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THE LINOLEIC ACID DERIVATIVE FR236924 FACILITATES HIPPOCAMPAL SYNAPTIC TRANSMISSION BY ENHANCING ACTIVITY OF PRESYNAPTIC α7 ACETYLCHOLINE RECEPTORS ON THE GLUTAMATERGIC TERMINALS

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Abstract—The present study aimed at understanding the effect of FR236924, a newly synthesized linoleic acid derivative with cyclopropane rings instead of *cis*-double bonds, on hippocampal synaptic transmission in both the *in vitro* and *in vivo* systems. FR236924 increased the rate of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor-mediated miniature excitatory postsynaptic currents, without affecting the amplitude, triggered by nicotine in CA1 pyramidal neurons of rat hippocampal slices, that is inhibited by GF109203X, a selective protein kinase C (PKC) inhibitor or α-bungarotoxin, an inhibitor of α7 acetylcholine (ACh) receptors. FR236924 stimulated glutamate release from rat hippocampal slices and in the hippocampus of freely behaving rats, and the effect was also inhibited by GF109203X or α-bungarotoxin. FR236924 induced a transient huge potentiation followed by a long-lasting potentiation in the slope of field excitatory postsynaptic potentials recorded from the CA1 region of rat hippocampal slices, and the latter effect was blocked by GF109203X or α-bungarotoxin. Likewise, the compound persistently facilitated hippocampal synaptic transmission in the CA1 region of the intact rat hippocampus. It is concluded from these results that FR236924 stimulates glutamate release by functionally targeting presynaptic α7 ACh receptors on the glutamatergic terminals under the influence of PKC, responsible for the facilitatory action on hippocampal synaptic transmission. This may provide evidence for a link between *cis*-unsaturated free fatty acids and presynaptic α7 ACh receptors in hippocampal synaptic plasticity.

Key words: FR236924, α7 acetylcholine receptor, glutamate release, facilitation, synaptic transmission, hippocampus.

Several lines of evidence have pointed to a pivotal role of brain nicotinic acetylcholine (ACh) receptors in cognitive functions; however, the underlying mechanism is not fully understood. In explanation of this, we propose that presynaptic nicotinic ACh receptors may function downstream of *N*-methyl-D-aspartate (NMDA) receptor signaling events in the pathway of long-term potentiation (LTP), a cellular model of learning and memory. This is based upon the data that NMDA receptor-dependent LTPs are inhibited by inhibitors of nicotinic ACh receptors; nicotine facilitates hippocampal synaptic transmission with saturation occluding the potentiation achieved by high frequency stimulation, to induce LTP, and vice versa; and the LTPs are still induced by nicotine treatment in the presence of a selective NMDA receptor inhibitor (Matsuyama et al., 2000; Nishizaki et al., 2001). *Cis*-unsaturated free fatty acids such as arachidonic, linoleic, and linolenic acid, alternatively, induce a long-lasting facilitation of hippocampal synaptic transmission that resembles LTP, as a result of enhancing activity of nicotinic ACh receptors via a protein kinase C (PKC) pathway (Ikeuchi et al., 1996; Nishizaki et al., 1997, 1998, 1999a). Interestingly, high frequency stimulation to hippocampal slices causes an increase in glutamate release followed by arachidonic acid release (Nishizaki et al., 1999b). *Cis*-unsaturated free fatty acids, thus, may modulate hippocampal synaptic transmission by functionally targeting nicotinic ACh receptors. Direct evidence for this in the *in vivo* systems, however, has not been shown, since when systemically applied, *cis*-unsaturated free fatty acids are promptly metabolized and decomposed before arriving in the brain, even though high concentrations of the free fatty acids are contained in the blood.

To resolve this problem, we synthesized a variety of derivatives of *cis*-unsaturated free fatty acids that exhibit stable bioactivities. In screening their action on *Torpedo* nicotinic ACh receptors expressed in *Xenopus* oocytes, the most potent positive hit was the linoleic acid derivative FR236924, that has cyclopropane rings instead of *cis*-double bonds (Tanaka and Nishizaki, 2003). The present study explored the effects of FR236924 on hippocampal synaptic transmission in both the *in vitro* and *in vivo* systems by monitoring excitatory postsynaptic currents (EPSCs), assaying glutamate, and recording field excitatory postsynaptic potentials (fEPSPs). We show here that FR236924 stimulates glutamate release as mediated via presynaptic α7 ACh receptors on the glutamatergic terminals, possibly responsible for the facilitatory action on hippocampal synaptic transmission.

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Abbreviations: ACh, acetylcholine; ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; DL-AP5, DL-2-amino-5-phosphonovaleric acid; DMSO, dimethylsulfoxide; DNQX, 6,7-dinitroquinoxaline-2,3-dione; eEPSC, evoked excitatory postsynaptic current; EPSC, excitatory postsynaptic current; fEPSP, field excitatory postsynaptic potential; HPLC, high-performance liquid chromatography; LTP, long-term potentiation; mEPSC, miniature excitatory postsynaptic current; NMDA, *N*-methyl-D-aspartate; PKC, protein kinase C; TTX, tetrodotoxin.

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EXPERIMENTAL PROCEDURES

Slice-patch recording

Slice patches were made from CA1 pyramidal neurons of rat hippocampal slices (male Wistar rat, six w) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor-mediated miniature EPSCs (AMPA-mEPSCs), that are abolished by 6,7-dinitroquinoxaline-2,3-dione (DNQX; 20 μM), were recorded in standard artificial cerebrospinal fluid (ACSF; in mM: 117 NaCl, 3.6 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 25 $NaHCO_3$, 11.5 glucose) oxygenated with 95% O_2 and 5% CO_2, containing tetrodotoxin (TTX; 0.5 μM), DL-2-amino-5-phosphonovaleric acid (DL-AP5; 100 μM), a selective inhibitor of NMDA receptors, and bicuculline (20 μM), a selective inhibitor of $GABA_A$ receptors, at 34 °C with an Axopatch-200 A amplifier (Axon Instruments, Inc., Union City, CA, USA). All drugs were bath-applied by switching three-way cock, and a recording chamber was continuously perfused with ACSF in the presence and absence of FR236924 (100 nM) at the flow rate of 2 ml/min. Evoked EPSCs (eEPSCs) were recorded from CA1 pyramidal neurons by electrically stimulating the Schaffer collateral in standard ACSF at 34 °C. The patch electrode-filling solution was (mM) 110 Cs_2SO_4, 5 TEACl, 2 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 5 HEPES, and 5 MgATP. Biocytin (0.3% v/v) was added to the patch electrode-filling solution for staining of patched cells.

Biocytin staining

After patch-clamp recordings, slices were fixed with 4% paraformaldehyde (v/v)/0.1 M phosphate buffer solution (pH 7.4) for 12 h. Then, slices were washed with 80% ethanol (v/v) followed by dimethylsulfoxide (DMSO). After rinsing with 0.1 M phosphate-buffered saline (pH 7.0), slices were treated with 0.5% Triton X-100 (v/v)/0.1 M Tris buffer (pH 7.0) and additional 0.1% extravidin–horseradish peroxidase conjugates (v/v) for 12 h. Finally, slices were reacted with diaminobenzidine (0.5 mg/ml) and 0.01% hydroxyperoxide (v/v), and mounted in DMSO after washing out with 0.1 M Tris buffer.

Glutamate assay

All experimental procedures were previously approved by the Hyogo College of Medicine Committee on Animal Research and carried out in accordance with the guidelines of NIH on animal care. We tried to minimize the number of animals used and their suffering for experiments. Rat hippocampal slices (male Wistar rat, six w) were incubated in standard ACSF containing TTX (0.5 μM) with and without nicotine (1 μM) in the presence and absence of FR236924 (100 nM) alone/or plus GF109203X (100 nM), α-bungarotoxin (100 nM), mecamylamine (3, 10, and 30 μM), or dihydro-β-erithroidine (10 μM) at 34 °C for 20 min. In a different set of experiments, slices were treated with choline (1 mM) or A85380 (1 μM) in the presence and absence of FR236924 (100 nM). Glutamate released into the medium was measured using a high-performance liquid chromatography (HPLC). For glutamate assay in freely behaving rats, a microdialysis probe was inserted and fixed in the CA1 region of the right hippocampus under urethane anesthesia. Perfusate was collected every 10 min by perfusing standard ACSF with and without GF109203X (10 μM) or α-bungarotoxin (1 μM) at a flow rate of 2 ml/min before and after injection with FR236924 (1 mg/kg, i.p.), linoleic acid (1 mg/kg, i.p.), or saline, and glutamate released was measured using an HPLC.

fEPSP recording

For rat hippocampal slices (male Wistar rat, six w), fEPSPs were recorded from the CA1 region by electrically stimulating the Schaffer collateral (0.03 Hz, 0.1 ms in duration) in standard ACSF oxygenated with 95% O_2 and 5% CO_2 at 34 °C before and after 10-min treatment with FR236924 (100 nM) in the presence and absence of GF109203X (100 nM), α-bungarotoxin (100 nM), or α-bungarotoxin (100 nM) plus mecamylamine (3 μM). For intact rats (male Wistar rat, six w) under urethane anesthesia, fEPSPs were recorded from the CA1 region of the hippocampus by electrically stimulating the Schaffer collateral (0.03 Hz, 0.1 ms in duration) before and after i.p. injection with FR236924 (1 mg/kg), linoleic acid (1 mg/kg), or saline.

Chemicals

AMPA, DNQ, DL-AP5, and GF109203X were purchased from Tocris (Bristol, UK); bicuculline, biocytin, α-bungarotoxin, mecamylamine, dihydro-β-erithroidine, and A85380 from Sigma (St. Louis, MO, USA); TTX from WAKO (Osaka, Japan); and all other chemicals from Nacalai (Kyoto, Japan).

RESULTS

Effects of FR236924 on synaptically released glutamate

Presynaptic α7 ACh receptors on the glutamatergic terminals regulate excitation of pyramidal neurons in the hippocampus (Gray et al., 1996; Wonnacott, 1997). We earlier found that FR236924 potentiates α7 ACh receptor responses via a PKC pathway (Tanaka and Nishizaki, 2003). Then, one would wonder whether FR236924 exerts its stimulatory action on glutamate release. To address this point, we made whole-cell patches in CA1 pyramidal neurons of rat hippocampal slices (Fig. 1) and monitored AMPA-mEPSCs. FR236924 (100 nM) significantly increased the rate of AMPA-mEPSCs triggered by nicotine ($P<0.01$, Kolmogorov-Smirnov test), without affecting the amplitude (Fig. 2A), while FR236924 by itself does not affect AMPA-mEPSCs in the absence of nicotine (Fig. 2D). The FR236924 action was blocked by GF109203X, a selective inhibitor of PKC, or α-bungarotoxin, an inhibitor of α7 ACh receptors (Fig. 2B, C), but not by mecamylamine (3 and 10 μM), a broad-spectrum inhibitor of non-α7 ACh receptors (data not shown), suggesting that FR236924 stimulates glutamate release via presynaptic α7 ACh receptors under the influence of PKC.

To obtain further evidence for this, we assayed glutamate released from rat hippocampal slices in the presence of TTX. FR236924 (100 nM) did not change glutamate release in the absence of nicotine (Fig. 3A). Nicotine-stimulated glutamate release (not significant), and FR236924 (100 nM) significantly increased nicotine-triggered glutamate release to three-fold of that in the absence of FR236924 (Fig. 3A). The increase was prevented by GF109203X (100 nM) or α-bungarotoxin (100 nM) but not by mecamylamine at concentrations of 3, 10, and 30 μM or dihydro-β-erithroidine (10 μM), a relative specific inhibitor of α4β2 ACh receptors (Fig. 3A). Furthermore, FR236924 (100 nM) significantly enhanced glutamate release induced by choline (1 mM), an agonist of α7 ACh receptors (Papke et al., 1996), but it otherwise had no effect on glutamate release induced by A85380 (1 μM) (Fujii and Sumikawa, 2001), an agonist of non-α7 ACh receptors (Fig. 3A). In freely behaving rats, FR236924 (1 mg/kg, i.p.) stimulated glutamate release in the CA1