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# Dopamine $D_2$, but not $D_4$, receptor agonists are emetogenic in ferrets

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**Abstract**

Agents that activate the dopamine $D_2$-like family of receptors elicit emesis in humans and other species with a vomiting/emetic reflex; however, the lack of dopamine receptor subtype selective agonists has hampered an understanding of which dopamine $D_2$-like receptor subtype(s) contributes to the emetic response. In this study, stable cell lines expressing the ferret dopamine $D_{2\text{-long}}$ ($D_{2L}$) and $D_4$ receptors were used to characterize known dopamine agonists via radioligand binding and calcium ion flux assays, while emetic activity of these dopamine receptor agonists was determined in male ferrets. Latencies to first emetic event, average number of emetic episodes, and stereotypical behaviors which may be indicative of nausea were also determined. Agonists at dopamine $D_1$-like and $D_4$ receptors had no emetic effect in ferrets. Conversely, stimulation of dopamine $D_2$ and/or $D_3$ receptors resulted in a robust emetic response characterized by a relatively short latency (<15 min) and multiple emetic events. Competitive antagonists of dopamine $D_2$-like receptors (domperidone, haloperidol) dose-dependently blocked the emetic response to PNU95666E, a dopamine $D_2$ receptor selective agonist. Thus, dopamine $D_2$ and/or $D_3$ receptor agonists elicit emesis, while dopamine $D_1$/$D_5$ or $D_4$ receptor-selective agonists are devoid of emetic properties.


*Keywords:* Calcium flux; Dopamine receptors; Dopamine agonists; Ferrets; Nausea; Vomiting

## 1. Introduction

Diverse stimuli can trigger an emetic response in mammals that possess a vomiting reflex. These stimuli include chemical, biological, and physical agents. Regardless of their nature, emetic stimuli elicit emesis through several neural mechanisms (Andrews and Hawthorn, 1988). While peripheral-acting emetic stimuli activate visceral afferent neurons that project to the nucleus tractus solitarius (NTS) and 'vomiting center', blood-borne centrally acting emetogens are detected by the area postrema, a circumventricular organ located in the brainstem (Miller and Leslie, 1994). Results from vagotomy and area postrema ablation studies demonstrate that emetic stimuli can display varying degrees of selectivity (or none at all) for peripheral or central emetic pathways. Dopaminergic, serotonergic, cholinergic, and tachykinin neurotransmitter systems have all been implicated in emetic pathways in the NTS and area postrema (Andrews and Rudd, 2004; Andrews et al., 1990). In this report, we have investigated which dopamine receptor subtypes are responsible for the emetic response in ferrets, a laboratory species with an emetic response to many emetogens that is similar to humans (Andrews et al., 1990; King, 1990).

The actions of the neurotransmitter dopamine are mediated by specific G-protein coupled receptors that can be divided into two major families based on their ability to stimulate ($D_1$-like) or inhibit ($D_2$-like) adenylate cyclase (Kebabian and Calne, 1979; Vallone et al., 2000). Two

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dopamine $D_1$-like receptors ($D_1$ and $D_5$) and three human dopamine $D_2$-like receptors ($D_2$, $D_3$ and $D_4$) have been identified by cDNA and genomic cloning (Vallone et al., 2000). Two isoforms of the dopamine $D_2$ receptor exist, $D_{2S}$ and $D_{2L}$ ("short" and "long", respectively), based on the absence or presence of a 29 amino acid segment in the third cytoplasmic loop of the receptor (Missale et al., 1998). The two isoforms display differences in G-protein coupling, sequestration rate, and regional distribution. The delineation of the role of specific dopamine receptors has been hampered by the lack of highly selective receptor ligands as well as a comparative pharmacology of dopaminergic receptors. Apomorphine is a nonselective dopamine receptor ligand that has historically been described as a dopamine $D_2$-like receptor agonist. Apomorphine is highly emetic in a variety of species including dogs (Niemegeers and Janssen, 1965), ferrets (Florczyk et al., 1982; Andrews et al., 1986; King 1988, 1990; Osinski et al., 2003) and humans (Schofferman, 1976). The emetic effect of apomorphine is thought to result from activation of dopamine receptors because dopamine $D_2$-like receptor antagonists prevent apomorphine-elicited emesis in dogs (Hsu et al., 1986), ferrets (King, 1988), and humans (Corsini et al., 1979). However, studies examining localization of dopamine $D_2$, $D_3$ and $D_4$ receptors in the NTS and area postrema in humans show the presence of all three receptors (Hyde et al., 1996). Further, a comparative pharmacology across human dopamine $D_2$-like receptors compared in the same assay system shows that apomorphine is a potent full agonist at dopamine $D_2$, $D_3$ and $D_4$ receptors and that domperidone can block all three receptors as well (Newman-Tancredi et al., 2002; Moreland et al., 2004).

Dopamine receptor agonists are important therapeutics for the treatment of several disorders including Parkinson's Disease, hyperprolactinemia, and to a lesser extent, heart failure. The nonselective dopamine receptor agonist apomorphine has been demonstrated to be efficacious for the treatment of erectile dysfunction (Bukofzer and Livesey, 2001). Unfortunately, nausea and vomiting are dose-limiting side effects. Recent preclinical data suggest that dopamine $D_4$ receptor activation is capable of producing pro-erectile activity in rats (Hsieh et al., 2004); however, the lack of vomiting reflex in rats leaves the question of emetogenicity of dopamine $D_4$ receptor agonists unanswered. Little is known about potential emetogenicity of dopamine $D_4$ receptor agonists due to the relative absence of dopamine $D_4$ subtype-selective agonists as well as an underappreciation of therapeutic uses of agonists of this pharmacologic class. In this report, the pharmacology of ferret dopamine $D_{2L}$ and $D_4$ receptors is characterized in vitro and correlated with in vivo behavioral results (i.e., emetic activity) in ferrets. A preliminary version of these results was presented in poster format at the 2004 Society for Neuroscience meeting held in San Diego, CA, USA.

## 2. Materials and methods

### 2.1. Cloning and expression of ferret dopamine $D_{2L}$ and $D_4$ receptors

Ferret dopamine $D_{2L}$ receptor c-DNA was cloned by RT-PCR from ferret whole brain poly-A RNA using primers designed from the published human sequence (GenBank accession no. AY394849). Ferret dopamine $D_4$ receptor c-DNA was cloned by RT-PCR from ferret whole brain poly-A RNA using primers designed from the published human sequence (GenBank accession no. AY394848). HEK293 cells were co-transfected with an expression vector for ferret dopamine $D_{2L}$ or $D_4$ receptor plus an expression vector for a $G\alpha_{qo5}$ chimeric G protein (Moreland et al., 2004). Stable colonies were selected with 200 mg/L Geneticin (ferret dopamine $D_{2L}$ or $D_4$ receptor selection) and 200 mg/L hygromycin B ($G\alpha_{qo5}$ selection).

### 2.2. Calcium flux assay

Pharmacological responses to dopaminergic receptor ligands were measured using a calcium imaging technique (Moreland et al., 2004). Fluo-4 AM, a fluorescent $Ca^{2+}$ chelating dye, was used as an indicator of the relative levels of intracellular $Ca^{2+}$ in a 96-well format with a Fluorescence Imaging Plate Reader (FLIPR, Molecular Devices, Sunnyvale, CA). All reagents were prepared in Dulbecco's phosphate-buffered saline (D-PBS) containing 0.004% ascorbic acid and 0.5 mM IBMX. Cells were grown to 90% confluence in 96-well black-walled clear bottom poly-D-lysine coated plates (Biocoat, BD Biosciences) and loaded with Fluo-4AM and Pluronic F-127 in D-PBS for 1–2 h at room temperature. Prior to assay, the cells were washed with D-PBS containing 0.004% ascorbic acid and 0.5 mM IBMX. In the case of antagonism experiments, antagonists were added 3 min before the addition of 1 μM dopamine. Data shown are based on the peak increase in relative fluorescence units as compared to 10 μM dopamine. A full agonist was defined as a compound that produced a response $\geq$80% of the response elicited by dopamine in this assay.

### 2.3. Radioligand binding assay

Membranes were prepared from a stable HEK293 cell line expressing the ferret dopamine $D_{2L}$ receptor. Confluent cells growing in a Cell Factory (VWR, Plainfield, NJ) were detached with cell dissociation buffer and the cell pellet homogenized using a Polytron for 10 s in 50 mM Tris–HCl, pH 7.4. After the homogenate was centrifuged for 30 min at 100,000 $\times g$, the membranes were stored at −80 °C until use. For dopamine $D_2$ receptor binding assays, the membranes were incubated with the dopamine $D_2$-like receptor agonist [$^{125}$I]-PIPAT 0.6 nM, 50 mM Tris–HCl, pH 7.4, 10 mM $MgSO_4$, and 1 mM EDTA as

previously described (Vessotskie et al., 1997; Moreland et al., 2004). Nonspecific binding was determined in the presence of 10 μM spiperone. The reaction was terminated by filtration using a Filtermate Harvester (Packard, Meriden, CT). Radioactivity was measured using a Top-count Microplate Scintillation Counter (Packard). Protein content was determined by BCA Protein Assay Kit (Pierce, Rockford, IL). Competition curves were analyzed by nonlinear regression using the curve-fitting program (Prism, GraphPad Software, San Diego, CA). All assays were performed in triplicate and $IC_{50}$ values were converted to $K_i$ values by the method of Cheng and Prusoff (1973).

### 2.4. Animals

All procedures involving animals were reviewed and approved by the Abbott Laboratories Animal Care and Use Committee. United States Department of Agriculture (USDA) regulations on the scientific use of laboratory animals were strictly followed. Castrated male ferrets (0.9–1.7 kg; fitch, albino, or Siamese coat coloration) were purchased from Marshall Farms (North Rose, NY) and were housed in groups of 3 in a temperature (20 °C) and humidity (50%) controlled environment. Animals were maintained on a 12-h light–dark cycle with lights on at 0600 hours. Food (Marshall Premium Ferret Diet; Marshall Pet Products, Wolcott, NY) and water were available ad libitum up until ~18 h prior to the beginning of a study, at which time food was withdrawn. Each ferret was tested no more than 4 times with a randomly assigned emetogen; a minimum of 14 days elapsed between emetic challenges. This challenge schedule is sufficient to prevent either desensitization of the emetic response or conditioning (Andrews et al., 1990; Abbott Labs, unpub. observ.).

### 2.5. Emesis studies

Emesis studies were carried out as previously described (Osinski et al., 2003). Briefly, animals were placed in individual polycarbonate cages with ventilated tops and allowed to acclimate to the experimental room for 1 h prior to commencing study. With the exception of apomorphine, drugs were dissolved in sterile saline and injected subcutaneously (1 mL/kg body weight) in the subscapular region. Vehicle for apomorphine was sterile saline with 0.1% ascorbic acid added as an antioxidant. Emesis experiments were also conducted with haloperidol and domperidone, competitive antagonists of dopamine $D_2$-like receptors. Each antagonist was tested at 0.1 μmol/kg, s.c. Pilot dose–response experiments demonstrated that this dose of either antagonist administered 30 min prior to emetic challenge with dopamine receptor agonist completely blocked the emetic and Nausea Index response to maximally emetic doses of apomorphine or PNU-95666E. After dosing, ferrets were observed in blinded fashion for 90 min for vomiting and the presence of a collection of stereotypical behaviors displayed by ferrets prior to vomiting that has been argued to be an index of the subjective sensation of nausea in this species (Zaman et al., 2000). The behaviors recorded were licking, mouth-clawing, backward walking, gagging, and burying of the head in cage bedding (Osinski et al., 2003). The number of these behaviors displayed by each animal during the observation period was recorded and used to calculate a Nausea Index as described in the next section. Although the animals were fasted for ~18 h prior to study, the ferrets would still expel gastric juice upon emetic challenge. Additional data recorded were latency to the first emetic episode and the number of emetic episodes. An emetic episode was defined as one or more vomits (i.e., the forceful oral expulsion of liquid or solid upper gastrointestinal contents by an individual ferret) that is temporally separated from a second episode by an emesis-free period of at least 30 s (Osinski et al., 2003).

### 2.6. Data analysis

In vitro data are presented as mean ± S.E.M. For the in vivo ferret studies, the incidence of vomiting in response to a given dose of drug was calculated as the percentage of animals that vomited one or more times during the observation period divided by the total number of animals tested. For drugs that caused emesis, $ED_{50}$ values and associated 95% confidence intervals were calculated by the method of Litchfield and Wilcoxon (1949) using Pharm/PCS software (v. 4.2; MicroComputer Specialists, Philadelphia, PA). A Nausea Index was defined as the mean (±SEM) number of nausea behaviors observed among all animals in a given dose group. Statistics were calculated with Minitab statistical software (Release 11; Minitab Inc., State College, PA). $P$ values $< 0.05$ were considered significant.

### 2.7. Reagents

Pluronic F-127 and Fluo-4AM were purchased from Molecular Probes (Eugene, OR). D-PBS (Cat No. 11965-084), Geneticin, hygromycin B and all tissue culture reagents were purchased from Invitrogen (Rockville, MD). *N*-[4-(2-Cyano-phenyl)-piperazin-1-ylmethyl]-3-methyl-benzamide maleate (PD168077; Glase et al., 1997), 5-fluoro-2-(4-pyridin-2-yl-piperazin-1-ylmethyl)-1H-indole (CP226269; Zorn et al., 1997), 5-methylamino-5, 6-dihydro-1H, 4H-imidazo [4,5,1-ij] quinoline-2-thione (PNU142774E; Meglasson et al., 2001) and 5-methylamino-5, 6-dihydro-1H, 4H-imidazo[4,5,1-ij] quinolin-2-one (PNU95666E; Heier et al., 1997) were synthesized at Abbott Laboratories. 7-hydroxy-*N*, *N*-di-*n*-propyl-2-aminotetralin (7-hydroxy-DPAT), R(+)-6-chloro-7, 8-dihydro-1-phenyl-2, 3, 4, 5-tetrahydro-1H-3-benzazephrine (SKF81297) and all other chemicals were purchased from Sigma.

## 3. Results

### 3.1. In vitro functional studies

In order to determine the selectivity of dopaminergic ligands at dopamine $D_{2L}$ and $D_4$ receptors, the ferret dopamine $D_{2L}$ and $D_4$ receptors were cloned and co-expressed with a chimeric G protein ($G\alpha_{qo5}$) in HEK293 cells. This allowed the $G\alpha_{i/o}$-coupled dopamine $D_{2L}$ and $D_4$ receptors to signal through $G_q$, resulting in a robust calcium signal that is readily measured with automated fluorometric detection. The pharmacology and selectivity of the ferret cell lines with representative selective agonists for dopamine $D_1$, $D_2$, $D_3$ and $D_4$ receptors are shown in Table 1. Dopamine was chosen as the reference agonist to which the efficacies of all other tested drugs were compared (Fig. 1). Dopamine produced a concentration-dependent increase in intracellular calcium with an $EC_{50}$ value of 8.9±0.8 nM at the ferret $D_{2L}$ receptor (Fig. 1A and Table 1). This is consistent with the $K_i$ value of 12.4±0.7 nM obtained from competitive binding with [$^{125}$I]-PIPAT. The nonselective dopamine $D_2$-like receptor agonists, apomorphine and quinpirole, also displayed full agonist activity (>85% efficacy in comparison to 10 μM dopamine) at the ferret dopamine $D_{2L}$ receptor while their respective potencies were 2.2±0.6 and 27.9±9.5 nM. These potencies were comparable to the competition binding $K_i$ values obtained with [$^{125}$I]-PIPAT of 2.9±0.9 and 53.9±4.2 nM.

The dopamine $D_1$-like selective agonist (SKF81297) was essentially inactive in both the ferret dopamine $D_{2L}$ calcium flux assay and binding ($EC_{50}$>10,000 nM). The dopamine $D_2$ receptor selective compounds, PNU95666E and PNU142774E (Meglasson et al., 2001), were full agonists in the ferret dopamine $D_{2L}$ calcium flux assay with $EC_{50}$ values of 46.1±8.6 and 34.6±8.5 nM, respectively. These potencies were comparable to competition binding $K_i$ values with [$^{125}$I]-PIPAT of 73.1±10.9 and 38.9±7.1 nM. The reportedly selective dopamine $D_3$ receptor agonist 7-OH DPAT showed full agonist activity at the ferret dopamine $D_{2L}$ receptor with an $EC_{50}$ value of 5.2±2.0 and a $K_i$ value of 9.8±0.6 nM obtained in competition binding with [$^{125}$I]-PIPAT. The dopamine $D_4$ receptor selective agonist PD168077 was inactive in the ferret dopamine $D_{2L}$ calcium flux assay and had a $K_i$ value of 828 nM in competitive binding experiments with [$^{125}$I]-PIPAT. Another reported dopamine $D_4$ receptor agonist, CP226269, displayed partial agonism (57% efficacy) with a potency of 320±50 nM and a $K_i$ value of 261±54 nM at the ferret dopamine $D_{2L}$ receptor.

In order to determine the dopamine $D_4$ receptor selectivity of the dopaminergic agonists tested above, they were also tested in the ferret dopamine $D_4$ calcium flux assay (Fig. 1B). As expected, dopamine was a full agonist at the ferret dopamine $D_4$ receptor with an $EC_{50}$ value of 2.8±0.6 nM. Apomorphine and quinpirole were also full agonists at the ferret dopamine $D_4$ receptor ($EC_{50}$ values of 1.5±0.1 and 22.8±2.9 nM, respectively). SKF81297, the dopamine $D_1$-like selective agonist, was inactive in both ferret $D_{2L}$ calcium flux assay and binding but showed weak activity at the ferret dopamine $D_4$ receptor (1750±83 nM). PNU95666E and PNU142774E, dopamine $D_{2L}$ selective agonists, were inactive at the ferret $D_4$ receptor. A reportedly dopamine $D_3$ selective agonist but demonstrated in our laboratory to be a full agonist at the ferret dopamine $D_{2L}$ receptor, 7-OH DPAT, was also a full agonist at the ferret $D_4$ receptor with an $EC_{50}$ value of 24.3±4.3 nM. As expected, PD168077 elicited full agonist activity at the dopamine $D_4$ receptor (16.6±2.4 nM), while CP226269 exerted partial agonist activity (72% efficacy) with a potency of 24.8±5.6 nM at the dopamine $D_4$ receptor.

All of the dopamine agonists were also tested in HEK293 cells or HEK293 cells transfected with $G_{q05,}$ and no signal was observed. The nonselective antagonists, haloperidol and domperidone, did not activate either ferret dopamine $D_{2L}$ or $D_4$ receptors (data not shown), but did inhibit the agonist activity of 1 μM dopamine at the both receptors. The $K_i$ value obtained with competitive binding for haloperidol (0.22±0.02 nM) correlates with the $K_i$ value from calcium flux experiments (0.30±0.09 nM); however, domperidone's $K_i$ value obtained from binding was 10-fold greater than the $K_i$ value determined by the calcium flux assay (Table 2).

Table 1
Summary of in vitro activities of dopamine receptor agonists in cloned ferret receptors measured in calcium flux assays

| Compound | Selectivity | $D_{2L}$ FLIPR assay | | $D_{2L}$ binding | $D_4$ FLIPR assay | |
|---|---|---|---|---|---|---|
| | | $EC_{50}$ (nM) | % Efficacy | $K_i$ (nM) | $EC_{50}$ (nM) | % Efficacy |
| Dopamine | – | 8.9±0.8 | 100 | 12.4±0.7 | 2.8±0.6 | 100 |
| Apomorphine | $D_2$-like | 2.2±0.6 | 91 | 2.9±0.9 | 1.5±0.1 | 84 |
| Quinpirole | $D_2$-like | 27.9±9.5 | 103 | 53.9±4.2 | 22.8±2.9 | 89 |
| SKF 81297 | $D_1$-like | >10,000 | 19 | >10,000 | 1750±83 | 65 |
| PNU 95666E | $D_2$ | 46.1±8.6 | 86 | 73.1±10.9 | >10,000 | – |
| PNU142774E | $D_2$ | 34.6±8.5 | 83 | 38.9±7.1 | >10,000 | – |
| 7-OH DPAT | $D_2/D_3$ | 5.2±2.0 | 95 | 9.8±0.6 | 24.3±4.3 | 91 |
| PD168077 | $D_4$ | >10,000 | – | 828±88 | 16.6±2.4 | 82 |
| CP226269 | $D_4$ | 320±50 | 57 | 261±54 | 24.8±5.6 | 72 |

Data expressed as mean±SEM ($n$=4–10). A dash indicates efficacy<10%.

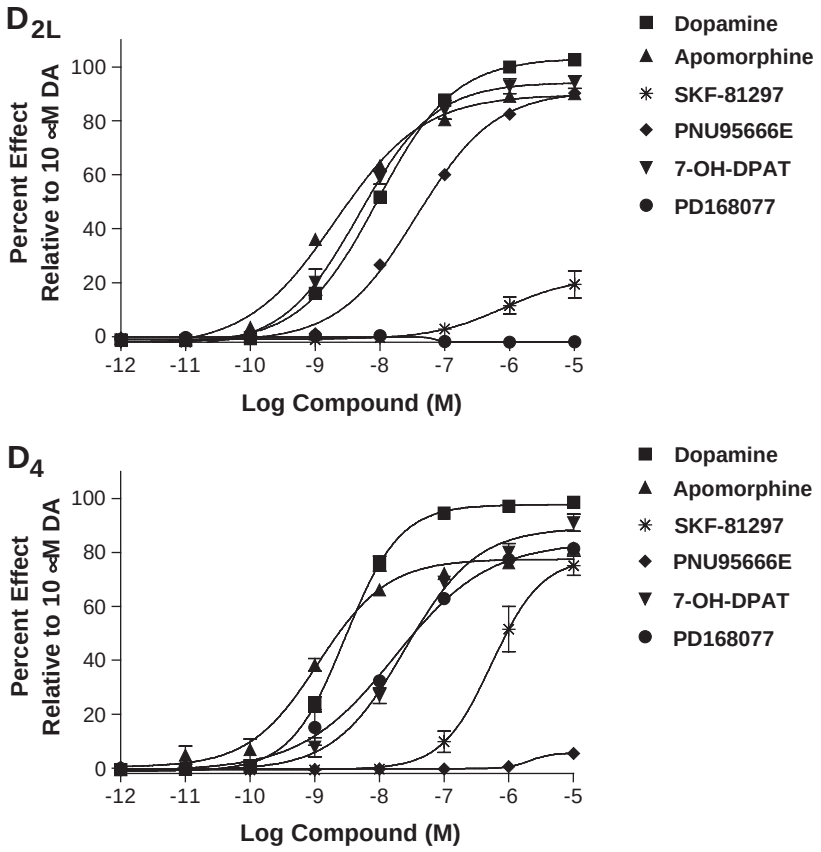


Fig. 1. Pharmacological activity of selected dopamine agonists in HEK293 cells co-expressing the ferret dopamine $D_{2L}$ (upper graph) or $D_4$ (lower graph) receptor and $G\alpha_{qo5}$. Cells were loaded with a calcium indicator dye (Fluo-4AM) and concentration-dependent increases in fluorescence were measured with FLIPR. Agonist responses were expressed as the mean±SEM percentage of the response to 10 μM dopamine ($n=4-8$).

### 3.2. Emesis

#### 3.2.1. Nonselective dopamine receptor activation

Apomorphine, a dopamine $D_2$-like receptor agonist produced a robust, reproducible emetic response in ferrets. The apomorphine emetic dose–response curve was bell-shaped, with emesis beginning at a dose of 0.03 μmol/kg and peaking at 0.3 μmol/kg (Table 3). The dopamine $D_2$-like receptor antagonist haloperidol (0.03–0.3 μmol/kg, s.c., given 30 min prior to apomorphine) dose-dependently reversed the emetic response to apomorphine (0.3 μmol/kg), with complete prevention of emesis at a haloperidol dose of 0.1 μmol/kg (data not shown). Neither antagonist elicited emesis or a non-zero Nausea Index value when administered in the absence of agonist (Table 2). The Nausea Index values produced in response to apomorphine mimicked the emetic response, dose-dependently rising to a maximum of 14.5±3.7 behaviors/group at the peak emetic apomorphine dose of 0.3 μmol/kg, and then gradually diminishing at higher apomorphine doses. Haloperidol reduced Nausea Index values to 0.

Dopamine $D_1$-like receptor activation. The dopamine $D_1/D_5$ receptor agonist SKF-81297 did not elicit vomiting/retching in ferrets at subcutaneous doses ranging from 0.1 to 3.0 μmol/kg. The SKF-81297 doses chosen are similar to those that modify a variety of behaviors in rats (Cohen et al., 1999). Therefore, dopamine $D_1$ receptor activation is unlikely to be emetic at behaviorally relevant doses. No prodromal behaviors were observed over the dose range tested, resulting in Nausea Index values of 0.

#### 3.2.2. Dopamine $D_2$-like receptor activation

A variety of agonists with varying degrees of selectivity at dopamine $D_2$-like receptors were tested for their emetic effects in ferrets (Table 3). Like apomorphine, the non-selective dopamine $D_2$-like receptor agonist quinpirole was a very potent emetogen, with an $ED_{50}$ value of 0.09 μmol/kg

Table 2
Summary of in vitro and in vivo activities of dopamine receptor antagonists

| Compound | Selectivity | Flux assay | Binding | Emesis | Nausea index |
|---|---|---|---|---|---|
| | | $K_i$ (nM) | $K_i$ (nM) | $ED_{50}$ (μmol/kg) | |
| Haloperidol | $D_2$-like | 0.30±0.09 | 0.22±0.02 | No emesis | – |
| Domperidone | $D_2$-like | 0.51±0.18 | 5.14±0.12 | No emesis | – |

In vitro data expressed as mean±SEM of 4–8 experiments. For the ferret emesis studies, each antagonist was administered at 0.1 μmol/kg, s.c. to groups of 6 animals.

Table 3
Emetic activity of dopaminergic receptor ligands in conscious ferrets

| Compound | Receptor subtype selectivity | Dose, s.c. (μmol/kg) | Incidence of emesis (%) | Latency (min) | Number of emetic episodes | Nausea index |
|---|---|---|---|---|---|---|
| Apomorphine | Non-selective | 0.01 | 0 | – | – | – |
| | | 0.03 | 8.3 | 9.5 | 1 | 0.1±0.1 |
| | | 0.1 | 25 | 16.3±2.7 | 1.0±0 | 6.9±1.2 |
| | | 0.3 | 79.2 | 10.2±1.5 | 2.5±0.5 | 14.5±3.7 |
| | | 1 | 50 | 11.2±8.2 | 2±0.6 | 11.2±2.4 |
| | | 3 | 33 | 12.7 | 1 | 7.0±1.0 |
| | | 10 | 33 | 1.5 | 1 | 1.2±0.5 |
| SKF81297 | $D_1/D_5$ | 0.1 | 0 | – | – | – |
| | | 0.3 | 0 | – | – | – |
| | | 1.0 | 0 | – | – | – |
| | | 3.0 | 0 | – | – | – |
| Quinpirole | $D_2/D_3/D_4$ | 0.03 | 0 | – | – | – |
| | | 0.06 | 17 | 20 | 1 | 2.0±1.0 |
| | | 0.1 | 67 | 18.1±4.1 | 2.0±1.0 | 6.8±3.8 |
| | | 0.3 | 100 | 7.7±0.6 | 5.0±0.8 | 28.7±4.5 |
| PNU95666E | $D_2$ | 0.1 | 0 | – | – | 0.2±0.2 |
| | | 0.3 | 0 | – | – | 0.3±0.3 |
| | | 0.6 | 0 | – | – | 1.2±0.7 |
| | | 1 | 83 | 8.1±1.6 | 3.8±0.7 | 18.2±6.1 |
| | | 3 | 100 | 8.0±2.4 | 5.2±1.1 | 22.8±4.0 |
| PNU142774E | $D_2$ | 0.1 | 0 | – | – | – |
| | | 0.3 | 17 | 5 | 1 | 1.7±1.7 |
| | | 0.6 | 67 | 7.0±1.3 | 2.2±0.9 | 8.7±3.4 |
| | | 1.0 | 83 | 12.5±5.5 | 5.6±1.2 | 26.7±7.9 |
| 7-OH-DPAT | $D_2/D_3$ | 0.03 | 17 | 14.5 | 1 | 1.2±0.5 |
| | | 0.1 | 17 | 9.5 | 5 | 8.3±6.6 |
| | | 0.3 | 83 | 13.7±3.1 | 8.2±2.1 | 26.7±6.1 |
| PD168077 | $D_4$ | 0.1 | 0 | – | – | 0.5±0.3 |
| | | 0.3 | 0 | – | – | 1.3±0.4 |
| | | 1 | 0 | – | – | 0.3±0.2 |
| | | 3 | 0 | – | – | – |
| | | 10 | 0 | – | – | 0.3±0.3 |
| CP226269 | $D_4$ | 0.1 | 0 | – | – | – |
| | | 0.3 | 0 | – | – | – |
| | | 1 | 0 | – | – | 0.2±0.2 |
| | | 3 | 0 | – | – | – |
| | | 10 | 0 | – | – | – |

Data are expressed as mean±SEM for results that contain at least 3 observations; otherwise, only the mean is displayed. Six to 24 animals were tested at each dose. Time to emesis and # of episodes only includes those animals which experienced emesis.

(95% CI: 0.06–0.13). Other, reportedly more selective dopamine $D_2$ agonists also were also highly emetic. Both PNU95666E and PNU142774E elicited dose-dependent emetic responses in ferrets, with respective $ED_{50}$ values of 0.89 and 0.52 μmol/kg (respective 95% CI: 0.67–1.17 and 0.32–0.84). Quinpirole, PNU95666E, and PNU142774E dose-dependently increased Nausea Index values; the maximum Nausea Index value for each of these compounds was approximately twice as large as the Nausea Index value obtained with apomorphine. The dopamine $D_2$-like receptor competitive antagonists haloperidol and domperidone dose-dependently prevented the emetic response to PNU95666E. The antagonists also reduced the Nausea Index values to 0. In total, these results confirm that dopamine $D_2$ receptor activation results in emesis.

The dopamine $D_2/D_3$ receptor selective compound 7-OH-DPAT evoked a dose-dependent emetic response when tested at doses ranging from 0.03 to 0.3 μmol/kg. The $ED_{50}$ value for emesis was 0.14 μmol/kg (0.05–0.37). A dose-dependent increase in the Nausea Index was observed with 7-OH-DPAT, with a maximum value (26.7±6.1 behaviors/group) similar to that produced by the selective dopamine $D_2$ receptor agonists quinpirole, PNU95666E, and PNU142774E. Two dopamine $D_4$ selective agonists, PD168077 and CP226229, were not emetic when administered to ferrets at doses ranging from 0.1 to 10 μmol/kg. These doses span the range deemed efficacious for eliciting penile erection in rats (Hsieh et al., 2004). Although a limited number of prodromal behaviors was observed in response to each of these compounds, the mean number of behaviors was quite small (<1.5 behaviors/group) and the behaviors did not display dose-dependency. Therefore, we conclude that dopamine $D_4$ receptor activation is not emetic in this species.

## 4. Discussion

In order to determine the specific dopamine receptor responsible for the emetic effects of dopamine receptor ligands, we cloned and expressed the ferret dopamine $D_{2L}$ and $D_4$ receptors in stable cell lines. Co-transfection of the cells with a chimeric $G\alpha_{qo5}$ resulted in a functional in vitro assay for assessing the dopamine receptor selectivity of a variety of dopamine receptor ligands. This assay relies upon a highly sensitive fluorometric calcium imaging technique that lends itself well to automated data collection (Chambers et al., 2003). The dopamine receptor ligands characterized in vitro were then tested in conscious ferrets for their emetic activity. The major finding of this study was that dopamine $D_4$ receptor selective agonists were not emetic in ferrets.

The results obtained with the functional calcium flux assay for the ferret dopamine $D_{2L}$ receptor correlate very well with the potencies obtained with competition binding using the dopamine receptor agonist radioligand [$^{125}$I]-PIPAT. The binding affinities for compounds that had full agonist activity at the ferret dopamine $D_{2L}$ receptor (dopamine, apomorphine, quinpirole, PNU-95666E, PNU-142774E) were highly correlated with the potencies obtained in the calcium flux assay. As expected, the dopamine $D_4$ selective agonist PD168077 was inactive in the ferret dopamine $D_{2L}$ calcium flux assay and had very weak binding affinity at the ferret dopamine $D_{2L}$ receptor ($K_i$=828 nM). CP226269, a reported dopamine $D_4$ agonist (Zorn et al., 1997), had a binding affinity of 261 nM and showed partial agonist activity at the ferret $D_{2L}$ receptor in the calcium flux assay (57% efficacy) with an $EC_{50}$ of 320 nM. These data agree with previous data obtained for human dopamine $D_{2L}$ receptor-transfected cell lines (Newman-Tancredi et al., 2002; Moreland et al., 2004). These results suggest that, in contrast to radioligand-binding assays, the high-throughput in vitro calcium flux assay can accurately predict functional dopamine $D_2$ receptor agonist activity of novel chemical entities.

While the emetic activity of dopamine $D_2$-selective agonists has been firmly established in a variety of species for decades (Eggleston and Hatcher, 1912; Costello and Borison, 1977; Florczyk et al., 1982; Darmani et al., 1999), most of these studies have used apomorphine as test drug. Numerous studies, including receptor-binding and in vitro functional assays, provide ample evidence that apomorphine is not a dopamine $D_2$-specific receptor agonist, but displays agonism at dopamine $D_2$, $D_3$, and $D_4$ receptor subtypes (Newman-Tancredi et al., 2002; Moreland et al., 2004). Using in vitro and in vivo experimental approaches, we have shown that dopamine $D_2$ receptor agonists are highly emetic in ferrets. Yoshikawa et al., (1996) have shown that 7-OH-DPAT is emetic in ferrets, an action attributed by these authors to dopamine $D_3$ receptor activation. Our results strongly suggest that dopamine $D_2$ receptor stimulation likely contributes to the emetic activity of agents that have been incorrectly classified as dopamine "$D_3$-selective" agonists. A definitive conclusion on the emetogenicity of dopamine $D_3$ receptor stimulation awaits the development of truly selective dopamine $D_3$ receptor agonist.

In agreement with previously published results (Andrews et al., 1986; Costall et al., 1989), apomorphine's dose–response curve with respect to emesis was bell-shaped. It has been suggested that apomorphine-elicited emesis in ferrets may depend on the rate of presentation of apomorphine to the area postrema since intravenously administered apomorphine is less effective at producing emesis than subcutaneously administered apomorphine (Andrews et al., 1986). Of particular interest is that fact that dopaminergic agonists produce biphasic dose–response relationships for many endpoints, including locomotion, pain sensitivity, penile erection, cardiovascular, endocrine and other biological responses (Calabrese, 2001). As dose increases, receptor selectivity of a given agonist likely decreases. Activation of related receptor subtypes (or perhaps even pharmacologically unrelated receptors) could result in anti-emetic activity that opposes the initial emetic stimulation by dopamine receptor agonists. Alternatively, higher drug doses could result in greater drug penetration into hindbrain structures, ultimately leading to anti-emetic activity through agonist effects at a site downstream from the area postrema. Such a mechanism has been hypothesized for the emetic and anti-emetic effects of opioid agonists in ferrets (Thompson et al., 1992).

The dopamine $D_4$ receptor agonist PD168077 increases locomotor activity in rats (Nayak and Cassaday, 2003), while Bernaerts and Tirelli (2003) have demonstrated that PD168077 increases mean step-through latency in mice, suggesting involvement of dopamine $D_4$ receptors in memory consolidation. Also, administration of PD168077 increases c-Fos expression in rat striatum (Trías et al., 2001). Perhaps even more intriguing is recent preclinical data that suggest dopamine $D_4$ receptor agonists may be clinically useful for the treatment of erectile dysfunction (Hsieh et al., 2004; Brioni et al., 2004). The PD168077 doses used in all of these studies overlap the dose range used in the present work, further underscoring the relevance of our finding that dopamine $D_4$ receptors are non-emetic. Although apomorphine is currently marketed in Europe for the treatment of erectile dysfunction, nausea occasionally occurs with its use (Bukofzer and Livesey, 2001). The data presented in the current study suggest that apomorphine's nauseogenic activity results from dopamine $D_2$ and/or $D_3$ receptor agonism. The recent demonstration that dopamine $D_4$ receptor agonists induce penile erection in rats suggests apomorphine's pro-erectile activity may be mediated through dopamine $D_4$ receptors (Hsieh et al., 2004; Brioni et al., 2004). The lack of emetogenicity of PD168077 and CP226269, as well as the novel dopamine $D_4$ agonist ABT-724 (Brioni et al., 2004) indicates that non-emetic, centrally-acting dopamine $D_4$ receptor agonists may be a valuable therapeutic approach for the treatment of erectile dysfunction.

A significant advantage of our experimental approach is that the same species (ferret) has been used for both in vivo and in vitro characterization, eliminating potential cross-species confounds. Moreover, the similarity between human and ferret dopamine receptor pharmacology, with respect to ligand-binding and in vitro functional activity (Moreland et al., 2004), provides additional credibility for the choice of ferrets for preclinical emesis testing (King, 1990). A further benefit emanating from these studies is the potential for reduction in animal use by pre-screening novel chemical entities in the in vitro dopamine receptor assays, reserving in vivo studies only for those compounds lacking activity at dopamine $D_{2L}$ receptors. In summary, these studies indicate that activation of dopamine $D_2$ receptors, but not dopamine $D_4$ receptors, is related to the emetic properties of dopamine agonists.

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