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L-152,804: Orally Active and Selective Neuropeptide Y Y5 Receptor Antagonist

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Neuropeptide Y (NPY) elicits food intake through the action of hypothalamic G-protein-coupled receptors. Previous publications indicate that the Y5 receptor may represent one of these postulated hypothalamic "feeding" receptors. Using a potent and orally available Y5 antagonist L-152,804, we evaluated the involvement of the Y5 receptor in feeding regulation. L-152,804 displaced [^{125}I]peptide YY (PYY) binding to human and rat Y5 receptors with K_i values of 26 and 31 nM, respectively, and inhibited NPY (100 nM)-induced increase in intracellular calcium levels via human Y5 receptors (IC_{50} = 210 nM). L-152,804 did not show significant affinity for human Y1, Y2, and Y4 receptors at a dose of 10 μM. Intracerebroventricular (ICV) (30 μg) or oral (10 mg/kg) administration of L-152,804 significantly inhibited food intake evoked by ICV-injected bovine pancreatic peptide (bPP, 5 μg; a moderately selective Y4, Y5 agonist) in satiated SD rats. However L-152,804 did not significantly inhibit ICV NPY (5 μg; a Y1, Y2, Y5 agonist)-induced food intake. These findings suggest that L-152,804 is a selective and potent nonpeptide Y5 antagonist with oral bioavailability and brain penetrability. In addition, the anorexigenic effects of L-152,804 on bPP-induced feeding revealed participation of the Y5 receptor in feeding regulation, while ICV administration of NPY does not appear to significantly contribute to Y5 stimulated food intake. We conclude that the potent and orally active Y5 antagonist, L-152,804, represents a useful tool to address the physiological role of the Y5 receptor.

Neuropeptide Y (NPY) is a 36-amino-acid polypeptide belonging to the pancreatic polypeptide family, which consists of NPY, peptide YY (PYY) and pancreatic polypeptide (PP) (1, 2). NPY is highly concentrated within the hypothalamus (2–4), and induces potent stimulation of feeding behavior via NPY receptors (5–9). Chronic administration of NPY into the brain results in hyperphagia and body weight gain, reduces energy expenditure, and increases lipogenic activity in the liver and adipose tissue (10, 11). Based on this data, it has been argued that NPY is one of the major regulators of energy metabolism.

Five distinct types of NPY receptors, Y1, Y2, Y4, Y5, and y6, have been cloned (12). Among these NPY receptors, the Y5 receptor is proposed to be one of the feeding receptors based on the correlation between the *in vitro* functional and binding activity of different peptide agonists and their potent stimulation of food intake in rodent models (13, 14). In support of this hypothesis, a significant reduction of food intake induced by Y5-prefering agonists has been reported in Y5 receptor deficient mice (15, 16). To confirm the participation of the Y5 receptor in feeding regulation, orally available and highly selective Y5 antagonists are of great significance.

We show in the present study that orally-active L-152,804 is a potent and selective antagonist of the Y5 receptor. Using this potent Y5 antagonist, we investigated the role of the Y5 receptor in feeding regulation.

MATERIALS AND METHODS

Reagents. Neuropeptide Y (NPY) was purchased from Peptide Institute (Osaka, Japan). Peptide YY (PYY) and pancreatic polypeptide (PP) were from Sigma (St. Louis, MO). [^{125}I]PYY and [^{125}I]PP were obtained from New England Nuclear-DuPont (Boston, MA). The culture reagents were from GIBCO (Grand Island, NY). All other chemicals were of analytical grade. L-152,804 (2-(3,3-dimethyl-1-oxo-4*H*-1*H*-xanthen-9-yl)-5,5-dimethyl-cyclohexane-1,3-dione) was synthesized by Banyu Pharmaceutical Co., Ltd. The structure of L-152,804 is shown in Fig. 1.

Cell culture. CHO-K1, LMtk- and COS-7 cells were obtained from ATCC (Rockville, MD). CHO-K1 cells expressing recombinant human Y1, Y2, and Y4 receptors were grown in Iscove's modified

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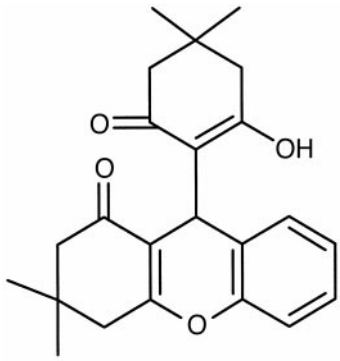

FIG. 1. Structure of L-152,804.

Dulbecco's medium supplemented with 10% fetal bovine serum (FBS), penicillin-G (100 IU/ml), streptomycin (100 μg/ml) and G418 (1 mg/ml). LMtk- cells expressing recombinant human Y5 receptor were grown in Dulbecco's modified Eagle's medium (high glucose) with 10% FBS, penicillin-G (100 IU/ml), streptomycin (100 μg/ml) and G418 (0.8 mg/ml). COS-7 cells transiently expressing recombinant rat Y5 receptor were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, penicillin-G (100 IU/ml) and streptomycin (100 μg/ml). These cells were grown in a 95% air, 5% CO_2 humidified atmosphere at 37°C.

Binding experiments. Cells were washed with 50 mM Hepes buffer (pH 7.4) containing 20% sucrose, homogenized, and centrifuged at 1000*g* for 15 min. The supernatant was centrifuged at 100,000*g* for 45 min. The pellets were resuspended in 5 mM Hepes buffer (pH 7.4) and centrifuged again. The membrane fraction was resuspended by a homogenizer in the same buffer and used for this study.

Binding of [^{125}I]PYY and [^{125}I]PP to membrane preparations was performed in 0.2 ml of 25 mM Tris buffer (pH 7.4) containing 10 mM $MgCl_2$, 1 mM PMSF, 0.1% bacitracin and 0.5% bovine serum albumin. The membranes (10–300 μg/ml) prepared from LMtk-, CHO-K1 and COS-7cells expressing human or rat NPY receptors, were incubated at 25°C for 120 min with [^{125}I]PYY or [^{125}I]PP (25 pM). Bound and free peptides were separated by filtration using a GF/C glass filter presoaked with 0.3% polyethylenimine. The remaining radioactivity on the filter was quantitated using a TopCount (Packard Instruments Co. Inc.). Specific binding was defined as the difference between total binding and nonspecific binding in the presence of 1 μM PYY or PP. Binding affinities are the average of more than 3 determinations.

Measurement of intracellular calcium ion concentrations. $[Ca^{2+}]_i$ was measured fluorometrically using a Ca^{2+}-sensitive fluorescent dye, fura-2. The cells expressing human NPY receptors were harvested using 0.25% trypsin and 0.02% EDTA. The cells (1.0×10^7 cells) were washed once with Krebs-Henseleit Hepes buffer containing 0.1% BSA (pH 7.4), suspended in 1 ml of the buffer and incubated with 2 μM fura-2 acetoxymethyl ester at 37°C for 60 min. The fura-2-loaded cells were washed with the buffer and resuspended in 10 ml of the buffer. 0.5 ml of the resultant suspension was stirred continuously at 37°C in a cuvette during the measurement. Test compounds or vehicle were added 5 min before the addition of NPY and the related ligands, and fluorescent intensity at an emission wavelength of 500 nm and excitation wavelengths of 340 and 380 nm was monitored with a CAF-110 intracellular ion analyzer (JASCO, Tokyo, Japan). $[Ca^{2+}]_i$ values were calculated according to a previously reported method (17).

In vivo experimental protocols. Adult male Sprague–Dawley rats (7 weeks old, Charles River Japan, Japan, 280-350 g) were maintained in individual cages under controlled conditions of temperature (23 ± 2°C), humidity (55 ± 15%) and light–dark cycle (0700–1900). Water and pellet food (CE-2, CLEA Japan Inc., Japan) were available *ad libitum*. SD rats were anesthetized with sodium pentobarbital (50 mg/kg ip, Dainabot, Japan) and a permanent 21-gauge stainless-steel cannula was stereotaxically implanted into the right lateral ventricle. After one week of recovery, satiated rats were used in experiments. A dose response to ICV NPY and bPP was determined in which groups of 6–12 animals received injections of 10 μl of NPY or bPP (1, 5, 10, or 20 μg ICV) or vehicle (10 mM phosphate-buffered saline containing 0.05% bovine serum albumin), and their food intake was monitored for 2 h. Compound was evaluated in groups of 8–10 animals which received injections of 10 μl of NPY (5 μg ICV), bPP (5 μg ICV), L-152,804 (30 μg ICV) + NPY (5 μg ICV), or L-152,804 (30 μg ICV) + bPP (5 μg ICV), and their food intake was monitored for 2 h. PO dosing of L-152,804 (suspended in 0.5% methylcellulose in distilled water) was done 1 h before ICV-agonist dosing. The experiments were performed between 9:00 and 11:30 AM.

All experimental procedures followed the Japanese Pharmacological Society Guideline for Animal Use. Results are given as means ± SE. Statistical analysis was performed using Student's *t* test.

RESULTS AND DISCUSSION

[^{125}I]PYY-specific binding to human and rat Y5 receptors was inhibited by L-152,804 with high affinities (K_i = 26 nM and 31 nM, respectively) (Table 1). In contrast, L-152,804 did not inhibit [^{125}I]PYY or [^{125}I]PP binding to the human Y1, Y2 or Y4 receptors at a dose of 10 μM (Table 1). L-152,804 inhibited NPY (100 nM)-induced $[Ca^{2+}]_i$ increase dose-dependently with an IC_{50} value of 210 nM in LMtk- cells expressing the human Y5 receptors (Table 1), while L-152,804 alone did not induce a $[Ca^{2+}]_i$ increase even at a dose of 10 μM (data not shown). Additionally, we could not detect any sig-

TABLE 1

In Vitro Profiles of L-152,804 for NPY Receptors

	Binding affinity (K_i, nM)					$[Ca^{2+}]_i$ response (IC_{50}, nM)
	hY5 [^{125}I]PYY	rY5 [^{125}I]PYY	hY1 [^{125}I]PYY	hY2 [^{125}I]PYY	hY4 [^{125}I]PP	hY5 100 nM NPY
NPY	0.70	0.82	0.45	0.09	180	—
bPP	1.9	1.6	42	>1000	0.18	—
L-152,804	26	31	>10000	>10000	>10000	210

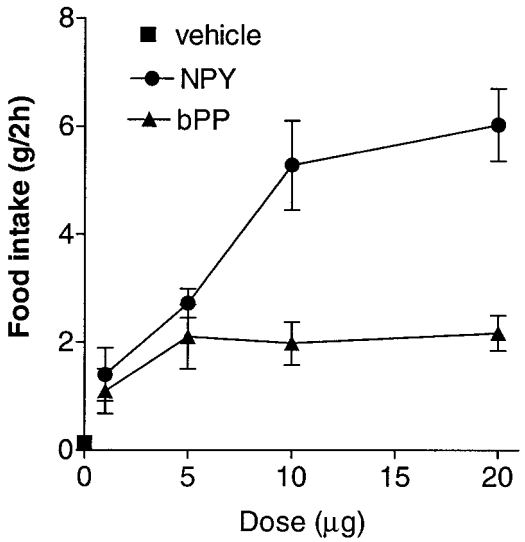

FIG. 2. Cumulative food intake induced by NPY and bPP in satiated Sprague–Dawley rats. Food intake was measured for 2 h after ICV injection of agonists. Data are reported as the mean ± SE. $n = 6–12$ rats/group.

nificant cross-reactivity with a wide variety of 120 other binding assays and 7 enzyme assays (data not shown). These findings demonstrate that L-152,804 is a potent and selective Y5 antagonist.

Intracerebroventricular (ICV) injection of NPY and bPP induced rapid and robust feeding in satiated SD rats in a dose-dependent manner (Fig. 2). Food intake stimulated by ICV bPP reached a plateau at ~5 μg of peptide, and was significantly less potent than NPY-induced food intake (Fig. 2). Subsequent food intake measurements (and evaluation of the inhibitory effects of L-152804) were made using 5 μg NPY or bPP administered ICV. In comparison with the respective vehicles, ICV L-152,804 (30 μg) administered in the absence of NPY or bPP did not change cumulative food intake, indicating that L-152,804 had no effect on food intake in satiated rats per se (data not shown). In addition, we did not observe any notable changes in other behaviors, including sedation or barrel-rolling, at any of the dosages used. Simultaneous injection of L-152,804 (30 μg) with bPP (5 μg) significantly suppressed food consumption induced by bPP in SD rats. In contrast L-152,804 had no effect on NPY-induced feeding (Fig. 3). Oral dosing of L-152,804 (10 mg/kg) also significantly and selectively inhibited bPP-induced food intake, but not NPY-induced food intake, although a significant amount of L-152,804 (2.9 μM) could be observed in the brain 2 h after dosing (Fig. 4). The brain levels of L-152,804 were 100-fold higher than the IC_{50} value of L-152,804 in the calcium functional assays.

It is well known that bPP is a potent Y4 and Y5 receptor agonist (Table 1; 13, 16). Because the Y4 specific agonist, rat PP failed to stimulate feeding behavior in rats, the Y5 receptor has been considered a major feeding receptor (13). Our findings suggest that bPP-induced food intake is caused by the activation of the Y5 receptor, because a Y5 selective antagonist, L-152,804, selectively inhibited bPP-induced feeding. We concluded that the Y5 receptor is one of the feeding receptors in rats. We also infer from the data that at a dose of 5 μg, NPY-induced food intake does not significantly involve Y5 receptor activation, since L-152,804

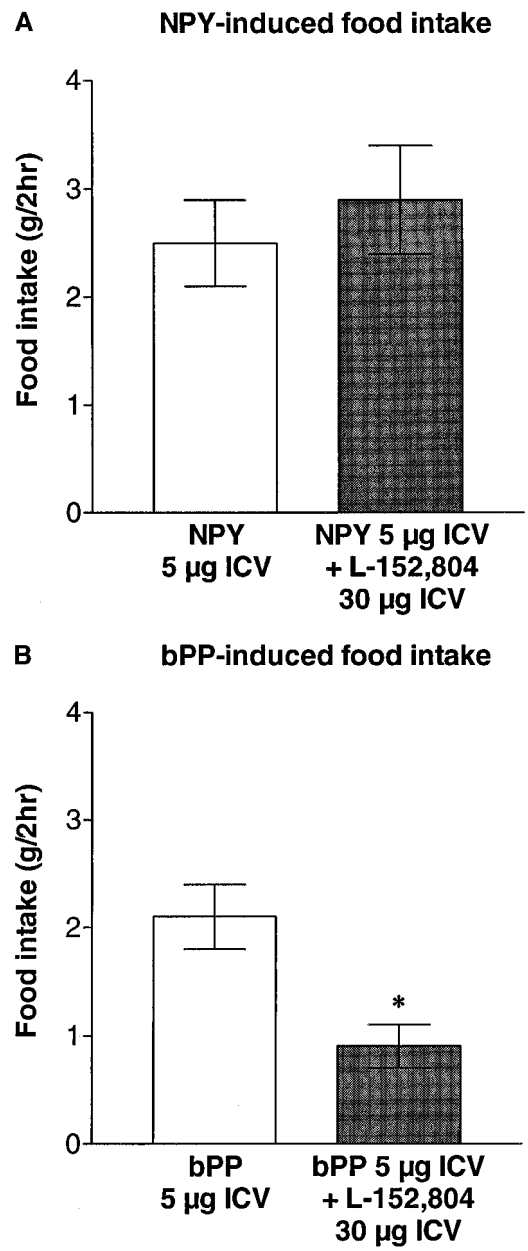

FIG. 3. Effects of L-152,804 on feeding response to NPY or bPP in satiated Sprague–Dawley rats. L-152,804 was ICV-injected with NPY or bPP, simultaneously. *$P < 0.01$ compared with rats injected with NPY or bPP alone. Data are expressed as the mean ± SE. $n = 8–10$ rats/group (Student's t test).

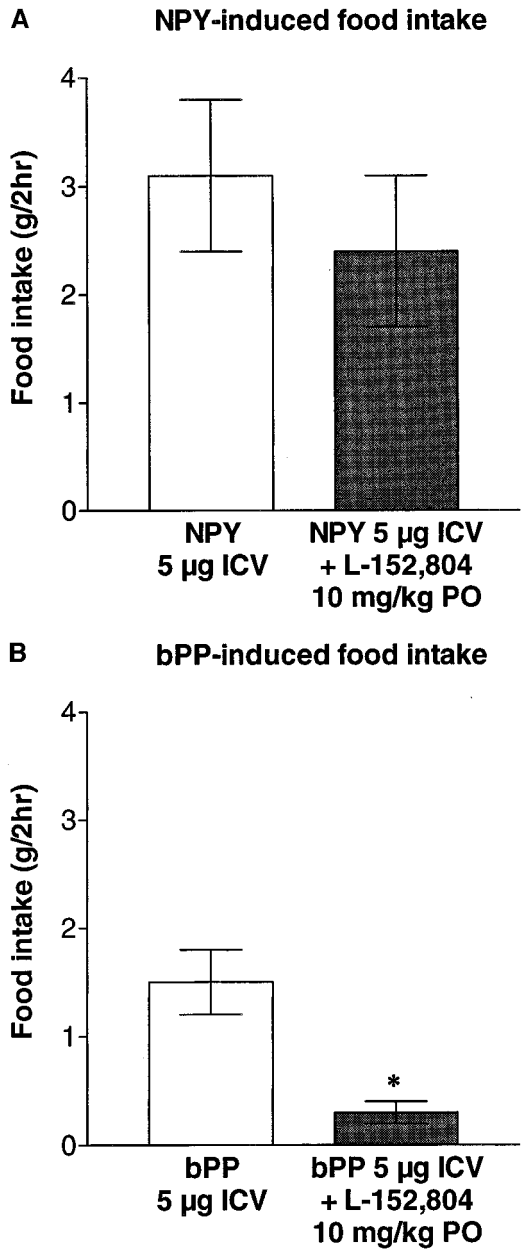

FIG. 4. Effects of L-152,804 on NPY or bPP-induced feeding in satiated Sprague–Dawley rats. L-152,804 was orally administered 1 h before ICV injection of NPY or bPP. $*P < 0.01$ compared with rats injected with NPY or bPP alone. Data are expressed as the mean ± SE. $n = 8–10$ rats/group (Student's t test).

did not show significant hypophagic effects in NPY-induced feeding.

Based on the *in vitro* affinity of NPY for the Y1 and Y5 receptor, one could predict that the Y5 receptor, at least in part, contributes to NPY-induced feeding. However, our data indicate that the role of the Y5 receptor is negligible in NPY induced models of food intake. To support this finding, inactivation of the Y5 receptor in knock out mice failed to lead to a reduction in the efficiency of ICV NPY-induced feeding (15, 16). We conclude that the Y5 receptor is not a major feeding receptor through which exogenously applied NPY elicits its effects on feeding in rodents. In contrast, our conclusion differs from a recent report in which a potent Y5 antagonist CGP71638A significantly inhibited NPY-induced feeding (18). Making use of Y5 −/− mice to determine whether Y5 antagonists applied truly work in a mechanism based manner will be essential to help further clarify this issue. In addition, comparing selected conditions, in which a compound shows varying efficacy, like we did in the comparison of L-152,804 with ICV bPP and NPY induced food intake, can help further define that overt toxicity does not contribute to the mechanism of food intake inhibition.

Several investigators showed that structurally diverse Y1 antagonists potently suppressed NPY-induced feeding (19–22) and that a reduction of NPY-induced feeding was observed in Y1 receptor-deficient mice (16, 23). These findings reveal that the Y1 receptor plays a key role in the NPY-mediated feeding. This finding is in agreement with our conclusion that the Y5 receptor represents a feeding receptor, though the Y5 receptor does not appear relevant in models of NPY-induced feeding. Our findings do not exclude a physiological role for the Y5 receptor in other models of feeding regulation, including a potential role in energy homeostasis (14).

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