0026-895X/92/061089-07$03.00/0

MOLECULAR PHARMACOLOGY, 41:1089-1095

[^{3}H]pBC 264, a Suitable Probe for Studying Cholecystokinin-B Receptors: Binding Characteristics in Rodent Brains and Comparison with [^{3}H]SNF 8702

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Received August 15, 1991; Accepted March 23, 1992

SUMMARY

[^{3}H]Propionyl-Tyr-(SO_3H)-gNle-mGly-Trp-(NMe)Nle-Asp-Phe-NH_2 ([^{3}H]pBC 264) (98–100 Ci/mmol), a new peptidase-resistant cholecystokinin (CCK) agonist that is 1000-fold more potent for CCK-B than for CCK-A receptors, interacts, with a similar subnanomolar affinity, with a single class of binding sites (K_d, 0.15–0.2 nM) in brain membranes of mouse, rat, guinea pig, and cat, in Tris and Krebs buffers. The concentration of CCK-A receptors in rodent brain was estimated to be 8–10 fmol/mg of protein, by measurement of the B_{max} values of the nonselective agonist [^{3}H] propionyl-CCK_8, with or without 10 nM pBC 264 to saturate CCK-B sites. In guinea pig and mouse brain, specific [^{3}H]pBC 264 binding was not affected by NaCl and/or guanyl-5′-yl-imidodiphosphate. In contrast, in rat brain the affinity of [^{3}H]pBC 264 was decreased and the maximal number of binding sites was increased by NaCl and the guanyl nucleotide or by alkaline treatment, suggesting that a proportion of CCK-B receptors are linked to guanine nucleotide-binding proteins. The B_{max} of a CCK_8 analog, [^{3}H]SNF 8702, was higher (57 fmol/mg of protein) than that of [^{3}H]pBC 264 (40 fmol/mg of protein) in guinea pig brain cortex but not in mouse brain. The relative potencies of various analogs differed among species. The CCK-B antagonist L365,260 was 18-, 30-, and 64-fold less potent than [^{3}H]pBC 264 in guinea pig, mouse, and rat, respectively. PD 134308, a CCK-B antagonist, was 20-fold less potent in rat brain than in guinea pig brain. Likewise, the cyclic analog BC 254 displayed a 30- and 60-fold lower affinity for mouse and rat than for guinea pig brain preparations. Together, these results suggest the presence of CCK-B receptor subtypes. In all tissues, the specific binding of [^{3}H]pBC 264 at its K_d values was very high (75–90%) and higher than that of the hydrophobic CCK-B probe [^{3}H]SNF 8702 (~50%). Therefore, unlike [^{3}H]SNF 8702, [^{3}H]pBC 264 can be used to study preparations with low receptor concentrations, such as rat brain, making this radiolabeled molecule the most appropriate ligand available to date for CCK-B receptor studies.

The octapeptide CCK_8, which derives from the large precursor preprocholecystokinin, constitutes the major CCK fragment in mammalian brain (1, 2). CCK_8 is widely distributed in different brain areas, where it appears to be released from neurons through a classical neurotransmitter mechanism (for review, see Ref. 3). CCK_8 has a similar nanomolar affinity for CCK-A sites (or peripheral receptor type), found in the gastrointestinal tract and in a few regions of the brain (4–6), and CCK-B sites (or central receptor type), largely distributed in the central nervous system (7–12). The role of CCK_8 in the brain is still unknown, although anxiolytic effects resulting from blockade of CCK-B receptors by the selective CCK-B antagonists L-365,260 and PD 134308 have recently been reported (13, 14). This suggests that stimulation of CCK-B receptors by endogenous CCK peptides could induce anxiogenic responses.

Therefore, a highly selective and potent radiolabeled CCK-B agonist is required to characterize brain CCK-B receptors fully and to relate the occupancy of these binding sites to pharmacological responses. The recently developed highly selective and peptidase-resistant CCK-B agonist BC 264 (15) has been used in various electrophysiological and behavioral experiments to study the role of CCK-B receptors (16–18). An analog

This study was supported by Rhône Poulenc Rorer. M.R.-G. is a recipient of a European Community Fellowship.

ABBREVIATIONS: CCK_8, Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2 (CCK_{26-33}); CCK_8NS, nonsulfated CCK_8; CCK_5, CCK_{29-33}; CCK_4, CCK_{30-33}; BC 264, Boc-Tyr(SO_3H)-gNle-mGly-Trp-(*N*-Me)Nle-Asp-Phe-NH_2; BC 254, Boc-γ-D-Glu-Tyr(SO_3H)Nle-D-Lys-Trp-Nle-Asp-Phe-NH_2; pCCK_8, propionyl-CCK_8; L-364,718, (3*S*)-(−)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-1*H*-indole-2-carboxamide; L-365,260, (3*R*)-(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-3-methylphenylurea; SNF 8702, Asp-Tyr-(*N*-Me)Nle-Gly-Trp-(*N*-Me)Nle-Asp-Phe-NH_2; PD 134308, [*R*-(*R**,*R**)]-4-[[2-[[(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-phenyl-ethyl]amino]-4-oxobutanoic acid; Gpp(NH)p, guanyl-5′-yl-imidodiphosphate; App(NH)p, adenyl-5′-yl-imidodiphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; G protein, guanine nucleotide-binding protein; CCK, cholecystokinin; pBC 264, propionyl-BC 264.

of BC 264 with a propionyl protecting group in place of the *N-tert*-butyloxycarbonyl group, pBC 264, was, therefore, synthesized and shown to be also highly potent and selective (K_I for CCK-A, 62 nM; K_I for CCK-B, 0.06 nM; selectivity ratio, 1038) (19) and able to cross the blood-brain barrier (20). Its tritiated derivative, [^{3}H]pBC 264, was proposed in a preliminary report as the first selective radiolabeled CCK-B agonist for receptor studies (19).

In this paper, we describe the properties of [^{3}H]pBC 264 binding to mouse brain membranes under different conditions and the determination of the apparent affinities of various selective and nonselective agonists and antagonists. Several studies have suggested that there are differences in the binding properties and distribution of CCK-B binding sites among closely related animal species (21–26); therefore, the biochemical characteristics of the binding sites labeled by [^{3}H]pBC 264 have been compared in mouse, rat, guinea pig, and cat.

Recently, a new selective CCK-B radioligand, [^{3}H]SNF 8702, has been reported but, in addition to its described nonoptimal properties for binding studies, it has not yet been established whether it is a full or a partial agonist (27). We have, therefore, compared binding characteristics of this ligand with those obtained using [^{3}H]pBC 264 or the nonselective agonist [^{3}H]pCCK$_8$, in rodent brains.

Experimental Procedures

Chemicals. CCK$_8$ and analogs were synthesized in the laboratory. The cyclic agonist BC 254 and BC 264 were prepared as described (15, 28). L364,718, L365,260, and PD 134308 were synthesized following the previously reported methods (29–31). [^{3}H]pBC 264 (98–100 Ci/mmol) was obtained as described (19). [^{3}H]pCCK$_8$ (60 Ci/mmol) was purchased from Amersham. [^{3}H]SNF 8702 (55 Ci/mmol) was purchased from NEN. Gpp(NH)p, App(NH)p, GTP, GDP, and GMP were from Boehringer Mannheim. HEPES, Tris, bovine serum albumin, and bacitracin were obtained from Sigma Chemical Co., (St Louis, MO). Stock solutions (10^{-3} M) were prepared in distilled water, with prior addition of ethanol (10%) when necessary or of dimethylformamide (50%) for L364,718, L365,260, and PD 134308.

Preparation of crude membranes. Sprague-Dawley rats (140–180 g) (Depré, Saint-Doulchard, France), Swiss mice (25–30 g) (Depré), and guinea pigs (300 g) (Ruvel, Thiron-Gardais, France) were killed by decapitation, and their brains quickly were removed. Cat cortex was a generous gift from Dr. André Bado, U 10 INSERM, France). Brain cortices of rats, guinea pigs, and cat and whole brain (minus cerebellum) of mice, guinea pigs, and rats were homogenized (12 ml/g of tissue wet weight) at 4° in 50 mM Tris·HCl buffer, pH 7.4, containing 5 mM $MgCl_2$ (except when indicated). The homogenate was centrifuged at 4° for 35 min at 100,000 × *g*, and the resulting pellet was rehomogenized in a large excess of ice-cold buffer and centrifuged under the same conditions. The final pellet was resuspended in 50 mM Tris·HCl buffer, pH 7.4, supplemented with 0.2 mg/ml bacitracin and 5 mM $MgCl_2$ (6–7 mg of protein/ml). Membranes were also prepared under the same conditions using a Krebs-HEPES buffer (118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM HEPES, pH 7.4). The homogenate was used immediately for binding assays. Protein concentration was determined by the method of Lowry *et al.* (32), using bovine serum albumin standards.

Binding assays. The binding assays were performed in 50 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, 0.2 mg/ml bacitracin. Some saturation studies were carried out using Krebs-HEPES buffer supplemented with 0.2 mg/ml bacitracin. Each assay contained, in a final volume of 1 ml, the membrane preparation (0.6–0.7 mg of protein) and the tritiated ligand. The nonspecific binding was determined in the presence of 1 μM CCK$_8$. Competition experiments were performed using 0.2 nM [^{3}H]pBC 264, in the presence of eight to ten different concentrations of the competitor. Incubations (60 min at 25° for [^{3}H]pBC 264 and [^{3}H]pCCK$_8$ and 90 min for [^{3}H]SNF 8702, at which time steady state conditions were reached) were terminated by filtration through Whatman GF/B filters that had been precoated by incubation in buffer containing 0.1% (w/v) bovine serum albumin. The filters were rinsed with 2 × 5 ml of ice-cold buffer, and the radioactivity was counted. For the experiments monitoring the association of 0.2 nM [^{3}H]pBC 264, the reaction was terminated by immediate filtration of the preparation (1 ml) at different times. For dissociation experiments, the radioligand (0.2 nM) was incubated for 60 min with membranes. Dissociation was initiated by addition of CCK$_8$ (final concentration, 1 μM), and the residual binding was measured at given times by filtration and counting. In some experiments, fresh membranes from rat brain cortex were diluted 20-fold with ice-cold 50 mM sodium phosphate buffer, pH 11.5, in the presence of 1 mM EDTA. This treatment has been shown to eliminate the guanyl nucleotide-binding component activity of the membrane (33–35). After a 45-min incubation on ice with intermittent vortex-mixing, the suspension was centrifuged at 100,000 × *g* for 35 min and resuspended in 50 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, 0.2 mg/ml bacitracin, in half the previous volume, for binding assays.

Data analysis. The dissociation constant (K_d) and the maximal number of binding sites (B_{max}) for the radioligands were determined from Scatchard analysis of saturation isotherms. In competition experiments, the concentrations of the analogs that decreased binding of the radioligand by 50% (IC_{50}) were obtained according to the Hill equation. The K_i value was calculated from the Cheng-Prusoff equation, assuming a competitive inhibition, $K_i = IC_{50}/(1 + L/K_d)$. Dissociation rate constants were determined by plotting $\ln[RL/RL_{eq}]$ versus time, with RL_{eq} and RL being the amounts of the radioligand specifically bound at equilibrium and at a given time, respectively. The slope of this plot is equal to $-k_{-1}$. The apparent on-rate constants ($k_{+1_{app}}$) were calculated as the slope of the plot $\ln[RL_{eq}/(RL_{eq} - RL)]$ versus time, according to the equation $\ln[RL_{eq}/(RL_{eq} - RL)] = k_{+1_{app}} \cdot t$, where $k_{+1_{app}} = (k_{+1} \cdot L) + k_{-1}$. All values are the mean ± standard error of at least three determinations, performed in triplicate.

Results

Binding parameters of [^{3}H]pBC 264. The specific binding of [^{3}H]pBC 264 increased linearly with protein concentrations of up to 1.15 mg/assay, in all species. Steady state conditions were established from the association kinetics of 0.2 nM [^{3}H]pBC 264, measured at 25°, using brain cortex membranes of guinea pig and whole brain minus cerebellum for mouse and rat. In each case, equilibrium was reached in 40–50 min, with an apparent pseudo-first order rate constant, $k_{+1_{app}}$, of 0.053 min^{-1} for guinea pig, 0.064 min^{-1} for mouse, and 0.076 min^{-1} for rat. An incubation time of 60 min was, therefore, selected for all further binding experiments. The calculated association rate constant in mouse brain, k_{+1}, was 3.3 ± 0.6 × 10^6 sec^{-1} M^{-1}. The dissociation curve of [^{3}H]pBC 264 was very slow ($t_{1/2}$ = 72 min) (Fig. 1). Assuming a monophasic dissociation, the off-rate constant was calculated to be 1.48 ± 0.25 × 10^{-4} sec^{-1}. The kinetically derived dissociation constant ($K_d = k_{-1}/k_{+1}$) was 0.45 × 10^{-10} M.

The binding of [^{3}H]pBC 264 to mouse brain, using concentrations between 4 × 10^{-11} and 1.7 × 10^{-9} M, in Tris·HCl buffer, was saturable (Fig. 2). Scatchard analysis of several binding isotherms showed a single class of binding sites characterized by the following parameters: K_d = 0.15 ± 0.01 nM and B_{max} = 29.9 ± 0.4 fmol/mg of protein (Fig. 3). The K_d value is slightly higher than the dissociation constant determined from kinetic experiments.

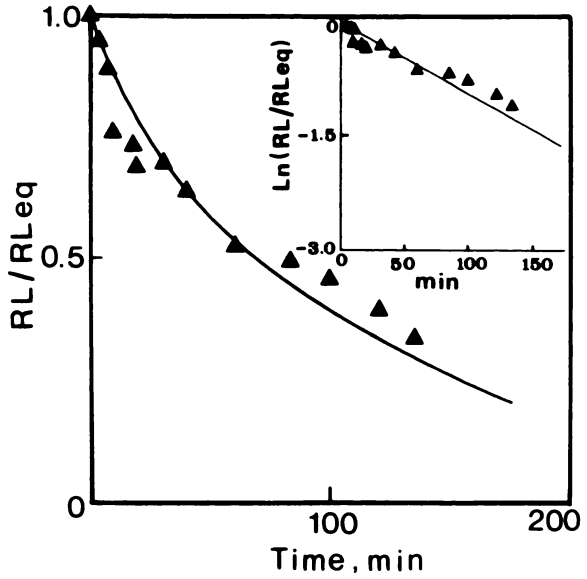

Fig. 1. Dissociation of 0.2 nM [^{3}H]pBC 264 from mouse brain membranes at 25°. The points shown were obtained in a single representative experiment, performed in triplicate. *Inset*, plot of the dissociation of the specific binding, in the same experiment, according to the first-order kinetic equation, as described in Experimental Procedures.

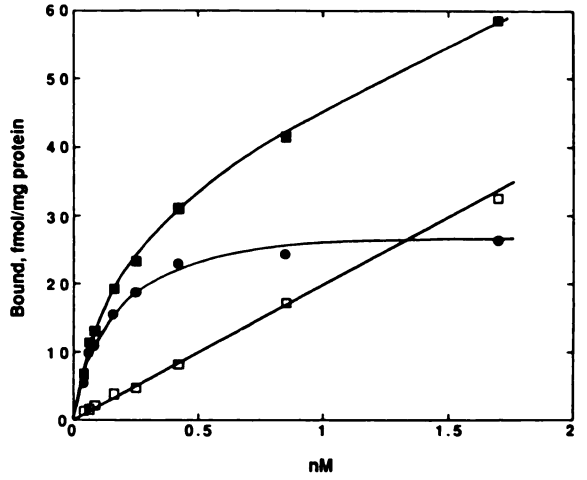

Fig. 2. Binding of [^{3}H]pBC 264 in mouse brain as a function of radioligand concentrations ranging from 0.04 to 1.7 nM. The points shown are from one experiment performed in triplicate. This experiment was replicated three times, with similar results. ■, Total binding; □, nonspecific binding; ●, specific binding, determined as the difference between total and nonspecific binding. Nonspecific binding was defined using 1 μM CCK$_8$.

The binding parameters of [^{3}H]pBC 264 were found to be identical, (K_d = 0.17 ± 0.02 nM, B_{max} = 31.6 ± 2.5 fmol/mg of protein) when Tris·HCl buffer was replaced by the rather more physiological Krebs-HEPES buffer. Hill plots of the [^{3}H]pBC 264 binding data gave Hill coefficients of 0.96 and 1.03 in Tris·HCl and Krebs-HEPES buffers, respectively, suggesting the absence of positive or negative cooperativity.

[^{3}H]pBC 264 also interacted with high affinity with a single class of binding sites in brain cortex membranes of rat (K_d = 0.24 ± 0.02 nM, B_{max} = 22.0 ± 1.6 fmol/mg of protein), guinea pig (K_d = 0.15 ± 0.01 nM, B_{max} = 39.8 ± 2.7 fmol/mg of protein) (Table 1), and cat (K_d = 0.70 ± 0.08 nM, B_{max} = 20 ± 1.1 fmol/mg of protein). The Hill coefficients were close to unity in each experiment.

When it was used in routine experiments at concentrations

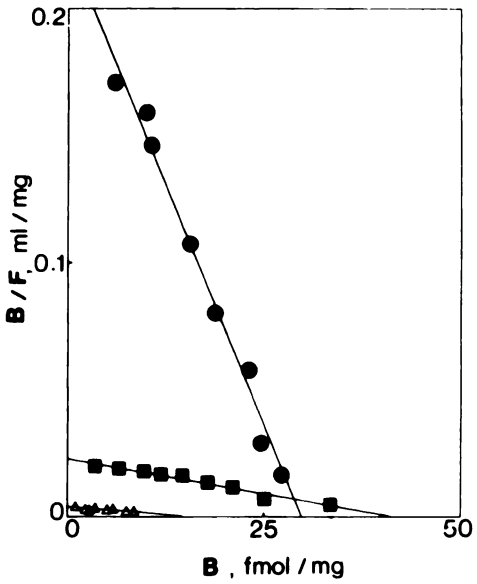

Fig. 3. Scatchard plots of specific binding, to mouse brain membranes, of [^{3}H]pBC 264 (●), [^{3}H]pCCK$_8$ (■), and [^{3}H]pCCK$_8$ (△) in the presence of 10 nM pBC 264. The data shown are from a single representative experiment, with each point in triplicate. For each ligand, the nonspecific binding was determined using 1 μM CCK$_8$.

around its K_d value (0.2 nM), the specific binding of [^{3}H]pBC 264 was very high in all species, representing 85% of the total binding in mouse brain, 75–80% in rat brain, and around 90% in guinea pig brain.

Evaluation of CCK-A and CCK-B sites by comparing [^{3}H]pBC 264 and [^{3}H]pCCK$_8$ binding. The binding capacities measured with [^{3}H]pBC 264 were then compared with those determined, under the same experimental conditions (Table 1), with [^{3}H]pCCK$_8$, which has a similar affinity, in the nanomolar range, for CCK-A and CCK-B rat receptors (16). The affinity of [^{3}H]pCCK$_8$ varied among species, from 0.2 nM for guinea pig brain to 1.5 nM for mouse and rat brain.

In brain cortex of guinea pig and rat, the maximal numbers of binding sites determined were similar for both ligands, 22.0 versus 20.9 fmol/mg of protein in rat and 39.8 versus 42.0 fmol/mg of protein in guinea pig with [^{3}H]pBC 264 and [^{3}H]pCCK$_8$, respectively. Under these conditions, only one class of binding sites could be detected with either ligand.

In contrast, in the whole brain of mouse, rat, and guinea pig, the maximal number of binding sites for [^{3}H]pBC 264 was significantly lower ($p < 0.05$) than the maximal number of binding sites determined using [^{3}H]pCCK$_8$, 29.9 versus 40.6 fmol/mg of protein in mouse, 17.2 versus 26.0 fmol/mg of protein in rat, and 22.3 versus 30.1 fmol/mg of protein in guinea pig with [^{3}H]pBC 264 and [^{3}H]pCCK$_8$, respectively.

In order to evaluate the binding parameters of CCK-A sites in mouse brain, saturation studies were performed using [^{3}H]pCCK$_8$ as a nonselective ligand, in the presence of 10 nM pBC 264, a concentration estimated from the law of mass action to bind about 98.5% of CCK-B sites. Under these conditions, [^{3}H]pCCK$_8$ interacted apparently with one class of binding sites, with a K_d of 3.8 ± 1.3 nM (of the same order of magnitude as that with [^{3}H]pCCK$_8$ alone) and a B_{max} of 11.4 ± 0.8 fmol/mg of protein (Fig. 3). Nevertheless, given the low concentration of CCK-A binding sites, the range of error in the determination of binding parameters was relatively high, with the standard deviation of the error of the raw data (36) reaching 30%.

TABLE 1

Comparison of B_{max} values obtained with [^{3}H]pBC 264, [^{3}H]pCCK$_8$, and [^{3}H]SNF 8702 in crude brain membrane fractions of different species

Results are the mean ± standard error of three or four determinations performed in triplicate.

Tissue	[^{3}H]pBC 264		[^{3}H]pCCK$_8$		[^{3}H]SNF 8702	
	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}
	nM	*fmol/mg of protein*	*nM*	*fmol/mg of protein*	*nM*	*fmol/mg of protein*
Mouse brain	0.15 ± 0.01	29.9 ± 0.4	1.50 ± 0.16	40.6 ± 3.0[a]	0.47 ± 0.03	31.4 ± 0.4
Rat cortex	0.24 ± 0.02	22.0 ± 1.6	1.25 ± 0.06	20.9 ± 1.1[b]	ND[c]	
Rat brain	0.29 ± 0.05	17.2 ± 0.8	1.48 ± 0.17	26.0 ± 1.0[a]	ND[c]	
Guinea pig cortex	0.15 ± 0.01	39.8 ± 2.7	0.18 ± 0.03	42.0 ± 3.8[b]	0.80 ± 0.10	57.2 ± 4.9[a]
Guinea pig brain	0.27 ± 0.02	22.3 ± 0.8	0.31 ± 0.01	30.1 ± 1.8[a]	NI[d]	

[a] $p < 0.05$, compared with values obtained with [^{3}H]pBC 264 (Student's *t* test).
[b] Data from Ref. 24.
[c] ND, not determined, due to the too high nonspecific binding.
[d] NI, not investigated.

Comparison between the binding parameters of [^{3}H]pBC 264 and [^{3}H]SNF 8702. The binding parameters of [^{3}H]pBC 264 were also compared with those obtained with [^{3}H]SNF 8702. Unfortunately, due to the low amount of total binding and the very high proportion of nonspecific binding obtained with [^{3}H]SNF 8702 in rat tissues, its affinity could not be determined in this species. In mouse brain and guinea pig brain cortex, the K_d values were 0.47 and 0.8 nM, respectively (Table 1). At these concentrations, the specific binding was around 50–55%. The maximal number of binding sites was 31.4 fmol/mg of protein in mouse and 57.2 fmol/mg of protein in guinea pig, which is significantly higher ($p < 0.05$) than values obtained with [^{3}H]pBC 264 (Table 1) or with [^{3}H]pCCK$_8$. Nevertheless, an accurate determination of the B_{max} value with [^{3}H]SNF 8702 was difficult, due to the low specific binding (only 17% at 77% saturation), leading to large standard deviations and preventing experiments from being carried out at concentrations approaching saturation. In contrast, under the same conditions [^{3}H]pBC 264 displayed 60% specific binding at 93% saturation.

Effect of ions, nucleotides, and high pH treatment. The effects of metal ions and of the stable GTP analog Gpp(NH)p on the specific binding of [^{3}H]pBC 264 were also investigated (Table 2). In the absence of magnesium, specific binding was significantly decreased in all species, especially in rat brain membranes, where it represented only 34%, compared with control. The binding of 0.2 nM [^{3}H]pBC 264 was not modified or was only very slightly modified by 100 mM NaCl in the three species studied. Gpp(NH)p (10 μM) had no effect in guinea pig and mouse brain and gave a slight but not significant inhibition in rat brain preparations (10%). The association of NaCl and Gpp(NH)p led to a significant decrease (~40%) of specific binding in the rat brain. Specific binding of [^{3}H]pBC 264 in rat brain membranes was also significantly reduced by 0.1 mM GTP and GDP (Table 3), whereas GMP and App(NH)p were without effect. To clarify the effects of ions and nucleotides on CCK-B binding sites, saturation studies were performed under different conditions, using rat brain cortex (Table 4). Scatchard analysis indicated that 10 μM Gpp(NH)p resulted in a nonsignificant 1.7-fold decrease in the affinity of [^{3}H]pBC 264 and no effect on the maximal number of binding sites. A nonsignificant 1.5-fold decrease in the affinity of [^{3}H]pBC 264 was also observed in the presence of 100 mM NaCl, but there was a 40% increase ($p < 0.05$) in the maximal number of binding sites. In the presence of NaCl, Gpp(NH)p led to a significant decrease in affinity (2.3-fold), compared with NaCl alone, without significantly affecting the maximum number of binding sites. After pretreatment of rat brain membranes at pH 11.5, the affinity of [^{3}H]pBC 264, in the presence of NaCl, was significantly reduced (2.8-fold), compared with membranes maintained at pH 7.4, and the B_{max} was significantly increased. Gpp(NH)p had no effect on the affinity of [^{3}H]pBC 264 with membranes pretreated at pH 11.5.

Pharmacological profile. The apparent affinities of CCK$_8$ and various analogs for [^{3}H]pBC 264 binding sites in different species are summarized in Table 5. The K_i values determined for pBC264 were identical to the equilibrium dissociation constants computed from saturation experiments and were similar in all species studied. Moreover, the affinities of the selective agonist pBC 264 for the CCK-B receptor were 18–60-fold better

TABLE 2

Effect of guanyl nucleotide and ions on specific binding of [^{3}H]pBC 264 in different species

Binding experiments were carried out using 0.2 nM [^{3}H]pBC 264, with 0.6–0.7 mg of protein, for 60 min at 25°. Nonspecific binding was determined in the presence of 1 μM CCK$_8$. The data are expressed as a percentage of control binding in routine buffer, which contained 50 mM Tris·HCl and 5 mM $MgCl_2$. The values are the mean ± standard deviation of three independent determinations performed in triplicate.

Tissue	Specific binding				
	Tris (50 mM), $MgCl_2$ (5 mM)	Tris (50 mM)	Tris (50 mM), $MgCl_2$ (5 mM), NaCl (100 mM)	Tris (50 mM), $MgCl_2$ (5 mM), Gpp(NH)p (10 μM)	Tris (50 mM), $MgCl_2$ (5 mM), Gpp(NH)p (10 μM), NaCl (100 mM)
	% of control				
Guinea pig cortex	100	66.4 ± 11.7[a]	103.5 ± 6.9	103.1 ± 3.7	104.0 ± 2.1
Mouse brain	100	57.1 ± 6.6[a]	97.3 ± 8.5	96.3 ± 6.6	104.2 ± 4.0
Rat cortex	100	33.8 ± 1.7[a]	94.5 ± 8.1	90.7 ± 9.2	61.2 ± 3.0[a]

[a] $p < 0.05$ (Student's *t* test).

TABLE 3

Effect of 0.1 mM nucleotides on specific binding of [^{3}H]pBC 264 in rat brain cortex membranes

Binding experiments were carried out using 0.2 nM [^{3}H]pBC 264, with 0.6–0.7 mg of protein, for 60 min at 25°. Nonspecific binding was determined in the presence of 1 μM CCK_8. The data are expressed as a percentage of control binding in routine buffer, which contained 50 mM Tris·HCl and 5 mM $MgCl_2$. The values are the mean ± standard deviation of three independent determinations performed in triplicate.

Nucleotides	Specific binding
	% of control
GTP	82 ± 5[a]
Gpp(NH)p	59 ± 4[a]
GDP	87 ± 1[a]
GMP	94 ± 6
App(NH)p	102 ± 4

[a] $p < 0.05$ (Student's t test).

TABLE 4

Effect of NaCl and Gpp(NH)p on the binding parameters of [^{3}H]pBC 264 in rat brain cortex membranes, with or without pretreatment at pH 11.5

The mean ± standard error from three to six independent determinations, each performed in triplicate, is shown. The assay conditions were as described in Experimental Procedures. All media contained 5 mM $MgCl_2$. Additions, where noted, were Gpp(NH)p at 10 μM and NaCl at 100 mM.

Addition	K_d	B_{max}
	nM	*fmol/mg of protein*
None	0.25 ± 0.02	22.0 ± 1.6
NaCl	0.36 ± 0.05	30.8 ± 2.3[a]
Gpp(NH)p	0.41 ± 0.12	26.0 ± 4.3
NaCl, Gpp(NH)p	0.83 ± 0.09[b]	39.0 ± 4.2
NaCl, pH 11.5 pretreated	1.01 ± 0.12[b]	47.7 ± 4.9[c]
NaCl, Gpp(NH)p, pH 11.5 pretreated	0.99 ± 0.04[b]	44.5 ± 3.5[c]

[a] $p < 0.05$, compared with control.
[b] $p < 0.01$, compared with NaCl group.
[c] $p < 0.05$, compared with NaCl group (Mann-Whitney U tests).

than those of the CCK-B antagonist L365,260, depending on the species. The apparent affinities of CCK_8 and $pCCK_8$ for [^{3}H]pBC 264 binding sites were about 2-fold lower in mouse brain and 4-fold lower in rat brain than in guinea pig brain. These affinities were 3–4-fold better than those determined with [^{3}H]$pCCK_8$ in saturation experiments. The apparent affinity of CCK_5 was 5.6- and 8.3-fold better in rat and mouse brains than in guinea pig brain. Compared with guinea pig, the apparent affinities of CCK_8NS and L364,718 were slightly weaker in rat brain and slightly higher in mouse; L364,718 was 10-fold less potent in rat brain than in mouse brain. PD 134308 was around 4-fold and 20-fold less potent in mouse brain and rat brain, respectively, than in guinea pig brain. CCK_4 exhibited the same affinity for rat and guinea pig brain but a higher affinity for mouse brain homogenates. A more pronounced difference was obtained with the cyclic analog BC 254, which appeared 30- and 60-fold less potent, respectively, in inhibiting [^{3}H]pBC 264 binding to mouse and rat brain than to guinea pig brain. A variety of drugs and peptides chemically unrelated to CCK, including enkephalins, substance P, and serotonergic and dopaminergic agonists, failed to inhibit the specific binding of [^{3}H]pBC 264 to mouse brain.

Discussion

Until now, the only available selective radiolabeled ligands endowed with high affinity for CCK-B receptors were the antagonists developped by Merck (4, 37). Radiolabeled selective agonists are needed to characterize parameters such as receptor-coupling mechanisms, levels of spare receptors, and possible variations in binding site concentrations under various physiopharmacological conditions. After the preliminary report on [^{3}H]pBC 264 binding (19), confirming the CCK-B selectivity of this peptidase-resistant peptide, the nonsulfated CCK_8 analog [^{3}H]SNF 8702, devoid of amino-terminal protection, was proposed as a CCK-B probe, but it is so far unclear whether this molecule is a full or a partial agonist (27). In contrast, the selective agonist pBC 264 behaves as a full and selective CCK-B agonist in various electrophysiological and pharmacological studies (16–18). [^{3}H]pBC 264 binds to rat, mouse, and guinea pig membranes with a very high affinity (K_d ~0.2 nM) and in a time-dependent, reversible, and saturable manner. Even in the rat brain, a preparation that has been shown to have the highest proportion of nonspecific binding and the lowest concentration of CCK receptors (22), the specific binding of [^{3}H]pBC 264 is high (80% at the K_d concentration), leading to accurate measurements of the binding parameters, whereas such experiments were not possible using [^{3}H]SNF 8702. The Scatchard and Hill transformations of the saturation data indicated that [^{3}H]pBC 264 apparently interacts, with a similar high affinity, with a single class of binding sites in the four species studied (guinea pig, rat, mouse, and cat), in contrast to the results obtained with [^{3}H]SNF 8702 in whole brain (27) and with [^{3}H]BDNL (Boc(Nle^{28,31})CCK_{27-33}) in cortex of guinea pig brain (38). The dissociation rate of [^{3}H]pBC 264 is very slow, which is an advantage not only in equilibrium binding

TABLE 5

Inhibitory potencies of CCK analogs on the specific binding of 0.2 nM [^{3}H]pBC 264 at CCK-B sites in different species

Results are mean ± standard error from three or four experiments performed in triplicate. Each determination was obtained from the analysis of Hill plots, using eight to ten concentrations of analogs.

	Guinea pig cortex		Rat cortex		Mouse brain	
	K_i	n_H	K_i	n_H	K_i	n_H
	nM		*nM*		*nM*	
CCK_8	0.23 ± 0.03	0.94 ± 0.08	1.01 ± 0.05	0.82 ± 0.07	0.48 ± 0.07	0.95 ± 0.09
$pCCK_8$	0.13 ± 0.01	0.96 ± 0.06	0.55 ± 0.15	0.90 ± 0.07	0.33 ± 0.04	0.91 ± 0.05
pBC 264	0.13 ± 0.01	0.90 ± 0.08	0.17 ± 0.03	0.92 ± 0.03	0.17 ± 0.04	0.97 ± 0.03
CCK_8NS	6.0 ± 1.1	0.89 ± 0.03	10.2 ± 3.1	0.71 ± 0.08	4.6 ± 0.1	0.76 ± 0.04
CCK_5	110 ± 20	0.78 ± 0.03	19.7 ± 2.8	0.63 ± 0.03	13.2 ± 2.4	0.84 ± 0.03
CCK_4	49.9 ± 9.9	0.81 ± 0.12	55.7 ± 10.2	0.65 ± 0.04	16.4 ± 3.4	0.78 ± 0.05
BC 254	0.76 ± 0.09	0.83 ± 0.04	43.9 ± 6.7	0.88 ± 0.08	23.3 ± 1.0	0.95 ± 0.01
L-364,718	132.0 ± 18.3	1.02 ± 0.04	549 ± 69	0.93 ± 0.08	62.4 ± 7.6	0.80 ± 0.05
L-365,260	2.32 ± 0.27	0.92 ± 0.06	10.9 ± 2.0	0.87 ± 0.01	5.2 ± 0.6	0.88 ± 0.07
PD 134308	0.28 ± 0.01	1.02 ± 0.01	6.1 ± 0.5	0.97 ± 0.08	1.2 ± 0.2	0.83 ± 0.07

studies but also in experiments aimed at isolating CCK-B receptors (39).

[^{3}H]pBC 264 displays a very high selectivity for CCK-B receptors in rodents. The maximal number of binding sites found in whole rat, mouse, and guinea pig brain was significantly lower using [^{3}H]pBC 264, compared with the nonselective ligand [^{3}H]pCCK8. In contrast, the maximal number of binding sites was found to be identical with both ligands in brain cortices of rat and guinea pig, which have been reported to contain only CCK-B sites (5, 6, 10–12). The binding sites evidenced with [^{3}H]pCCK$_8$ in the presence of BC 264 could, therefore, correspond to CCK-A sites. The presence of this rather high level of CCK-A receptors in the brain was unexpected, because, so far, autoradiographic studies have indicated the existence of peripheral-type CCK receptors in only a few structures of rodent brain, such as the area postrema, nucleus tractus solitarius, and interpeduncular nucleus (5, 6). These results suggest that CCK-A sites could be present in other brain regions but with a distribution too diffuse or with a concentration too low to be detected by autoradiography. This would be in agreement with the CCK-A-related electrophysiological and pharmacological responses observed after local injection of CCK$_8$ in brain structures apparently devoid of CCK-A binding sites, such as the nucleus accumbens (40, 41).

Unlike the pancreatic receptor (42), the interactions between CCK-B binding sites and coupling effectors remain unknown. We have, therefore, studied the influence of ions and guanyl nucleotides on the binding of [^{3}H]pBC 264. The divalent cation Mg^{2+} has been shown to increase the specific binding of all radiolabeled nonselective CCK agonists in both brain and pancreas membrane preparations (7, 21, 43). This study, performed with a tritiated CCK-B-selective agonist in brain tissues containing both types of CCK sites, clearly shows that binding to CCK-B receptors is increased by magnesium ions. The specific binding of [^{3}H]pBC 264 was not modified by sodium in guinea pig and mouse brain. In rat brain, the main effect of Na^+ ions was an increase in the maximal number of binding sites, which was more pronounced in the presence of Gpp(NH)p. A possible explanation for these findings could be the existence of a population of sites that are in a low affinity state under control conditions and, therefore, are not detectable. These sites may be transformed into a high affinity state by Na^+, and this effect may be potentiated by Gpp(NH)p or by alkaline treatment. Gpp(NH)p had no effect on the specific binding of [^{3}H]pBC 264 in mouse and guinea pig brain. In contrast, in rat brain, in the presence of NaCl, the specific binding was selectively reduced by guanine nucleotides, with the order of potency being Gpp(NH)p > GTP > GDP, whereas GMP and App(NH)p had no effect. As shown in Table 4, the reduction in specific binding of [^{3}H]pBC 264 induced by Gpp(NH)p in the presence of NaCl is due to an approximately 2-fold decreased affinity. It has previously been shown that alkalinization of membrane preparations results in the uncoupling of receptors from their associated G proteins (33–35). After treatment of rat brain membranes at pH 11.5 and subsequent neutralization, the affinity of [^{3}H]pBC 264 decreased significantly. Under these conditions, no difference in the binding parameters was observed in the presence or the absence of Gpp(NH)p, confirming that the G proteins were decoupled. The reduction in affinity of [^{3}H]pBC 264 induced by alkaline treatment was similar to that observed when Gpp(NH)p was added to untreated membranes. Taken together, these results support the hypothesis that at least a proportion of CCK-B receptors are coupled to G proteins. However, the second messengers associated with central CCK-B receptors remain to be determined, because neither phosphoinositide turnover nor adenylate cyclase modifications have been reported. It is interesting to note that the sensitivity of the various radiolabeled CCK agonists to these agents differed considerably. Thus, unlike its propionylated derivative, [^{3}H]CCK$_8$ binding remained unchanged in rat in the presence of sodium or nucleotides (10). On the other hand, binding of ^{125}I-CCK$_{33}$ and [^{3}H]pentagastrin has been shown to be strongly reduced by guanyl nucleotides and sodium, using guinea pig and mouse brain tissues (7, 8, 43). Moreover, [^{3}H]SNF 8702 has also been shown to be sensitive to Gpp(NH)p, especially in guinea pig cortex (27).

Our study indicates that, in guinea pig brain cortex, [^{3}H]SNF 8702 binds to a larger population of CCK-B sites (57 fmol/mg of protein) than does [^{3}H]pBC 264 (39.8 fmol/mg of protein), which is not the case in mouse brain. These results could reflect the presence of several CCK-binding subtypes having different sensitivities to ions and nucleotides. Thus, most of the receptors labeled by [^{3}H]pBC 264 in guinea pig brain may be insensitive to these reagents, unlike the additional sites bound by [^{3}H]SNF 8702. Unfortunately, owing to the low specific binding of [^{3}H]SNF 8702, it was not possible to compare the B_{max} values of these two radioligands in rat brain cortex. However, a population of CCK-B receptors labeled by [^{3}H]pBC 264 in this species is sensitive to Gpp(NH)p, which could suggest that the proportion of receptors sensitive to guanyl nucleotides is larger in rat brain than in guinea pig brain cortex.

The apparent affinity for [^{3}H]pBC 264 binding sites displayed by CCK$_8$ and derivative peptides varied among species. Whereas pBC 264 showed the same very high affinity in the three species, L364,718 was found to be almost 9-fold more potent in mouse brain than in rat brain, and PD 134308 was 20-fold more potent in guinea pig brain than in rat brain. Likewise, as already reported (24), the cyclic CCK analog BC 254 displayed a 30- and 60-fold lower affinity for mouse and rat brain, respectively, than for guinea pig brain preparations. These results could be explained either by a slight structural difference in the receptors of guinea pig and rat brain or by the presence of two or more CCK-B receptor subtypes present in different concentrations in each species.

In conclusion, [^{3}H]pBC 264 has not only high affinity, in the subnanomolar range, and a high selectivity for CCK-B receptors but also, compared with ligands currently available, high specific binding, good water solubility, and a high resistance to peptidases. In addition to these properties, its easy preparation and its high specific radioactivity make it an ideal molecule for *in vitro* as well as autoradiographic studies, especially in tissues with low receptor densities. Such experiments are in progress in our laboratory. Studies on the bioavailability of [^{3}H]pBC 264 have shown that at least 0.016% of the administered dose is present in the brain 15 min after peripheral injection in the mouse and that the tritiated compound is very resistant to peptidases *in vivo* (20). Moreover, this radiolabeled ligand has been useful for *in vivo* binding after intravenous or intracerebroventricular injection (44). The characterization of binding parameters and the pharmacological profile under *in vivo* con-

ditions, using [^{3}H]pBC 264, has allowed pharmacological responses and CCK-B receptor occupancy to be correlated.

Acknowledgments

We wish to thank Dr. A. Beaumont for critical reading of the manuscript and C. Dupuis for typing it.

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