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MODULATION OF NEURONAL MAO ACTIVITY, 5-HT UPTAKE AND IMIPRAMINE BINDING BY ENDOGENOUS SUBSTANCES IN DOG CEREBROSPINAL FLUID

TORU EGASHIRA,* RITSUKO TAKANO and YASUMITSU YAMANAKA
Department of Pharmacology, Medical College of Oita, 1-1506, Idaigaoka, Hazama-cho, Oita, 879-56 Japan

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Abstract—Addition of small amounts of dog cerebrospinal fluid (CSF) inhibited both type A and type B monoamine oxidase (MAO) in dog brain mitochondria. The inhibition was competitive with 5-HT as substrate, but non-competitive with β-phenylethylamine as substrate. Tricyclic antidepressants also exhibited competitive inhibition with type A MAO, but were non-competitive with type B MAO. The endogenous materials in CSF activate [^{3}H]-imipramine specific, dose-dependent binding in dog brain preparations. The maximum number of binding sites (B_{max}) increased, but the dissociation constant (K_d) was altered significantly in the presence of CSF. Addition of CSF induced a marked activation of uncompetitive [^{14}C]-5-HT uptake in dog brain preparations. Moreover, there were reversibilities of the inhibition of MAO activity or of the activation of imipramine binding and 5-HT uptake by CSF substance after dilution experiment. These results indicate the possible presence of an endogenous psychotic drug-like substance in CSF.

There has been much interest in monoamine oxidase (MAO) activity since the many reports [1–5] which associated platelet MAO activity and depressive symptoms. Furthermore, there are many reports on the possible presence of MAO modulators in the soluble fraction of various tissues [6–8], in plasma [9–11] and urine [12–14]. Although, they have possible *in vivo* roles as physiological regulators of MAO activity, their properties and structure have not been fully studied.

In this paper, we report that dog cerebrospinal fluid (CSF) inhibits both type A and type B MAO of dog brain mitochondria as well as human CSF, which was reported earlier [15]. We also report changes in imipramine binding and serotonin (5-HT) uptake in dog cerebral cortex after addition of CSF *in vitro*.

MATERIALS AND METHODS

Dogs were anesthetized with ketalar, given s.c., and the brains were quickly removed after withdrawing CSF by cisterna cerebello medullaris puncture. The brains and CSF samples were stored at −60° until used.

MAO activity. Mitochondrial fractions were prepared by differential centrifugation as described earlier [16]; the mitochondria suspended in 0.32 M sucrose were used as the enzyme preparations for determination of MAO activity. MAO activity was estimated by radioisotopic assay with [^{14}C]-5-HT (substrate for type A MAO) and [^{14}C]-β-phenylethylamine(β-PEA) (substrate for type B MAO) as described earlier [16]. The incubation medium contained a suitable amount of the enzyme to give a linear reaction for at least 40 min in a total volume of 275 μl of 0.1 M phosphate buffer, pH 7.2. The reaction was started by adding 25 μl of labeled substrate; incubation was carried out for 20 min at 37°. Then the reaction was stopped by adding 2 N HCl. The reaction products were extracted with ethyl acetate–benzene (1:1, v/v). Samples of the extract were mixed with Triton x-100-toluene scintillation liquid and their radioactivity were measured by liquid scintillation spectrometry.

Imipramine binding. [^{3}H]-imipramine binding was carried out with minor modification of a published method [17]. The dog cortex was homogenized in 50 vol. of ice-cold buffer (50 mM Tris–HCl, 100 mM NaCl and 5 mM KCl pH 7.4). The homogenate was centrifuged for 10 min at 2000 *g*, the pellet discarded and the supernatant further centrifuged for 30 min at 30,000 *g*. The supernatant was discarded and the pellet (P_2) was rehomogenized in the same buffer. This P_2 fraction obtained by centrifugation was used as the crude membrane preparation for the imipramine binding. Membrane suspensions (100 μg protein) were incubated at 0° for 60 min with [^{3}H]-imipramine (51.5 Ci/mmole) (final concentration 0.1 nM–8.0 nM) in a final volume of 250 μl. After incubation, 250 μl of the incubation medium was diluted in 3 ml of ice-cold buffer and rapidly filtered through Whatman GF/B glass fiber filters. The filters were rapidly washed three times with 5 ml of ice-cold buffer and then dried and the radioactivity determined in Triton x-100-toluene scintillation fluid in a liquid scintillation spectrometer. Specific binding was defined as the difference between the total binding minus binding in the presence of 100 μM desipramine.

* To whom correspondence should be addressed.

5-HT uptake. The uptake procedure of [^{14}C]-5-HT into the P_2 fraction was carried out by the method of Nomura and Segawa [18]. The dog cortex was homogenized in 9 vol. of 0.32 M sucrose; the homogenate was centrifuged for 10 min at 2000 *g*, the pellet discarded, and the supernatant further centrifuged for 30 min at 30,000 *g*. The pellet (P_2) was rehomogenized with buffer (Krebs–Ringer solution, pH 7.2 containing EDTA, pargyline and ascorbic acid). The P_2 fraction (3 mg protein) was incubated for 10 min at 37° (for total uptake) or 0° (for non-specific uptake) with [^{14}C]-5-HT (46.0 mCi/mmole) (final concentration 0.26–1.0 M) in a final volume of 2.0 ml. At the end of the incubation period, 6 ml of ice-cold saline were added to the reaction medium and centrifuged at 11,500 *g* for 20 min at 4°; the supernatant fluid was decanted. The pellet was resuspended in 8 ml of ice-cold saline. After recentrifugation at 11,500 *g* for 20 min, 0.5 ml of NCS solubilizer was added to the pellet, left at 50° for 1 hr, and neutralized with 15 μl acetic acid. Radioactivity was determined in 10 ml of Triton x-100-toluene scintillation liquid by a liquid scintillation spectrometer.

Estimation of protein. Protein concentrations of the preparations were measured by the method of Lowry *et al.* [19] with bovine serum albumin as the standard.

Chemicals. The Radiochemical substances [^{14}C]-β-phenylethylamine HCl (β-PEA) (48 mCi/mmole) and [^{3}H]-imipramine HCl (51.5 Ci/mmole) were purchased from New England Nuclear (Boston, MA) and [^{14}C]-5-hydroxy tryptamine creatinin sulphate (5-HT) (46 mCi/mmole) was from Amersham Japan Co. Imipramine HCl and desipramine HCl were obtained from Sigma Chemical Co. Amitrityline HCl and nortriptyline HCl were gifts from Yamanouchi Pharmaceutical Co., Ltd. and Dainippon Pharmaceutical Co. Ltd., respectively.

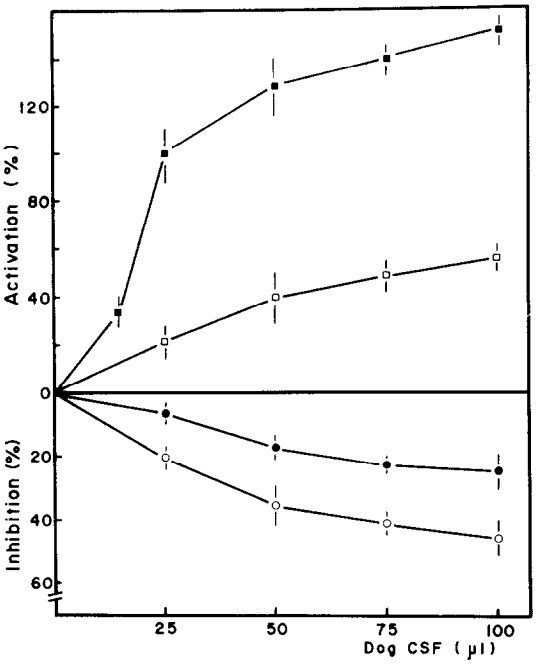

Fig. 1. Effects of addition of CSF on dog brain MAO activity, imipramine binding and 5-HT uptake. MAO activities to 5-HT (○—○) and β-PEA (●—●) as substrates were measured with addition of CSF at 37° for 20 min. The results of imipramine binding (■—■) and 5-HT uptake (□—□) with addition of CSF are as illustrated. Each point represents the mean percentages (±SE) of the control values in triplicate experiments.

RESULTS AND DISCUSSION

The effect of adding varying amounts of dog CSF to dog brain mitochondrial MAO *in vitro* was studied using 5-HT and β-PEA as substrates. When the brain mitochondria were incubated with increasing amounts of CSF, the increases in the inhibition of MAO activity with 5-HT and β-PEA were observed. Addition of 100 μl CSF inhibited MAO activity about 45% and 20% with 5-HT and β-PEA as substrates, respectively (Fig. 1). In addition, there was a reversibility of the inhibition of MAO activity by CSF substance after the dilution technique (data not shown). A kinetic study of the inhibition of MAO by CSF addition was investigated with Lineweaver–Burk double reciprocal plots. The results with 5-HT and β-PEA as substrates are shown in Fig. 2. The inhibition was competitive at least toward 5-HT (Fig. 2, upper). With β-PEA as substrate, the inhibition was non-competitive with a decrease in the V_{max} and no change in the K_m (Fig. 2, bottom). Similar results have been reported using an endogenous MAO modulator, e.g. an inhibitor or activator of MAO in plasma [9–11], urine [12–14], in heart [8] and CSF [15]; these modulators exhibited competitive inhibition or activation to type A MAO but were non-competitive to type B MAO. Moreover, the effects of the tricyclic antidepressants, imipramine, amitriptyline and nortriptyline on dog brain mitochondrial MAO were investigated. The tricyclic antidepressants were found to inhibit dog brain mitochondrial MAO; these drugs also exhibited competitive inhibition with the substrate 5-HT (Fig. 3, upper) but non-competitive inhibition with β-PEA (Fig. 3, bottom). From these results, it can be presumed that the endogenous materials in the dog CSF possess an antidepressive effect as well as the tricyclic antidepressants. It is commonly known that tricyclic antidepressants inhibit the uptake of biogenic amines and also increase the level of biogenic amines at synaptic receptors. This results from the blockade of the uptake of these amines [20–23]. Moreover, pharmacologically active tricyclic antidepressants are all potent inhibitors of [^{3}H]-imipramine binding [23–25].

To determine whether or not this CSF possesses truly antidepressant-like action, the effects of adding varying amounts of dog CSF on [^{3}H]-imipramine binding and [^{14}C]-5-HT uptake were studied. When the P_2 fractions were incubated with increasing amounts of CSF, the activation in the binding of imipramine was observed; the binding of [^{3}H]-imipramine increased by about 125% in the presence of 50 μl CSF (Fig. 1). In addition, the activating effect

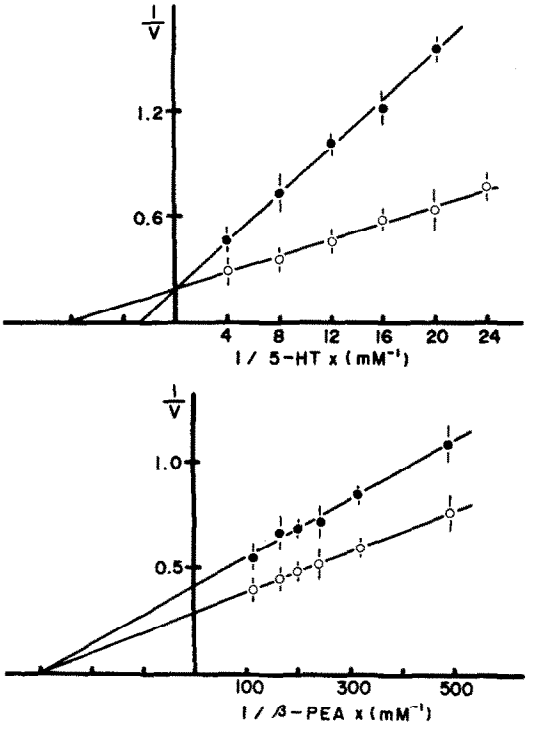

Fig. 2. Effect of CSF on MAO activity in dog brain mitochondria. Lineweaver–Burk plots of the reciprocal of the initial velocity of 5-HT and β-PEA oxidation against the reciprocal of the substrate concentration in the presence of dog CSF. Abscissa: 1/substrate concentration in mM, Ordinate: 1/initial velocity in nmole/min/mg of protein. Substrates used were 5-HT (upper) and β-PEA (bottom) assays in the absence (○—○) and presence (●—●) of 100 μl dog CSF. Each point represents the mean MAO activity (±SE, N = 3) assayed in triplicate experiments.

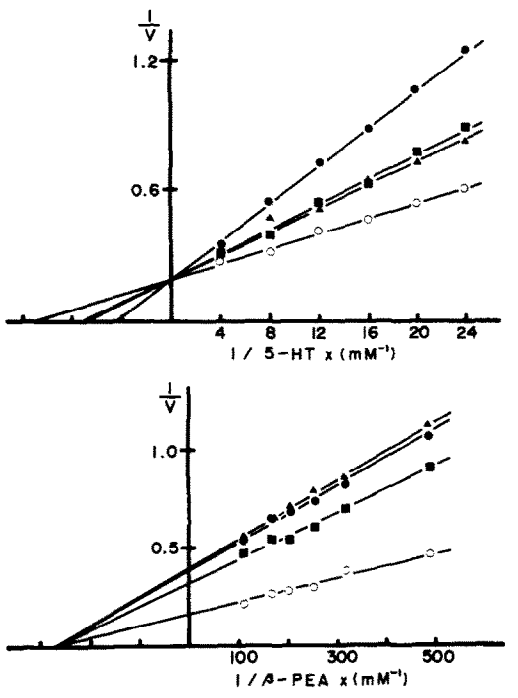

Fig. 3. Effect of various tricyclic antidepressants on MAO activity in dog brain mitochondria. Lineweaver–Burk plots of the reciprocal of the initial verocity of 5-HT and β-PEA oxidation against the reciprocal of the substrate concentration in the presence of antidepressants. Abscissa: 1/substrate concentration in mM; ordinate: 1/initial velocity in nmole/min/mg of protein. Each point represents the mean of triplicate determinations. Substrates used were 5-HT (upper) and β-PEA (bottom) assay in the absence (○—○) and presence of imipramine 5×10^{-5} M (●—●), amitriptyline 5×10^{-5} M (▲—▲) and nortriptyline 1×10^{-5} M (■—■). Each point represents the mean MAO activity assayed in triplicate experiments.

of imipramine binding by CSF substance was a reversible after dilution technique. The specific binding of [^{3}H]-imipramine to dog cerebral cortex was saturable in the presence and absence of CSF (Fig. 4, inset). Scatchard analysis of specific [^{3}H]-imipramine binding indicated a single population of binding sites with an apparent dissociation constant, K_d, of 8.92 ± 0.41 nM with CSF and 8.26 ± 0.33 nM without CSF. The maximal concentration of binding sites (B_{max}) was estimated to be 1.68 ± 0.08 pmole/mg of protein with CSF and 1.10 ± 0.12 pmole/mg of protein without CSF. The maximum number of binding sites increased in the presence of dog CSF; however, K_d was not altered significantly.

Addition of dog CSF induced a marked activation of [^{14}C]-5-HT uptake (Fig. 1). Kinetic analysis of [^{14}C]-5-HT uptake into dog cerebral cortex and the effect of dog CSF were determined over the concentration range of 0.26 μM–1.0 μM at 37° and are shown in Fig. 5. The uptake was concentration dependent and exhibited apparent saturation (K_m = 0.56 ± 0.03 μM and V_{max} = 1.28 ± 0.15 pmole/min/mg of protein). Dog CSF activated the uptake uncompetitively with an apparent K_m of 0.66 ± 0.12 μM and a V_{max} of 1.79 ± 0.11 pmole/min/mg of protein; e.g. there was an apparent change in the affinity and V_{max} of 5-HT uptake to its transporter. Moreover, this activation of 5-HT uptake by CSF substance was a reversible by dilute technique.

Thus the endogenous materials in CSF do not directly compete with [^{14}C]-5-HT for the transport recognition sites, but rather interact with the transport system through another mechanism.

The [^{3}H]-imipramine binding site appears to be associated with the neuronal uptake process of 5-HT, since there is a highly significant correlation between the potency of tricyclic antidepressants for the inhibition of [^{3}H]-imipramine binding and the inhibition of [^{14}C]-5-HT uptake [23, 26, 27]. It is also known that tricyclic antidepressants *in vitro* generally inhibit MAO activity [28–30]. James *et al.* [31] also reported that clorgyline, known as a type A MAO inhibitor, is a more specific inhibitor of 5-HT uptake, while deprenyl, known as type B MAO inhibitor, is an inhibitor of norepinephrine uptake. But this material in CSF inhibited exactly type A and B MAO activity, while activating the imipramine binding and 5-HT uptake. These results do not indicate the possibility that this material in CSF may be an endogenous antidepressant-like substance.

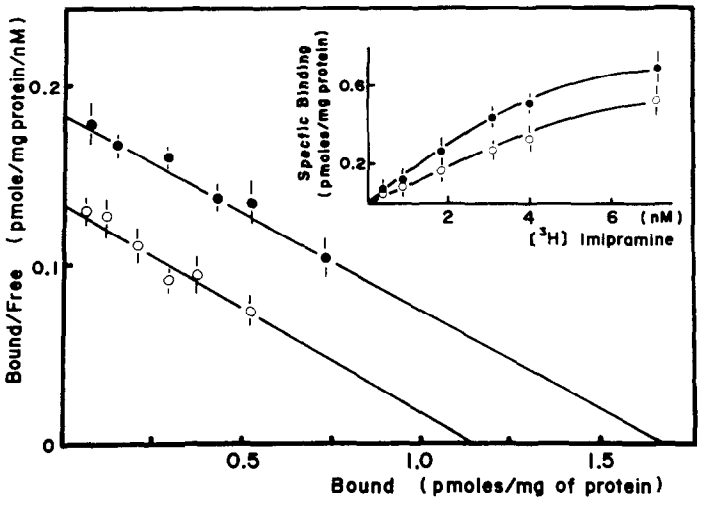

Fig. 4. Saturation curve and Scatchard analysis of specific [^{3}H]-imipramine binding to dog cerebral cortex. Specific binding represents total binding minus binding in the presence of 100 μM desipramine, at free imipramine concentrations ranging from 0.1 nM to 8.0 nM, in the absence (○—○) and presence (●—●) of dog CSF (inset). The saturation curves were then transformed into a linear plot by Scatchard analysis. Each point represents the mean value of both the total and non-specific binding assayed in triplicate experiments.

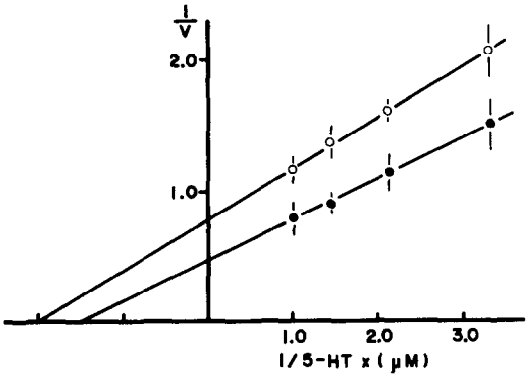

Fig. 5. Lineweaver–Burk plots of [^{14}C]-5-HT uptake with or without dog CSF. P_2-fraction of dog brain was pre-incubated for 20 min in the absence (○—○) or presence of dog CSF (●—●) and then incubated with various concentrations of [^{14}C]-5-HT 0.26–1.0 μM). Values are means ± SE for three separate preparations.

Few have reported on the activation of 5-HT uptake and imipramine binding *in vitro*, and the possible physiological significance of the activating materials is unknown, while many investigators have reported a stimulation of norepinephrine uptake by lithium administration *in vivo* [32–34]. Kuriyama *et al.* [35] also reported that lithium administration increased the synaptosomal uptake of norepinephrine in the presence of an MAO inhibitor. From these reports, since preliminary experiments suggest an inhibition of MAO activity and both an activation of [^{14}C]-5-HT uptake and [^{3}H]-imipramine binding to dog cerebral cortex membrane, the materials in this CSF may represent endogenous lithium-like substances. These drugs are used in the therapy of manic-depressive illnesses and have a role in the regulation of serotonergic neurons.

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