Some Selective Serotonin Reuptake Inhibitors Inhibit Dynamin I Guanosine Triphosphatase (GTPase)

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Eukaryotic cells take up extracellular materials and recycle their membranes by endocytosis, which involves the formation of numerous types of membrane vesicles at the plasma membrane. 1—3 Vesicles occur in various sizes, ranging from large phagosomes, to smaller clathrin-coated vesicles, to tiny synaptic vesicles. Endocytic mechanisms have many cellular functions, including the uptake of extracellular nutrients, regulation of cell surface receptor expression and signaling, antigen presentation, and maintenance of synaptic transmission.

Synaptic transmission is dependent on the continuous recruitment of synaptic vesicles via local membrane recycling. 4,5 Although the precise mechanisms of synaptic vesicle reformation remain a matter of debate, 6—10 there is strong evidence for a key role of the guanosine triphosphatase (GTPase) dynamin in this process. 11—15 as well as in a variety of endocytic reactions in all cell types. 12,16—19 Dynamin (Dyn) is thought to oligomerize at the neck of endocytic pits and to mediate neck constriction and fission. 1,12,14

Receptor-mediated endocytosis (RME) and synaptic vesicle endocytosis (SVE) utilize many proteins and lipid cofactors. 20 SVE occurs when nerve terminals retrieve empty synaptic vesicles after stimulated exocytosis to enable refill of these vesicles with neurotransmitters for a new round of exocytosis. Overexpression of GTPase-defective Dyn mutants inhibits both RME and SVE in a variety of cells. 21 Mammals have three dynamins with different expression patterns. 22,23 Dyn I, II and III are all found in neurons, but Dyn I is neuron-specific and is expressed much more strongly than either of the others. 23 Several observations 22,23,25 strongly suggest that Dyn I plays a dedicated and essential role in the recycling of synaptic vesicles and, thus, has a critical role in nervous system function. All dynamins have four functional domains: an N-terminal GTPase domain, a pleckstrin homology (PH) domain, a proline-rich domain (PRD), and an assembly domain also known as the GTPase effector domain (GED). 3

1-Phosphatidylycerine (PS) or phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) binds the PH domain of Dyn, enhances its GTPase activity, 26,27 and induces cooperative helix assembly. 28 Myristyl trimethyl ammonium bromide (MiTMAB) is surface-active, and it alters protein–lipid interactions. 29,30 At high concentrations, MiTMAB is a cationic surfactant, as observed for other pharmacologically active cationic amphiphilic compounds, including chlorpromazine, an antipsychotic, and imipramine, an antidepressant. 31 Further, chlorpromazine is well known as an endocytosis inhibitor. 32 Thus, the inhibition of Dyn I GTPase activity by various psychotropic drugs was investigated to explore potentially clinically useful endocytosis inhibitors and to understand their mechanism of action.

MATERIALS AND METHODS

Materials PfuUltraTM II Fusion HS DNA Polymerase was purchased from Stratagene. Restriction enzymes and the DNA ligation kit ver.2 were purchased from Takara Bio Co., Ltd. TALON Metal Affinity Resin was purchased from Clontech Co., Ltd. The Mono Q 5/50 GL column was purchased from Merck Co., Ltd. PET21a expression vectors and Escherichia coli Rosetta2 (DE3) were purchased from Merck Co., Ltd. Quant-iT Protein Assay kit and Thermo-X reverse transcriptase were purchased from Invitrogen. Other chemicals in this study were of analytical or higher grade.

Construction of Expression Vector for Mus musculus Dyn I and GTPase Domain of Dyn I Three fragments of the Dyn I gene (gi: 116063569) were obtained by PCR from mouse brain cDNA, which was synthesized by Thermo-X reverse transcriptase. The GTPase domain (amino acid residues (aa), 1—230) of Dyn I was amplified by using primer 1 (5’-GGAATTCAGATCTCATATGGGAATTCAGATCTCATATGGCAACC-
same sites in pET21a to create an expression vector for His₆
mazine) and 1-[3-(dimethylamino)propyl]-1-(4-flu-
pan-1-amine, (clomipramine)
(3,4-dihydrobenzo[
b]thienepin-10-yl)-N,N-dimethylpropan-1-amine (nimipramine) were from Astellas Pharma Inc. Methyl 2-phenyl-2-(2-piperidyl)acetate (methylene
diphenyl) and 3-(5,6-dihydrobenzol[b][1]benzazepin-11-
yl)-N,N-dimethylpropan-1-amine (imipramine) were from Novartis Pharma K.K. 1,2,3,4,10,14b-Hexahydro-2-meth-
yldibenzol[c,f]pyrazino[1,2-a]azepine (mianserin) was from
Organon, (1R,2R)-2-(aminomethyl)-N,N-diethyl-1-phenylcy-
clopropane-1-carboxamide (milnacipran) was from Asahi
Kasei Pharma and, (35-trans)-3-(1,3-benzodioxol-5-yl)-
methy1)-4-(4-fluorophenyl)piperidine (paroxetine), was from
GlaxoSmithKline K.K. 2-(5-Methoxy-1-[3-(trifluorometh-
yl)phenyl]penty-lidine)aminoxyethanamine (fluvoxamine)
was from Solvay Seiyaku K.K. and, 3-(10,11-dihydro-5H-
dibenzol[a,d]cyclohepten-5-ylidene)-N-methyl-1-propan-
amine (nortriptyline) was from Dainippon Sumitomo Pharma
Co., Ltd. The drugs and MiTMAB were made up as stock
solutions in 100% DMSO or 50% (v/v) DMSO, 30 mM Tris–
HCl pH 7.4 and diluted in 30 mM Tris–HCl pH 7.4 prior to
use in assay. Stocks were stored at −20 °C for up to several
months.

**GTPase Assay**

The Malachite Green GTPase assay was used for the sensitive colorimetric detection of orthophos-
phate (Pi) according to the method reported by Quan et
al. 33,34 Purified 20 nM Dyn-His₆ (diluted in dynamin diluting
buffer: 6 mM Tris–HCl, 20 mM NaCl, 0.02% Tween 80, pH
7.4) was incubated in GTPase buffer (10 mM Tris–HCl,
10 mM NaCl, 2 mM Mg²⁺, 0.05% Tween 80, pH 7.4, 1 µg/ml
leupeptin and 0.1 mM PMSF) and guanosine 5’-triphosphate
(GTP) 0.3 mM in the presence of test compound for 30 min
at 30 °C. The final assay volume was 40 µl. The assay was
conducted in round-bottomed 96-well plates. The incubations
of the plate were performed in a dry heating block with shaking
at 300 rpm (Eppendorf Thermomixer). Dynamin activity was
measured as phospholipid release stimulated with addition of
different concentrations of PS liposomes. The reaction was
terminated with 10 µl of 0.5 M EDTA pH 8.0. To each well
was added 150 µl of Malachite Green solution (2% (w/v)
ammonium molybdate tetrahydrate, 0.15% (w/v) malachite
green and 1 M HCl): the solution was passed through 0.45 
µm filters. Color was allowed to develop for 20 min, and the
absorbance of samples in each plate was determined on a mi-
croplate spectrophotometer (Wallac 1420 ARVOxs from
PerkinElmer) at 650 nm. Phosphate release was quantified by
comparsion with a standard curve of sodium dihydrogen or-
thophosphate monohydrate which was run in each experi-
ment. KaleidaGraph 4.0 (Synergy Software) was used for
plotting data points and analysis of enzyme kinetics using
non-linear regression. The curves were generated using the
Michaelis–Menten equation \( v = \frac{V_{\text{max}} [S]}{K_m + [S]} \) where
\( S = \) PS activator or GTP substrate. After the \( V_{\text{max}} \) and \( K_m \) val-
ues were determined, the data were transformed using the
Lineweaver–Burke equation \( \frac{1}{v} = \frac{1}{V_{\text{max}}} + (\frac{K_m}{V_{\text{max}}}) \left( \frac{1}{[S]} \right) \)

**RESULTS**

**Recombinant Dyn I from** E. coli **Has GTPase Activity**
Although native Dyn I and recombinant Dyn I from the bac-
ulovirus expression system have been employed to investi-
gate the GTPase activity of Dyn I,[34,35] it has not previously
been confirmed that recombinant Dyn I from E. coli shows
GTPase activity. We expressed and purified dynamin I with a
His_6 tag fused to the C-terminus (Dyn-His_6) and the GTPase
domain of Dyn I from E. coli Rosetta2 (DE3) with a His_6 tag
fused to the C-terminus by transforming the cells with pET-
Dyn1 and pETDynGTP (Fig. 1A). Purified Dyn-His_6 hy-
drolyzed GTP and released orthophosphate Pi time-depend-
ently. In contrast, release of Pi was not observed in the case
of the purified GTPase domain of Dyn I (Fig. 1B). These re-
results clearly show that purified Dyn-His_6 from E. coli has
GTPase activity, while the GTPase domain of Dyn I alone
does not.

Some Selective Serotonin Reuptake Inhibitors (SSRIs)
Inhibit the GTPase Activity of Dyn I
The IC_{50} values of MiTMAB (used as a standard inhibi-
tor of Dyn I) and nineteen psychotropic drugs were esti-
ated as shown in Table 1. MiTMAB inhibited Dyn-His_6 GTPase with an IC_{50} of
24.1 ± 9.4 μM in this study. Quan et al. reported an IC_{50} value of
3.1 ± 0.2 μM,[33] which is rather different from that deter-
dined here. This discrepancy may be explained by the differ-
ence between native Dyn I from sheep brain used in their
study, and recombinant Dyn-His_6 from E. coli used in ours.
Therefore, we evaluated the Dyn I inhibitory activity of the
nineteen psychotropic drugs by comparing their IC_{50} values
with that of MiTMAB in the same system.

Serotonin/noradrenaline reuptake inhibitor (milnacipran),
anticonvulsant (carbamazepine) and the antipsychotics
(chlorpromazine, haloperidol, sulphiride, zotepine and
tiapride) all showed little inhibition of dynamin I GTPase ac-
tivity compared with MiTMAB (Table 1). However, the IC_{50}
values for two antidepressants (clomipramine and mapro-
tilone) and two SSRIs (fluoxetine and paroxetine) were greater
than or similar to the IC_{50} for MiTMAB (Table 1). It is note-
worthy that the two SSRIs showed lower IC_{50} values (7.3 ±
1.0 μM for sertraline and 14.7 ± 1.6 μM for fluvoxamine,
Table 1) than MiTMAB. The full concentration response
curves are shown in Fig. 2. Other psychotropic drugs did not
inhibit dynamin I GTPase activity (Table 1).

How Do Sertraline and Fluvoxamine Inhibit Dynamin I
GTPase? To investigate whether sertraline and fluvoxam-
ine interfer with the activity of dynamin I GTPase, kinetic
analysis of dynamin I GTPase activity in the presence of var-ious concentrations of PS liposomes or GTP was conducted
(Fig. 3).

Kinetic analysis with increasing concentrations of GTP re-
vealed that the maximal velocity of dynamin I GTPase activ-
ity, V_{max}, was 573 nmol/mg/min and the Michaelis–Menten
constant, K_{m}, was 25.3 μM, as calculated from the line for
0 μM fluvoxamine in Fig. 3B. In the presence of sertraline or
fluvoxamine, the V_{max} decreased and K_{m} increased with in-
creasing concentrations of SSRI. The Lineweaver–Burke plots
show mixed type inhibition (Figs. 3A—D).

With PS liposomes, the V_{max} was 593 nmol/mg/min, and
the K_{m} was 0.445 μM, as calculated from the line for 0 μM
fluvoxamine in Fig. 3F. In the presence of fluvoxamine, there
was no effect on V_{max}. On the other hand, K_{m} increased with
increasing concentrations of fluvoxamine. These data show
that fluvoxamine competes with PS for binding to Dyn I.

DISCUSSION

Ramachandran et al. reported that the Dyn middle domain
Fig. 3. Sertraline Does Not Compete with PS, But Fluvoxamine Competes with PS

The effect of fluvoxamine is shown in Michaelis–Menten (A) and Lineweaver–Burke (B) plots of the GTPase activity of purified Dyn-His<sub>6</sub> (20 μM) with increasing concentrations of GTP and a fixed concentration of PS (5.0 μM). The panels (C) and (D) show Michaelis–Menten and Lineweaver–Burke plots of the GTPase activity in the case of sertraline. The panels (E) and (F) show Michaelis–Menten and Lineweaver–Burke plots of the GTPase activity of purified Dyn-His<sub>6</sub> in the absence and presence of the indicated concentrations of fluvoxamine with increasing concentrations of PS liposomes and a fixed concentration of GTP (300 μM). The panels (G) and (H) show Michaelis–Menten and Lineweaver–Burke plots of the GTPase activity in the case of sertraline. All results are representative of at least 2 independent experiments.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristyl trimethyl ammonium bromide (MtMAB)</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>24.1±9.4</td>
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<tr>
<td>3-(2-Chloro-10H-phenothiazin-10-yl)-N,N-dimethyl-propan-1-amine (chlorpromazine)</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>47.2±23.1</td>
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<tr>
<td>4-[4-(4-Chlorophenyl)-4-hydroxy-1-piperidyl]-1-(4-fluorophenyl)-butan-1-one (haloperidol)</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>&gt;100</td>
</tr>
<tr>
<td>N-[1-Ethylpyrrolidin-2-yl]methyl]-2-methoxy-5-sulfamoyl-benzamide (sulpiride)</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>&gt;100</td>
</tr>
<tr>
<td>2-((8-Chlorodibenzo(b,f)thiepin-10-yl)oxy)-N,N-dimethyl-ethylamine (zotepine)</td>
<td><img src="image5.png" alt="Structure" /></td>
<td>37.2±17.3</td>
</tr>
<tr>
<td>N-(2-Diethylaminoethyl)-2-methoxy-5-methylsulfonyl-benzamide (tiapride)</td>
<td><img src="image6.png" alt="Structure" /></td>
<td>&gt;100</td>
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<tr>
<td>Methyl 2-phenyl-2-(2-piperidyl)acetate (methylphenidate)</td>
<td><img src="image7.png" alt="Structure" /></td>
<td>&gt;100</td>
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<tr>
<td>3-(5,6-Dihydrobenzo[b][1]benzazepin-11-yl)-N,N-dimethylpropan-1-amine (imipramine)</td>
<td><img src="image8.png" alt="Structure" /></td>
<td>&gt;100</td>
</tr>
<tr>
<td>3-(9-Chloro-5,6-dihydrobenzo[b][1]benzazepin-11-yl)-N,N-dimethylpropan-1-amine (clomipramine)</td>
<td><img src="image9.png" alt="Structure" /></td>
<td>29.9±5.9</td>
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<tr>
<td>10,11-Dihydro-5-[3-(methylamino)propyl]-5H-dibenz[b,f]azepine monohydrochloride (desipramine)</td>
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<td>&gt;100</td>
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<tr>
<td>N-Methyl-9,10-ethanoantracene-9(10H)-propanamine (maprotiline)</td>
<td><img src="image11.png" alt="Structure" /></td>
<td>21.1±3.6</td>
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<td>3-(10,11-Dihydro-5H-dibenzo[a,d]cyclohepten-5-ylidene)-N-methyl-1-propanamine (nortriptyline)</td>
<td><img src="image12.png" alt="Structure" /></td>
<td>64.0±38.0</td>
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<tr>
<td>1,2,3,4,10,14b-Hexahydro-2-methyldibeno(c,f)pyrazino[1,2-a]azepine (mianserin)</td>
<td><img src="image13.png" alt="Structure" /></td>
<td>&gt;100</td>
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<tr>
<td>5H-Dibenz[b,f]azepine-5-carboxamide (carbamazepine)</td>
<td><img src="image14.png" alt="Structure" /></td>
<td>&gt;100</td>
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<tr>
<td>(1R,2R)-2-(Aminomethyl)-N,N-diethyl-1-phenyl-cyclopropane-1-carboxamide (milnacipran)</td>
<td><img src="image15.png" alt="Structure" /></td>
<td>&gt;100</td>
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</table>
is necessary for tetramerization and higher-order self-assembly. Dyn exhibits GTPase activity upon self-assembly and undergoes appreciable conformational changes during its GTP hydrolysis cycle. These observations are consistent with our finding that the GTPase domain of Dyn I did not show GTPase activity. On the other hand, purified Dyn-His6 from E. coli did show GTPase activity.

It is known that Dyn I is dephosphorylated by the calcium-dependent phosphatase calcineurin and is subsequently rephosphorylated by cyclin-dependent kinase 5 (cdk5) on Ser774 and Ser778 during SVE. Although Dyn-His6 from E. coli would not have undergone post-translational modification, such as phosphorylation, it should be suitable for the present purpose, since it retains GTPase activity at least.

The IC50 values for sertraline and fluvoxamine (7.3 ± 1.0 μM and 14.7 ± 1.6 μM, Table 1) were less than the IC50 of MiTMAB (Table 1), so these SSRIs are inhibitors of dynamin I GTPase. The IC50 value of chlorpromazine was 47.2 ± 23.1 μM (Table 1) in this study. Given that chlorpromazine is a well-known endocytosis inhibitor, it seems likely that sertraline and fluvoxamine inhibit endocytosis by repressing dynamin I GTPase activity.

MiTMAB is a surface-active compound that competes with PS for binding to the dynamin I PH domain, like MiTMAB. Fluvoxamine has a methoxypentylidene group, which may behave like the alkyl chain of MiTMAB.

Sertraline showed mixed type inhibition with respect to GTP and PS (Fig. 3). We cannot explain the inhibition mechanism of sertraline from the results of this study, but it is clearly different from that of fluvoxamine.

Most of the SSRIs (fluoxetine, paroxetine, sertraline and fluvoxamine) inhibited dynamin I GTPase (Table 1). Dyn I is expressed at much higher levels than Dyn II and III in neurons, and plays a critical role in nervous system function. We speculate that these SSRIs regulate the transportation of neurotransmitters through the modulation of the synaptic vesicle endocytosis via the inhibition of dynamin I GTPase.

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