Stimulation of Endocannabinoid Formation in Brain Slice Cultures through Activation of Group I Metabotropic Glutamate Receptors

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ABSTRACT

Activation of group I metabotropic glutamate (mGlu) receptors drives the endocannabinoid system to cause both short- and long-term changes of synaptic strength in the striatum, hippocampus, and other brain areas. Although there is strong electrophysiological evidence for a role of endocannabinoid release in mGlu receptor-dependent plasticity, the identity of the endocannabinoid transmitter mediating this phenomenon remains undefined. In this study, we show that activation of group I mGlu receptors triggers the biosynthesis of the endocannabinoid 2-arachidonoylglycerol (2-AG), but not anandamide, in primary cultures of corticostriatal and hippocampal slices prepared from early postnatal rat brain. Pharmacological studies suggest that 2-AG biosynthesis is initiated by activation of mGlu5 receptors, is catalyzed by phospholipase C (PLC) and 1,2-diacylglycerol lipase (DGL) activities, and is dependent on intracellular Ca2+ ions. Real-time polymerase chain reaction and immunostaining analyses indicate that DGL-β1 is the predominant DGL isoform expressed in corticostriatal and hippocampal slices and that this enzyme is highly expressed in striatal neurons, where it is colocalized with PLC-β1. The results suggest that 2-AG is a primary endocannabinoid mediator of mGlu receptor-dependent neuronal plasticity.

The endocannabinoid system serves important functions in the regulation of brain synaptic transmission (for review, see Freund et al., 2003). One prominent example of this function is a form of short-term synaptic plasticity termed depolarization-induced suppression of inhibition (DSI), in which depolarization of a postsynaptic neuron induces the transient suppression of neurotransmitter release from presynaptic nerve terminals impinging on that neuron (for review, see Alger, 2002). Based on electrophysiological and pharmacological studies, it has been proposed that DSI may be mediated through the Ca2+-dependent formation of an endocannabinoid messenger, which might be produced postsynaptically and travel across the synaptic space to activate CB1 receptors on adjacent axon terminals (Alger, 2002; Freund et al., 2003).

Another important form of endocannabinoid-mediated neural plasticity involves group I metabotropic glutamate (mGlu) receptors. Activation of these Gq/11 protein-coupled receptors depresses synaptic transmission in a variety of brain regions, including the striatum (Gerdean et al., 2002), hippocampus (Varma et al., 2001; Ohno-Shosaku et al., 2002; Robbe et al., 2002; Brown et al., 2003; Chevaleyre and Castillo, 2003; Rouach and Nicoll, 2003), midbrain (Mels et al., 2004), and amygdala (Mariscano et al., 2002; Azad et al., 2004) through a mechanism that requires CB1 receptor activation. Furthermore, mGlu receptor-dependent forms of synaptic modulation mediated by endocannabinoids have been implicated in prolonged changes in synaptic strength.
such as those occurring during the induction of long-term depression at corticostriatal synapses (Robbe et al., 2002; Gerdenman et al., 2003).

Although the contribution of the endocannabinoid system to depolarization- and receptor-dependent synaptic plasticity is supported by a large body of electrophysiological data, the chemical identity of the endocannabinoid molecule(s) mediating these processes remains elusive. Two lipid-derived endocannabinoid substances have been characterized thus far: anandamide (arachidonoylthanolamid) and 2-arachidonoylglycerol (2-AG) (for review, see Piomelli, 2003). Anandamide may be generated by hydrolysis of the phospholipid precursor N-arachidonoylphosphatidylethanolamine, catalyzed by a selective phospholipase D (Sugiura et al., 1996b; Cadas et al., 1997; Okamoto et al., 2004). On the other hand, 2-AG may be produced by PLC-mediated cleavage of membrane phosphoinositides, which yields 1,2-diacylglycerol (1,2-DAG), followed by diacylglycerol lipase (DGL)-catalyzed conversion of 1,2-DAG to 2-AG (Stella et al., 1997; Bisogno et al., 2003; Hashimoto et al., 2005).

To fully understand the roles played by the endocannabinoid system in synaptic plasticity, it is essential to determine both the identity of the endocannabinoid(s) involved and the molecular mechanisms responsible for their production. In the present study, we have used direct biochemical analyses to characterize mGlu receptor-dependent endocannabinoid formation in organotypic cultures of rat corticostriatal and hippocampal slices. We found that activation of mGlu5 receptors, which are expressed at high levels in both striatum and hippocampus (Testa et al., 1994), rapidly stimulates the biosynthesis of 2-AG, but not anandamide, suggesting that 2-AG plays a key role in mGlu5 receptor-initiated signaling events.

### Materials and Methods

**Chemicals.** (S)-3,5-Dihydroxyphenyleglycine (DHPG), 2-methyl-6-(phenylethyl)lyridine hydrochloride and LY367385 were obtained from Tocris (Ellisville, MO); ionomycin, BAPTA-AM, and thapsigargin from Sigma-Aldrich (St. Louis, MO); RHC80267 and U73122 from BIOMOL Research Laboratories (Plymouth Meeting, PA). Testa et al. (1994) used 3,4-diethylpyridine hydrochloride and LY367385 as agonists for DGL and 2-AG. We used a mixture of protease inhibitors (Roche, Rockford, IL). For Western blotting, lysates from corticostriatal slices were prepared in a buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.25% Nonidet P-40, and 2 mM EDTA supplemented with a mixture of protease inhibitors (Roche Diagnos-
tics, Indianapolis, IN). The slices were homogenized in lysis buffer and the homogenates were centrifuged at 14,000 g for 10 min. Proteins (30 μg) were separated on 4 to 15% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and subjected to Western blotting.

**Statistical Analyses.** Results are expressed as the mean ± S.E.M. of n separate experiments. Statistical significance was evaluated using Student’s t test or, when appropriate, one-way analysis of variance followed by Dunnett’s test.

**Results**

**Activation of Group I mGlu Receptors Increases 2-AG Levels in Brain Slice Cultures.** To measure receptor-dependent changes in brain endocannabinoid levels, we used primary cultures of corticostriatal or hippocampal slices prepared from 7-day-old rat pups (Stoppini et al., 1991). The slice cultures maintained an essentially normal morphology for at least 7 days in vitro, as assessed by light microscopy and immunostaining for the neuron-specific marker class III β-Tubulin (Fig. 1A), and contained detectable amounts of 2-AG. A representative HPLC/MS tracing illustrating the presence of a diagnostic ion for this compound (m/z = 401, [M+Na]+) in extracts of corticostriatal slices is reported in Fig. 1B. Figure 1B also shows a second HPLC component, which was identified as 1(3)-AG from its retention time and mass-to-charge ratio (m/z = 401, [M+Na]+). Because 1(3)-AG arises from the nonenzymatic isomerization of 2-AG, which occurs during sample preparation, we included it in our calculations of total 2-AG levels (Stella et al., 1997). On average, corticostriatal slices contained 253.5 ± 18.5 pmol of 2-AG per mg protein (n = 44), whereas hippocampal slices contained 440.0 ± 35.2 pmol of 2-AG per mg protein (n = 8). Anandamide was also detectable in slice extracts, although its levels were lower than those of 2-AG [6.4 ± 0.5 and 13.0 ± 0.9 pmol/mg of protein in corticostriatal (n = 44) and hippocampal (n = 8) slices, respectively].

Incubation of corticostriatal slices for 10 min at room temperature in the presence of the group I mGlu receptor agonist DHPG (100 μM), which was previously shown to induce endocannabinoid-mediated long-term depression of transmitter release in the ventral striatum (Robbe et al., 2002), significantly increased 2-AG content compared with controls (Fig. 1C). The treatment also augmented the levels of 2-OG, a monoacylglycerol that does not activate cannabinoid receptors (Sugiura et al., 1996a). In contrast, DHPG did not affect the formation of anandamide (Fig. 1E) or noncannabinoid fatty-acid ethanolamides such as oleoylethanolamide (Rodriguez de Fonseca et al., 2001; Fu et al., 2003; control, 89.2 ± 8.6 pmol/mg of protein; DHPG, 91.2 ± 7.9 pmol/mg of protein, n = 44) and palmitoylethanolamide (Calignano et al., 1998; control, 440.0 ± 33.9 pmol/mg of protein; DHPG, 460.9 ± 35.6 pmol/mg of pro-
tein, n = 44). We obtained similar results using hippocampal slice cultures (Fig. 1F and Table 1).

**mGlut5 Receptor Activation Mediates 2-AG Formation.** Further pharmacological analyses, conducted in corticostriatal slice cultures, indicated that DHPG stimulates 2-AG formation by activating mGlut5 receptors. Preincubation with MPEP (5 μM, 5 min), a selective mGlut5 receptor antagonist (Gasparini et al., 1999), but not with LY 367385 (100 μM, 5 min), an mGlut1 antagonist (Clark et al., 1997), abrogated the ability of DHPG to stimulate 2-AG biosynthesis (Fig. 2). Neither antagonist exerted any significant effect on 2-AG levels when applied alone (Fig. 2). These results corroborate previous electrophysiological data, suggesting that DHPG stimulates endocannabinoid formation in acutely prepared corticostriatal slices by activating mGlut5 receptors (Robbe et al., 2002).

**2-AG Is Produced through the PLC-DGL Cascade.** mGlut5 receptors are coupled to Gq/11 proteins and signal through PLC-mediated breakdown of inositol phospholipids (Hannan et al., 2001). Therefore, incubation with the PLC inhibitor U73122 (10 μM, 15 min) prevented the stimulation of 2-AG formation by DHPG in corticostriatal slices (Fig. 3A). The nonselective DGL inhibitor RHC80267 (60 μM, 15 min) exerted a similar effect (Fig. 3A), whereas neither agent significantly affected 2-AG levels when applied alone (Fig. 3A).

We next investigated the Ca^2+ dependence of DHPG-induced 2-AG production. As shown in Fig. 3B, treatment with the Ca^2+ ionophore ionomycin (2 μM, 15 min) markedly increased 2-AG levels, supporting the idea that intracellular Ca^2+ rises can trigger 2-AG biosynthesis (Fig. 3B). Furthermore, the Ca^2+-ATPase inhibitor thapsigargin (5 μM, 15 min) slightly enhanced DHPG-induced 2-AG formation, whereas the cell-permeable calcium chelator BAPTA-AM (50 μM, 15 min) blocked this response (Fig. 3B). An even more marked reduction in 2-AG levels was elicited by EGTA (5 mM, 15 min), which lowered such levels below those of control slices (Fig. 3B).

**DGL Is Expressed in Slice Cultures.** To gather further information on the molecular mechanism of 2-AG production, we examined whether corticostriatal and hippocampal slice cultures express DGL, an intracellular lipid hydrolase that catalyzes the first committed step in neuronal 2-AG biosynthesis (Stella et al., 1997). Two DGL isoforms have been molecularly cloned, DGL-α and DGL-β (Bisogno et al., 2003). Real-time PCR analyses revealed that both isoforms are present in corticostriatal slices (Fig. 4A), where DGL-β mRNA was approximately 150 times more abundant than

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**TABLE 1**

Levels of anandamide (AEA), oleoylethanolamide (OEA), and palmitoylethanolamide (PEA) in hippocampal slices incubated with DHPG (100 mM) or vehicle

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Control</th>
<th>DHPG</th>
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<tr>
<td>AEA (pmol/mg protein)</td>
<td>11.7 ± 1.6</td>
<td>10.2 ± 1.9</td>
</tr>
<tr>
<td>OEA (pmol/mg protein)</td>
<td>8.5 ± 1.8</td>
<td>9.7 ± 1.1</td>
</tr>
<tr>
<td>PEA (pmol/mg protein)</td>
<td>65.5 ± 11.9</td>
<td>68.0 ± 10.8</td>
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**Fig. 2.** mGlut5 receptors mediate DHPG-induced increase of 2-AG levels in corticostriatal slice cultures. The slices were incubated with mGlut5 antagonist MPEP (5 μM) or mGlut1 antagonist LY367385 (100 μM) for 5 min and with DHPG (100 μM) for an additional 10 min. Changes in tissue 2-AG levels are expressed as percentage of control (287.7 ± 24.9 pmol/mg of protein; n = 18). ***, P < 0.001.

**Fig. 3.** mGlut5 receptors trigger Ca^2+-dependent formation of 2-AG through the PLC-DGL cascade. A, corticostriatal slice cultures were incubated with PLC inhibitor U73122 (10 μM) or DGL inhibitor RHC80267 (60 μM) for 5 min and with DHPG (100 μM) for an additional 10 min. B, the slice cultures were incubated with thapsigargin (5 μM), EGTA (5 mM), or BAPTA-AM (50 μM) for 5 min and with DHPG (100 μM) for an additional 10 min. Ionomycin (10 μM) treatment was 15 min. Changes in 2-AG levels are expressed as percentage of control [439.8 ± 52.5 pmol/mg of protein in A (n = 11); 307.4 ± 34.2 pmol/mg of protein in B (n = 8–10)]. **, P < 0.01; ***, P < 0.001.
DGL-α mRNA (ratio DGL mRNA/glyceraldehyde 3-phosphate dehydrogenase mRNA × 10³; DGL-α, 0.33 ± 0.06; DGL-β, 50.73 ± 9.48, n = 10). DGL-β mRNA levels in hippocampal slices (60.67 ± 3.99, n = 10) were similar to those measured in striatal slices, whereas DGL-α mRNA levels were considerably higher (17.27 ± 2.29, n = 10) (Fig. 4B).

We confirmed the high expression of DGL-β in slice cultures using an affinity-purified polyclonal antibody, which we raised using a peptide antigen comprising 20 amino acid residues of the rat DGL-β C terminus. The antibody recognized a protein with an apparent molecular mass of approximately 70 kDa on SDS-polyacrylamide gel electrophoresis, as expected for DGL-β (Bisogno et al., 2003; Fig. 5A). The band disappeared after preabsorption with the immunizing peptide (Fig. 5A). To further characterize the specificity of our antibody, we used human embryonic kidney 293 cells that heterologously expressed a modified DGL-β containing a V5-His tag fused to the protein’s C terminus. Double immunofluorescence staining with anti-DGL-β and anti-V5 antibodies followed by confocal imaging showed colocalization of the two signals in cytosol and plasma membrane (Fig. 5B). Preabsorption with the antigen selectively abrogated the DGL-β signal, confirming its specificity (Fig. 5B). Additional immunostaining studies revealed the presence of immunoreactive DGL-β throughout the striatum, cortex, and hippocampus of 7-day-old pups (Fig. 5C). In particular, anti-DGL-β antibody selectively stained neuronal elements in the striatum (Fig. 5D) that were also stained by an antibody that recognizes the 65-kDa isoform of glutamic acid decarboxylase (GAD-65), a marker of GABAergic neurons (data not shown). The DGL-β staining was eliminated by preabsorption with DGL-β peptide (Fig. 5D) and was absent when the primary antibody was omitted (data not shown).

Finally, because of the postulated role of PLC-β in 2-AG formation, we sought to determine whether this enzyme was colocalized with DGL-β. Double immunofluorescence labeling confirmed the presence of PLC-β1 in striatum (Hernan-

![Fig. 4.](image1)

![Fig. 5.](image2)
dez-Lopez et al., 2000) (Fig. 5E) and revealed that expression of this protein largely coincides with that of DGL-β (Fig. 5F).

Discussion

The main finding of the present study is that activation of glutamate mGlu5 receptors stimulates 2-AG formation in rat corticostriatal and hippocampal slice cultures. mGlu5 receptors belong to the group I mGlu receptor subfamily, which includes the mGlu1 and mGlu5 subtypes (Conn and Pin, 1997). mGlu5 receptors are highly expressed in the striatum, nucleus accumbens, and hippocampus and more moderately in the neocortex (Testa et al., 1994). Within the striatum, these receptors are localized to GABAergic projection neurons (Testa et al., 1994), where they may contribute to both short- and long-term forms of synaptic plasticity (Gerdenman et al., 2003; Gubellini et al., 2004) as well as to the addictive properties of psychostimulant drugs (Chiamulera et al., 2001). On the other hand, mGlu1 receptors are predominantly expressed in the cerebellum, amygdala, and brainstem (Testa et al., 1994). Thus, the brain distribution of these receptor subtypes tallies well with our results, which show that DHPG-induced 2-AG formation in corticostriatal slices is prevented by the selective mGlu5 receptor antagonist MPEP.

Two points are important, however. First, the role of striatal mGlu1 and mGlu5 receptors should not be understood as a simple dichotomy, because mGlu1 receptors are also expressed in the striatum (Kerner et al., 1997) and might be involved in local forms of long-term depression (Gubellini et al., 2001; Sung et al., 2001). Second, in other brain areas mGlu5 receptors participate in endocannabinoid-mediated plasticity (Brown et al., 2003; Galante and Diana, 2004; Azad et al., 2004), suggesting that they might be responsible for the generation of 2-AG in these areas. Thus, it would be interesting to test whether mGlu5 receptor activation triggers 2-AG release in mGlu1-rich structures such as the cerebellum or the amygdala (Azad et al., 2004; Galante and Diana, 2004).

Group I mGlu receptors are linked through Gq to the activation of PLC-β, which catalyzes the hydrolysis of membrane phosphatidylglycerol bisphosphate to produce the pleiotropic intracellular second messengers inositol 1,4,5-trisphosphate and 1,2-DAG (Conn and Pin, 1997). The effects of 1,2-DAG, which are mostly mediated by protein kinase C activation, are terminated through DAG kinase-mediated inactivation (Chevaleyre and Castillo, 2003; Galante and Diana, 2004). Cells that express DGL can convert phosphorylation of 1,2-DAG to phosphatidic acid (Topham et al., 1994). Thus, the brain distribution of these receptor isoforms largely coincides with that of DGL-β (Fig. 5F).

In conclusion, our experiments provide the first biochemical demonstration that mGlu5 receptors are linked to the biosynthesis of 2-AG, but not anandamide, in the rat brain. These findings should help differentiate the functions served by these two endocannabinoid lipids in synaptic modulation.

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References


Gerdenman GL, Partridge JG, Lupica CR, and Lovinger DM (2003) It could be habit formation (Lupica CR, and Lovinger DM, 2003). Our results, showing Ca2+ mobilization and receptor-dependent 2-AG formation, are consistent with these data. There are, however, several forms of endocannabinoid-mediated plasticity that do not require Ca2+ for their expression, such as those triggered by muscarinic acetylcholine receptors in the hippocampus (Maejima et al., 2001; Kim et al., 2002; Chevaleyre and Castillo, 2003; Azad et al., 2004; Galante and Diana, 2004). The identity of the endocannabinoid mediator(s) released by activation of these receptors and the molecular mechanism underlying Ca2+-independent endocannabinoid release are important questions, which should be addressed in the future.

In conclusion, our experiments provide the first biochemical demonstration that mGlu5 receptors are linked to the biosynthesis of 2-AG, but not anandamide, in the rat brain. These findings should help differentiate the functions served by these two endocannabinoid lipids in synaptic modulation.