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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 Ca^{2+} ions. Real-time polymerase chain reaction and immunostaining analyses indicate that DGL- β 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- β 1. 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 Ca^{2+} -dependent formation of an endocannabinoid messenger, which might be produced postsynaptically and travel across the synaptic space to activate CB_1 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 $G_{q/11}$ protein-coupled receptors depresses synaptic transmission in a variety of brain regions, including the striatum (Gerdeman 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 (Melis et al., 2004), and amygdala (Marsicano et al., 2002; Azad et al., 2004) through a mechanism that requires CB_1 receptor activation. Furthermore, mGlu receptor-dependent forms of synaptic modulation mediated by endocannabinoids have been implicated in prolonged changes in synaptic strength,

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ABBREVIATIONS: DSI, depolarization-induced suppression of inhibition; mGlu, metabotropic glutamate; 2-AG, 2-arachidonoylglycerol; PLC, phospholipase C; 1,2-DAG, 1,2-diacylglycerol; DGL, diacylglycerol lipase; DHPG, (S)-3,5-dihydroxyphenylglycine; LY367385 (S)-(+)- α -amino-*m*-methylbenzeneacetic acid; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid, acetoxymethyl ester; RHC80267, 1,6-bis-(cyclohexyloximino carbonylamino)-hexane; U73122, 1-(6-[17 β -3-methoxyestra-1,3,5-(10)triene-17-yl]amino)hexyl) 1*H*-pyrroledione; HPLC, high-performance liquid chromatography; MS, mass spectrometry; 2-OG, 2-oleoylglycerol; PCR, polymerase chain reaction; MPEP, 2-methyl-6-phenylethynyl-pyridine; 1,2-DAG, 1,2-diacylglycerol.

such as those occurring during the induction of long-term depression at corticostriatal synapses (Robbe et al., 2002; Gerdeman 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 (arachidonylethanolamide) and 2-arachidonylethanolamide (2-AG) (for review, see Piomelli, 2003). Anandamide may be generated by hydrolysis of the phospholipid precursor *N*-arachidonylethanolamine, 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- β -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-Dihydroxyphenylglycine (DHPG), 2-methyl-6-(phenylethynyl)pyridine hydrochloride and LY367385 were obtained from Tocris (Ellisville, MO); ionomycin, BAPTA-AM, EGTA, and thapsigargin from Sigma-Aldrich (St. Louis, MO); RHC80267 and U73122 from BIOMOL Research Laboratories (Plymouth Meeting, PA). Test compounds were dissolved in either water (DHPG, MEPE, LY367385, and EGTA) or dimethyl sulfoxide (ionomycin, BAPTA-AM, thapsigargin, RHC80267, and U73122) and used on the same day of preparation. Final dimethyl sulfoxide concentration did not exceed 0.5%.

Slice Cultures. We prepared organotypic slice cultures from Wistar rat pups, as described previously (Stoppini et al., 1991). In brief, the pups were sacrificed on postnatal day 7 by decapitation after halothane anesthesia. The brains were cut into 0.4-mm coronal slices using a vibratome (Campden Instruments, Leicestershire, UK) in a bath of ice-cold, high-glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA). Corticostriatal or hippocampal slices were placed on Millicell culture inserts (Millipore, Billerica, MA) in six-well plates and covered with basal Eagle's medium with Earle's salts containing heat-inactivated horse serum (25%; v/v), L-glutamine (1 mM), and glucose (0.5%; w/v) supplemented with Earle's balanced salt solution and antibiotics (Invitrogen). Slices were incubated at 37°C with 5% CO₂ for 6 to 7 days before use.

Lipid Analyses. We incubated the slices with various pharmacological agents or their vehicles as described under *Results*. After a brief wash with ice-cold methanol (50%), the slices were scraped into 0.4 ml of 50% methanol and homogenized on ice with a Teflon pestle. We extracted lipids with chloroform/methanol (1:1) and analyzed

lipid products by high-performance liquid chromatography/mass spectrometry (HPLC/MS) as described previously (Fegley et al., 2005). 2-[²H₈]AG (Cayman Chemical, Ann Arbor, MI) and 1(3)-heptadecanoylglycerol (500 pmol; NuCheck Prep, Elysian, MN) were used as standards for the quantification of 2-AG and 2-oleoylglycerol (2-OG), respectively. Standards for fatty-acid ethanolamides (²H₄]anandamide, [²H₄]oleylethanolamide, and [²H₄]palmitylethanolamide) were synthesized in the laboratory (Fegley et al., 2005). We normalized the quantity of lipids by the amount of protein, measured using a BCA protein assay (Pierce, Rockford, IL).

Quantitative Polymerase Chain Reaction. We extracted total RNA from corticostriatal or hippocampal brain slices cultured for 7 days at 37°C with 5% CO₂ with TRIzol (Invitrogen) and synthesized first-strand complementary DNA using Superscript II RNaseH reverse transcriptase (Invitrogen). Reverse transcription of total RNA (0.2 μ g) was carried out using oligo(dT)12–18 primers for 50 min at 42°C. Real-time quantitative PCR was conducted using a PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). We designed primer/probe sets with Primer Express software (Applied Biosystems) and rat DGL sequences obtained from NCBI database based on reported DGL sequences (Bisogno et al., 2003). Primers and fluorogenic probes were synthesized by TIB Molbiol (Adelphia, NJ). DGL mRNA levels were normalized using glyceraldehyde-3-phosphate dehydrogenase as an internal standard. The primer/probe sequences were as follows: for rat DGL- α (GI: 54312093): forward, 5'-CCAGGCCCTTTGGGCG-3'; reverse, 5'-GCC-TACCACAATCAGGCCAT-3'; TaqMan probe, 5'-ACCTGGGCCGTG-GAACCAACA-3'; for rat DGL- β (GI: 34870416): forward, 5'-AGG-ACTGCGTGGCCAC-3'; reverse, 5'-CGTTGACCAGTCTTCGATA-AATGT-3'; TaqMan probe, 5'-AGGGAATTGCTCAAGCGCCAGA-3'.

Plasmids. Coding sequences for the full-length rat DGL β were amplified by PCR from a rat brain cDNA library using High Fidelity PCR Master (Roche, Indianapolis, IN). The primers used were 5'-DGL β (5'-CGTAATGCCGGGGATGGTGCTGTTTC-3') and 3'-DGL β (5'-AGGGACGTTAGAGCCGCTTGGCC-3'). The PCR product was subcloned into a pEF-V5/His vector by TOPO cloning (Invitrogen). Constructs were fully verified by DNA sequencing.

Immunostaining and Western Blotting. We raised a rabbit polyclonal antibody against a glutathione *S*-transferase-linked peptide comprising residues 661 to 680 in the C-terminal sequence of rat DGL- β (ETEFKILIGPKMLIDHMPD, GI:34870417). The antigen affinity-purified antibody was used at a dilution of 1:2500 for Western blotting and 1:500 for immunostaining, which were conducted as described previously (Jung et al., 2003). We used frozen brain sections prepared from 7 day-old rat pups for all the immunostaining experiments, except Fig. 1A, inset, in which we used corticostriatal slice cultures. Fixed sections were blocked for 4 h at room temperature with 4% normal goat serum in phosphate-buffered saline and subsequently incubated overnight at 4°C with primary antibody under the following conditions: monoclonal anti-neuronal class III β -Tubulin (TUJ1, 1:500; Covance, Berkeley, CA); monoclonal anti-V5 (1:5000; Invitrogen); monoclonal anti-PLC β 1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) in 4% normal goat serum; and 0.2% Triton X-100 in phosphate-buffered saline. Sections were stained by using Elite ABC (Vector Laboratories, Burlingame, CA) and diaminobenzidine substrate (Vector Laboratories) kits and counterstained with hematoxylin before mounting. Alexa 488-labeled anti-rabbit or Alexa 568-labeled anti-mouse secondary antibodies (1:1000; Invitrogen) were used for fluorescence detection. For confocal microscopy, we used fluorescein isothiocyanate-labeled anti-rabbit or Texas Red-labeled anti-mouse secondary antibodies (1:150; Jackson ImmunoResearch Laboratories, West Grove, PA). Slides were mounted and images were captured using a confocal or fluorescence microscope equipped with a digital camera (Diagnostic Instruments, Sterling Heights, MI). 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 14,000g for 10 min. Proteins (30 μ g) 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 \pm 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 μ M), 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-

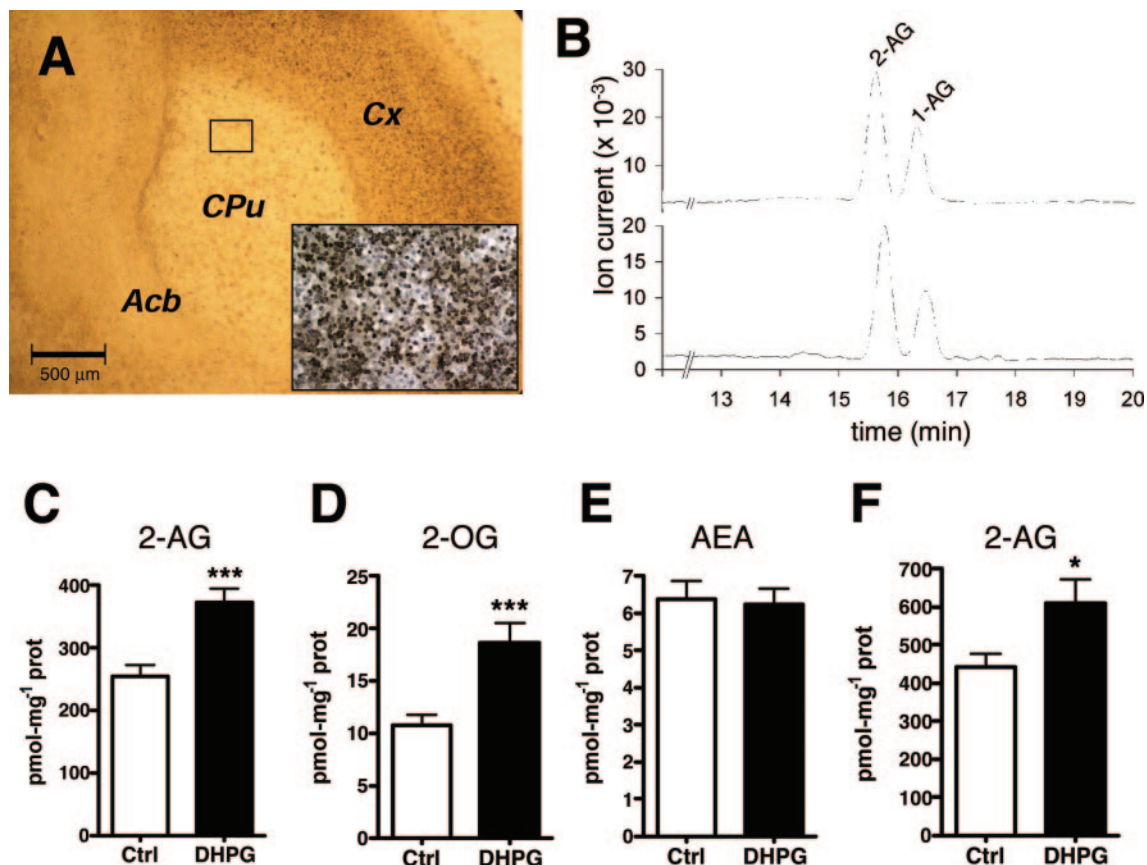


Fig. 1. Pharmacological activation of group I mGlu receptors increases 2-AG levels in rat brain slice cultures. A, inverted microscope image of a 7-day-old corticostriatal slice in culture. Acb, nucleus accumbens; CPu, caudate putamen; Cx, cortex. Inset shows staining for neuronal class III β -Tubulin in the marked region. B, representative HPLC/MS tracing showing the presence of endogenous 2-AG in corticostriatal slice cultures (bottom, $m/z = 401$); also shown is the internal standard 2-[²H₈]AG (top, $m/z = 409$). Effects of group I mGluR agonist DHPG (100 μ M) on 2-AG levels (C), 2-OG levels (D), and anandamide (AEA) (E) levels in corticostriatal slice cultures ($n = 44$). F, effects of DHPG (100 μ M) on 2-AG levels in hippocampal slice cultures ($n = 8$). *, $P \leq 0.05$; ***, $P \leq 0.001$.

tein, $n = 44$). We obtained similar results using hippocampal slice cultures (Fig. 1F and Table 1).

mGlu5 Receptor Activation Mediates 2-AG Formation. Further pharmacological analyses, conducted in corticostriatal slice cultures, indicated that DHPG stimulates 2-AG formation by activating mGlu5 receptors. Preincubation with MPEP (5 μ M, 5 min), a selective mGlu5 receptor antagonist (Gasparini et al., 1999), but not with LY 367385 (100 μ M, 5 min), an mGlu1 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 mGlu5 receptors (Robbe et al., 2002).

2-AG Is Produced through the PLC-DGL Cascade. mGlu5 receptors are coupled to $G_{q/11}$ proteins and signal through PLC- β -mediated breakdown of inositol phospholipids (Hannan et al., 2001). Therefore, incubation with the PLC inhibitor U73122 (10 μ M, 15 min) prevented the stimulation of 2-AG formation by DHPG in corticostriatal slices (Fig. 3A). The nonselective DGL inhibitor RHC80267 (60 μ M, 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 μ M, 15 min) markedly increased 2-AG levels, supporting the idea that intracellular Ca^{2+} rises can trigger 2-AG biosynthesis (Fig. 3B). Further-

more, the Ca^{2+} -ATPase inhibitor thapsigargin (5 μ M, 15 min) slightly enhanced DHPG-induced 2-AG formation, whereas the cell-permeable calcium chelator BAPTA-AM (50 μ M, 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

TABLE 1

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

Lipid levels are presented as mean \pm S.E.M. ($n = 8$).

	AEA	OEA	PEA
	<i>pmol/mg protein</i>		
Control	11.7 \pm 1.6	8.5 \pm 1.8	65.5 \pm 11.9
DHPG	10.2 \pm 1.9	9.7 \pm 1.1	68.0 \pm 10.8

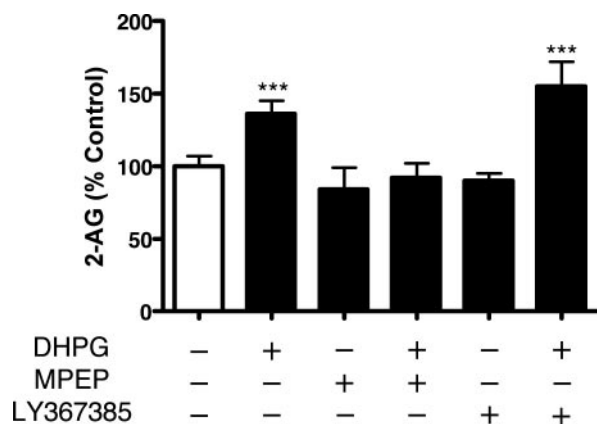


Fig. 2. mGlu5 receptors mediate DHPG-induced increase of 2-AG levels in corticostriatal slice cultures. The slices were incubated with mGlu5 antagonist MPEP (5 μ M) or mGlu1 antagonist LY367385 (100 μ M) for 5 min and with DHPG (100 μ M) for an additional 10 min. Changes in tissue 2-AG levels are expressed as percentage of control (287.7 \pm 24.9 pmol/mg of protein; $n = 18$). ***, $P < 0.001$.

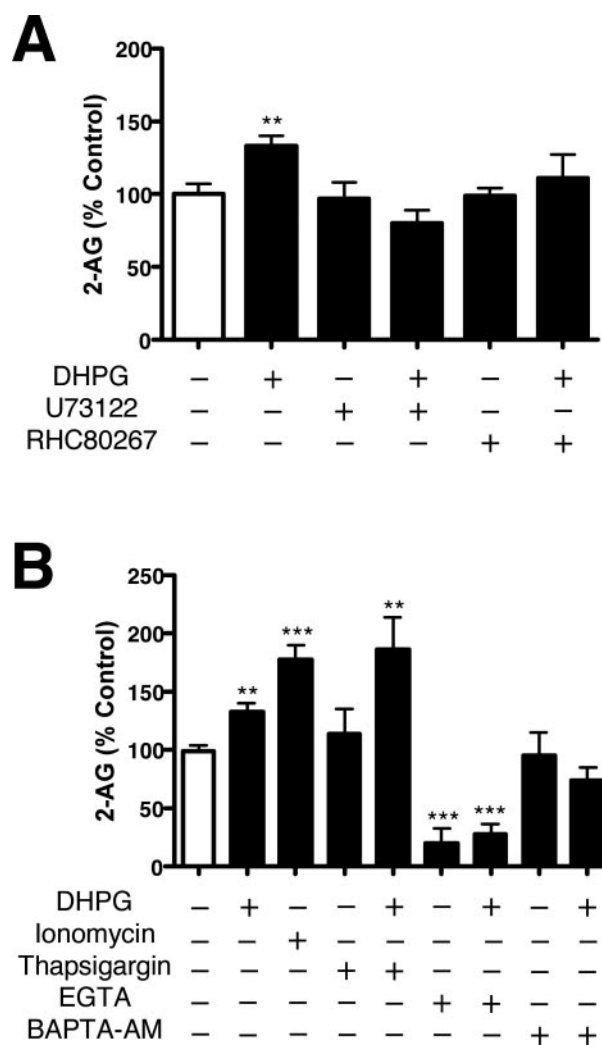


Fig. 3. mGlu5 receptors trigger Ca^{2+} -dependent formation of 2-AG through the PLC-DGL cascade. A, corticostriatal slice cultures were incubated with PLC inhibitor U73122 (10 μ M) or DGL inhibitor RHC80267 (60 μ M) for 5 min and with DHPG (100 μ M) for an additional 10 min. B, the slice cultures were incubated with thapsigargin (5 μ M), EGTA (5 mM), or BAPTA-AM (50 μ M) for 5 min and with DHPG (100 μ M) for an additional 10 min. Ionomycin (10 μ M) treatment was 15 min. Changes in 2-AG levels are expressed as percentage of control [439.8 \pm 52.5 pmol/mg of protein in A ($n = 11$); 307.4 \pm 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 $\times 10^3$; 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 approx-

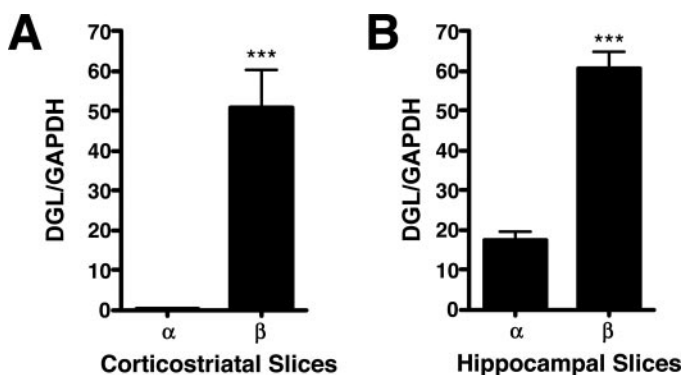


Fig. 4. DGL- α and DGL- β mRNA levels in corticostriatal (A) and hippocampal (B) slices cultured for 7 days in vitro, as assessed by quantitative PCR. ***, $P < 0.001$ ($n = 10$).

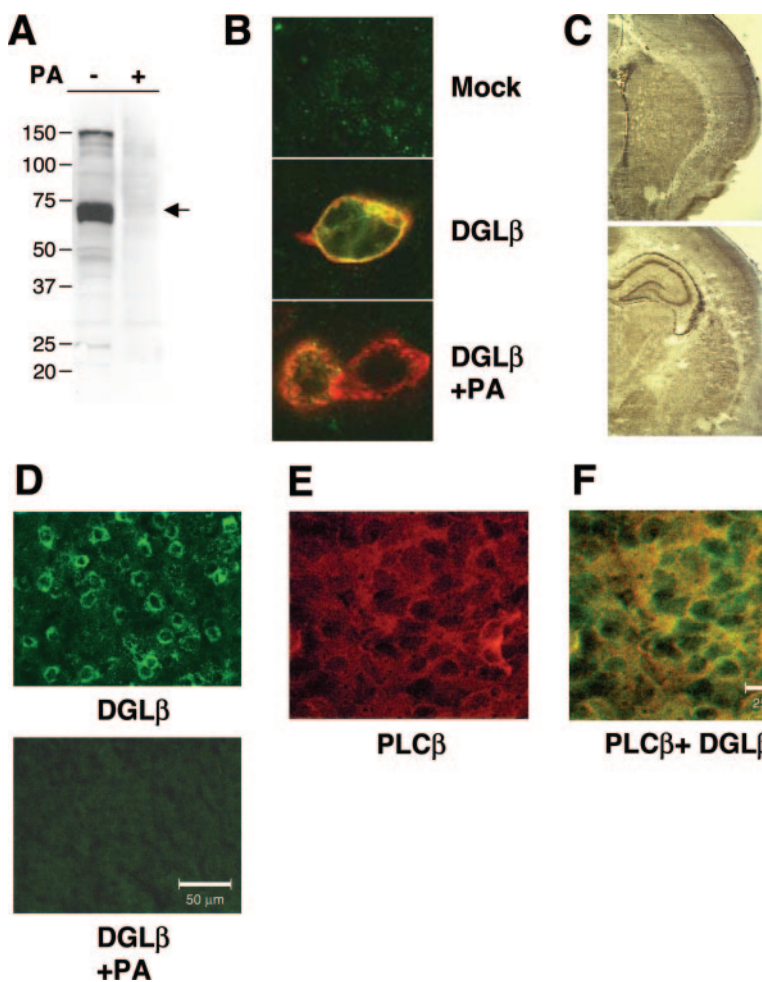


Fig. 5. DGL- β is expressed in rat corticostriatal slices and neonatal striatal neurons. A, Western blot analysis of slice extracts with an affinity-purified anti-DGL- β antibody. Arrow indicates the 70-kDa protein band recognized by the antibody. PA, preabsorption with immunizing peptide. B, confocal microscope image of human embryonic kidney 293 cells expressing a DGL- β -V5-His fusion protein. Double immunostaining was performed with anti-DGL- β (green) and anti-V5 (red) antibodies. C, diaminobenzidine immunostaining of coronal section of neonatal rat brain with anti-DGL- β . D, fluorescence immunostaining of neonatal striatum with anti-DGL- β . E, fluorescence immunostaining of neonatal striatum with anti-PLC- β 1 (E) and double labeling of DGL- β (green) and PLC- β 1 (red) (F).

imately 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- β 1 in striatum (Hernan-

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 (Gerdeman 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 mGlu1 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 mGlu1 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 $G_{q/11}$ to the activation of PLC- β , which catalyzes the hydrolysis of membrane phosphatidylinositol biphosphate 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 phosphorylation of 1,2-DAG to phosphatidic acid (Topham and Prescott, 1999). Cells that express DGL can convert 1,2-DAG to 2-AG and other unsaturated 2-acylglycerols (Prescott and Majerus, 1983). Our experiments indicate that nonselective pharmacological inhibitors of PLC and DGL, which were previously shown to block mGlu-mediated plasticity (Chevalere and Castillo, 2003; Galante and Diana, 2004), prevent DHPG-induced 2-AG formation. The results also show that the β isoform of DGL is expressed in striatal neurons, where it colocalizes with the $\beta 1$ isoform of PLC, an essential component of mGlu- and endocannabinoid-dependent plasticity in hippocampal neurons (Hashimoto et al., 2005). Together, these findings support the view that the PLC- β -DGL- β pathway is responsible for mGlu5-induced 2-AG generation in corticostriatal slice cultures. Future experiments should further test this hypothesis, determining in

particular the roles of different PLC- β and DGL isoforms in depolarization- and receptor-dependent 2-AG formation.

In the striatum, endocannabinoid-mediated changes in synaptic strength are dependent on intracellular Ca^{2+} (Gerdeman et al., 2002; Robbe et al., 2002) and mGlu5 receptor activation mobilizes Ca^{2+} from internal stores (Robbe et al., 2002; Mao and Wang, 2003). Our results, showing Ca^{2+} chelators block that mGlu5-induced 2-AG biosynthesis, are consistent with these data. There are, however, several forms of endocannabinoid-mediated plasticity that do not require Ca^{2+} for their expression, such as those triggered by muscarinic acetylcholine receptors in the hippocampus (Maejima et al., 2001; Kim et al., 2002; Chevalere and Castillo, 2003; Azad et al., 2004; Galante and Diana, 2004). The identity of the endocannabinoid mediator(s) released by activation of such receptors and the molecular mechanism underlying Ca^{2+} -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.

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