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[^{3}H]PD 140376: A Novel and Highly Selective Antagonist Radioligand for the Cholecystokinin$_B$/Gastrin Receptor in Guinea Pig Cerebral Cortex and Gastric Mucosa

J. C. HUNTER, N. SUMAN-CHAUHAN, K. G. MEECHAM, V. U. K. DISSANAYAKE, D. R. HILL, M. C. PRITCHARD, C. O. KNEEN, D. C. HORWELL, J. HUGHES, and G. N. WOODRUFF

Departments of Biology (J.C.H., N.S.-C., K.G.M., V.U.K.D., D.R.H., J.H., G.N.W.) and Medicinal Chemistry (M.C.P., C.O.K., D.C.H.), Parke-Davis Neuroscience Research Centre, Cambridge CB2 2QB, United Kingdom

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SUMMARY

The specific binding characteristics of the novel cholecystokinin (CCK)$_B$/gastrin receptor-selective peptoid antagonist radioligand [^{3}H]PD 140376 were investigated using membrane homogenates prepared from guinea pig cerebral cortex and gastric fundic mucosa. [^{3}H]PD 140376 (0.01–10 nM) bound to both cerebral cortex and gastric gland homogenates with comparable high affinity (K_d, 0.1–0.2 nM) and to an apparent single population of sites with B_{max} values of 119 and 296 fmol/mg of protein, respectively. The level of specific binding, defined as that displaced by unlabeled CCK sulfated octapeptide, was routinely between 60 and 70% in the cortex and between 50 and 60% in the fundic mucosa. Pharmacological characterization of the [^{3}H] PD 140376-labeled binding sites with a series of agonist and antagonist ligands selective for each of the CCK receptor subtypes demonstrated, in both preparations, an affinity profile consistent with that of the CCK$_B$/gastrin receptor. However, Hill slopes for the competition curves for the unlabeled agonist ligands against specific [^{3}H]PD 140376 binding were significantly less than unity, whereas those for the antagonist ligands, including unlabeled PD 140376, were close to unity. The affinity and Hill slope for PD 140376 and the related CCK$_B$/gastrin antagonist CI-988 were unaffected by the presence of the nonhydrolyzable GTP analogue guanylyl-5′-imidodiphosphate. In contrast, guanylyl-5′-imidodiphosphate caused a characteristic decrease in affinity and an increase in the Hill slopes towards unity for the agonist ligands CCK sulfated octapeptide and pentagastrin. The binding characteristics of unlabeled PD 140376 were also unaffected by the presence of the monovalent cation sodium. In conclusion, the present study has demonstrated that [^{3}H]PD 140376 is the most potent and selective antagonist radioligand yet described for the characterization of CCK$_B$/gastrin receptors in the central and peripheral nervous systems.

The CCK/gastrin family of neuropeptides have a ubiquitous distribution throughout the central and peripheral nervous systems and have been implicated in many pathophysiological events including anxiety and panic disorders, psychoses, satiety, and gastric acid and pancreatic enzyme secretions (1). These effects appear to be mediated through specific interactions with at least two major distinct types of receptors, previously termed "peripheral" and "central" CCK receptors but now referred to as CCK$_A$ and CCK$_B$, respectively (2). A third type of receptor, termed the gastrin receptor (3, 4), has also been described but differentiation between it and the CCK$_B$ receptor remains at best equivocal, with no clear evidence to suggest that the two sites may be distinguishable (5, 6).

Each of the major types of receptors has been found to have a distinct anatomical distribution, with CCK$_A$ receptors predominating in the gastrointestinal tract (7) and pancreas (8–10) but also localized in discrete brain areas (2, 11, 12). In contrast, CCK$_B$ and gastrin receptors predominate in the brain and gastric glands, respectively (13–17), although there is also evidence in support of gastrin receptors in gastrointestinal smooth muscle (18–20) and pancreas (3, 4). CCK$_A$ and CCK$_B$ receptors have been separated on a pharmacological basis using agonist and antagonist selectivity profiles in a diversity of *in vitro* radioligand binding studies (6, 8, 9, 21–23), electrophysiological (24–27), neurochemical (28, 29), and smooth muscle bioassays (4, 7, 18–20), and behavioral models (30–36). Although CCK$_{8S}$ has a similarly high affinity at both receptor types, the CCK$_A$ receptor can now be clearly distinguished from the CCK$_B$ or gastrin receptor using selective agonists such as A71623 (37) and the selective antagonists devazepide (formerly L-364,718 and MK-329) (22), lorglumide (38), and PD 140548 (39). Conversely, PG and CCK$_{8US}$ (5, 6), BC 264 (40), and SNF

ABBREVIATIONS: CCK, cholecystokinin; CCK$_{8S}$, cholecystokinin sulfated octapeptide; CCK$_{8US}$, cholecystokinin unsulfated octapeptide; BH-CCK$_{8S}$, Bolton-Hunter-labeled cholecystokinin sulfated octapeptide; PG, pentagastrin; Gpp(NH)p, guanylyl-5′-imidodiphosphate; CCK$_4$, cholecystokinin tetrapeptide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; gastrin$_{1-17US}$, gastrin unsulfated peptide 1–17.

8702 (41) are selective agonists and L-365,260 (23), LY 247348 (42), and CI-988 (6) are selective antagonists for the CCK_B/gastrin receptor, although the degree of selectivity can be species dependent (42).

Until recently many of the *in vitro* binding studies depended upon the availability of the various radiolabeled forms of CCK_{8S} and related peptide fragments for the characterization of CCK_A and CCK_B or gastrin receptors (8–10, 15, 21, 41, 43). Such an approach relied either on the tissue source containing a predominance of one type of receptor or on the inclusion of selective unlabeled antagonists to suppress cross-reactivity with the opposite site (11, 12). Furthermore, even when selective agonist radioligands such as [^{3}H]PG (21), [^{3}H]SNF8702 (41), and [^{3}H]BC264 (40) were used, these labeled only high and not low affinity states of the receptor. This situation has improved considerably with the recent availability of the radiolabeled forms of the respective CCK_A- and CCK_B-selective antagonists [^{3}H]devazepide (44) and [^{3}H]L-365,260 (5). However, in contrast to devazepide, which has extremely high, subnanomolar affinity and selectivity for the CCK_A receptor, L-365,260, although having affinity in the nanomolar range, has only moderate selectivity for the CCK_B receptor (6, 22, 23).

CI-988 and PD 140376 are members of a peptoid series that have recently been shown to have the highest affinity and selectivity yet described for nonpeptide ligands interacting with the CCK_B or gastrin receptor (6, 45). CI-988 and related peptoids have been shown, *in vitro*, to be antagonists of the action of CCK_{8S} and PG at the CCK_B receptor (6, 27). Similarly, PD 140376 has been shown to antagonize PG-mediated depolarizations of rat hypothalamic neurons, *in vitro*, with an affinity constant of 2 nM.[1] Peptoids of this class are potent anxiolytic agents (6) and have also been shown to reverse PG-induced anxiogenic-like behavior in rodent models (33, 34). These ligands are also potent inhibitors of PG-induced gastric acid secretion (46). In the present study, we have therefore characterized CCK_B/gastrin receptors in the cerebral cortex and gastric glands of the guinea pig by comparing the binding profile of [^{3}H]PD 140376 with those of ^{125}I-BH-CCK_{8S} and ^{125}I-gastrin.

Materials and Methods

Membrane homogenate preparation. Male Dunkin-Hartley guinea pigs (250–350 g) were killed by cervical dislocation, and the cerebral cortices were rapidly removed and homogenized in 20 volumes (original wet weight/volume) of ice-cold buffer A (50 mM Tris·HCl, pH 6.9 at 22°) for 10 sec, using a Polytron (setting 6). The homogenate was then centrifuged at 40,000 × *g* for 15 min. The supernatant was discarded, and the pellet was washed by resuspension in 10 volumes (original wet weight/volume) of ice-cold buffer A and was recentrifuged as described above. The final pellet was then resuspended in assay buffer C (10 mM HEPES, 130 mM NaCl, 4.7 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.025% bacitracin, pH 7.2 at 22°) to a concentration of 10 mg (original wet weight)/ml.

Gastric gland homogenates were prepared by quickly removing and opening the stomach, emptying the contents, and rinsing the stomach with ice-cold buffer B (20 mM HEPES, 1 mM EGTA, 0.01% bacitracin, pH 7.4 at 4°). Using only tissue from the stomach fundus, the mucosa was scraped down to the muscle layer with a glass slide, washed by repeated pipetting in 5 ml of buffer B, and then centrifuged at 250 × *g* for 3 min at 4°. The supernatant was discarded together with a surface layer of mucous, and the pellet was washed by resuspension in 5 ml of buffer B and was homogenized for 30 sec using a Polytron (setting 6). The homogenate was then recentrifuged at 250 × *g* for 3 min and both the supernatant and the surface layer of mucous were removed. The pellet was resuspended in 40 ml of buffer B and centrifuged at 40,000 × *g* for 15 min. This step was then repeated to yield a final pellet for resuspension to a concentration of 10 mg (original wet weight)/ml in assay buffer C.

Assay incubation conditions. Aliquots (0.4 ml) of either cortical or fundic mucosa membrane homogenates were incubated for 45 min at 25°, in a final volume of 0.5 ml of assay buffer C containing the appropriate concentrations of radioligands and unlabeled drugs. After incubation, the assay was terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filter strips, and the filters were washed three times with 4 ml of ice-cold 50 mM Tris·HCl, pH 6.9 at 22°. The filters were transferred to vials containing 5 ml of Beckman HP scintillation cocktail, and bound radioactivity was determined by liquid scintillation counting. All experiments were performed in duplicate. Protein content was determined by the method of Lowry *et al.* (47).

Saturation binding studies were performed with increasing concentrations of [^{3}H]PD 140376 (0.01–10 nM; specific activity, 51 Ci/mmol; Amersham International). Competition experiments were conducted with a single concentration of either 0.2–0.4 nM [^{3}H]PD 140376 (both tissues), 50 pM ^{125}I-CCK_{8S} (cortex), or 50 pM ^{125}I-gastrin (gastric glands) and at least seven concentrations of unlabeled ligands. Nonspecific binding was always defined as the amount of radioligand binding in the presence of 1 μM unlabeled CCK_{8S}.

Preliminary competition experiments, using assay incubation conditions identical to those described above, yielded a K_i value for unlabeled PD 140376 of 350 (240–580) nM against ^{125}I-CCK_{8S} binding to rat pancreas (CCK_A sites) membranes. When compared with the K_i value (0.35 nM, see Table I) for PD 140376 obtained against ^{125}I-CCK_{8S} in the guinea pig cortex (CCK_B sites), this extrapolated to a CCK_A:CCK_B selectivity ratio of 1000.

The time course for association of [^{3}H]PD 140376 to cortical membrane homogenates was determined by incubation at 22° for various time intervals in the absence or presence of unlabeled CCK_{8S} (1 μM), to define total and nonspecific binding, respectively. The reaction was terminated by immediate filtration at the specified times and specific binding was calculated by subtraction of the nonspecific binding from the total binding. Each time point was measured in duplicate. For dissociation experiments, [^{3}H]PD 140376 was incubated with cortical membrane preparations at 22° for 45 min, at which time dissociation was initiated by addition of an excess concentration of unlabeled CCK_{8S} (1 μM). The residual binding was then measured at each time point after immediate filtration, with each determination being performed in duplicate.

Analysis of binding data. Saturation and competition binding data were transformed and analyzed by the Graphpad INPLOT program (Graphpad Software Inc.). In the saturation binding experiments K_d and B_{max} values were obtained by either analysis of untransformed specific binding data by nonlinear regression or analysis of a Scatchard plot by linear regression. The two methods yielded identical results. Competition data were transformed by nonlinear regression to obtain IC_{50} values, which were then converted by the program to K_i values by using the Cheng-Prusoff equation.

Kinetic data were analyzed according to the procedure of Weiland and Molinoff (48). The apparent association rate constant (k_{obs}) was calculated as the slope of the plot ln $[B_e/(B_e - B_t)]$ versus time, according to the equation $B_t = B_e[1 - e^{-(k_{obs} \cdot t)}]$, where k_{obs} is $k_1[L][B_T]/B_e$, B_t is the amount of radioligand specifically bound at time t, B_e is the amount of radioligand specifically bound at equilibrium, B_T is the total concentration of binding sites, and L is the total ligand concentration. The dissociation rate constants were determined according to the equation $B_t = B_0 e^{-(k_{-1} \cdot t)}$, by plotting ln (B_t/B_0) versus time; the slope of this plot is equal to k_{-1}.

Synthesis of [^{3}H]PD 140376. A full description of the preparation

[1] P. R. Boden and R. D. Pinnock, personal communication.

of [^{3}H]PD 140376 has been submitted elsewhere (49). Briefly, the final step in the synthesis involved a solution of the di-iodo precursor (20 mg, 0.024 mmol) in methanol (5 ml) being treated for 3 hr with tritium gas (10 Ci) in the presence of di-isopropylamine (0.1 ml) and 10% palladium on carbon (25 mg). After filtration, unreacted tritium was removed by repeated evaporation from ethanol. The product was purified by high performance liquid chromatography with elution with methanol/water/triethylamine, and the purity was confirmed by thin layer chromatography with elution with butan-1-ol/water/acetonitrile, to give tritiated PD 140376 with a specific activity of 51 Ci/mmol and a radiochemical purity of >98% (Fig. 1).

Materials. [^{3}H]PD 140376 was supplied to Parke-Davis as a custom synthesis by Amersham International, UK. ^{125}I-CCK$_{8S}$ (2200 Ci/mmol) and ^{125}I-gastrin (2200 Ci/mmol) were also supplied by Amersham.

The following drugs were obtained from external sources: CCK$_{8S}$, CCK$_{8US}$, and gastrin$_{1-17US}$ were obtained from Cambridge Research Biochemicals (UK); PG, CCK$_4$, and caerulein were obtained from Bachem (UK).

CI-988 [[*R*-(*R**,*R**)]-4-[[2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo[3.3.1.1^{3,7}]dec-2-yloxy)carbonyl]amino]propyl]amino]-1-phenylethyl]amino]-4-oxobutanoic acid], PD 140376 [L-3-[(4-aminophenyl)methyl]-*N*-[α-methyl-*N*-[(tricyclo[3.3.1.1^{3,7}]dec-2-yloxy)carbonyl]-D-tryptophyl]-β-alanine], and PD 140548 [*N*-[α-methyl-*N*-[(tricyclo[3.3.1.1^{3,7}]dec-2-yloxy)carbonyl]-L-tryptophyl-D-3-(phenylmethyl)-β-alanine] were all synthesized by the Department of Medicinal Chemistry, Parke-Davis Neuroscience Research Centre (Cambridge, UK). L-365,260 [(*R*)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-[1,4]benzodiazepin-3-yl)-*N*′-(3-methylphenyl)urea], devazepide [(*S*)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-[1,4]benzodiazepin-3-yl)-1*H*-indole-2-carboxamide], and LY 247348 [2-[2-(5-bromo-1*H*-indol-3-yl)-ethyl]-3-[3-(1-methylethoxy)phenyl]-4-(3*H*)-quinazolinone] were synthesized by the Department of Medicinal Chemistry, Parke-Davis Research Division (Ann Arbor, MI). Lorglumide was a gift from Rotta Pharmaceuticals (Italy).

Results

General binding characteristics of [^{3}H]PD 140376. Total, specific, and nonspecific binding of [^{3}H]PD 140376 increased linearly with the concentration of membrane protein, up to 2 mg/ml in the cortex and 0.4 mg/ml in the fundic mucosa (data not shown). At the final protein concentrations used routinely in the assay (between 0.1 and 0.2 mg/ml), the specific binding of [^{3}H]PD 140376 was between 60 and 70% in the cortex and between 50 and 60% in the fundic mucosa.

Kinetic studies. The binding kinetics of [^{3}H]PD 140376 appeared to follow those of a simple bimolecular reaction, with rates of both association and dissociation being monophasic after transformation of the specific binding data (Fig. 2). Specific [^{3}H]PD 140376 (0.2–0.5 nM) binding reached equilibrium within 30 min and was stable for up to 200 min. The rate of association (Fig. 2A) was transformed according to the pseudo-first-order rate equation because <10% of the total radioligand concentration was bound at equilibrium (48). The data were resolved into a single-exponential function from which the association rate constant, k_1, was calculated as 2.73 ± 0.37 × 10^8 M^{-1} min^{-1} (four experiments). The rate of dissociation of [^{3}H]PD 140376 was also resolved into a single-exponential function when the specific binding data were transformed according to the first-order rate reaction (Fig. 2B), from which the dissociation rate constant, k_{-1}, was calculated as 0.026 ± 0.002 min^{-1} (four experiments). The mean K_d determined from these studies was 0.10 ± 0.01 nM.

Saturation binding analysis. In both guinea pig cerebral cortex and fundic mucosal membranes, [^{3}H]PD 140376 demonstrated saturable, high affinity binding to an apparent single population of noninteracting sites when the nonspecific binding was defined as the amount of radioligand remaining in the presence of 1 μM unlabeled CCK$_{8S}$ (Fig. 3). The equilibrium dissociation constant (K_d) and maximal capacity of binding sites (B_{max}) obtained from the specific binding data were 0.11 ± 0.02 nM and 119 ± 15 fmol/mg of protein, respectively, in the cortex (four experiments) and 0.12 ± 0.01 nM and 296 ± 44 fmol/mg of protein, respectively, in the fundic mucosa (four experiments). In comparison, the K_d and B_{max} values for ^{125}I-BH-CCK$_{8S}$ in the cortex were 0.22 ± 0.05 nM and 137 ± 12 fmol/mg of protein (four experiments), respectively, and for ^{125}I-gastrin binding to fundic mucosa were 1.26 ± 0.19 nM and 175 ± 30 fmol/mg of protein (four experiments), respectively.

Competition studies. All unlabeled ligands, whether agonists or antagonists, produced a concentration-dependent inhibition of specific [^{3}H]PD 140376 binding to membranes prepared from both tissues. A close correlation was found between the rank order of potencies for agonist and antagonist inhibition of [^{3}H]PD 140376 binding in the cortex and the corresponding profiles in the fundic mucosa (Table 1). In both tissues, the rank order for agonists was CCK$_{8S}$ > caerulein > PG ≃ gastrin ≃ CCK$_{8US}$ ≫ CCK$_4$ and for the CCK$_B$/gastrin receptor-selective antagonists was PD 140376 > CI-988 ≃ L-365,260 ≫ LY 247348. The CCK$_A$ receptor-selective antago-

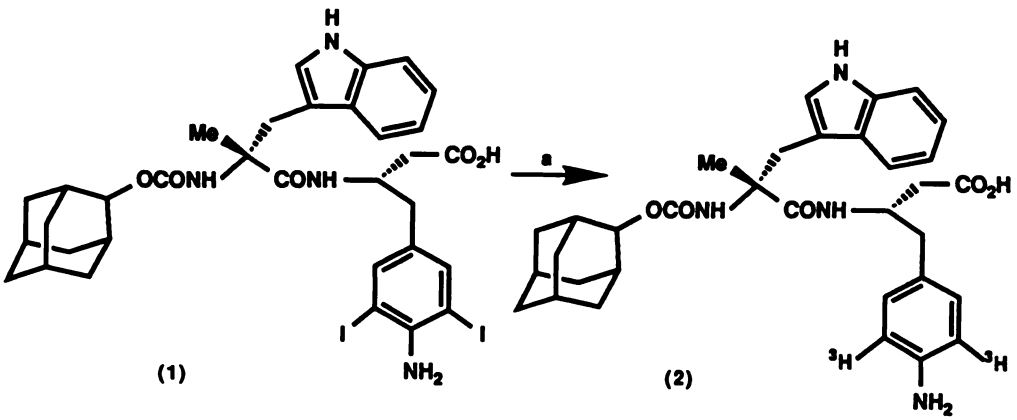

Fig. 1. Preparation of [^{3}H]PD 140376. Tritiated PD 140376 (2) was synthesized after treatment of a solution of the di-iodo precursor (1) in methanol with tritium gas in the presence of reagents di-isopropylamine and 10% palladium on carbon (*a*) (see Materials and Methods for details).

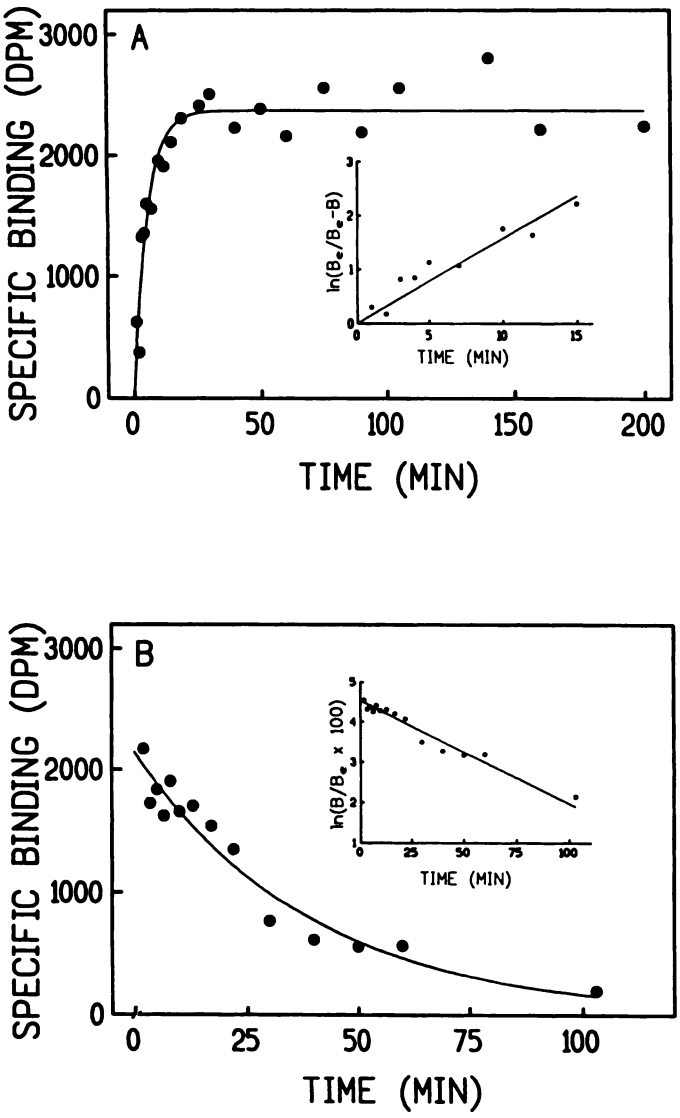

Fig. 2. Kinetic analysis of specific [^{3}H]PD 140376 (0.2 nM) binding to guinea pig cortical membranes at 25°. A, Time course of association of [^{3}H]PD 140376 binding. *Inset*, transformation of association data appropriate for pseudo-first-order conditions. B, Time course of dissociation of [^{3}H]PD 140376 binding initiated with 1 μM unlabeled CCK_{8S}. *Inset*, transformation of specific binding data according to the first-order dissociation rate equation. The data are representative of four such experiments performed as described in Materials and Methods.

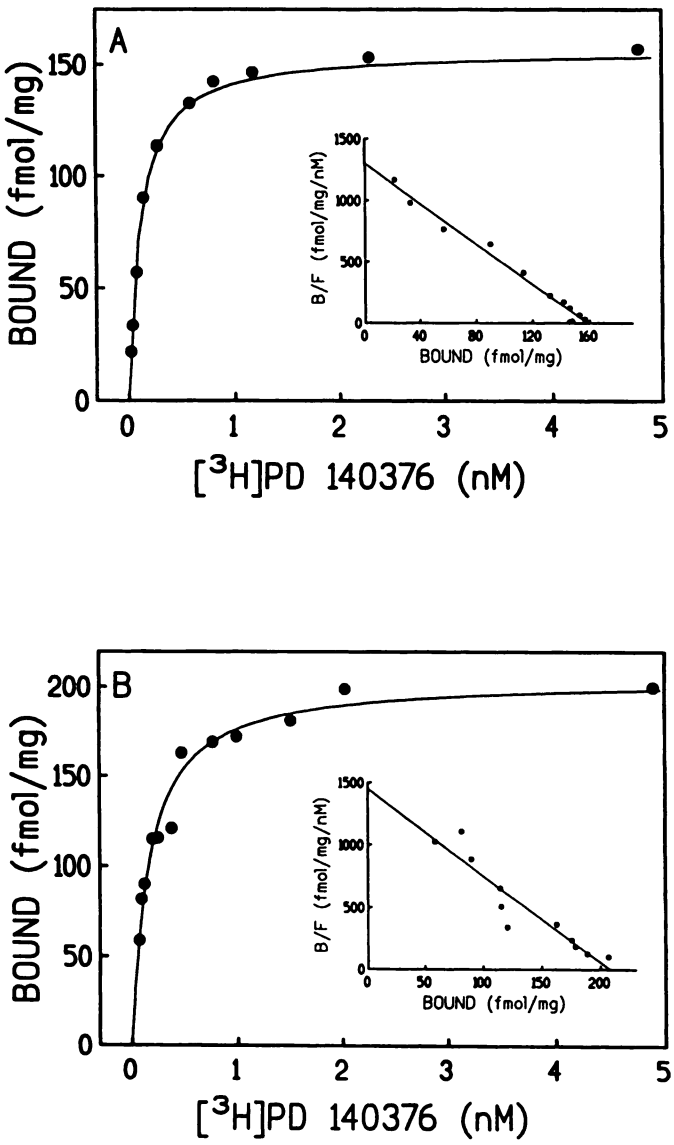

Fig. 3. Saturation analysis and Scatchard transformation (*inset*) of [^{3}H]PD 140376 binding to guinea pig cortical (A) and gastric fundic mucosa (B) membranes. Nonspecific binding to each tissue was defined with 1 μM unlabeled CCK_{8S}. Data shown are from a single representative experiment performed in duplicate. Similar results were obtained in four separate experiments. *B/F*, bound/free.

nists L-364,718, PD 140548, and lorglumide, irrespective of the radioligand, had considerably higher K_i values than did either CI-988 or L-365,260 (Table 1). In contrast, PD 140548 and lorglumide were equipotent with LY 247348, and L-364,718 had a consistently lower K_i value than did LY 247348 (Table 1).

Although the corresponding K_i values for each of the antagonists were again similar between tissues, with <2-fold variation, the potencies of the agonists were consistently higher in the gastric glands. In particular, the K_i values for PG, $gastrin_{1-17US}$, and CCK_4 were 5–10-fold more potent in the gastric tissue (Table 1).

The slope values for agonist, but not antagonist, inhibition of specific [^{3}H]PD 140376 binding were in general significantly lower than unity in both tissues, although the effect was more pronounced in the fundic mucosa (Table 2). Consistent with this observation, the presence of Gpp(NH)p (30 μM), a non-hydrolyzable analogue of GTP, caused a 7–8-fold decrease in apparent affinities of the agonist ligands CCK_{8S}, PG, and $gastrin_{1-17US}$ for [^{3}H]PD 140376-labeled sites in the gastric membranes, coupled with an increase in the individual Hill slopes for these agonists towards unity (Table 2). In comparison, whereas a similar qualitative effect was observed in the cortex, in that case there was only a nonsignificant 2-fold increase in agonist K_i values associated with the increase in the Hill slopes to unity. The net effect of Gpp(NH)p on agonist binding, however, was to almost completely eradicate the small differences observed between the K_i values obtained for the agonist ligands in the cortex and those obtained in the gastric mucosa. Gpp(NH)p (30 μM) had no effect on either K_i values or gradients of Hill plots for the unlabeled antagonist ligands CI-988 and PD 140376 (Table 2).

A close correlation was observed between the K_i values ob-

TABLE 1

K_i values for CCK receptor agonists and antagonists at CCK_B and gastrin receptors in guinea pig cerebral cortex and gastric mucosa membranes

Each value represents the geometric mean of the K_i, with the range given in parentheses, from four experiments. Values with no range represent the mean K_i from two experiments.

Compound	K_i			
	Guinea pig cerebral cortex		Guinea pig gastric mucosa	
	^{125}I-CCK_{8S}	[^{3}H]PD140376	[^{3}H]PD140376	^{125}I-Gastrin
	nM			
Agonists				
CCK_{8S}	0.82 (0.70–1.0)	0.65 (0.24–1.5)	0.18 (0.10–43)	0.24 (0.14–0.38)
CCK_{8US}	20 (12–29)	29 (23–36)	8.4 (6.4–13)	3.3 (0.7–6.1)
CCK_4	170 (120–260)	190 (130–340)	35 (26–46)	105 (40–169)
PG	6.5 (5.9–6.9)	22 (15–36)	2.0 (1.5–3.4)	2.6 (0.6–4.4)
Gastrin	15 (8.0–35)	25 (13–42)	3.2 (2.0–4.0)	0.89 (0.53–1.65)
Caerulein	2.4 (1.5–3.6)	2.6 (1.1–3.9)	0.81 (0.58–1.3)	NT[a]
Antagonists				
CI-988	1.7 (1.1–3.0)	0.97 (0.6–1.7)	0.82 (0.55–1.5)	1.1 (0.5–1.6)
PD 140376	0.35 (0.23–0.79)	0.15 (0.1–0.2)	0.22 (0.20–0.24)	0.42 (0.41–0.43)
L-365,260	1.5 (1.3–1.6)	1.8 (1.2–3.8)	2.0 (0.93–5.8)	11.2 (8.4–19.6)
LY 247348	670 (570–900)	610 (560–640)	340 (200–580)	NT
L-364,718	79 (62–110)	62 (52–71)	44 (34–67)	121 (90–158)
PD 140548	1100 (780–1600)	400 (230–690)	240 (200–290)	360 (280–570)
Lorglumide	760 (660–1000)	360 (270–540)	790	132

[a] NT, not tested in this assay.

TABLE 2

Influence of Gpp(NH)$_p$ on K_i values for CCK receptor agonist and antagonist inhibition of [^{3}H]PD 140376 binding in guinea pig cortex and gastric mucosa

Each value represents the geometric mean, with the range given in parentheses.

	Control		+ 30 μM Gpp(NH)$_p$		Ratio
	K_i	nH	K_i	nH	
	nM		nM		
Cortex					
CCK_{8S}	0.65 (0.24–1.5)	0.79	1.2 (0.7–2.8)	0.90	1.8
PG	22 (15–36)	0.67	30 (22–36)	1.00	1.4
Gastrin	24 (13–42)	0.74	27 (10–52)	0.75	1.1
CI-988	0.97 (0.61–1.7)	0.98	1.15 (0.72–2.1)	1.35	1.2
L-365,260	1.8 (1.2–3.8)	1.18	1.2 (0.9–1.5)	1.25	0.7
Gastric glands					
CCK_{8S}	0.18 (0.10–0.43)	0.82	1.2 (1.0–1.3)	0.90	6.7
PG	2.0 (1.5–3.4)	0.66	15 (13–19)	1.00	7.5
Gastrin	3.2 (2.0–4.0)	0.65	25 (15–36)	0.89	7.8
CI-988	0.82 (0.55–1.50)	1.16	0.77 (0.22–1.3)	1.13	0.9
L-365,260	2.0 (0.9–5.8)	0.81	2.2 (1.2–3.3)	1.02	1.1

tained for the unlabeled agonists and antagonists at [^{3}H]PD 140376-labeled sites and those obtained at either ^{125}I-CCK_{8S}- or ^{125}I-gastrin-labeled sites in the cerebral cortex or fundic mucosa, respectively (Table 1). Slope values, however, for agonist-mediated inhibition of ^{125}I-CCK_{8S} and ^{125}I-gastrin$_{1-17US}$ binding were always not significantly different from unity (data not shown).

Removal of sodium ions from the normal assay incubation buffer caused, in general, a characteristic increase in the apparent affinities for the agonist ligands CCK_{8S} and gastrin (Table 3). In comparison, whereas sodium had little effect on the binding of PD 140376, its removal from the assay buffer caused either a 3–4-fold increase or 4–5-fold decrease in the K_i values for L-365,260 and CI-988, respectively, in both cerebral cortex and gastric mucosa (Table 3).

In addition to the single, high affinity, [^{3}H]PD 140376-labeled site observed when the nonspecific binding was defined using unlabeled CCK_{8S}, both PD 140376 and CI-988, at concentrations above 1 μM, further inhibited [^{3}H]PD 140376 binding to below the level defined by CCK_{8S}. This additional low affinity site was only recognized by the peptoid ligands and accounted for approximately 10% of the specific [^{3}H]PD 140376 binding under these conditions (data not shown).

Discussion

PD 140376 is a novel ligand from a peptoid series of compounds that have been shown to interact with high affinity and selectivity at the CCK_B/gastrin receptor, where the main pharmacological function has been demonstrated as a competitive antagonism of CCK_{8S} and/or gastrin$_{1-17}$ and related analogues (1, 6, 27, 33, 34). In its radiolabeled form, [^{3}H]PD 140376 was found to bind to a single population of noninteracting CCK-sensitive sites. The relatively high affinity of the previously described CCK_B or gastrin receptor-selective agonists PG, gastrin$_{1-17US}$, and CCK_{8US} (6) and the antagonists CI-988 (6) and L-365,260 (23) and the relatively lower potency of the CCK_A-selective antagonists devazepide (22), PD 140548 (39), and lorglumide (38) were indicative of [^{3}H]PD 140376 labeling CCK_B or gastrin sites in the guinea pig cerebral cortex and fundic mucosa. This was confirmed by the close correlation observed between the rank order of potencies and absolute affinities of the unlabeled compounds for ^{125}I-CCK_{8S}-labeled sites in the cortex and ^{125}I-gastrin$_{1-17US}$-labeled sites in the fundic mucosa and the respective [^{3}H]PD 140376 sites in each tissue. The much lower than expected potency of the quinazolinone CCK_B/gastrin antagonist LY 247348, relative to previously reported affinity estimates of 9 nM in mouse cortex (42), would appear to simply reflect a species differences that is restricted to this chemical series. The CCK_B/gastrin affinities of either the peptoids or benzodiazepine derivatives are not markedly altered between rodent species (5, 6, 42).

Recent evidence has been provided in the literature to suggest that the CCK_B receptor in the central nervous system may be different from the gastrin receptor present in peripheral tissues such as the stomach or pancreas (3, 20, 50). Indeed, there is a

TABLE 3

Effect of sodium ions on K_i values derived for specific CCK receptor ligands at ^{125}I-BH-CCK$_{8s}$-labeled sites in the guinea pig gastric mucosa and cerebral cortex

Each value represents the geometric mean, with the range in parentheses, of between three and 11 separate experiments.

Drug	Specific ^{125}I-BH-CCK$_{8s}$ binding (K_i)			
	Guinea pig gastric mucosa		Guinea pig cerebral cortex	
	+Na$^+$	−Na$^+$	+Na$^+$	−Na$^+$
	nM			
CCK$_{8s}$	0.37 (0.09–1.21)	0.22 (0.19–0.26)	0.80 (0.67–1.02)	0.13 (0.09–0.17)
Gastrin	4.56 (3.22–6.68)	0.33 (0.31–0.37)	17.9 (12.5–23.0)	3.70 (1.89–7.94)
CI-988	2.45 (1.13–5.01)	0.41 (0.31–0.61)	1.46 (0.94–3.03)	0.31 (0.29–0.35)
PD 140376	1.14 (0.75–1.38)	0.86 (0.69–1.28)	0.14 (0.10–0.17)	0.12 (0.09–0.17)
L-365,260	2.03 (1.32–3.71)	7.95 (6.51–9.01)	1.65 (1.31–2.90)	5.42 (4.00–7.36)

disproportionate abundance of CCK$_{8s}$ and CCK$_4$ in the brain, in comparison with the relative paucity of gastrin in its various forms (51, 52), so it is not unreasonable to suggest that the sites in the brain may represent a distinct type (CCK$_B$) of CCK receptor. Conversely, it is possible that the sites found in the gastric mucosa may represent a closely related gastrin receptor. The present study, however, by using the radiolabeled form of PD 140376, an antagonist with binding that is unaffected by manipulation through the use of, for example, monovalent cations or guanyl nucleotides, has shown that the sites in the cortex and fundic mucosa remain pharmacologically indistinguishable.

This conclusion is supported by the recent evidence provided by the molecular cloning of the CCK$_B$/gastrin receptor from human brain and gastric fundus (53). That study found that the primary amino acid sequences deduced from the cloned receptor cDNA from both areas were identical (53). It is also consistent with the only previous study of CCK$_B$/gastrin receptors to use an antagonist radioligand, [^{3}H]L-365,260 (5), but is in contrast to most other attempts to differentiate brain and peripheral CCK$_B$/gastrin binding sites, which have relied on the use of agonist radioligands to make comparisons between the affinities of competing agents. The data presented in this study reveal clear differential sensitivity to guanyl nucleotides of agonist binding to cortical and fundic mucosa receptors. Consequently, apparent differences in binding affinity measured in the absence of guanyl nucleotides, as were observed for the agonist ligands PG, CCK$_8$, and gastrin and which might be taken to reflect different receptors, were abolished when the nucleotide was included in the assay medium. This serves to highlight once again the caution that is required when discriminating receptor subtypes using agonist radioligands, which may reflect only the selective labeling of high affinity states of the receptor.

Because [^{3}H]PD 140376 is an antagonist radioligand, it should be possible for it to recognize low affinity "ground" states of the CCK$_B$/gastrin receptor in addition to the high affinity sites labeled by agonists such as ^{125}I-CCK$_{8s}$ and ^{125}I-gastrin$_{1-17}$ (4, 12, 54). In this respect, it was interesting that the binding of PD 140376, as an antagonist, was not affected by the presence of monovalent cations or guanine nucleotide analogues, whereas sodium ions caused either a reduction or an increase in the affinity of CI-988 and L-365,260, respectively, with the latter effect being consistent with a previous observation with this ligand (5).

Moreover, the lack of effect of the stable GTP analogue Gpp(NH)p on the affinity of PD 140376 and CI-988 was consistent with the functional studies showing peptoid compounds to be antagonists (6, 27, 33, 34, 46). In comparison with the cortex, the greater degree of sensitivity of the agonist-mediated inhibition of [^{3}H]PD 140376 binding in the fundic mucosa to the effect of Gpp(NH)p was also consistent with previous studies that provided evidence to suggest that the post-receptor coupling mechanism for gastrin sites in the stomach may be different from that observed for CCK$_B$ sites in the brain (55, 56). Thus, the larger and more consistent GTP shifts in agonist potency have generally appeared to be associated with those types of receptors coupled through a guanine nucleotide-binding protein principally to second messenger systems involving the production of either cAMP or inositol phosphates. In this respect, pancreatic CCK$_A$ receptors (57, 58) and gastrin sites in the gastric mucosa (55, 56) have been reported to be linked to phosphoinositidase C and the production of inositol phosphates, whereas as yet there is no conclusive evidence to support the existence of any such second messenger system being coupled to the CCK$_B$ sites in the brain.

[^{3}H]PD 140376 can be distinguished from the only other previously described radiolabeled antagonist for the CCK$_B$/gastrin receptor, the benzodiazepine derivative [^{3}H]L-365,260 (5), on the basis of possessing a much higher affinity and selectivity, relative to CCK$_A$ sites. In addition, the combination of high subnanomolar affinity and high specific activity for a tritiated radioligand achieved by the introduction of two atoms of tritium into the phenyl ring is exemplified by a high reproducibility of specific binding as a consequence of low background levels of nonspecific binding even at concentrations well in excess of the K_d for [^{3}H]PD 140376. These properties should be particularly advantageous for autoradiographic studies, in which a prime concern may be the detection of limited populations of CCK$_B$/gastrin sites in, for example, subnuclei of the brain. Indeed, to reduce nonspecific binding of [^{3}H]L-365,260 to an acceptable level we have found it necessary to perform the experiments at 4°,[2] rather than at 25° as described in Ref. 5.

In addition to the high affinity population of CCK-sensitive sites, [^{3}H]PD 140376 also labeled a low affinity site recognized only by the peptoid ligands CI-988 and PD 140376 and not by any of the peptide ligands, the benzodiazepine derivative L-365,260, or the quinazolinone LY 247348 (data not shown). The contribution of these sites corresponded to a level of

[2] D. R. Hill, personal communication.

binding insufficient to allow for any further definitive examination. They are, however, clearly not related to any CCK/gastrin receptors and appear to be peculiarly associated with just the peptoid ligands. They may well represent the 'specific nonspecific' binding sites that are observed with many radioligands including [^{3}H]L-365,260.

In conclusion, [^{3}H]PD 140376 is a novel, highly potent, and selective antagonist radioligand for the CCK$_B$ or gastrin receptor that will be an important tool for use in future studies on the role of the CCK/gastrin family of neuropeptides in the pathophysiology of major brain dysfuctions.

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Send reprint requests to: J. C. Hunter, Department of Biology, Parke-Davis Neuroscience Research Centre, Addenbrookes Hospital Site, Hills Road, Cambridge CB2 2QB, UK.