A COMPARISON OF THE INHIBITORY EFFECTS OF CERTAIN ANTIPARKINSONIAN AGENTS ON DOPAMINE ACCUMULATION INTO THE RAT STRIATUM

Takashi NOSE and Tomio SEGAWA
Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto
Accepted September 22, 1973

Abstract—The inhibitory effect of certain centrally acting drugs, including antiparkinsonian agents, tricyclic antidepressant and phenothiazine, was studied in vitro on the accumulation of dopamine into a crude mitochondrial fraction (P₂-fraction) from rat striatal homogenate, and the site of inhibition was studied by further fractionating the P₂-fraction into synaptic vesicles fraction (P₂V-fraction) and supernatant fluid (P₂S-fraction). Many antiparkinsonian agents, such as benztropine, trihexyphenidyl, ethopropazine, diphenhydramine or 6, 6, 9-trimethyl-9-azabicyclo[3, 3, 1]non-3β-yl α, α-di(2-thienyl) glycolate hydrochloride monohydrate (PG-501), inhibited dopamine accumulation into P₂-fraction from rat striatal homogenate in concentrations of 10⁻⁵–10⁻⁴ M, though atropine did not inhibit the amine accumulation even in a concentration of 10⁻⁴ M. Furthermore, benztropine, one of the most potent inhibitors of dopamine accumulation into P₂-fraction, inhibited the accumulation into P₂V-fraction to a greater extent than that into P₂S-fraction and PG-501 inhibited the accumulation into P₂S-fraction to a greater extent than that into P₂V-fraction. The possible site of action of these two agents is discussed herein.

In patients with Parkinson's Disease, it has been hypothesised that there is antagonistic interaction between dopamine and acetylcholine in the striatum and disturbance of the balance in the direction of cholinergic dominance (1). Coyle and Snyder (2) have pointed out that a variety of antiparkinsonian agents inhibit dopamine uptake into synaptosomes obtained from rat striatum. The authors have speculated that inhibition of the uptake would intensify the synaptic actions of dopamine and represent the mode of action of these agents.

In the present investigation, the inhibitory effect of certain antiparkinsonian agents on dopamine accumulation into P₂-fraction from rat striatal homogenate has been studied and the site of the inhibitory effect by subfractionating P₂-fraction to synaptic vesicles and cytoplasm was also investigated.

MATERIALS AND METHODS

Wistar male rats (150–200 g) were sacrificed by decapitation. The brains were rapidly removed and the striatum including nuclei of caudate and putamen was rapidly dissected. All procedures prior to incubation were carried out at 0°C to 4°C. The tissue samples were weighed and homogenized for 2 min in 10 volumes of 0.32 M sucrose with a Teflon-glass homogenizer. The P₂-fraction, a crude mitochondrial fraction was prepared by
Suspension of P₂-fraction in Incubation Medium (pH 7.4 Tris Buffer)

Pheniprazine $5 \times 10^{-5}$ M →

Drug

Pre-incubated for 15 min (In Air, 37°C)

Dopamine $5 \times 10^{-6}$ g/ml →

Incubated for 20 min

Centrifuged at $11,500 \times g$, 20 min

Resuspended in 5 ml Ice Cold 0.32 M Sucrose

Centrifuged at $11,500 \times g$, 20 min (Twice)

Resuspended in 0.32 M Sucrose, Add 3 Volumes of Water

11,500 x g, 20 min

P₂D Mitochondria, Myelin,
NIEPs Ghost

P₂V Synaptic Vesicle,
Membrane

P₂S Cytoplasm

Fig. 1. Separation method of P₂V- and P₂S-fraction incubated with dopamine.

The centrifugation method described previously (3). The P₂-fraction was suspended in 4.6 ml of modified Krebs solution (7.7 ml/0.1 g of original tissue). Unless otherwise specified, this solution had the following composition: 113 mM NaCl, 5.1 mM KCl, 1.5 mM CaCl₂, 0.32 mM MgSO₄, 2.4 mM NaH₂PO₄, 23 mM NaHCO₃, 11.1 mM glucose, 20 mM Tris buffer (pH 7.4). The suspension was transferred to a 30 ml Erlenmeyer flask and pre-incubated with 0.1 ml of varying concentrations of the test drugs and 0.1 ml of pheniprazine (final concentration of $5 \times 10^{-6}$ M) for 15 min in air at 37°C. After the pre-incubation, dopamine dissolved in 0.2 ml of modified Krebs-Tris solution was added to the flask so as to achieve a final concentration of 5 μg/ml and the incubation was continued for another 20 min. The incubation mixture was then centrifuged at 11,500 x g for 20 min at 4°C, and this procedure was repeated. The washed pellet was subjected to protein assay (4) and dopamine determination (5).

When the effect of the test drugs on dopamine accumulation in subfractions of P₂-fraction was investigated, P₂-fraction was further separated to P₂V and P₂S according to the method described previously (6). Thus the washed pellet was submitted to an osmotic shock in ice-cold distilled water and centrifuged at 11,500 x g for 20 min. The supernatant fluid was further separated by centrifugation (150,000 x g, 30 min) into the vesicles (P₂V-fraction) and the final supernatant fluid (P₂S-fraction).

The following test drugs were studied: ouabain, chlorpromazine hydrochloride, imi-
pramine hydrochloride, atropine sulphate, trihexyphenidyl hydrochloride, benztropine mesylate, ethopropazine hydrochloride, diphenhydramine hydrochloride, 6, 6, 9-trimethyl-9-azabicyclo[3, 3, 1]non-3(3-yl a, a-di(2-thienyl) glycolate hydrochloride monohydrate (PG-501). PG-501 has been found to have pronounced central anticholinergic activity relative to its peripheral one (7). The doses used refer to the form mentioned.

RESULTS

1. Accumulation of dopamine into P2-fraction from rat striatal homogenate

The P2-fraction prepared from rat striatal homogenate was incubated for time intervals varying from 5 to 60 min, and was then assayed for ng dopamine per mg protein (Fig. 2). Zero time value was determined by incubating the mixture at 37°C for 15 min and centrifuging it immediately after the pre-incubation. Dopamine accumulation continued approximately 20 min, after which the concentration decreased gradually and the amine concentration at 60 min was less than that at 20 min (P<0.05, as determined by Student's t-test).

Fig. 3 shows several factors affecting the accumulation of dopamine into P2-fraction prepared from rat striatal homogenate. When P2-fraction was incubated for 20 min at varying temperatures from 0°C to 37°C, the accumulation of dopamine into P2-fraction increased in proportion to increasing temperature at incubation. When P2-fraction was incubated for 20 min

Fig. 2. Time course of dopamine accumulation into P2-fraction from rat striatal homogenate. Each point is the mean of 4 determinations. Vertical bars represent s.e.m.

Fig. 3. Influence of temp., ouabain and calcium concentration on dopamine accumulation into P-fraction from rat striatal homogenate. Each column represents the mean value±s.e.m. from 4 determinations.
at 37°C, the accumulation was suppressed by ouabain and enhanced by calcium ion in a concentration of 1.5 mM. However, larger doses of calcium decreased the accumulation of the amine into P₂-fraction of rat striatal homogenate.

2. Effect of various drugs on the dopamine accumulation into P₂-fraction from rat striatal homogenate

Various drugs were examined for inhibitory activities on dopamine accumulation into P₂-fraction from rat striatal homogenate (Fig. 4). Both benztropine and PG-501 inhibited the accumulation of dopamine in a concentration of 10⁻⁵ M. Trihexyphenidyl also inhibited the accumulation in the same concentration, although it was considerably less effective in inhibiting dopamine accumulation than benztropine or PG-501. Similarly ethopropazine and diphenhydramine also inhibited dopamine accumulation in a concentration of 10⁻⁴ M, but the inhibitory effect of ethopropazine could not be observed in a concentration of 10⁻⁵ M. Atropine, in contrast to the other drugs examined, did not cause any blockade of dopamine accumulation in concentrations of 10⁻⁵–10⁻⁴ M. Chlorpromazine and imipramine also inhibited the amine accumulation in 10⁻⁴ M, although the inhibitory effect of these drugs was markedly decreased in a concentration of 10⁻⁵ M.

![Graph showing the effect of various drugs on dopamine accumulation into P₂-fraction from rat striatal homogenate.](image)

Fig. 4. Effect of drugs on dopamine accumulation into P₂-fraction from rat striatal homogenate. Results are expressed as the percentage of dopamine accumulation in the absence of drugs after subtracting a blank value of P₂-fraction incubated without dopamine. Each column represents the mean value ± s.e.m. from 4 experiments.
3. Effect of benztropine and PG-501 on the dopamine accumulation into P2V-fraction from rat striatal homogenate

In order to acquire further information concerning the inhibitory activities of benztropine and PG-501 in dopamine accumulation, P2-fraction was subfractionated to P2V- and P2S-fractions which contained predominantly synaptic vesicles and cytoplasm of nerve ending particles, respectively. As indicated in Fig. 1, after the incubation of P2-fraction in the presence of benztropine or PG-501, the washed pellet was separated to P2V- and P2S-fractions, and both of these fractions were subjected to protein assay and dopamine determination.

As shown in Fig. 5, benztropine evoked a dose dependent inhibition of the amine accumulation into both P2V- and P2S-fractions, and the inhibitory effect of benztropine on the accumulation into P2V-fraction was stronger than that into P2S-fraction. On the other hand, the inhibitory effect of PG-501 on dopamine accumulation was more predominant on P2S-fraction than on P2V-fraction in contrast to that of benztropine.

**DISCUSSION**

It is suggested that P2-fraction from rat striatal homogenate takes up exogenous dopamine utilizing an energy-dependent transport mechanism, since dopamine accumulation was temperature-dependent and was suppressed by ouabain. In a preliminary experiment, the highest concentration of dopamine was found after a 20 min incubation of P2-
fraction at 37°C in modified Krebs-Tris solution in which the calcium concentration was 1.5 mM and the concentration of dopamine at 60 min was less than that at 20 min. This may be caused by enzymatic metabolism of dopamine in nerve ending particles and/or by destruction of nerve ending particles during the incubation.

Philippu and Heyd (8) have demonstrated that calcium enhances the release of dopamine dose dependently from isolated dopamine vesicles of the caudate nucleus. These results would explain why a decrease in dopamine accumulation was observed in the higher concentration of calcium, and also suggests that calcium is not essential to the accumulation of the amine into P2-fraction since dopamine accumulation was evident even in medium to which calcium had not been added.

It has been reported that certain psychotropic drugs such as imipramine or chlorpromazine are potent inhibitors of noradrenaline uptake but less effective in inhibition of dopamine uptake (9, 10). In this experiments, imipramine and chlorpromazine showed a strong inhibition on dopamine accumulation in 10⁻⁴ M, but in a concentration of 10⁻⁵ M, the inhibitory effects of these drugs were weaker than those of benztropine and PG-501.

In agreement with the result reported by Coyle and Snyder (2), benztropine, trihexyphenidyl and diphenhydramine showed the potent inhibition of dopamine accumulation into the striatal P₂-fraction. However atropine, a potent anticholinergic agent, did not inhibit dopamine accumulation in concentrations of 10⁻³–10⁻⁴ M. Coyle and Snyder (2) have suggested that the inhibition of dopamine uptake caused by benztoprine-like agents would intensify the synaptic actions of dopamine and represent the mode of action of these drugs as antiparkinsonian agents. This hypothesis however would be reasonable when the drug-induced inhibition of dopamine uptake takes place at synaptic membranes of dopaminergic neuron. In the present investigation, benztropine inhibited dopamine accumulation into P₂-V-fraction more strongly than that into P₂-S-fraction. On the other hand, PG-501 inhibited dopamine accumulation into P₂-S-fraction to a greater extent than that into P₂-V-fraction. It is therefore reasonable that the reduced accumulation of dopamine in rat striatal P₂-fraction following the treatment of benztropine depends more greatly on a blockade of the amine accumulation at the synaptic vesicles than at synaptic membranes, whereas the reduced accumulation of dopamine induced by PG-501 depends on a blockade of the amine accumulation at membrane of dopaminergic neuron. In light of these findings, PG-501 more than benztropine was likely to exert its therapeutic effect in parkinsonian patients by potentiating the synaptic actions of dopamine in addition its own anticholinergic property.

Acknowledgments: The authors wish to thank Professor H. Takagi, Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kyoto University, for helpful suggestions and encouragement. Thanks are also due to Tanabe Seiyaku Co. for the supply of PG-501.
REFERENCES

1) KLAWANS, H.L.: Dis. nerv. Sys. 29, 805 (1968)
2) COYLE, J.T. AND SNYDER, S.H.: Science, 166, 899 (1969)
3) NOSE, T., SEGAWA, T. AND TAKAGI, H.: Japan. J. Pharmacol. 22, 867 (1972)
4) LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. AND RANDAL, R.J.: J. biol. Chem. 193, 265 (1951)
5) ANTON, A.H. AND SAYRE, D.F.: J. Pharmacol. exp. Ther. 145, 326 (1964)
6) SEGAWA, T., KURUMA, I. AND TAKAGI, H.: Site of action of drugs, Edited by TAKAGI, H., p. 127, Nankodo, Tokyo (1968) (in Japanese)
7) KOJIMA, M., NOSE, T., SHINTOMI, K. AND YONEDA, N.: Japan. J. Pharmacol. 21, 276 (1971)
8) PHILIPPU, A. AND HEYD, W.: Life Sci. 9, 361 (1970)
9) HORN, A.S., COYLE, J.T. AND SNYDER, S.H.: Mol. Pharmacol. 7, 66 (1971)
10) CARLSSON, A., FUNE, K., HAMBERGER, B. AND LINDQUIST, M.: Acta physiol. scand. 67, 481 (1966)