental detection of the spread of retrograde transmission to nondepolarized cells likely depends on one or more uncontrolled factors such as the degree of dendritic overlap.

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**References**


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