ル分画の分析

Table 1 抑肝散構成生薬抽出物のβセクレターゼ阻害活性

	β-secretase activity(%)				β-secretase activity (%)		
Cnidium of f icinale	0.5	5.0	50 μg/mL	*Uncaria sinensis*	0.5	5.0	50 μg/mL
MeOH	<5%	<5%	<5%	MeOH	<5%	<5%	<5%
EtOAc	<5%	<5%	17%	EtOAc	<5%	<5%	<5%
H_2O	<5%	<5%	<5%	H_2O	<5%	<5%	<5%
Poria cocos				*Uncaria rhynchophylla*			
MeOH	<5%	<5%	<5%	MeOH	<5%	<5%	<5%
EtOAc	<5%	<5%	<5%	EtOAc	<5%	<5%	<5%
H_2O	<5%	<5%	<5%	H_2O	<5%	<5%	<5%
Bupleurum f alcatum				*Angelica acutiloba*			
MeOH	<5%	<5%	<5%	MeOH	<5%	<5%	<5%
EtOAc	<5%	<5%	45%	EtOAc	<5%	7%	<5%
H_2O	<5%	<5%	<5%	H_2O	11%	17%	9%
Atractylodes lancea				*Glycyrrhiza uralensis*			
MeOH	13%	14%	15%	MeOH	8%	<5%	<5%
EtOAc	<5%	7%	11%	EtOAc	<5%	<5%	<5%
H_2O	10%	6%	<5%	H_2O	19%	15%	12%

Table 2 酢酸エチル分画（分画 5）中に観察された成分

Peak No	Observed m/z	Observed Ion	MW	Compound
1	985	$[M+CH_3COO^-]^{1-}$	926	Saikosaponin c/h/i
2	987	-	-	-
3	811	$[M-H]^{-1}$	812	Saikosaponin b_3/b_4
4	779	$[M-H]^{-1}$	780	Saikosaponin $a/b_1/b_2/d/g$
5	779	$[M-H]^{-1}$	780	Saikosaponin $a/b_1/b_2/d/g$
6	822	$[M-H]^-$	823	Acetylated Saikosaponin $a/b_1/b_2/d/g$
	881	$[M+CH_3COO^-]^{1-}$	780	Saikosaponin $a/b_1/b_2/d/g$
7	779	$[M-H]^{-1}$	780	Saikosaponin $a/b_1/b_2/d/g$
8	822	$[M-H]^{-1}$	823	Acetylated saikosaponin $a/b_1/b_2/d/g$
	881	$[M+CH_3COO^-]^{1-}$	780	Acetylated saikosaponin $a/b_1/b_2/d/g$

分担研究報告書

統合失調症に有効な抑肝散構成成分の薬理解析と単一成分固定

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研究要旨

認知症に伴う行動心理学的症状（BPSD）の治療には非定型抗精神病薬が効果的を示す。また、抑肝散もBPSDに対して効果を示すことが報告されている。そこで、本研究では抑肝散に含まれる天然アルカロイド成分から、非定型抗精神病薬と類似した作用を示す単体の薬物を検索し、統合失調症に有効な新しい薬剤を開発するのが目的である。平成22年度までに、抑肝散の天然成分中からセロトニン受容体に作用を示すアルカロイド成分をスクリーニングし、ガイソシジンメチルエーテルがセロトニン（5-HT）1A受容体に対してはパーシャルアゴニストとして、5-HT2A、2C、5-HT7受容体にはアンタゴニストとして作用することを見いだした。本年度はドーパミン受容体に対するガイソシジンメチルエーテルの効果を調べた。ガイソシジンメチルエーテルは、D2受容体に対してパーシャルアゴニストとして働き、D1受容体に対しては影響がなかった。これらの特性は非定型抗精神病薬のアリピプラゾールと類似しており、抑肝散の一成分の構造を元に非定型精神病薬の開発を行える可能性がある。

A．研究目的

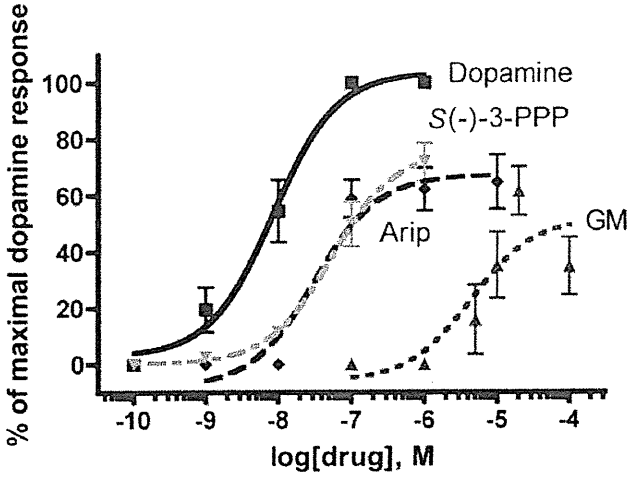
認知症に伴う行動心理学的症状（BPSD）の治療において非定型抗精神病薬が効果を示すことがしられている。また、抑肝散もBPSDに対して効果を示す報告がある。そこで、本研究では抑肝散に含まれる天然アルカロイド成分から、非定型抗精神病薬と類似した作用を示す単体の薬物を検索し、統合失調症に有効な新しい薬剤を開発につなげるのが目的である。

B．研究方法

抑肝散に含まれるアルカロイドの一成分であるガイソシジンメチルエーテルがドーパミン受容体に対してどの様に作用するかを、細胞内Ca^{2+}イメージング法を用いて解析した。本研究ではドーパミン受容体の中でも特に中枢神経系において主要な機能を担っているD1受容体とD2受容体について検討した。D1受容体はGs蛋白質と、D2受容体はGi蛋白質と共役するため、単純にHEK293T細胞に各タイプのドーパミン受容体を発現させただけでは、Ca^{2+}イメージング法で反応が得られない。本研究ではこの点を解消するため、それぞれのタイプのドーパミン受容体と選択的に共役するGα蛋白とGα16蛋白との間でキメラ蛋白を作成することにより、全ての種類のドーパミン受容体のシグナル伝達をPI turnover系に集約し、Ca^{2+}イメージング法で解析を行った。また、実験に用いるD1、D2受容体は、RT-PCR法により脳組織から単離した。本研究では抑肝散の構成生薬の釣籐鈎のアルカロイドの一成分であるガイソシジンメチルエーテルがドーパミン受容体に与える影響について検討した。

C．研究結果

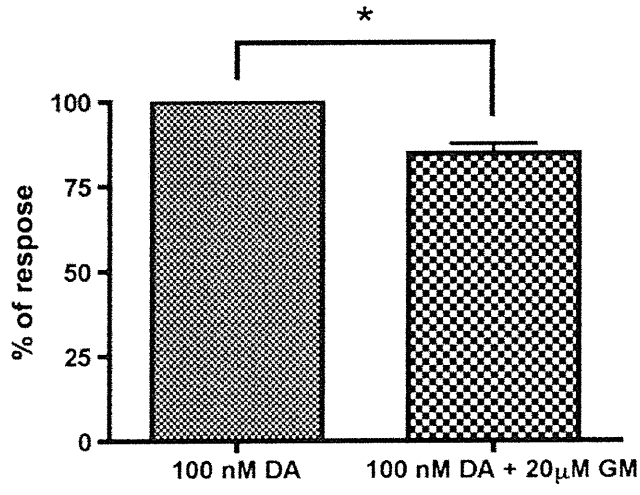
HEK293T細胞にD2受容体とキメラG蛋白質であるG16/oやG16/i3を強制発現させ、カルシウムイメージング法でガイソシジンメチルエーテルの感受性を検討した。



【図１】

図１はドーパミン、S(-)-3PPP（D2 パーシャルアゴニスト)、アリピプラゾール（D2 パーシャルアゴニスト)、ガイソシジンメチルエーテルの D2 受容体に対する応答性を調べた濃度依存曲線であるが、S(-)-3PPP やアリピプラゾールに比べると親和性は下がるが、ガイソシジンメチルエーテルは D2 受容体にパーシャルアゴニストとして作用した（pEC50：5.36±0.74、Emax:50±15%)。

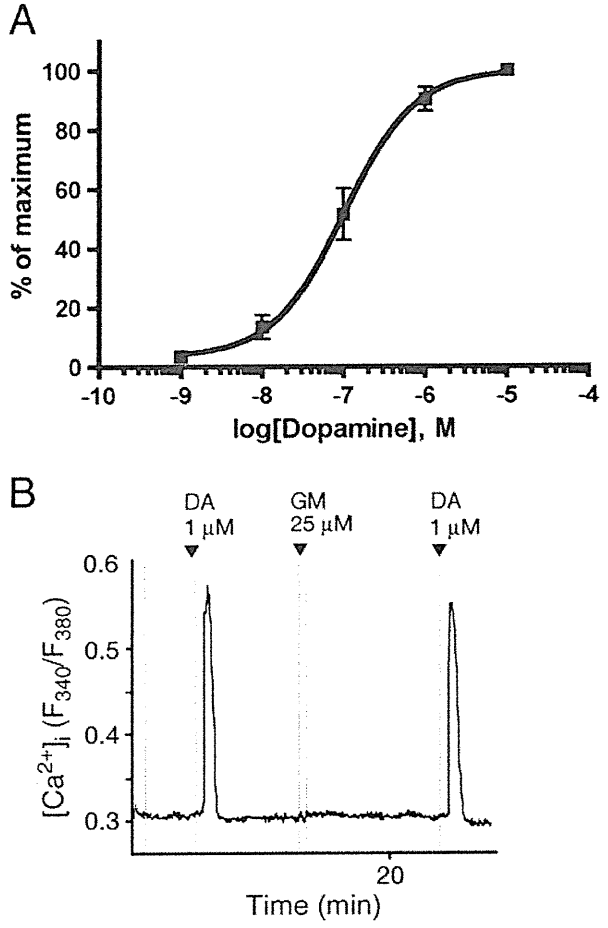
ガイソシジンメチルエーテルは D2 受容体のパーシャルアゴニストであることが分かったので、ガイソシジンメチルエーテルの D2 受容体に対するパーシャルアンタゴニストとしての作用を検討した。



【図２】

図２はD2受容体に対して100nMのドーパミンが示す応答をガイソシジンメチルエーテルが部分的に阻害することを示した図である。このように、ガイソシジンメチルエーテルはD2受容体に対してパーシャルアンタゴニストとして働いた。

次に、ガイソシジンメチルエーテルのD1受容体に対して、影響があるのかどうかを、HEK293T細胞にD1受容体とGα15蛋白質やGα16蛋白質を強制発現させて検討した。



【図３】

図３aは、この実験系を用いてドーパミンのD1受容体に対する濃度依存性の応答曲線を示したものである。D1受容体をGα15蛋白質やGα16蛋白質と共役させた系においても、D1受容体をGαs蛋白質と共役させた場合と同様の親和性が得られた。図３bは、カルシウムイメージング法を用いて、D1受容体の応答を示したものであるが、D1受容体はドーパミンには応答するが、ガイソシジンメチルエーテルに対しては全く応答を示さなかった。

D．考察

今回の研究から、抑肝散含有アルカロイドの一成分であるガイソシジンメチルエーテルはD2受容体に対してはパーシャルアゴニストとして作用し、D1受容体に対しては影響を与えなかった。

平成２２年度の本研究ではガイソシジンメチルエーテルがセロトニン（5-HT）1A受容体に対してはパーシャルアゴニストとして、5-HT2A、2C、5-HT7受容体にはアンタゴニストとして作用することを示した。これらのセロトニン受容体に対するプロフィールと平成２３年度に得られたドーパミン受容体に対する結果とを照らし合わせると、ガイソシジンメチルエーテルは、非定型抗精神病薬のアリピプラゾールと類似した薬理学特性を有してお

り、ガイソシジンメチルエーテルをリード化合物とした新しい抗精神病薬の開発にも繋がる可能性がある。

E．結論

抑肝散に含まれる天然アルカロイドの一成分であるガイソシジンメチルエーテルのドーパミン受容体に対する影響を調べた。その結果、ガイソシジンメチルエーテルはドーパミンD2受容体に対してパーシャルアゴニストとして作用することが分かった。昨年度までの研究結果と照らし合わせて考えると、ガイソシジンメチルエーテルは非定型抗精神病薬のアリピプラゾールと類似した薬理学特性を有することが分かった。

F．健康危険情報

該当なし

G. 研究発表

1. 論文発表

Ueda T, Ugawa S, Ishida Y, Shimada S. Geissoschizine methyl ether has third-generation antipsychotic-like actions at the dopamine and serotonin receptors. Eur J Pharmacol. 査読有り2011;671(1-3):79-86.

2. 学会発表

なし

H. 知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Behavioural Pharmacology

Geissoschizine methyl ether has third-generation antipsychotic-like actions at the dopamine and serotonin receptors

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Aripiprazole

ABSTRACT

Aripiprazole has made a significant contribution to the treatment of schizophrenia and related disorders. It has improved its safety and tolerability profiles, and these effects have been attributed to its pharmacological profile at the serotonin 5-HT and dopamine D_2 receptors. To discover compounds that have a similar pharmacological profile, we introduced a generic single-cell-based calcium imaging assay that standardizes the readouts from various assays used in previous studies on aripiprazole. In the present assay, the efficacy and potency of known ligands of serotonin 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_7 and dopamine D_{2L} receptors were comparable to those found in previous studies using a variety of readouts. The developed assay was also able to reproduce the partial agonist activity, the low intrinsic activity and the selective activation of aripiprazole at the dopamine D_{2L} receptors. Under identical experimental conditions, geissoschizine methyl ether (GM), a plant indole alkaloid, behaved as a partial agonist at the serotonin 5-HT_{1A} receptor, a partial agonist/antagonist at the dopamine D_{2L} receptor and an antagonist at the serotonin 5-HT_{2A}, 5-HT_{2C} and 5-HT_7 receptors. Interestingly, GM showed a relatively low intrinsic activity and evoked a partial activation response in a subset of cells expressing the dopamine D_{2L} receptor; both of these effects were similarly observed for aripiprazole. Although GM is far less potent at the dopamine receptor than aripiprazole at dopamine D_{2L} receptors (EC_{50} = 4.4 μM for GM *vs.* EC_{50} = 56 nM for aripiprazole), GM and GM derivatives may comprise a new set of candidates for atypical antipsychotics.

1. Introduction

Atypical antipsychotic drugs have revolutionized the treatment of schizophrenia and related disorders. Most approved antipsychotics to date behave as antagonists for the dopamine D_2 and serotonin 5-$HT_{2A/2C}$ receptors. However, an off-base dopamine D_2 receptor blockade in the dorsal striatum and pituitary is thought to cause extrapyramidal symptoms and hyperprolactinemia, respectively (Miyamoto et al., 2005). In contrast, aripiprazole acts as a partial agonist against dopamine D_2 receptors with a 'functionally selective' action, in addition to a partial agonist and antagonist effects at the serotonin 5-HT_{1A} receptors and serotonin 5-HT_{2A} and 5-HT_7 receptors, respectively (Jordan et al., 2007a, 2007b; Shapiro et al., 2003; Urban et al., 2007). Because aripiprazole is effective against the positive and negative symptoms of schizophrenia (Tadori et al., 2005), drugs with similar pharmacological profiles at these receptors are sought to advance the treatment of schizophrenia and related disorders.

For this purpose, we focused on *Yokukansan*, a Japanese *kampo* medicine that has been clinically reported to ameliorate the behavioral and psychological symptoms of dementia (BPSD) in patients with Alzheimer's disease, dementia with Lewy bodies, other forms of senile dementia, borderline personality disorder or schizophrenia (Iwasaki et al., 2005a, 2005b; Miyaoka et al., 2008, 2009; Monji et al., 2009; Shinno et al., 2007, 2008). We hypothesized that *Yokukansan* contains a major substance that affects a variety of serotonin and dopamine receptors.

G protein-coupled serotonin and dopamine receptors differentially stimulate a variety of intracellular signaling pathways. The measurement of cAMP levels is most appropriate for the G protein-coupled receptors linked to $G\alpha_s$ and $G\alpha_i$, whereas assays that measure either the inositol trisphosphate (IP_3) levels or the intracellular calcium levels are optimal for the G protein-coupled receptors linked to $G\alpha_q$. The [^{35}S]GTPγS binding assay is widely used to characterize all types of G protein-coupled receptor functions at one of the earliest receptor-mediated events. However, a previous study showed that aripiprazole, a known D_2 receptor partial agonist, is inactive in the [^{35}S]GTPγS binding assay using both Chinese Hamster Ovary (CHO) cell membranes expressing the cloned human dopamine D_{2L} receptor and CHO-D_{2L} cells, highlighting the limitation of this method for identifying dopamine D_2 receptor partial agonists (Jordan et al., 2007a). Due to this limitation, we introduced a single-cell-based assay that can analyze all types of G protein-coupled receptors on a single platform by

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integrating all of the downstream G protein-coupled receptor-Gα interactions into calcium flux (Ueda et al., 2009).

During screening for antipsychotic agents among the constituents of *Yokukansan*, we found that geissoschizine methyl ether (GM), an indole alkaloid that contains a β-carboline structure (Pengsuparp et al., 2001), inhibited the binding of radioligand to G protein-coupled serotonin receptors in a concentration-dependent manner. In the present study, we investigated the effect of GM on serotonin 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} and 5-HT_7 receptors and dopamine D_{2L} receptors using the developed assay system.

2. Materials and methods

2.1. Chemicals

5-Hydroxytryptamine (serotonin; 5-HT), 8-hydroxy-2-(di-n-propylamine)tetralin (8-OH-DPAT), 3-hydroxytyramine (dopamine), quinpirole, *S*(-)-3-(3-hydroxy-phenyl)-N-propylpiperidine (*S*(-)-3-PPP), haloperidol and WAY100635 were purchased from Sigma-Aldrich (St Louis, MO). Aripiprazole was obtained from Toronto Research Chemical, Inc. (TRC; North York, ON, Canada). Geissoschizine methyl ether (GM; $C_{22}H_{26}N_2O_3$) (Fig. 1) was provided from Tsumura Research Laboratories (Tsumura & Co, Ibaraki, Japan). Three different lots of GM were used in the present study, and there were no significant differences in the efficacy and potency on the dopamine and serotonin receptors between the different lots.

2.2. Construction of the Gα proteins and chimeras

A variety of G protein α subunits (Gα) were obtained from a human cell line and mouse brain tissues using reverse transcriptase-polymerase chain reaction (RT-PCR). Human $G\alpha_{16}$ was obtained from HL60 cells, whereas mouse $G\alpha_{15}$ was obtained from mouse blood. The $G\alpha_o$, $G\alpha_{i2}$ and $G\alpha_{i3}$ cDNAs were all cloned from mouse brain tissue. All of the chimeras were constructed by PCR using the human $G\alpha_{16}$ and the mouse-appropriate Gα cDNAs as templates. We constructed $G\alpha_{16}$-based chimeras by replacing the 44-residue C-terminal tail of the $G\alpha_{16}$ with those of $G\alpha_o$ and $G\alpha_{i3}$ ($G_{16/o}$ and $G_{16/i3}$) (Ueda et al., 2009). All full-length α-subunit cDNAs were subcloned into the pcDNA3.1(+) mammalian expression vector (Invitrogen).

2.3. Construction of the serotonin and dopamine receptors

Each open reading frame with a 5′ non-coding sequence of amplified mouse cDNA coding for the serotonin 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} and 5-HT_7 receptors and the dopamine D_1 and D_{2Long} (D_{2L}) receptors was subcloned into the pcDNA3.1(+) mammalian expression vector.

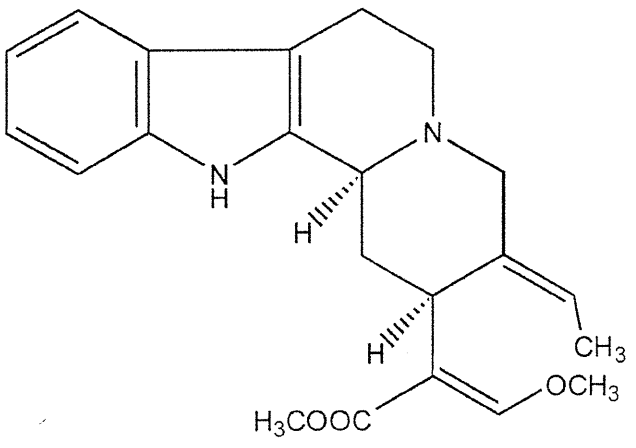

Fig. 1. Chemical structure of geissoschizine methyl ether.

2.4. Transfection of HEK293T cells

Human embryonic kidney 293 T (HEK293T) cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS; v/v) at 37 °C in humidified air with 5% CO_2. For transfection, the cells were seeded onto 100-mm dishes or uncoated glass coverslips in 35-mm chambers. After 24 h at 37 °C, the cells were washed in DMEM and transiently transfected with the Gα subunits and a receptor of interest using Lipofectamine 2000 (Invitrogen). We used $G\alpha_{15}$ and $G\alpha_{16}$ for $G\alpha_s$-coupled serotonin 5-HT_7 and dopamine D_1 receptors, $G_{16/o}$ and $G_{16/i3}$ for $G\alpha_i$-coupled serotonin 5-HT_{1A} and dopamine D_{2L} receptors, or no Gα for $G\alpha_q$-coupled serotonin 5-HT_{2A} and 5-HT_{2C} receptors. The transfection efficiencies were estimated by cotransfection with a GFP reporter plasmid or by immunohistochemistry and were typically >70%.

2.5. Calcium imaging analysis

A single-cell-based reporter system was used to examine the effects of the drugs on the G protein-coupled receptors (Ueda et al., 2009). Transfected cells on glass coverslips were moved 24 h after transfection to an assay chamber and loaded with 5 μM Fura-2/AM (Invitrogen, USA) for 30 min at room temperature. The cells were washed in 500 μl of assay buffer (10 mM HEPES, 130 mM NaCl, 10 mM glucose, 5 mM KCl, 2 mM $CaCl_2$ and 1.2 mM $MgCl_2$, pH 7.4) and stimulated with test compounds for 20 s using a bath perfusion system at a flow rate of 5 ml/min. To ensure that the cells were not desensitized as a result of previous ligand applications, a 180- to 420-s interval was maintained between each application of test compounds. The antagonistic effects of the compounds were tested by simultaneous application of the drugs with dopamine, 5-HT or GM for 20 s. We randomly selected Fura-2-loaded cells (100 cells/assay), and the effect of the test compounds on the internal calcium mobilization was measured with the commercially available ARGUS/HisCa system (Hamamatsu Photonics, Japan). The system was set for bottom-up reading with two alternative excitation wavelengths (340 nm and 380 nm) and a 510 nm emission wavelength. Ratios (340 nm/380 nm) were obtained by calculating the fluorescent intensities at 510 nm using the 340 nm and 380 nm excitation wavelengths with the ARGUS/HisCa software v1.65.

For further analysis, we selected cells that exhibited concentration-dependent internal calcium mobilization following the application of compounds (n = 10–15 cells per assay). The cells showing spontaneous and oscillating activities in response to 5-HT or dopamine were omitted. The specificities of all of the compounds to the receptors were confirmed by performing similar assays in non-transfected cells or cells transfected with Gα chimeras only. All of the compounds examined in the present study did not show any significant Ca^{2+} mobilization in non-transfected cells or cells transfected with Gα chimeras.

2.6. Data analysis

A three-parameter logistic equation was fit to the data to calculate the EC_{50}, pEC_{50} and E_{max} values (Prism version 4.0; GraphPad, Inc., San Diego, CA). Data are reported as the mean ± standard error of the mean (S.E.M.) using the traces of Ca^{2+} responses for each cell showing serotonin or dopamine responses in 3 to 5 separate experiments (n = 10–15 cells per experiment). Statistical comparisons were performed using paired *t*-test. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. The present cell-based assay at the serotonin 5-HT_{2A}, 5-HT_{2C}, 5-HT_{1A}, 5-HT_7 and the dopamine D_{2L} receptors

We first evaluated all types of serotonin and dopamine receptors of interest in the present assay system. Fig. 2 shows the dose–response curves of known ligands of the serotonin 5-HT_{2A} and 5-HT_{2C} receptors ($G\alpha_q$-coupled receptors) (Fig. 2A), the serotonin 5-HT_{1A} receptor (Fig. 2B), the dopamine D_{2L} receptor (Fig. 2C) ($G\alpha_i$-coupled receptors) and the serotonin 5-HT_7 receptor ($G\alpha_s$-coupled receptors) (Fig. 2D). In Table 1, the results obtained with the present method were compared with the findings from previous studies, and the results show that the pEC_{50} values obtained using the known ligands in the present assay were comparable to those determined from previous assays, such as phosphoinositide (PI) hydrolysis, the cAMP assay and the cell-based ELISA measurements of MAPK activation (Braden et al., 2006; Dunlop et al., 1998; Jordan et al., 2007a, 2007b; Lovenberg et al., 1993; Muntasir et al., 2006; Shapiro et al., 2003; Urban et al., 2007)(Table 1). In the present assay, aripiprazole behaved as a dopamine D_{2L} receptor partial agonist, as did S(-)-3-PPP (Fig. 2C), which indicated that the present assay was sufficient to search for dopamine D_2 receptor partial agonists with relatively low intrinsic activity.

3.2. Geissoschizine methyl ether (GM) inhibits the activation of serotonin 5-HT_{2A}, 5-HT_{2C} and 5-HT_7 receptors

Previous *in vivo* studies suggested that GM acted as an antagonist for the serotonin 5-HT_{2A} and 5-HT_{2C} receptors (Pengsuparp et al., 2001). The inhibitory effect of GM was next examined on the serotonin 5-HT_{2A} and 5-HT_{2C} receptors, as well as the serotonin 5-HT_{1A} receptor. When 5-HT was used at concentrations of 10 nM (5-HT_{1A}) or 100 nM (5-$HT_{2A/2C}$), GM significantly inhibited the 5-HT-induced responses of the serotonin 5-HT_{2A} and 5-HT_{2C} receptors in a concentration-dependent manner (Fig. 3A). The IC_{50} values were 13 ± 8.6 μM and 8.5 ± 5.2 μM, respectively (Table 2). Similarly, aripiprazole slightly suppressed 5-HT-induced Ca^{2+} mobilization at the serotonin 5-HT_{2A} and 5-HT_{2c} receptors (Fig. 3B). The IC_{50} values were comparable to those of GM (6.2 μM for 5-HT_{2A} and 6.4 μM for 5-HT_{2C}). In contrast, both GM and aripiprazole failed to suppress 5-HT-induced intracellular calcium ($[Ca^{2+}]_i$) mobilization by the serotonin 5-HT_{1A} receptor even at concentrations as high as 40 μM (Fig. 3A and B). In our assay system, spiperone, a serotonin 5-HT_{1A} receptor antagonist, inhibited the response of the serotonin 5-HT_{1A} receptor at 10 nM 5-HT in a concentration-dependent manner (data not shown), which indicated that GM was an antagonist of both the serotonin 5-HT_{2A} and 5-HT_{2C} receptors but not the serotonin 5-HT_{1A} receptor.

We examined whether GM could also act as a partial agonist on the serotonin 5-HT_{2A} and 5-HT_{2C} receptors. It had previously been reported that aripiprazole acted as a partial agonist with an EC_{50} of 48 nM and an intrinsic activity 12.7% that of 5-HT in GF62 cells (Shapiro et al., 2003). At higher concentrations (20 μM), GM caused a slight but significant increase of $[Ca^{2+}]_i$ in cells expressing the serotonin 5-HT_{2A} or 5-HT_{2C} receptors (data not shown). Therefore, GM acted as a partial agonist on these serotonin receptors.

In addition to behaving as an antagonist at the serotonin 5-HT_{2A} and 5-HT_{2C} receptors, it has been suggested that the inhibition of the serotonin 5-HT_7 receptor could be clinically useful for the treatment of positive symptoms in schizophrenia (Galici et al., 2008). Therefore, the effects of GM on the serotonin 5-HT_7 receptor were also determined. GM failed to activate the serotonin 5-HT_7 receptor (data not shown), but it significantly inhibited the 5-HT-induced calcium flux in a concentration-dependent manner (IC_{50} = 610 ± 290 nM) (Fig. 3A). This effect of GM was comparable to that of aripiprazole (IC_{50} = 980 ± 660 nM) (Fig. 3B) (Table 2).

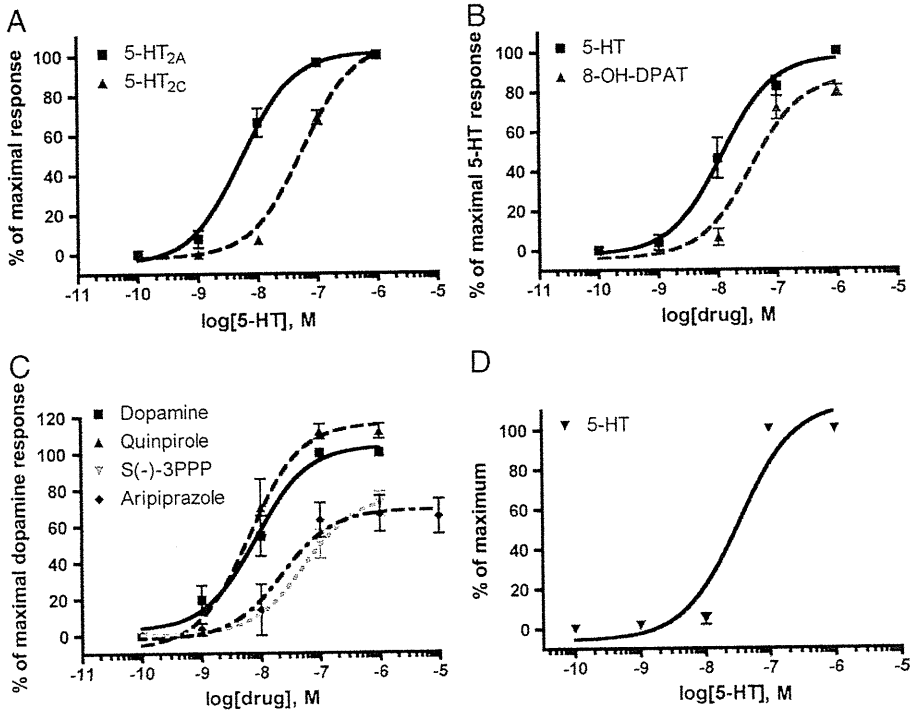

Fig. 2. Concentration–response curves obtained from the present calcium imaging assay for $G\alpha_q$-, $G\alpha_i$- and $G\alpha_s$-coupled serotonin and dopamine receptors. (A) Concentration-dependent ligand-induced stimulation of calcium mobilization by the $G\alpha_q$-coupled serotonin 5-HT_{2A} and 5-HT_{2C} receptors, (B) the $G\alpha_i$-linked serotonin 5-HT_{1A} receptor, (C) the dopamine D_{2L} receptor and (D) the $G\alpha_s$-coupled serotonin 5-HT_7 receptor. Data represent the mean ± S.E.M. from the Ca^{2+} response data for each cell showing serotonin or dopamine responses in 3 to 5 separate experiments (n = 10–15 cells per experiment). Table 1 compares the data from this study with previous assays.

Table 1
Estimates of pEC_{50} and E_{max} (±S.E.M.) for known ligands at the serotonin 5-HT and dopamine D_2 receptors.

Receptors	Ligands	Previous assays			Present assay	
		pEC_{50}	E_{max} (%)	Assays	pEC_{50}	E_{max} (%)
$5\text{-}HT_{2A}$	5-HT	8.29±0.08	–	PI hydrolysis[a]	8.25±0.08	–
$5\text{-}HT_{2C}$	5-HT	7.17±0.03	–	PI hydrolysis[b]	7.44±0.08	–
$5\text{-}HT_{1A}$	5-HT	8.35	–	cAMP assay[c]	8.01±0.11	–
	8-OH-DPAT	8.70±0.07	98±1	cAMP assay[d]	7.53±0.14	80±3
D_{2L}	Dopamine	7.22±0.09	106.7±3.5	cAMP assay[e]	8.08±0.14	103.6±10.3
		8.06±0.04	100[f]	MAPK assay[g]		
		7.50±0.17	100[h]	[^{3}H] AA release[g]		
	Quinpirole	7.87±0.11	120[f]	MAPK assay[g]	8.19±0.14	116.1±12.3
		7.76±0.04	120[h]	[^{3}H] AA release[g]		
	S(-)-3-PPP	6.28±0.26	20.3±2.9	cAMP assay[e]	7.27±0.15	75.9±11.5
		7.03±0.06	75[f]	MAPK assay[g]		
		7.15±0.08	70[h]	[^{3}H] AA release[g]		
	Aripiprazole	8.36±0.54	10.6±2.0	cAMP assay[e]	7.64±0.33	68.6±13.7
		6.77±0.09	70[f]	MAPK assay[g]		
		8.82±0.11	60[h]	[^{3}H] AA release[g]		
$5\text{-}HT_7$	5-HT	6.81±0.11	–	cAMP assay[i]	7.47±0.1	–

The estimates of pEC_{50} and E_{max} in the present study were determined by the data from the Ca^{2+} responses of each cell showing serotonin or dopamine responses in 3–5 separate experiments.
[a] Braden et al., 2006.
[b] Muntasir et al., 2006.
[c] Shapiro et al., 2003.
[d] Dunlop et al., 1998.
[e] Jordan et al., 2007a, 2007b.
[f] Relative E_{max} values to maximal dopamine response (MAPK assay).
[g] Urban et al., 2007.
[h] Relative E_{max} values to maximal dopamine response ([^{3}H] AA release assay).
[i] Lovenberg et al., 1993.

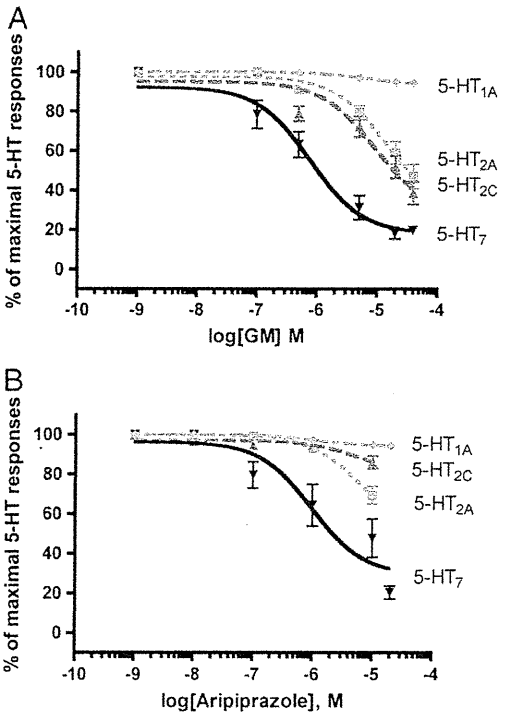

Fig. 3. The antagonistic effect of geissoschizine methyl ether (GM) and aripiprazole at the serotonin $5\text{-}HT_{2A}$, $5\text{-}HT_{2C}$ and $5\text{-}HT_7$ receptors. (A) The effect of GM on 5-HT-stimulated $[Ca^{2+}]_i$ mobilization in HEK293T cells transiently transfected with the indicated serotonin 5-HT receptor. (B) The effect of aripiprazole on 5-HT-stimulated $[Ca^{2+}]_i$ mobilization in HEK293T cells transiently transfected with the indicated serotonin 5-HT receptor. The cells showing a concentration-dependent response to 5-HT were selected and analyzed. All data represent the mean ± S.E.M. from the Ca^{2+} response data for each cell showing a serotonin response in 3 to 5 separate experiments (n = 10–15 cells per experiment).

3.3. GM activates the serotonin $5\text{-}HT_{1A}$ receptor

A variety of preclinical data suggested that the serotonin $5\text{-}HT_{1A}$ receptor was a therapeutic target for the development of improved antipsychotic drugs (Meltzer, 1999; Millan, 2000). Fig. 4A shows the effect of adding aripiprazole and GM on calcium mobilization by the serotonin $5\text{-}HT_{1A}$ receptor expressed in HEK293T cells transfected with $G_{16/o}$ and $G_{16/i3}$. In our assay, aripiprazole exhibited partial agonist activity (EC_{50} = 210 ± 150 nM; E_{max} = 80 ± 5% of the effect of 1 μM 5-HT on $[Ca^{2+}]_i$ increase) and was consistent with previous studies (Jordan et al., 2002; Shapiro et al., 2003). Similarly, GM stimulated an increase of $[Ca^{2+}]_i$ by the serotonin $5\text{-}HT_{1A}$ receptor with a potent EC_{50} value of 4.6 ± 7 μM and an E_{max} of 85 ± 3% (Fig. 4A) (Table 2). We also confirmed that 5-HT- and the GM-induced activations were inhibited by WAY100635, a selective serotonin $5\text{-}HT_{1A}$ receptor antagonist, in a dose-dependent manner (Fig. 4B and C).

3.4. GM is an unusual partial agonist/antagonist of the dopamine D_{2L} receptor

Aripiprazole partially activated the dopamine D_2 receptor-mediated inhibition of cAMP accumulation, although this action was determined to be system specific (Lawler et al., 1999; Shapiro et al., 2003). Consequently, the effect of GM on the dopamine D_{2L} receptor was analyzed using the presented single-cell-based calcium flux assay in HEK293T cells expressing the dopamine D_{2L} receptor, $G_{16/o}$ and $G_{16/i3}$ in the presence of aripiprazole. As shown in Figs. 1C and 5, aripiprazole acts as a partial agonist on the dopamine D_{2L} receptor in the present assay system. The EC_{50} was 56.0 ± 51.2 nM and the E_{max} was 68.6 ± 13.7% of the maximal dopamine response, both of which fell within the range of values from previous reports (Table 1). Under identical assay conditions, GM displayed a partial agonist activity with an EC_{50} of 4.4 ± 3.6 μM and an intrinsic activity that was 50 ± 15% that of dopamine (Fig. 5) (Table 2). It was noted that GM had a bell-shaped concentration response curve at a higher concentration (100 μM) (Fig. 5), which suggested that strong desensitization and/or antagonism may have occurred.

Table 2
Estimates of pIC_{50}, pEC_{50} and E_{max} (±S.E.M.) for aripiprazole and geissoschizine methyl ether (GM) at the serotonin and dopamine receptors.

Drugs	Receptors						
	5-HT_{2A}	5-HT_{2C}	5-HT_7	5-HT_{1A}		D_{2L}	
	(pIC_{50})	(pIC_{50})	(pIC_{50})	(pEC_{50})	E_{max} (%)	(pEC_{50})	E_{max} (%)
Aripiprazole	5.21 ± 0.76	5.19 ± 1.66	6.01 ± 0.49	6.83 ± 0.39	80 ± 5	7.64 ± 0.33	68.6 ± 13.7
GM	4.87 ± 0.44	5.07 ± 0.41	6.12 ± 0.36	5.34 ± 0.49	85 ± 3	5.36 ± 0.74	50 ± 15

The estimates of pIC50 at the serotonin receptors were derived from the inhibitory responses to 100 nM 5-HT-induced activations.

In addition to the partial agonist activity observed, GM caused an unusual response at the dopamine D_{2L} receptor, similar to aripiprazole. As shown in Fig. 6, the GM-induced $[Ca^{2+}]_i$ activation was only found in a subset of the dopamine-responsive cells and not in all of the cells (Fig. 6A). This partial activation was also observed when aripiprazole was used (data not shown). In addition, the responses obtained from the GM and aripiprazole applications were different from the other dopamine D_2 receptor agonists. For example, activations induced by *S*(-)-3-PPP were observed in all of the dopamine-responsive cells (nearly 100% of the cells), whereas GM activated 53.9% of the dopamine-reactive cells (Fig. 6B). Haloperidol, a potent D_{2L} antagonist, dose-dependently inhibited 10 nM dopamine-induced Ca^{2+} influx (Fig. 6C) in the present assay. Furthermore, it significantly suppressed the number of cells activated by the administration of 10 μM GM (Fig. 6B). These activations were only observed in the dopamine D_{2L} receptor-transfected cells, not in the cells expressing the dopamine D_1 receptor (Fig. 7), indicating that GM selectively activated the dopamine D_{2L} receptor.

We examined the antagonistic effect of GM on the dopamine D_{2L} receptor. As shown in Fig. 8, 20 μM GM conferred a slight but significant inhibition of the dopamine-induced (100 nM) calcium response of the dopamine D_{2L} receptor. These results suggested that GM could be a novel compound that acts as a partial agonist/antagonist on the dopamine D_{2L} receptor with low intrinsic activity and partial activation. These findings raised the possibility that GM functions as a dopamine system stabilizer.

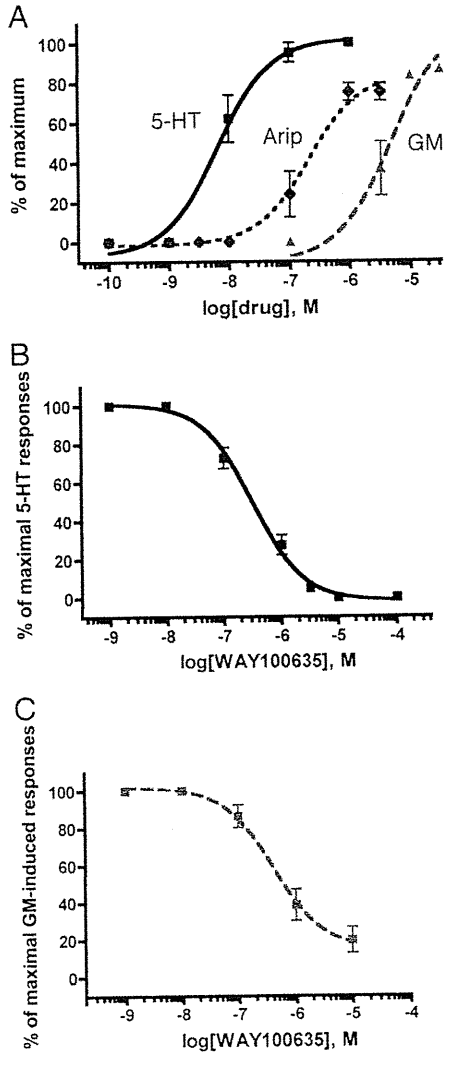

Fig. 4. The effect of geissoschizine methyl ether (GM) and aripiprazole (Arip) on $[Ca^{2+}]_i$ mobilization in HEK293T cells transiently transfected with the serotonin 5-HT_{1A} receptors, $G_{16/o}$ and $G_{16/i3}$. (A) Concentration–response curve of 5-HT, Arip and GM at the serotonin 5-HT_{1A} receptors. (B) The effect of WAY100635 on 5-HT-induced 5-HT_{1A} activation. (C) The effect of WAY100635 on GM-induced 5-HT_{1A} activation. All data represent the mean ± S.E.M. from the Ca^{2+} response data for each cell showing a serotonin response in 3 to 5 separate experiments (n = 10–15 cells per experiment).

4. Discussion

Atypical antipsychotics act on multiple serotonin and dopamine receptors coupled pleiotropically to various Gα and Gβγ protein subunits, affecting a wide array of signal transduction pathways. In the present study, we introduced a single-cell-based assay system that measures intracellular calcium mobilization to analyze candidate compounds with atypical antipsychotic-like action using $G\alpha_{15/16}$ and their chimeras on a single platform. The efficacy and potency of known ligands generated in the present assay were comparable to those found in previous studies using a variety of readouts (Table 1). Our calcium flux assay could also reproduce the partial agonistic action of aripiprazole with a lower intrinsic activity than *S*(-)-3-PPP at the dopamine D_{2L} receptor (Jordan et al., 2007a, 2007b; Urban et al., 2007), which suggests that the present assay could be useful for rapid screening of a library of compounds for development of new antipsychotic drugs.

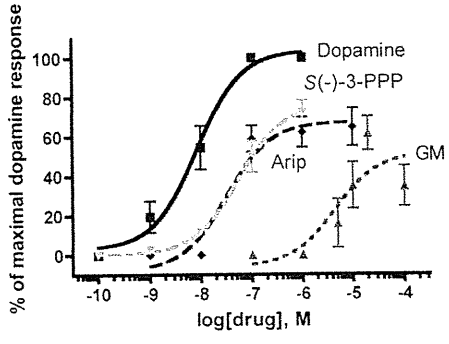

Fig. 5. The effect of dopamine, *S*(-)-3-PPP, aripiprazole (Arip) and geissoschizine methyl ether (GM) on $[Ca^{2+}]_i$ mobilization in HEK293T cells transiently transfected with the dopamine D_{2L} receptors, $G_{16/o}$ and $G_{16/i3}$. All data represent the mean ± S.E.M. from the Ca^{2+} response data for each cell showing a dopamine response in 3 to 5 separate experiments (n = 10–15 cells per experiment).

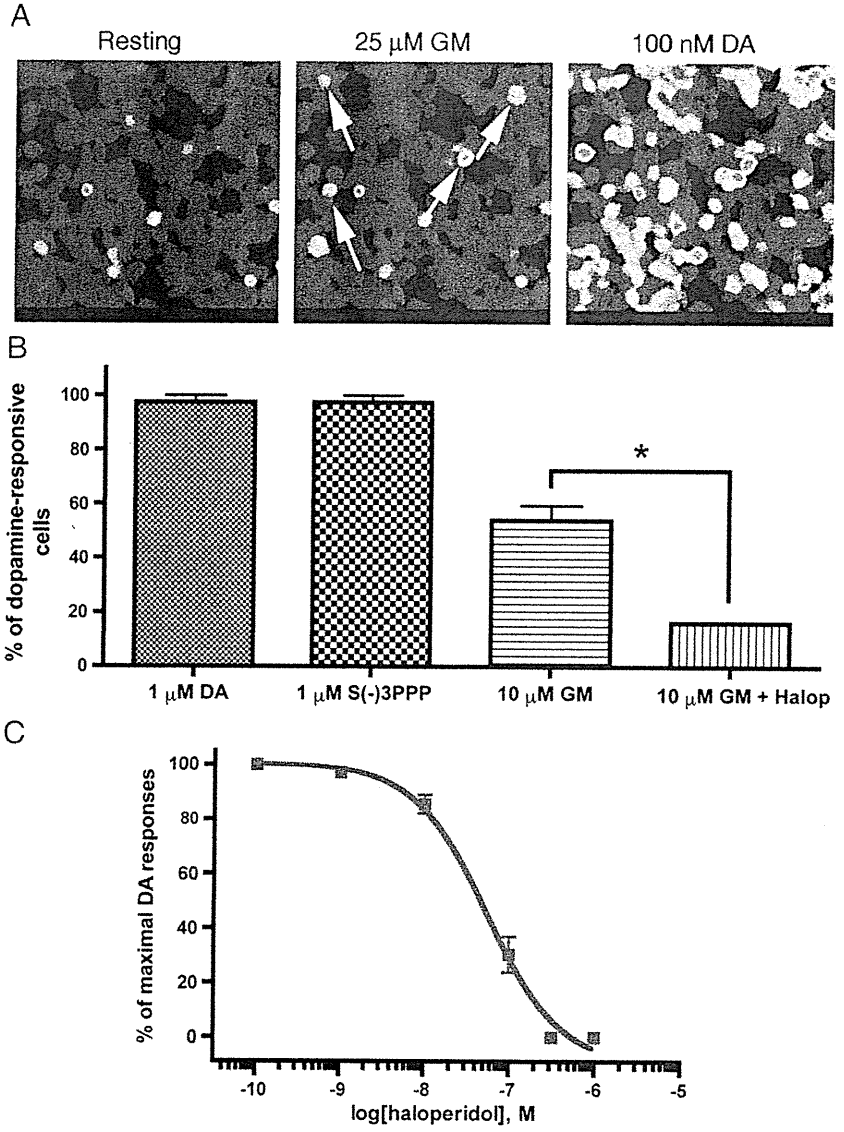

Fig. 6. Partial activations induced by geissoschizine methyl ether (GM) in cells transiently transfected with the dopamine D_{2L} receptor with $G_{16/o}$ and $G_{16/i3}$. (A) Representative images at 1 min after stimulation show that GM caused a partial increase of $[Ca^{2+}]_i$ in a subset of the dopamine-responsive cells (arrows), whereas dopamine (DA) substantially activated a large number of cells. (B) The quantitative analysis showed that *S*(-)-3-PPP activated all of the dopamine-responsive cells (100% of DA-responsive cells), whereas GM partially stimulated these cells (53.9% of DA-responsive cells). The activation induced by GM was significantly suppressed by the simultaneous administration of haloperidol (Halop, 10 μM). (C) Concentration-dependent inhibitory effect of haloperidol on DA-induced Ca^{2+} influx in these cells. All data represent the mean ± S.E.M. from the Ca^{2+} response data for each cell showing a dopamine response in 3 to 5 separate experiments (n = 10–15 cells per experiment). *P<0.01 *vs.* 10 μM GM.

Dopamine D_2 receptors are important targets for pharmaceuticals used in the management of schizophrenia, Parkinson's disease and drug abuse. Most approved antipsychotic drugs behave as antagonists at the dopamine D_2 receptors, whereas aripiprazole is a dopamine D_2 receptor partial agonist approved as an effective treatment for positive and negative symptoms of schizophrenia and schizoaffective disorder (Jordan et al., 2007a, 2007b). The present study demonstrated that GM is a partial agonist at dopamine D_{2L} receptors with similar intrinsic activity to aripiprazole. Moreover, GM, similar to aripiprazole, partially activated dopamine-responsive cells among the cells transfected with dopamine D_{2L} receptor and Gα chimeras. Because this partial activation was not observed in quinpirole and *S*(-)-3-PPP, GM could share certain characteristics of aripiprazole. However, the molecular mechanism underlying this phenomenon is still unknown. Aripiprazole is postulated to function as a regionally specific modulator of dopamine tone by decreasing high "basal" dopaminergic tone (antagonist) in regions with low dopamine D_2 receptor reserves and increasing low "basal" dopaminergic tone (agonist) in regions with high dopamine D_2 receptor reserves (Tadori et al., 2009). In addition, it has been accepted that aripiprazole has functionally selective actions at dopamine D_2 receptor-mediated signaling pathways (Urban et al., 2007). Thus, the details of GM-evoked partial activation at dopamine D_2 receptors should be addressed in future research.

Atypical antipsychotics commonly exhibit antagonist properties to the serotonin 5-HT_{2A} and 5-HT_{2C} receptors. The present study directly showed that GM has an antagonist activity at the serotonin 5-HT_2 receptors *in vitro*. GM can access the brain through the blood–brain barrier (Imamura et al., 2011). Previously, an *in vivo* study showed that GM reduced the 5-hydroxy-L-tryptophan (*l*-5-HTP) plus clorgyline-induced head switch response thought to be mediated by the serotonin 5-$HT_{2A/2C}$ receptors in a dose-dependent manner (>10 mg/kg i.p.) (Pengsuparp et al., 2001). Although correlations between the *in vitro* and *in vivo* studies across species are confounded by the extensive metabolism of the compound of interest (Wood et al., 2006), it is

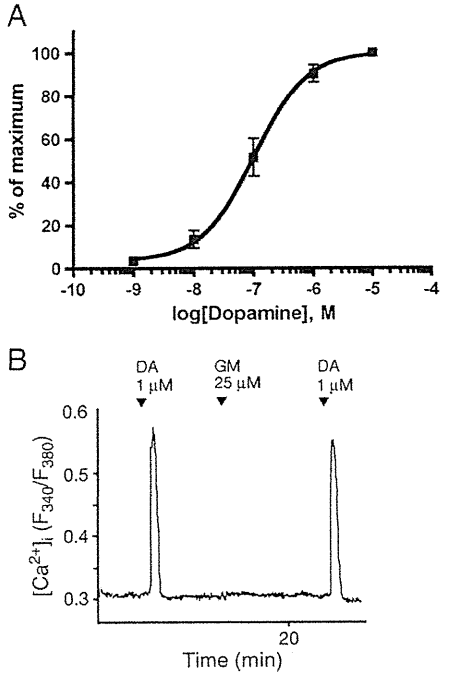

Fig. 7. The results obtained from the cells transiently transfected with the dopamine D_1 receptor with G_{15} and G_{16} as the $G\alpha_s$-coupled receptors. (A) Concentration-dependent dopamine-induced stimulation of $[Ca^{2+}]_i$. All data represent the mean ± S.E.M. from the Ca^{2+} response data for each cell showing a dopamine response in 3 separate experiments (n = 10–15 cells per experiment). (B) A representative trace showing that GM did not cause any calcium response in dopamine (DA)-responsive cells.

possible that GM is effective in living organisms despite the relatively high IC_{50} values obtained in our *in vitro* assay (serotonin 5-HT_{2A} receptor, 13 μM; serotonin 5-HT_{2C} receptor, 8.5 μM).

The importance of the agonist activity at the serotonin 5-HT_{1A} receptor in antipsychotic drug action has been suggested due to the extensive evidence in rodent models that indicates that the activation of these receptors prevents extrapyramidal symptoms (EPS) induced by dopamine D_2 receptor blockade, favors dopaminergic neurotransmission in the frontal cortex, has a positive influence on mood and opposes NMDA receptor antagonist-induced cognitive and social interaction deficits (Newman-Tancredi, 2010). In this study, we found that GM was a partial agonist at the serotonin 5-HT_{1A} receptor with a relatively high intrinsic activity. It has also been reported that GM has an affinity for [^{3}H]8-OH-DPAT binding in mouse cerebral cortex membranes (Ki of 0.8 μM) and that intraperitoneal administration of GM elicited a hypothermic response (Pengsuparp et al., 2001) primarily due to the activation of the serotonin 5-HT_{1A} receptor (Bill et al., 1991; Martin et al., 1992). Therefore, it is conceivable that GM acts as a functionally mixed serotonin 5-HT_{1A} receptor agonist/serotonin 5-$HT_{2A/2C}$ receptor antagonist *in vitro* and *in vivo*.

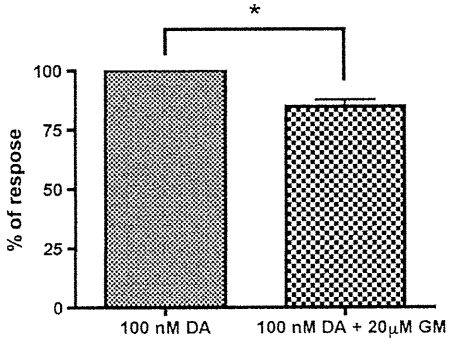

Fig. 8. Geissoschizine methyl ether (GM) partially inhibited the dopamine (DA, 100 nM)-induced $[Ca^{2+}]_i$ response in cells transiently transfected with the dopamine D_{2L} receptor with $G_{16/o}$ and $G_{16/i3}$. All data represent the mean ± S.E.M. from the Ca^{2+} response data for each cell showing a dopamine response in 3 separate experiments (n = 10–15 cells per experiment). *P < 0.01 *vs.* 100 nM.

The serotonin 5-HT_7 receptor is a more recently discovered G protein-coupled receptor. As such, the functions and possible clinical relevance of this receptor are not fully understood. Anxiety and schizophrenia models have yielded mixed results with no clear role for the serotonin 5-HT_7 receptor. However, a recent study reported that the blockade of the serotonin 5-HT_7 receptor could be clinically useful for the treatment of the positive symptoms of schizophrenia (Galici et al., 2008). Moreover, there is a considerable amount of evidence that supports a role for the serotonin 5-HT_7 receptor in depression (Abbas et al., 2009; Bonaventure et al., 2007). Therefore, some atypical antipsychotics, including aripiprazole, carry FDA-approved indications for acute mania, bipolar depression, psychotic agitation, and bipolar maintenance, among other indications. Thus, the antagonist activity of GM at the serotonin 5-HT_7 receptor (comparable to aripiprazole) suggested that GM could be an atypical antipsychotic-like compound and may have additional applications in other psychotic disorders.

GM is a plant indole alkaloid and an ingredient of *Chotoko* contained in *Yokukansan* (also referred to as *Yi-gan san*), a traditional Japanese medicine. We previously performed constituent analysis of *Yokukansan* using a competitive binding assay in CHO cell membranes that stably expressed human recombinant serotonin receptors (5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} and 5-HT_7) and found that compounds with affinities for these serotonin receptors were exclusively contained in *Chotoko* (Terawaki et al., 2010). In support of this finding, the present study demonstrated that GM, a component of *Chotoko*, behaves as a partial agonist at the serotonin 5-HT_{1A} receptor and an antagonist at the serotonin 5-HT_{2A}, 5-HT_{2C} and 5-HT_7 receptors. In addition, the potency of GM as an antagonist at the serotonin 5-HT_{2A}, 5-HT_{2C} and 5-HT_7 receptors was comparable to aripiprazole (Table 2). Moreover, this compound was a partial agonist/antagonist at the dopamine D_{2L} receptor with a relatively low intrinsic activity and partial activation. Thus, the pharmacological profile of GM at the serotonin and dopamine receptors was similar to that of aripiprazole. In agreement with these findings, our preliminary *in vivo* study showed that GM effectively suppressed an aggressive behavior in socially isolated mice (unpublished data). Although GM is far less potent at the dopamine D_{2L} receptors than aripiprazole, further *in vitro* and *in vivo* studies on GM and GM derivatives may contribute to the development of a new treatment for schizophrenia and related disorders. This study also provided new insights into the use of compounds from traditional medicines for the development of novel antipsychotics.

In conclusion, the present study demonstrated that geissoschizine methyl ether is a partial agonist to the serotonin 5-HT_{1A} receptor, a partial agonist/antagonist to the dopamine D_{2L} receptor and an antagonist to the serotonin 5-HT_{2A}, 5-HT_{2C} and 5-HT_7 receptors. Because the pharmacological profiles closely resemble aripiprazole, geissoschizine methyl ether and derivatives may comprise a new set of candidates for atypical antipsychotics.

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