U-46619, a Selective Thromboxane A₂ Mimetic, Inhibits the Release of Endogenous Noradrenaline From the Rat Hippocampus In Vitro

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ABSTRACT—Possible roles of thromboxane A₂ (TXA₂) in the release mechanism of hippocampal noradrenaline (NA) were examined in vitro. Slices or crude synaptosomes prepared from the rat hippocampus were superfused with modified Krebs-Ringer solution. Application of 20 mM KCl for 5 min increased the release of NA from the slices, and this release was consistently reproduced. Application of U-46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin F₂₀), a specific TXA₂ mimetic, just before the second KCl (20 mM) stimulation decreased the KCl-evoked NA release in a concentration-dependent manner (10–100 μM). This U-46619 (50 μM)-induced inhibition of NA release was abolished by 10 μM SQ29548, a specific TXA₂ receptor antagonist. In experiments with hippocampal crude synaptosomes, however, KCl (20 and 40 mM)-evoked release of NA was not attenuated by U-46619 (100 μM). Furthermore, the inhibitory effect of U-46619 (50 μM) in the sliced preparations was not modified by 100 μM (−)-bicineulline, a GABA_A-receptor antagonist. The present results indicate that U-46619 inhibits the release of NA from the rat hippocampus by activation of TXA₂ receptors. Activation of TXA₂ receptors probably excites an unidentified but not GABAergic neuron system, thereby inhibiting the NA release from the rat hippocampus.

Keywords: Thromboxane A₂ (TXA₂), Noradrenaline release, Hippocampus, U-46619, TXA₂ mimetic

Thromboxane A₂ (TXA₂), an active metabolite of arachidonate, is well-known as an endogenous vasoconstrictor and platelet aggregator (1). However, several lines of recent evidence suggest that TXA₂ plays roles in the brain. Reperfusion of the rat forebrain after ischemia elevates the levels of TXB₂, a stable TXA₂ metabolite, in the dorsal hippocampus (2, 3): excessive amount of hippocampal glutamate released under ischemia activates N-methyl-D-aspartate (NMDA) receptors (4); NMDA releases TXA₂ in the rabbit hippocampus (5). In our previous study, intracerebroventricular administration of TXA₂ antagonists or TXA₂ synthase inhibitors abolished the nitric oxide-induced elevation of plasma levels of catecholamines in rats (6). Furthermore, we have just found that application of NMDA into the hypothalamus produces TXA₂ and activates central adrenomedullary outflow (7).

Glutamatergic NMDA receptors are abundant in the hippocampus (8, 9), and the release of noradrenaline from the hippocampus is modulated by NMDA, γ-aminobutyric acid (GABA) and some other neurotransmitters (10–12). Activation of NMDA receptors induces long-term potentiation in the hippocampus (13), and noradrenaline enhances this long term potentiation (14–17). These reports together with our findings suggest a possibility that TXA₂ is involved in modulation of noradrenaline release from the hippocampus.

In the present study, therefore, we examined effects of U-46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin F₂₀), a stable TXA₂ mimetic, on the release of noradrenaline from the rat hippocampus, in vitro.

MATERIALS AND METHODS

Experiments with hippocampal slices

Male Wistar rats weighing approximately 350–400 g were provided with food and water ad libitum and were maintained on a constant day-night cycle at a constant temperature (22–24°C) for at least 2 weeks. The animal was sacrificed by decapitation, and the brain was quickly removed into ice-cold modified Krebs-Ringer bicarbonate solution (MKR) (pH 7.4). Then the brain was sliced into 300-μm coronal sections by a microslicer (DSK-1000; Dosaka, Kyoto), and hippocampal regions were carefully isolated by scissors under a stereoscopic microscope. Four slices were placed on a paper disk held in a chamber (Swinnex SX-13; Millipore Co., Bedford, MA, USA) and

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superfused with MKR (pH 7.4, 37°C), bubbled with a mixture of 95% O₂ / 5% CO₂, at a constant flow rate of 0.5 ml/min. MKR was composed of 118 mM NaCl, 4.7 mM KCl, 1.2 mM CaCl₂, 1.1 mM MgCl₂, 1.1 mM Na₂HPO₄, 25 mM NaHCO₃, 11.1 mM glucose, 10 μM pargyline and 1 μM desipramine. After an equilibration period of 60 min, the superfusate was collected into an ice-cold glass tube every 5 min.

In each experiment, KCl (20 mM)-stimulation was performed twice for 5 min at 65 min and 95 min after the start of superfusion. KCl was substituted for an equivalent amount of NaCl. U-46619 (Cayman Chemical Co., Ann Arbor, MI, USA), a selective TXA₂ mimetic, was applied during the second KCl-stimulation. SQ29548 (Cayman Chemical Co.), a TXA₂ receptor antagonist was applied 5 min before the second KCl-stimulation and continued during the second KCl-stimulation. Pretreatment with (−)-buculline methiodide (Sigma Chemical Co., St. Louis, MO, USA), a GABAₐ-receptor antagonist, was started 25 min before the first KCl-stimulation and continued by the end of the experiment. U-46619 was dissolved in 100% methyl acetate, and the final concentration of methyl acetate in the perfusion medium was 0.35%. SQ29548 was dissolved in 100% dimethylsulphoxide (DMSO), and the final concentration of DMSO in the perfusion medium was 0.03%. In the control experiments, perfusion medium containing 0.03% DMSO and/or 0.35% methyl acetate was applied. (−)-Bicuculline methiodide was dissolved in the perfusion medium.

**Experiments with hippocampal crude synaptosomes**

Crude synaptosomes were prepared by the method of Leslie et al. (18) with slight modifications. The hippocampus quickly removed on an ice-cold glass plate (19), was homogenized in 9 vol (w/v) of 0.32 M sucrose (buffered at pH 7.0 with 10 mM Tris). The supernatant was centrifuged for 10 min at 3,000 rpm at 4°C to remove nuclei and debris. The supernatant was centrifuged for 30 min at 11,000 rpm at 4°C, and the crude synaptosomal pellet (P2 fraction) was resuspended in 1 ml of 0.32 M sucrose. Then 0.5 ml aliquots of suspension were slowly injected into a chamber held on a glass microfiber filter (GF/C). The chamber was superfused with oxygenated MKR (95% O₂ / 5% CO₂, pH 7.4, 37°C) at a flow rate of 0.5 ml/min. After 30 min, the superfusate was collected into an ice-cold glass tube every 5 min. Each superfusate (2.3 ml) was used for noradrenaline assay. In each experiment, KCl (20 mM or 40 mM) was applied for 5 min once at 40 min after the start of superfusion. KCl was substituted for an equivalent amount of NaCl. U-46619 (dissolve in 0.35% methyl acetate) was applied during the KCl-stimulation.

**Noradrenaline assay**

Noradrenaline released into the medium and that remaining in the hippocampal preparations at the end of experiment was extracted by the method of Anton and Sayre (20), with slight modifications, and assayed electrochemically using high-performance liquid chromatography (HPLC) (21). For extraction of tissue noradrenaline, hippocampal slices or synaptosomes with microglass fiber filters were homogenized in 2 ml of cold 0.1 N perchloric acid containing 2 μmol disodium ethylenediamine-tetra-acetic acid (EDTA), 10 ng 3,4-dihydroxyamphetamine (DHBA) and 200 μl of 2% sodium pyrosulfite solution using a Polytron homogenizer. The homogenate was centrifuged for 10 min at 10,000 rpm at 4°C. The supernatant (1 ml) thus obtained or superfusion medium (2.3 ml) was put in a cold glass tube containing 30 mg activated alumina and 3 ml of 1.5 M Tris-HCl buffer solution (pH 8.6) and 2 μmol disodium EDTA. DHBA (1 ng) was also added in the glass tube for superfusion medium. The glass tube was shaked for 10 min and alumina was washed three times with 4 ml of ice-cold deionized water. Then noradrenaline was eluted with 200 μl of 4% acetic acid containing 2 μmol disodium EDTA. A pump (EP-300; Eicom Co., Kyoto), a sample injector (model-231XL; Gilson Co., Villiers-le-Bel, France) and electrochemical detector (ECD-300, Eicom Co.) equipped with a graphite electrode were used with HPLC. Analytical conditions were as follows: detector, + 450 mV potential against a Ag/AgCl reference electrode; column, Eicopack CA-50DS 2.1 × 150 mm (Eicom Co.); mobile phase, 0.1 M NaH₂PO₄-Na₂HPO₄ buffer (pH 6.0) containing 0.13 mM disodium EDTA, 8.09 mM sodium 1-octane sulfate (Nacalai Tesque, Inc., Kyoto) and 15% methanol at a flow rate of 0.22 ml/min. The amount of noradrenaline in each sample was calculated using the peak height ratio relative to DHBA, an internal standard. By this assay method, 0.5 pg of noradrenaline could be accurately determined.

**Evaluation and statistics**

The amount of noradrenaline released from the hippocampal preparations (slices or synaptosomes) was expressed as a percentage of its tissue content. In experiments with the sliced preparation, the amount of noradrenaline released for 15 min by the first or second KCl-stimulation was expressed as S1 or S2. U-46619 was applied by the second KCl-stimulation and its effect on the release of noradrenaline was evaluated as S2/S1 ratios. In the crude synaptosomal preparations, on the other hand, single KCl-stimulation was performed. The effect of U-46619 on the KCl-induced noradrenaline release was evaluated by comparison to that treated with vehicle alone. All values are expressed as the mean ± S.E.M. Data were analyzed by one-way ANOVA, followed by post-hoc analysis with the Bon-
ferroni method for comparing a control to all other means (Fig. 2). When only two means were compared, Student’s t-test was employed (Figs. 3 and 5). P values less than 0.05 were taken to be significant.

RESULTS

KCl-evoked release of endogenous noradrenaline from hippocampal slices

The content of noradrenaline remaining in the hippocampal slices (4 slices) was 2028 ± 88 pg (n = 41). Application of 20 mM KCl for 5 min significantly increased the release of noradrenaline. This KCl-evoked noradrenaline release was consistently reproduced (Fig. 1). The S2/S1 ratio for the release of noradrenaline was 0.78 ± 0.06 (n = 7).

Effect of U-46619, a TXA2 mimetic, on the 20 mM KCl-evoked release of noradrenaline from hippocampal slices

Application of U-46619 for 5 min during the second 20 mM KCl-stimulation decreased the KCl-evoked noradrenaline release in a concentration-dependent manner (Fig. 2). The S2/S1 ratios in the U-46619 (10, 30, 50, 100 μM)-treated groups were 0.76 ± 0.01 (n = 3), 0.62 ± 0.04 (n = 3), 0.55 ± 0.03 (n = 6) and 0.52 ± 0.02 (n = 5), respectively. The values with 50 and 100 μM U-46619 were significantly lower than that of the vehicle-treated controls (0.78 ± 0.06, cited from Fig. 1).

Effect of SQ29548, a TXA2 receptor antagonist, on the U-46619-induced inhibition of noradrenaline release from hippocampal slices

SQ29548 (10 μM) was applied for 10 min before and during the second 20 mM KCl-stimulation. SQ29548 alone did not alter the 20 mM KCl-evoked noradrenaline release (Fig. 3B). However, this TXA2-receptor antagonist abolished the inhibition by U-46619 (50 μM) of the KCl-evoked noradrenaline release (Fig. 3B). The S2/S1 ratios were 0.81 ± 0.05 for the SQ29548-treated groups (n = 5) and 0.74 ± 0.04 for the SQ29548- plus U-46619-treated group (n = 5), respectively.

Effect of U-46619 on the KCl-evoked release of endogenous noradrenaline from hippocampal crude synaptosomes

The content of NA remaining in the hippocampal crude synaptosomes was 1084 ± 86 pg (n = 12). Mean basal level of noradrenaline released into the superfusate was 116.2 ± 6.6 pg/sample (n = 12). KCl in concentrations of 20 or 40 mM was applied for 5 min, 40 min after the start of superfusion (Fig. 4). U-46619 (100 μM) simultaneously applied with KCl (20 or 40 mM KCl) did not affect the KCl-evoked release of noradrenaline (Fig. 4: A and B).

Fig. 1. Release of endogenous noradrenaline from rat hippocampal slices. After an equilibration period of 60 min, noradrenaline released into the perfusion medium was measured every 5 min and expressed as % of tissue content. KCl (20 mM) was applied twice, each time for 5 min at 65 min and 95 min after the start of superfusion. Basal level of noradrenaline released in 5 min immediately before the first or second KCl-stimulations (the first and seventh fractions) was not detectable (N.D.). Values are each a mean ± S.E.M. (n = 7).

Fig. 2. Effect of U-46619, a TXA2 mimetic, on the KCl-evoked release of noradrenaline from hippocampal slices. KCl (20 mM) was applied twice, each time for 5 min, as in Fig. 1. The amount of noradrenaline released within 15 min (3 fractions) by the first and second KCl-stimulation was expressed as S1 and S2, respectively. U-46619 (10, 30, 50 and 100 μM) was applied during the second KCl-stimulation. Effect of U-46619 was expressed as the ratio of S2 to S1. Values are each a mean ± S.E.M. The number of the experiments is indicated in each column. *P<0.05 (significantly different from the vehicle-treated control).
Fig. 3. Effect of SQ29548, a TXA2 receptor antagonist, on the U-46619-induced inhibition of noradrenaline release from rat hippocampal slices. KCl (20 mM) was applied twice, each time for 5 min, as in Fig. 1. SQ29548 (10 μM) was applied for 10 min before and during the second KCl-stimulation. A: Without SQ29548 (these data were cited from Fig. 2). B: With SQ29548. Other conditions were the same as for Fig. 2.

Fig. 4. Effect of U-46619 on the KCl-induced release of endogenous noradrenaline from rat hippocampal crude synaptosomes. KCl (20 mM or 40 mM) was applied for 5 min once at 40 min after the start of superfusion. The release of noradrenaline was expressed as % of tissue content. A: 20 mM KCl was applied for 5 min; ○, vehicle (n = 3); ●, 100 μM of U-46619 (n = 3). B: 40 mM KCl was applied for 5 min; □, vehicle (n = 3); ■, 100 μM of U-46619 (n = 3).

Fig. 5. Effect of (-)-bicuculline methiodide, a GABA_A-receptor antagonist, on the U-46619-induced inhibition of noradrenaline release from rat hippocampal slices. KCl (20 mM) was applied twice, each time for 5 min, as in Fig. 1. U-46619 (50 μM) was applied during the second KCl-stimulation. (-)-Bicuculline methiodide (100 μM) was applied throughout the experiment. Other conditions were the same as for Figs. 2 and 3.

*Effect of (-)-bicuculline, a GABA_A-receptor antagonist, on the U-46619-induced inhibition of noradrenaline release from hippocampal slices*

(-)-Bicuculline was applied throughout the experiments. This GABA_A-receptor antagonist alone in a concentration of 100 μM did not alter the 20 mM KCl-evoked noradrenaline release (Fig. 5). (-)-Bicuculline even in this relatively large dose did not attenuate the U-46619 (50 μM)-induced inhibition of noradrenaline release from hippocampal slices (Fig. 5). The S2/S1 ratio in the bicuculline-plus U-46619 (50 μM)-treated group (0.51 ± 0.02, n = 3) was significantly lower than that in the control treated with bicuculline alone (0.71 ± 0.01, n = 4).
DISCUSSION

In the hippocampus, activation of presynaptic TXA2 receptors stimulates the release of glutamate, and activation of postsynaptic TXA2 receptors leads to inhibition of synaptic transmission due to a decrease in the membrane input resistance in the CA1 neurons (22). It is therefore likely that TXA2 receptors are present in the hippocampal CA1 neurons. However, no evidence for the presence of these receptors in the hippocampus is yet available. In the present study with hippocampal slices, U-46619, a stable TXA2 mimic (23), inhibited the high K⁺-evoked release of noradrenaline from hippocampal slices. This inhibitory effect of U-46619 was abolished by SQ29548, a TXA2 receptor antagonist (24). These results indicate that activation of TXA2 receptors by U-46619 inhibits the release of noradrenaline from the hippocampus.

Then, in the next series, whether or not presynaptic TXA2 receptors are present in the hippocampal noradrenergic nerve terminals was examined. In contrast to the effects of U-46619 in the sliced preparations, this TXA2 receptor mimic did not inhibit the release of hippocampal noradrenaline even in its higher concentration, 100 μM. It is therefore likely that hippocampal TXA2 receptors are probably localized in areas other than noradrenergic nerve terminals.

Schwartz-Bloom et al. have reported that TXA2 inhibits GABA_A-receptor functions in the cerebral cortex (25). GABA is an inhibitory neurotransmitter in various brain regions. For example, GABA reduces high K⁺-evoked glutamate release from rat hippocampal slices (26). However, it has been reported that GABA enhances release of noradrenaline in several brain regions including the rat hippocampus (10, 27). Then, we finally examined possibilities that the U-46619-induced inhibition of noradrenaline release in the sliced preparation was due to GABA released by this TXA2 mimetic or due to inhibition of GABA_A receptor function. Even in the presence of (-)-bicuculline, a GABA_A-receptor antagonist, the U-46619-induced inhibition of noradrenaline release was not affected. These results indicate that GABA_A receptors are not involved in the U-46619-induced inhibition of noradrenaline release.

It has been reported that TXA2 receptors exist in the cultured rabbit astrocytes (28). Furthermore, TXA2 receptor cDNA has been cloned from the cultured rat astrocytes (29). It is tempting to assume that activation of TXA2 receptors on glial cells in the hippocampus modulates the release of noradrenaline by unidentified mechanisms.

In conclusion, U-46619 inhibited the high K⁺-evoked release of noradrenaline by activation of TXA2 receptors in the rat hippocampus. This inhibition is probably due to activation of a yet unidentified inhibitory neuronal system.

REFERENCES


