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PHARMACOLOGY

Dopamine transporter interaction with blockers and transportable substrates: insights from kinetics study

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Abstract. Competition kinetic analysis was performed to examine the interaction mechanism of the dopamine transporter with dopamine, (S)-amphetamine, and cocaine, which play a central role in drug abuse phenomena connected with the dopaminergic system. Efficient dopamine transporter inhibitor [^{3}H]PE2I was used as a reporter ligand for this analysis as this compound initiates slow isomerization of the transporter–ligand complex and thus ensures reliable results of the filtration radioligand assay. It was shown that the three investigated compounds do not initiate slow isomerization of their complexes with the transporter, but their presence inhibits the isomerization step of the radioactive reporter ligand. Secondly, it was shown that (S)-amphetamine and dopamine do not interfere with the fast step of the inhibitor ligand binding, pointing to the formation of the ternary complex, including transporter protein, reporter ligand, and an unlabeled compound. This is possible if the two molecules bind to non-overlapping sites on the transporter. Binding of cocaine results in slightly improved binding of the reporter ligand, pointing to a positive allosteric interaction between these binding processes.

Keywords: dopamine transporter, ligand interaction mechanism, competition kinetic analysis, amphetamine, cocaine, dopamine.

Abbreviations:

PE2I – methyl-8-[(2*E*)-3-iodoprop-2-en-1-yl]-3-(4-methylphenyl)-8-azabicyclo[3.2.1]octane-2-carboxylate, a potent dopamine transporter inhibitor

INTRODUCTION

Dopamine transporter removes dopamine (***I***, Fig. 1) from synaptic spaces and thus controls the action of this monoamine neurotransmitter at G-protein coupled dopamine receptors [1]. Disruption of this transport has serious consequences on dopaminergic signaling pathways and has been linked to several disorders [2]. Therefore, this transporter has been an attractive target for the design of antidepressants and stimulants as well as a target for recreational drugs [3,4].

Dopamine transporter is a target for drugs of abuse, such as amphetamine (***II***, Fig. 1) and cocaine (***III***, Fig. 1), which induce increased dopamine concentration in the synaptic cleft, thus increasing dopaminergic firing [4]. Although the effects of these compounds are similar, the mechanism of their action is different as cocaine inhibits dopamine transport [5], while amphetamine is transported, like dopamine, across the cell membrane and stimulates the efflux of intracellular dopamine through secondary mechanisms [6].

These addictions are major medical and public health problems, and efforts to block the action of the addictive drugs without affecting the functioning of the dopamine transporter have not been successful [1,7], although a significant number of studies on the transporter structure and possible binding sites have been made [1], including computational docking studies [8], with crystallographic X-ray data for the leucine transporter as a model, and data on the dopamine transporter from *Drosophila melanogaster* [9].

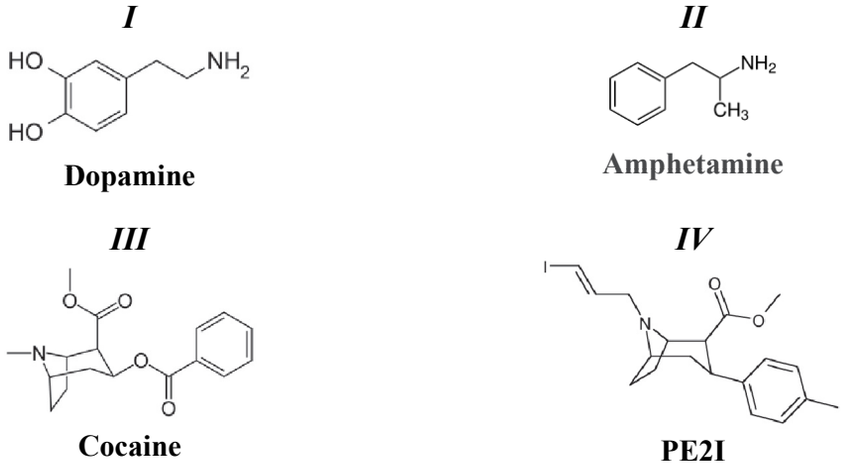


Fig. 1. Compounds interacting with dopamine transporter. ***I*** – Dopamine – 4-(2-aminoethyl)benzene-1,2-diol; ***II*** – Amphetamine – 1-phenylpropan-2-amine; ***III*** – Cocaine – methyl(1R,2R,3S,5S)-3-(benzoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate; ***IV*** – PE2I – methyl-8-[(2*E*)-3-iodoprop-2-en-1-yl]-3-(4-methylphenyl)-8-azabicyclo[3.2.1]octane-2-carboxylate.

Nevertheless, reports on the cocaine binding and inhibition mechanism are still considered inconsistent [1]. Therefore, this analysis returns to the question of the binding mechanism of these compounds with the dopamine transporter.

However, as an alternative to all previous attempts, the presented study approach relies on the methods of chemical kinetics [10]. The basic principles of the kinetic analysis approach have been outlined in 1976 by Strickland et al. [11] and were further used to investigate the ligand binding mechanisms of various membrane-associated neurotransmitter receptors [12] and the dopamine transporter [13].

In this work, we investigate the interaction of dopamine, (S)-amphetamine, and cocaine with the dopamine transporter through their influence on the binding characteristics of [^{3}H]PE2I (***IV***, Fig. 1) – a potent and selective tropane-based dopamine transporter inhibitor [14] and, as such, a suitable "reporter" ligand for the kinetic analysis [18].

THEORETICAL BACKGROUND OF THE KINETIC ANALYSIS METHOD

The prerequisite of the kinetic analysis method is the availability of a potent and selective "reporter" ligand, which can be used for the quantitative determination of the target sites using a conventional assay procedure, for example, the filtration technique [15]. These conditions are met in the case of [^{3}H]PE2I (tritium labeled methyl-8-[(2*E*)-3-iodoprop-2-en-1-yl]-3-(4-methylphenyl)-8-azabicyclo[3.2.1]octane-2-carboxylate) that interacts with the dopamine transporter in a two-step process: first by a fast binding equilibrium step, then by slow isomerization of the initially formed complex [13]:

$$\underset{\mathbf{R}}{\overset{0}{}} \rightleftharpoons \underset{\mathbf{RA}}{\overset{1}{}} \rightleftharpoons \underset{\mathbf{(RA)}}{\overset{2}{}}. \qquad (1)$$

In this scheme, R stands for the ligand binding protein (dopamine transporter), A is the reporter ligand ([^{3}H]PE2I), and RA is the fast complex, the formation of which is characterized by the rate constants k_{0-1} and k_{1-0}, the dissociation constant being $K_{0-1} = k_{1-0}/k_{0-1}$. (RA) is the "isomerized" complex, the formation of which is characterized by the rate constants k_{1-2} and k_{2-1}. The rate constants k_{1-2} and k_{2-1} characterize the isomerization step and allow calculation of the equilibrium constant for this process, $K_{isom} = k_{2-1}/k_{1-2}$. The off-rate (effective dissociation) of the radioactive reporter ligand from its complex with the binding protein is governed by the slow "de-isomerization" process, characterized by the rate constant k_{2-1}. Therefore, the interaction process can be quantitatively monitored by the conventional filtration assay, described by the pseudo-first order rate constant k_{obs} under the assumption of [R] << [A] [10]:

$$[(RA)]_t = [(RA)]_{eq}\,(1 - e^{k_{obs}t}). \qquad (2)$$

In this rate equation, $[(RA)]_t$ is the concentration of the isomerized complex at time moment t, and $[(RA)]_{eq}$ stands for the isomerized complex concentration at the end of the reaction when the equilibrium for the complex formation has been attained. The former parameter can be used for assaying the influence of an additional (non-radioactive) ligand B on the reporter ligand binding as the plots of

$[(RA)]_{eq}$ vs [B] can be used for calculating the parameter IC_{50} for this ligand by analogy with the conventional radioligand displacement method [15].

For the reaction scheme (1), the plot of the k_{obs} values vs the radioligand A concentration is described by a hyperbolic function [11]:

$$k_{obs} = \frac{k_{on}[A]}{K_A + [A]} + k_{off}. \quad (3)$$

This allows for the determination of the kinetic parameters k_{on}, K_A and k_{off}. In the case of the reaction mechanism (1), these experimentally determined parameters correspond to k_{1-2}, K_{0-1} and k_{2-1}, respectively.

Importantly, conventional equilibrium binding studies do not reveal the presence or absence of an isomerization step, and, therefore, the dissociation constant K_D, calculated from such data, can be a complex parameter [10]:

$$K_D = \frac{K_{0-1} \cdot K_{1-2}}{(K_{1-2} + 1)}. \quad (4)$$

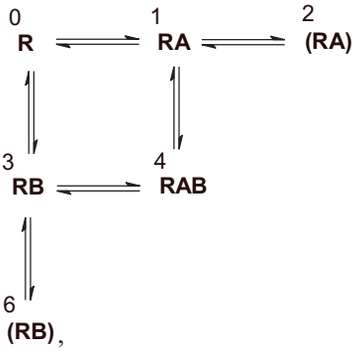
Kinetic studies of the reporter ligand (A) interaction with the target protein in the presence of another compound (B) significantly extended the possibilities of the kinetic approach and allowed greater insights into the mechanism of interaction between the target and the unlabeled ligand [10].

Firstly, it is possible to determine whether the ligand B also induces a slow isomerization step, following the initial fast complex formation.

Secondly, it is possible to analyze how the ligand B affects the binding equilibrium of the reporter ligand, i.e., answer the question whether the ligand B and the reporter ligand A compete for the same binding site or their binding is non-exclusive.

Thirdly, it is possible to analyze how the ligand B affects the isomerization step of the reporter ligand A.

Some of these options can be formalized by the following reaction scheme:



(5)

where R stands for the ligand binding protein (dopamine transporter), A is the radioactive reporter ligand ([^{3}H]PE2I), RA is the fast complex, and (RA) is the slow "isomerized" complex, formed between the protein and the reporter, respectively; B denotes the non-radioactive ligand, RB is the complex of the ligand B with the target protein, and (RB) denotes the putatively isomerized complex of the non-radioactive ligand and the binding protein. This model also takes into consideration the possibility of formation of the ternary complex RAB, which includes both the ligands A and B as well as the protein R.

For the mechanism (5), the dependence of k_{obs} on the concentration of the ligands A and B can be represented by the following rate equation:

$$k_{obs} = \frac{\frac{k_{1-2} \cdot [A]}{\left(1 + \frac{[B]}{K_{1-4}}\right)}}{K_A + A} + \frac{\frac{k_{3-6} \cdot [B]}{\left(1 + \frac{[A]}{K_{3-4}}\right)}}{K_B + B} + k_{2-1}. \quad (6)$$

For the simplification of data analysis, a constant concentration of one of these ligands is used in a series of experiments [10]. If k_{obs} vs A concentration plots are measured at the constant B concentration, the observed K_A value has the following meaning:

$$K_A = \frac{K_{0-1} \cdot \left(1 + \frac{[B]}{K_{0-3}} + \frac{[B]}{K_{0-3} \cdot K_{3-6}}\right)}{\left(1 + \frac{[B]}{K_{1-4}}\right)}. \quad (7)$$

In summary, the kinetic analysis of the reporter ligand interaction with the target protein, made in the presence of B, allows for the description of the interaction mechanism for this ligand and calculation of the relevant rate and equilibrium parameters.

METHODS

The [^{3}H]PE2I (specific activity 74 Ci/mmol) was synthesized from [^{3}H]iodomethane (Amersham, UK) and desmethyl PE2I precursor (PharmaSynth AS, Estonia). The exact labeling and synthetic procedure for the synthesis of PE2I (*N*-(3-iodoprop-(2*E*)-enyl-2β-carbomethoxy-3β-4-(methylphenyl) nortropane) is described elsewhere [16]. The cocaine hydrochloride was obtained from Tamro AS, Estonia; (S)-amphetamine hydrochloride (Cayman Chemical, USA) was a gift from Professor A. Zarkovski, Chair of Pharmacology, University of Tartu. Other reagents, such as dopamine, HEPES, and buffer salts of the highest commercial grade, were purchased from Sigma-Aldrich, USA. The composition of the assay buffer used throughout the study was as follows: 30 mM HEPES, 120 mM NaCl, 5 mM KCl, pH 7.4.

For the preparation of the striatal tissue suspension, female Wistar rats (4 months old, total of 17 animals) were decapitated, the striatum regions of the brain were rapidly dissected, homogenized in the ice-cold incubation buffer, and centrifuged at 30.000 g for 20 minutes 4 times. Super-

natant was discarded and the pellet was resuspended in the incubation buffer after each centrifugation. The procedures were performed in accordance with the guidelines established in the Directive 86/609/EEC and local guidelines approved by the Ethical Committee of the University of Tartu.

The membrane-bound [^{3}H]PE2I was determined by the conventional filtration method, using Whatman GF/B filters, pretreated with 0.3% polyethylenimine [13].

Kinetic analysis of the [^{3}H]PE2I association with striatal membranes in the absence and in the presence of cocaine, amphetamine, and dopamine was performed at 25° C by adding tracer and non-radioactive ligands simultaneously to the suspension of the rat striatal tissue. The process of [^{3}H]PE2I association was monitored by taking 100 µL aliquots from the reaction mixture, filtering them on Whatman GF/B filters, and washing with 15 mL of ice-cold phosphate buffer. The first samples were taken 5–15 seconds after initiating the process, and association was followed for 3–10 minutes (depending on the radioligand concentration). The influence of cocaine and amphetamine on [^{3}H]PE2I association with the dopamine transporter was measured varying both the radioligand and non-radioactive ligand concentrations.

Data processing, including linear and non-linear regression analysis, was performed with GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, California, USA).

RESULTS

Under the applied experimental conditions, [^{3}H]PE2I interaction with the dopamine transporter was characterized by the two-step interaction mechanism (1) as the k_{obs} vs [^{3}H]PE2I concentration plot is hyperbolic (Figs 5 and 6); the values K_A, k_{on} and k_{off} calculated from this plot are listed in Table 1. Considering these kinetic properties and due to high selectivity of this ligand against the dopamine transporter [14], it is concluded that [^{3}H]PE2I is a suitable reporter ligand for kinetic studies.

The influence of various concentrations of dopamine, amphetamine, and cocaine on kinetics of [^{3}H]PE2I interaction with the dopamine transporter was studied at a constant reporter ligand concentration (inserts in Figs 2–4).

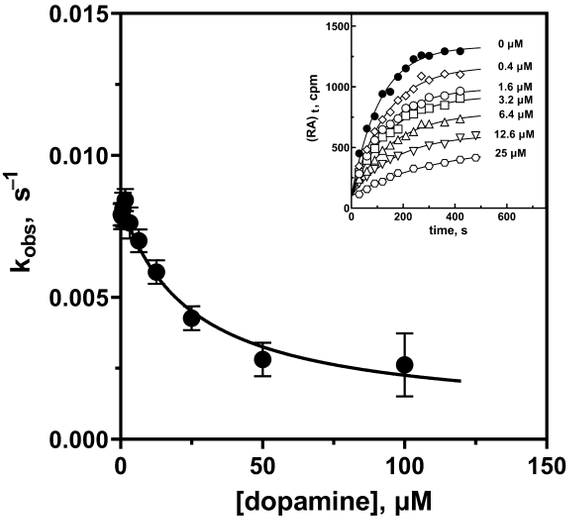


Fig. 2. Influence of dopamine on kinetics of [^{3}H]PE2I (3.4 nM) interaction with dopamine transporter.

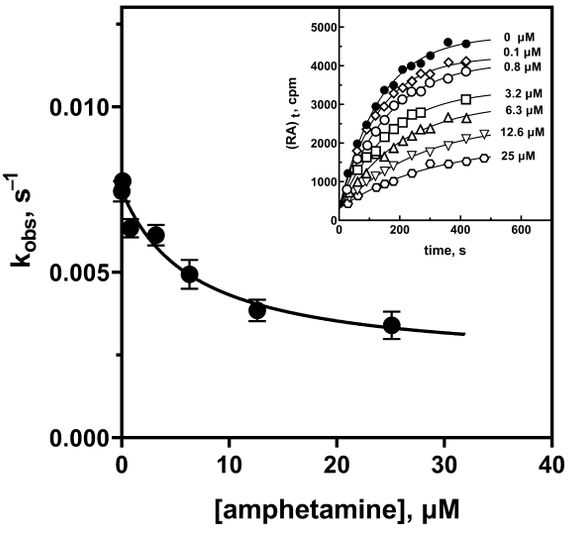


Fig. 3. Influence of (S)-amphetamine on kinetics of [^{3}H]PE2I (3.4 nM) interaction with dopamine transporter.

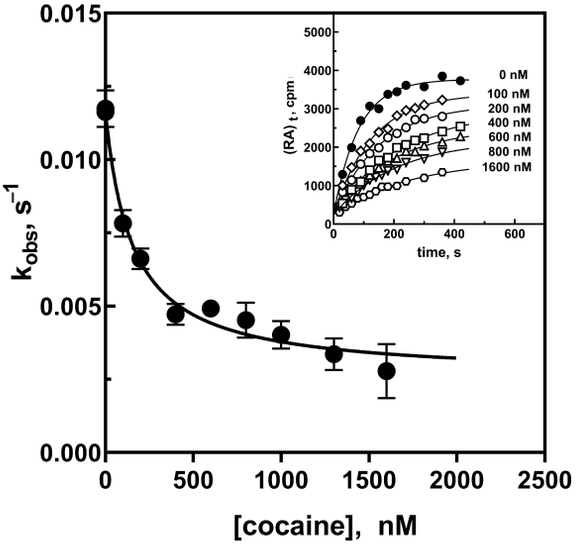


Fig. 4. Influence of cocaine on kinetics of [^{3}H]PE2I (4.1 nM) interaction with dopamine transporter.

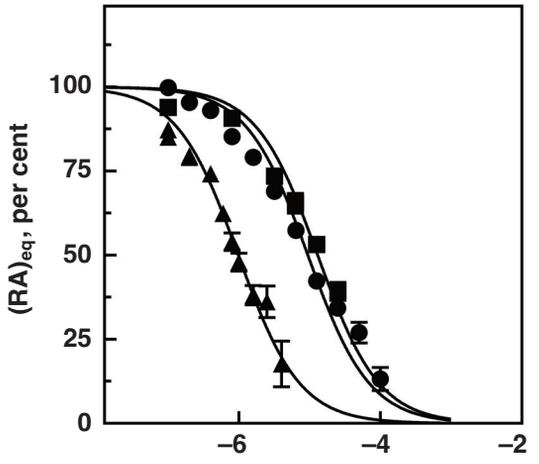


Fig. 5. Influence of dopamine (●), (S)-amphetamine (■), and cocaine (▲) on [^{3}H]PE2I binding with dopamine transporter under equilibrium conditions.

The results reveal that all these drugs slow down the reporter binding rate, as dose-dependent decrease of the k_{obs} values is observed in all these cases.

In parallel, a reduction of the radioligand–transporter complex concentration present at the equilibrium of the complex formation reaction in the presence of B was also observed, allowing the calculation of the IC_{50} values 9.45 ± 0.09 μM, 13.0 ± 0.12 μM, and 0.98 ± 0.01 μM for dopamine, (S)-amphetamine, and cocaine, respectively. Using the K_D value 1.2 nM for [^{3}H]PE2I, calculated from Eq. (4), these IC_{50} values were converted into the K_D values

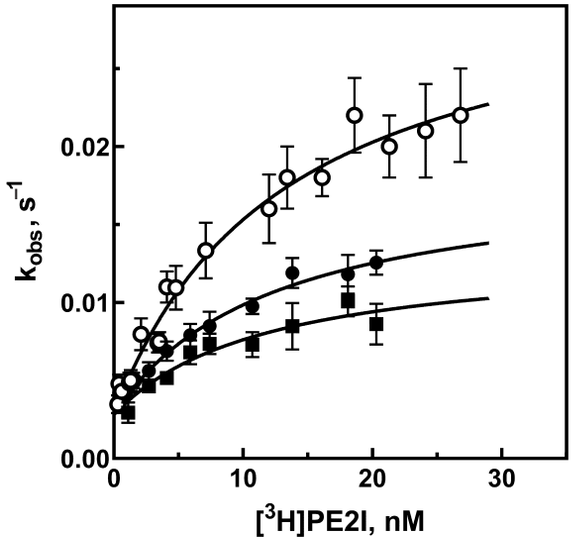


Fig. 6. Influence of (S)amphetamine on kinetics of [^{3}H]PE2I binding with dopamine transporter: ○ – no added drug, ● – 30 μM drug, ■ – 80 μM drug.

3.4 μM, 4.6 μM, and 200 nM, using the Cheng–Prusoff equation [17].

Additionally, the influence of fixed (S)-amphetamine and cocaine concentrations on [^{3}H]PE2I binding kinetics with the dopamine transporter was also investigated at different concentrations of [^{3}H]PE2I. The analysis allowed characterization of the influence of these compounds on reporter ligand binding (K_A) and on the isomerization and de-isomerization rate constants. The results are summarized in Figs 5 and 6. These dependencies also follow a hyperbolic pattern, as predicted by Eq. (3), with the exact mathematical interpretation of the calculated parameters k_{on}, K_A, and k_{off} in Table 1, depending on the presence or absence of the non-radioactive drug (the error margins shown denote a standard deviation of the fit parameters).

DISCUSSION

The kinetic analysis approach described above was used to analyze for the first time the kinetic details of the interaction mechanism of transportable compounds, dopamine and (S)-amphetamine, and transport inhibitor – cocaine, with the dopamine transporter and compare their binding modes.

It can be seen in Figs 2, 3 and 4 that in the presence of dopamine, (S)-amphetamine, and cocaine, the rate of [^{3}H]PE2I binding with the dopamine transporter decreases in a dose-dependent manner. This means that these compounds do not initiate the slow isomerization of the complex RB into (RB) and $k_{3–6} = 0$ in the reaction scheme (5). This conclusion is reliable as in the case of formation of the isomerized complex (RB), the dose-dependent increase in the k_{obs} values must be observed in the presence of the added unlabeled compound B [17]. This situation has been analyzed in detail before [10,17].

Secondly, the kinetic approach allows further specification of the interaction mechanism of (S)-amphetamine and cocaine with the dopamine transporter. The analysis was made in a straightforward way by studying the influence of these compounds on the kinetics of [^{3}H]PE2I interaction with the transporter. The results of these experiments are shown in Figs 6 and 7, and the kinetic parameters k_{on}, K_A, and k_{off} were calculated from the data (Table 1). The results reveal that both compounds inhibit isomerization of the [^{3}H]PE2I complex with the dopamine transporter (k_{on}), while the de-isomerization rate (k_{off}) is not affected. On the other hand, however, these drugs do not inhibit the reporter ligand binding step as no decrease of the K_A value for [^{3}H]PE2I binding is observed. On the contrary, the binding effectiveness of [^{3}H]PE2I was even somewhat increased in the presence of cocaine, while (S)-amphetamine had no influence on the reporter ligand binding effectiveness. This provides direct evi-

Table. 1. Influence of (S)-amphetamine and cocaine on kinetic parameters characterizing [^{3}H]PE2I interaction with the dopamine transporter

Drug concentration \ Kinetic parameters for [^{3}H]PE2I binding		k_{on}, s^{-1}	K_A, nM	k_{off}, s^{-1}
No drug*		0.029 ± 0.002	12.5 ± 1.9	0.0028 ± 0.0005
(S)-amphetamine	30 μM	0.016 ± 0.002	12.2 ± 2.0	0.0029 ± 0.0007
	80 μM	0.011 ± 0.002	12.0 ± 3.0	0.0030 ± 0.0006
Cocaine	75 nM	0.014 ± 0.002	7.8 ± 2.2	0.0033 ± 0.0008
	175 nM	0.009 ± 0.002	5.9 ± 1.5	0.0030 ± 0.0008

* In this case, the parameters k_{on}, k_{off}, and K_A correspond to k_{1-2}, k_{2-1}, and K_{0-1} in the reaction scheme (1). The calculated parameters are characterized with standard deviations.

dence for the non-exclusive binding mechanism of these ligands and the formation of the ternary complex, denoted as RAB in the model (5).

The absence of the isomerization step of the RB complex simplifies the model (5) as it may be omitted altogether. Formally, this corresponds to the situation where $k_{3-6} = 0$ and $K_{3-6} \to \infty$. Under these conditions, the kinetic parameters k_{on} and K_A can be presented by the following equations:

$$k_{on} = \frac{k_{1-2}}{\left(1 + \frac{[B]}{K_{1-4}}\right)}, \quad (8)$$

$$K_A = K_{0-1} \frac{\left(1 + \frac{[B]}{K_{0-3}}\right)}{\left(1 + \frac{[B]}{K_{1-4}}\right)}. \quad (9)$$

Accordingly, the rate constant k_{on} decreases in the dose-dependent manner in the presence of B, showing that the ternary complex RAB is formed. This explains the inhibitory effect of B on [^{3}H]PE2I binding kinetics as this process is kinetically limited by the isomerization step. The same inhibition effect can be seen in the classical study of the radioligand displacement by the ligand B.

However, the influence of B on the radioligand binding equilibrium, described by the parameter K_A in Eq. (9), is a more complex phenomenon as this parameter depends on both equilibrium constants K_{0-3} and K_{1-3}. If these parameters are equal, the K_A value does not change in the presence of the added drug B. On the other hand, if $K_{0-3} > K_{1-3}$, the increase in the observed binding effectiveness of the reporter ligand can be observed in the presence of B. Interestingly, both these phenomena were observed in this study with (S)-amphetamine and cocaine.

It is important to emphasize that the non-exclusive ligand binding mechanism, described as the ternary complex formation between the target protein and two ligands, also includes the conventional competitive and non-competitive interaction mechanisms, which are, firsthand, connected with the availability of the distinct binding sites. As this kinetic analysis allows determination of the equilibrium constants K_{0-3} and K_{1-3}, investigation into the specificity pattern of these different binding options is now possible.

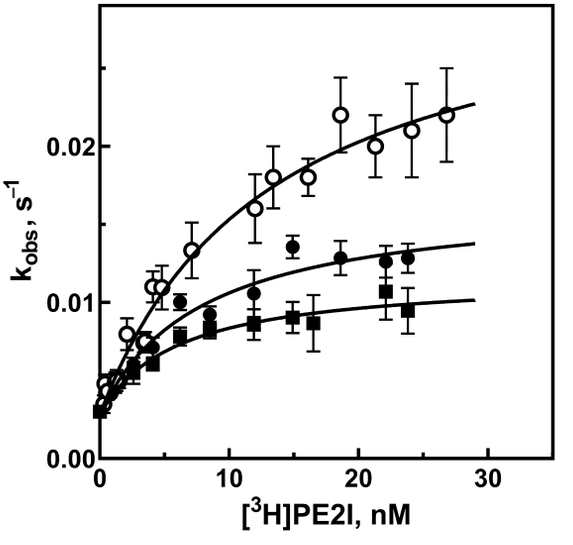


Fig. 7. Influence of cocaine on kinetics of [^{3}H]PE2I binding with dopamine transporter: ○ – no added drug, ● – 75 nM drug, ■ – 175 nM drug.

CONCLUSIONS

Competition kinetic analysis allowed investigation of the kinetic mechanism of the interaction of the dopamine transporter with dopamine, (S)-amphetamine, and cocaine, using [^{3}H]PE2I as a reporter ligand. The kinetic analysis

reveals that these drugs bind with the transporter protein reversibly without initiating kinetically distinguishable isomerization of the bound complex that is typical for potent nortropane derivatives, including [^{3}H]PE2I. Although both (S)-amphetamine and cocaine displace [^{3}H]PE2I from its complex with the transporter under equilibrium conditions, the reversible binding step of [^{3}H]PE2I with the transporter is not affected by the presence of these compounds. However, they do inhibit the isomerization process of the radioactive reporter ligand and transporter complex. These results provide evidence for the formation of the ternary complex, consisting of the transporter, the radiolabeled reporter ligand, and an unlabeled compound. At the same time, the unlabeled compound binding controls the isomerization rate of the [^{3}H]PE2I–transporter complex, pointing to cooperative interaction between these separate binding sites. This non-exclusive binding mechanism presents several interesting possibilities for data-driven ligand design that could alter certain aspects of dopamine transport functionality without gross impairment of its overall function.

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Dopamiini transporteri koostoime inhibiitorite ning transporditavate substraatidega: kineetika uuringutest järelduv molekulaarne mehhanism

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Dopamiini, (S)-amfetamiini ning kokaiini toime molekulaarsete mehhanismide selgitamine on oluline mõistmaks nende ainete mõju aju dopamiinergilise süsteemi komponentidele ning sellega seotud narkosõltuvuse ilmingutele, mis põhjustavad nii meditsiinilisi kui ka tõsiseid sotsiaalseid probleeme. Selles töös uuriti nende ainete interaktsiooni hiire aju juttkeha dopamiini transportvalguga, kasutades selleks erinevalt eelnevatest uuringutest kineetilise analüüsi meetodit radioaktiivse reporterligandi [^{3}H]PE2I toimel, mis on tõhus dopamiini transporteri inhibiitor. Töö tulemused näitavad, et ükski kolmest uuritud ühendist ei algata nende ainete ja transportvalgu kompleksi aeglast isomerisatsiooni, mis on iseloomulik paljudele madala molekulmassiga ühendite sidumisprotessidele valkudega. Teiseks näidati, et (S)-amfetamiin ning dopamiin ei mõjuta reporterligandi ja transporteri vahelise kompleksi moodustumise kiiret tasakaalu. Seega on reporterligandi nende ühendite seostumine transportvalgu erinevatele ja omavahel mitte-kattuvatele sidumiskohtadele ilmne. Samal ajal aga kõik kolm ainet aeglustavad [^{3}H]PE2I seostumise kiirele staadiumile järgnevat kompleksi aeglase isomerisatsiooni protsessi, viidates nende sidumiskohtade vaheliste allosteeriliste interaktsioonide olemasolule.