Autoradiographic Localization and Biochemical Characterization of Peripheral Type CCK Receptors in Rat CNS Using Highly Selective Nonpeptide CCK Antagonists

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Two potent and highly selective nonpeptide antagonists, L-365,031 [1-methyl-3-(4-bromobenzoyl)amino-5-phenyl-3H-1,4 benzodiazepin-2-one] and ³H-L-364,718 [1-methyl-3-(2-indoloyl)amino-5-phenyl-3H-1,4 benzodiazepin-2-one] were used to localize “peripheral” CCK receptors in rat brain. In autoradiographic experiments, L-365,031 displaced ¹²⁵I-Bolton Hunter CCK-8 binding from the interpeduncular nucleus (IPN) ($IC_{50} = 7 \times 10^{-8}$ M), the area postrema (AP), and the nucleus tractus solitarius (NTS) without influencing specific binding to other areas, such as the cerebral cortex or the spinal tract of the trigeminal nerve. Desulfated CCK preferentially inhibited ¹²⁵I-CCK binding to cerebral cortex ($IC_{50} = 7 \times 10^{-8}$ M) rather than IPN ($IC_{50} > 1 \times 10^{-6}$ M) or AP-NTS. In the medulla the localization of ³H-L-364,718 binding was similar to L-365,031-sensitive ¹²⁵I-CCK-8 binding and was found in the AP and medial, but not lateral, aspects of the NTS. In membranes prepared from IPN, NTS, and AP, ³H-364,718 binding was of high affinity ($K_d = 0.14$ nM), saturable ($B_{max} = 20$ fmol/mg protein), and inhibited by compounds previously shown to act at pancreatic CCK receptors. The receptors labeled by ³H-364,718 were modulated by guanyl nucleotide, which reduced agonist affinity 10-fold without affecting antagonist binding. The localization and high density of CCK receptors in AP and NTS suggest that these receptors may play an important role in processing sensory afferent information.

Cholecystokinin (CCK) is present in high concentrations in the mammalian CNS (Vanderhaeghen et al., 1975, 1980; Dockray, 1976; Innis et al., 1979). Indeed, as the sulfated octapeptide (sCCK-8), it is found in certain brain regions in higher concentrations than any other neuropeptide (see Crawley, 1985, for review). The distribution of sCCK-8 throughout the CNS, its localization in neuronal perikarya and nerve fibers, and its coexistence with other putative neurotransmitters (Hökfelt et al., 1980; Greenwood et al., 1981; Vanderhaeghen et al., 1981; Kubota et al., 1983; Hamill et al., 1984) suggest that this peptide may act as either a neurotransmitter or a neuromodulator. Additionally, the demonstration of binding sites with high affinity for sCCK-8 and related peptide fragments throughout the brain further supports the notion that sCCK-8 might be considered as an important mediator of information transfer between neurons.

Biochemical measurements of the affinity of CCK fragments at brain CCK receptors have shown that these differ in their pharmacological specificity when compared with CCK receptors in peripheral organs such as the pancreas or gallbladder (Innis and Snyder, 1980; Saito et al., 1980). For example, “peripheral” CCK receptors display lower affinity for desulfated CCK-8 and gastrin than do “central” CCK receptors found in the brain. Despite such differences between receptors in anatomically separate tissues, those within the CNS have generally been considered as being homogeneous, with no evidence of subtypes in different brain regions. Recently, however, Moran and colleagues (1986) reported that CCK receptors in the area postrema (AP), the nucleus tractus solitarius (NTS), and the interpeduncular nucleus (IPN) display relatively low affinity for desulfated CCK and may therefore represent “peripheral” type CCK receptors despite their location in central nervous tissue.

Recent developments in our laboratories have yielded a number of highly potent nonpeptide CCK-receptor antagonists that are selective for peripheral CCK receptors (Chang and Lotti, 1986; Evans et al., 1986). As such, these compounds provide very specific probes for determining whether peripheral type CCK receptors do exist in areas of mammalian brain. The present report describes the use of 2 such selective CCK antagonists in demonstrating the existence of CCK receptors in rat brain which resemble those in the periphery. In the first part of the study, autoradiography was employed to visually demonstrate these sites, with 2 different approaches to detection: first, indirectly, by labeling receptors using ¹²⁵I-Bolton Hunter CCK-8 (¹²⁵I-BH-CCK) and displacing with an unlabeled antagonist L-365,031 [1-methyl-3-(4-bromobenzoyl)amino-5-phenyl-³H-1,4-benzodiazepin-2-one], which shows high selectivity for peripheral CCK receptors (Chang and Lotti, personal communication); second, by direct visualization using a tritium-labeled antagonist of even greater selectivity L-364,718 [1-methyl-3-(2-indoloyl) amino-5-phenyl-3H-1,4-benzodiazepin-2-one] (Chang and Lotti, 1986; Chang et al., 1986a). Furthermore, the high affinity and selectivity of ³H-L-364,718 has permitted us to characterize these receptors using membrane preparations. Our results suggest that the CCK receptors localized in the AP, NTS, and IPN of rat brain are of the peripheral type, termed CCK-A receptors by Moran et al. (1986).

Materials and Methods

Autoradiography

Brains were removed from male Sprague-Dawley rats (200–250 gm) and rapidly frozen. Sections 10 μ m thick were cut on a cryostat and then mounted onto glass slides. The sections were allowed to dry and were stored at -20°C until required. On the day of use, sections were

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Figure 1. Inhibition of ^{125}I -BH-CCK binding to (A) guinea pig brain and (B) rat pancreas by sulfated CCK-8 (sCCK-8), desulfated CCK-8 (dCCK-8), and L-365,031. Membrane homogenates were incubated with 0.1 nM ^{125}I -BH-CCK-8 for 120 min at room temperature alone or with increasing concentrations of test compound. Nonspecific binding was determined by 1 μM sCCK-8, which was subtracted from total to give the specific portion of binding. Data are mean values from 2 experiments. L-365,031 displayed 1000-fold higher affinity for CCK receptors in the pancreas than in the brain. In contrast, desulfated CCK-8 showed a lower degree of selectivity for central receptors relative to sCCK, on the order of 2- to 10-fold.

thawed and then incubated in an assay buffer containing either ^{125}I -CCK-8 labeled with Bolton and Hunter reagent (0.6 nM; Amersham International) or ^3H -L-364,718 (5 nM; prepared by Dr. Avery Rosegay of MSD Laboratories, Rahway, NJ). Sections were incubated either in the presence of ligand alone or with varying amounts of unlabeled compound. The composition of the assay buffer for all incubations was 20 mM HEPES (pH 6.5), 5 mM MgCl_2 , 150 mM NaCl, 1 mM EGTA, and 0.025% bacitracin. The incubation was allowed to proceed for 60 min at room temperature, after which the sections were washed 4 times for 2 min in fresh buffer and then dried in a stream of air. Autoradiographs were generated by apposing sections to LKB-Ultrofilm for 7 d (for ^{125}I -CCK-8) or 8 weeks (for ^3H -L-364,718). The resulting negatives were analyzed using a Quantimet 920 image analyzer (Cambridge Instruments), and calibration was by means of brain paste standards prepared according to Clark and Hall (1986).

In order to verify the localization of binding in sections of medulla, sections labeled with ^{125}I -CCK were apposed for 7 d to coverslips that had been dipped in Ilford K5 emulsion. These were then developed, fixed, and washed several times before allowing them to dry. The tissue sections were stained using cresyl fast violet and the coverslips fixed in position with DPX mounting medium. The slides were viewed under normal illumination for histological examination and under dark-field illumination for visualization of silver grains.

Tissue preparations

Guinea pig cortex. The brains were removed from adult guinea pigs (300–400 gm) and the cortex dissected free of other tissue. This was then homogenized in 20 vol of ice-cold 0.32 M sucrose using a glass

homogenizer and Teflon pestle. The homogenate was centrifuged at $1000 \times g$ for 10 min, after which the supernatant was recentrifuged at $48,000 \times g$ for 20 min. The resulting pellet was resuspended in standard assay buffer (SAB) (20 mM HEPES, pH 6.5, 5 mM MgCl_2 , 150 mM NaCl, 1 mM EGTA, and 0.025% bacitracin) at a tissue concentration of 1 gm original wet weight to 40 ml assay buffer.

Rat pancreas. The pancreas was removed from male Sprague-Dawley rats and homogenized for 15 sec in 20 vol of ice-cold buffer comprising 10 mM HEPES, pH 7.4, plus 0.01% soybean trypsin inhibitor (SBTI) using a Polytron homogenizer. The tissue was centrifuged for 15 min at $48,000 \times g$ and the pellet resuspended in SAB supplemented with 0.01% SBTI and 0.2% BSA. The final tissue concentration in the assay was 1 gm original wet weight of tissue to 4000 ml of assay buffer.

Rat, AP, NTS, and IPN. After removal of the brains from Sprague-Dawley rats, a 2 mm slice of tissue containing the IPN was cut just caudal to the hypothalamus using a pair of parallel-mounted razor-blades. Similarly, a slab of tissue was cut from the medulla, 2 mm rostral to the beginning of the obex. From each of these slices, the appropriate area was dissected away from surrounding tissue, and for each assay the combined tissue from 13 rats was used. After dissection, the tissue was transferred immediately and maintained in ice-cold Krebs-Henseleit prior to homogenization in 10 mM HEPES, pH 7.4, with a glass homogenizer fitted with a Teflon pestle. The tissue homogenate was then centrifuged for 15 min at $48,000 \times g$, and the pellet was finally resuspended in SAB at a tissue concentration of approximately 10 mg original wet weight/ml buffer.

Binding assays

The protocol for each binding assay was essentially the same, with only details differing. Aliquots of tissue were incubated at 20°C for either 60 min with ^3H -L-364,718 (0.2–0.3 nM with AP, NTS, and IPN) or 120 min with ^{125}I -BH-CCK (0.1 nM with guinea pig brain or rat pancreas). Test compounds were included over a range of concentrations, and nonspecific binding was determined using either 1 μM sCCK-8 for “central” type receptors or 1 μM L-365,031 for “peripheral” type receptors. Saturation analysis was performed in a similar way, with the concentration of ligand being increased from 0.02 nM upwards. After each incubation, assays were terminated by filtration onto Whatman GF/C filters using a Brandel cell harvester, and the filters were washed a further 4 times with 3 ml of 20 mM HEPES, pH 6.5. The radioactivity associated with the filters was then counted using either a gamma-counter or liquid scintillation counter.

Materials

^{125}I -Bolton Hunter CCK was from Amersham International U.K. (specific activity ~ 2000 Ci/mmol). $^3\text{H}(\pm)$ -L-364,718 (specific activity, 67.4 Ci/mmol) was synthesized by Dr. A. Rosegay of M.S.D. Laboratories (Rahway, NJ), and L-365,031 was prepared by M.S.D. Laboratories (West Point, PA). CCK-8 and related peptides were purchased from Bachem U.S.A. or CRB U.K. (R) and (S) Tifluadom were synthesized by Dr. M. Bock (M.S.D., West Point, PA). All other reagents were of analytical grade and purchased from commercial sources.

Results

^{125}I -CCK-8 binding in rat pancreas and guinea pig brain

Figure 1 shows the selectivity of the antagonist L-365,031 for CCK receptors in pancreas versus cerebral cortex. In agreement with Chang and Lotti (1986), the degree of selectivity shown by this compound was on the order of 1000-fold in favor of the pancreatic receptor ($\text{IC}_{50} = 4 \times 10^{-9}$ M) compared with sites present in the cortex ($\text{IC}_{50} = 5 \times 10^{-6}$ M). Desulfated CCK displayed a much smaller degree of selectivity, having an affinity about 10-fold less potent than the sulfated form against guinea pig brain binding (IC_{50} sCCK = 8×10^{-10} M, IC_{50} dCCK = 1×10^{-8} M) and 70-fold less against pancreatic binding (IC_{50} sCCK = 1×10^{-9} M, IC_{50} dCCK = 8×10^{-6} M).

Autoradiography in rat brain

In light of these data, a series of autoradiographic experiments were performed to determine the ability of L-365,031 and dCCK

Figure 2. Autoradiographs showing selective displacement of ^{125}I -CCK binding to rat cortex and IPN by desulfated CCK and L-365,031. Sections of rat brain were incubated with ^{125}I -CCK (0.6 nM) either alone (A) or in the presence of 1 μM desulfated CCK (B) or 1 μM L-365,031 (C) for 60 min at room temperature. The sections were washed 4 times for 2 min each and used to generate autoradiographs. These were used as negatives to produce the photographs shown here. Areas of high ^{125}I -CCK binding are represented by white grains. Desulfated CCK (dCCK) selectively inhibited binding in the cortex (B) rather than the IPN, which can still clearly be seen. In contrast, L-365,031 displaced binding only in the IPN and was without effect in the cortex (C).

Figure 3. Selective inhibition of specific ^{125}I -Bolton-Hunter CCK-8 binding from sections of rat brain containing cortex and interpeduncular nucleus by desulfated CCK (dCCK) and L-365,031. Pairs of sections 10 μm thick were incubated for 60 min with 0.6 nM ^{125}I -BHCK, either alone or in the presence of the indicated concentrations of dCCK or L-365,031. After development of the autoradiographs, the density of binding in the cerebral cortex and IPN was measured using a Quantimet 920 image analyzer. Nonspecific binding was determined by 10^{-6} M CCK-8, which was subtracted from total to give the specific portion. Desulfated CCK displayed greatly affinity for CCK receptors in the cortex than IPN, whereas L-365,031 failed to inhibit binding to cortical sites at concentrations that blocked binding in the IPN. Data are mean values from 2 (L-365,031) or 3 (dCCK) separate experiments.

to displace ^{125}I -CCK binding from CCK receptors in cortex and IPN. Figure 2 shows an example of inhibition of ^{125}I -CCK binding to coronal sections of rat brain by dCCK (10^{-6} M) and L-365,031 (10^{-6} M). In addition, Figure 3 presents a quantitative estimate of the degree of inhibition produced by each compound in the IPN and cortex. L-365,031 failed to inhibit binding by more than 10% in the cerebral cortex at concentrations that were found to be maximally effective in pancreas binding assays (compare Figs. 3 and 1B). In the IPN, however, L-365,031 produced a dose-dependent reduction in specific ^{125}I -CCK binding (Fig. 3B). The data shown in this figure were fitted by a single hyperbolic function, which yielded an IC_{50} value of 6.6×10^{-8} M for L-365,031, together with a Hill coefficient of 1.00. By contrast, dCCK displayed greater affinity for receptors in the cortex than in the IPN (Fig. 3), thus agreeing with the observations of Moran et al. (1986). An IC_{50} value of 7×10^{-8} M (Hill slope, 0.74) was obtained for the data shown, which is close to that found in guinea pig cortex (see above) and that determined autoradiographically (2×10^{-8} M) for dCCK by Moran and colleagues (1986).

Another series of experiments was performed to determine the localization of ^{125}I -CCK binding sites in hindbrain, and the sensitivity of these sites to L-365,031. Figures 4 and 5 show

autoradiographs of rat medulla made using emulsion-coated coverslips or LKB-Ultrofilm, respectively. These sections were taken at approximately bregma -14 mm according to the atlas of Paxinos and Watson (1982). Specific ^{125}I -CCK binding was localized principally to the regions of the area postrema, the nucleus tractus solitarius and also to the spinal tract of the trigeminal nerve (Sp5). The presence of L-365,031 inhibited binding in the AP but failed to modify binding to the trigeminal nerve tract (Fig. 5A). Sulfated CCK-8 itself displaced binding to AP, NTS, and Sp5 (data not shown).

The density of binding in the area postrema and medial NTS increased progressively in a rostral direction but remained sensitive to inhibition by L-365,031 (Figs. 4, B, C; 5, B, C). However, binding in the lateral aspects of the NTS was refractory to the antagonist (Figs. 4, E, F; 5D), suggesting that these sites are of the classical brain type.

L-364,718 displays even greater affinity and selectivity for peripheral CCK receptors than does L-365,031 (Chang and Lotti, 1986), and it has been used in tritiated form to label pancreatic CCK receptors directly (Chang et al., 1986a). We have therefore used this compound in autoradiographic experiments to directly label peripheral CCK receptors present in caudal regions of rat brain.

Specific binding of ^3H -L-364,718 in the rat medulla was localized exclusively to regions of the AP and medial aspects of the NTS (Fig. 6). Neither the spinal tract of the trigeminal nerve nor lateral aspects of the NTS were labeled by the radioligand, agreeing with the pattern of displacement seen with ^{125}I -CCK and L-365,031 (compare Figs. 5 and 6). In more rostral sections of medulla, bilateral areas of relatively heavy labeling could be detected, which represented binding in the medial nucleus of the NTS (Fig. 6, C, D), but lateral aspects of the NTS were not labeled by ^3H -L-364,718 (compare Figs. 5D and 6D).

^3H -L-364,718 binding to AP, NTS, and IPN membranes

The extremely high affinity and selectivity of ^3H -L-364,718 for peripheral type CCK receptors also allowed this compound to be used as a radioligand to characterize "peripheral" sites present in brain using a more convenient membrane binding assay. For this purpose, the AP, NTS, and IPN were dissected and used to prepare a crude membrane preparation.

Binding of ^3H -L-364,718 to membrane fragments prepared from AP, NTS, and IPN was both saturable and of very high affinity, with maximum binding capacity (B_{max}) of 20.3 ± 3.9 fmol/mg protein ($n = 3$) and dissociation constant (K_d) of 0.14 nM ($\text{p}K_d = 9.58 \pm 0.22$, $n = 4$). At concentrations used for displacement studies (0.2–0.3 nM), the specific portion of binding represented between 50–60% of total binding and was highly reproducible between assays. Binding was displaced by a variety of CCK-related peptide fragments and Table 1 shows mean pIC_{50} values for these. Sulfated CCK-8 was the most potent peptide tested ($\text{pIC}_{50} = 7.8 \pm 0.08$), being equipotent with the nonpeptide antagonist L-365,031 ($\text{pIC}_{50} = 7.9 \pm 0.3$). Desulfated CCK was about 100 times less potent than the sulfated octapeptide. Gastrin, CCK-4, and CCK-6 were essentially inactive as inhibitors of binding ($\text{pIC}_{50} < 4.0$). The opiate agonist tifluadom, which possesses a benzodiazepine structure and has been shown to have antagonist activity at peripheral CCK receptors in pancreas and gallbladder (Chang et al., 1986b), was also active at central CCK receptors labeled by ^3H -L-364,718.

CCK receptors in pancreatic membranes have been shown to be sensitive to modulation by guanyl nucleotides such as GTP

Figure 4. Light microscope dark-field autoradiographs and corresponding stained sections showing the localization of ^{125}I -BH-CCK binding to the medial and lateral aspects of the nucleus tractus solitarius. Sections of rat medulla were incubated with 0.2 nM ^{125}I -BH-CCK alone or in the presence of 10^{-7} M L-365,031. After washing and drying, the sections were exposed to emulsion-coated coverslips for 7 d. The emulsion was then developed, the tissue sections stained using cresyl fast violet, and the coverslip fixed in position using DPX mounting medium. Stained tissue sections (shown in *A* and *D* and equivalent to autoradiographs *C* and *F*, respectively) were viewed under normal illumination, and the autoradiographs were viewed using dark-field illumination, with white areas representing silver grains. Sections shown in *A-C* are caudal to those shown in *D-F*. Binding in the medial aspects of the nucleus tractus solitarius was sensitive to L-365,031 (*B* and *C*), whereas binding in lateral regions of the nucleus was not inhibited by the presence of the antagonist (*E* and *F*). A small degree of binding to medial NTS can just be discerned in the more rostral section (*E*), and this was displaced by L-365,031. mNTS, medial nucleus tractus solitarius; INTS, lateral nucleus tractus solitarius; Cer, cerebellum; 4V, fourth ventricle.

Figure 5. Localization of ^{125}I -BH-CCK-8 binding to sections of rat medulla and its displacement by 10^{-7} M L-365,031. Sections ($10\ \mu\text{m}$) of rat medulla were incubated for 60 min with $0.6\ \text{nM}$ ^{125}I -BH-CCK alone or in the presence of 10^{-7} M L-365,031. After washing and drying, the sections were apposed to LKB Ultrafilm for 6 d for generation of autoradiographs. On these photographs, the binding of ^{125}I -BH-CCK-8 is represented by white grains. Specific binding was localized to the area postrema (AP), the nucleus tractus solitarius (NTS), and spinal tract of the trigeminal nerve (sp5). Binding in all regions was displaced by 10^{-6} M sCCK-8. L-365,031 inhibited binding to the AP and medial aspects of the NTS (mNTS) (A–C) but failed to displace binding in either sp5 (A) or lateral NTS (lNTS) (D).

and its more stable analogs (Innis and Snyder, 1980). To determine whether the binding sites present in the AP and IPN also shared this property, the effects of $100\ \mu\text{M}$ GppNHp on ^3H -364,718 binding and on the affinity of CCK as an inhibitor of binding were determined.

The inclusion of GppNHp in the assay medium had no effect on the binding of the antagonist itself. However, the affinity of sCCK-8 for the binding sites was reduced by approximately an order of magnitude from $\text{pIC}_{50} = 7.7 \pm 0.03$ under control conditions to $\text{pIC}_{50} = 6.5 \pm 0.12$ in the presence of GppNHp

Figure 6. Localization of ^3H -L-364,718 binding to sections of rat medulla. Sections ($10\ \mu\text{m}$) of rat medulla were incubated either with $5\ \text{nM}$ ^3H -L-364,718 alone or in the presence of $10^{-7}\ \text{M}$ L-365,031. After washing and drying, the sections were apposed to LKB Ultrofilm for generation of autoradiographs. ^3H -L-364,718 binding is shown by white grains. Binding of ^3H -L-364,718 that was displaceable by L-365,031 or CCK ($10^{-6}\ \text{M}$) was localized to the area AP and medial portions of the NTS (*A* and *B*). Bilateral areas of relatively heavy labeling were localized to the medial LTS (*mNTS*), but no displaceable binding could be detected in the spinal tract of the trigeminal nerve or lateral aspects of the NTS. Compare with Figures 4*D–F* and 5*D*, for instance.

Figure 7. Effect of GppNHp on the affinity of sCCK-8 as an inhibitor of ^3H -L-364,718 binding to rat brain membranes. Freshly prepared membranes were incubated for 60 min with ^3H -L-364,718 and a range of concentrations of sCCK-8 in the absence (*open symbols*) and presence (*closed symbols*) of 100 μM GppNHp. Nonspecific binding was defined by 1 μM L-365,031. The inclusion of the guanyl nucleotide in the incubation medium had no effect on the binding of ^3H -L-364,718 itself but reduced the affinity of sCCK-8 for the receptor sites approximately 10-fold. Additionally, the Hill slope was raised to near unity. Data are the mean values from 3 separate experiments.

(Fig. 7). Furthermore, the Hill slope increased from 0.5 ± 0.06 to 0.9 ± 0.17 in the presence of the nucleotide.

Discussion

We have shown that, in discrete areas of rat brain, there exist CCK receptors that are sensitive to compounds having high affinity and selectivity for peripheral CCK receptors. A clear distinction between receptors for CCK present in the IPN and also the AP-NTS complex and those located in most other areas of the brain is the high affinity shown by AP, IPN, and NTS sites for the nonpeptide antagonists L-365,031 and L-364,718. The present work therefore confirms and enlarges upon the original observations of Moran et al. (1986). These authors reported that binding of ^{125}I -CCK-33 in the AP-NTS, IPN, and also posterior hypothalamic nuclei was less sensitive to inhibition by desulfated CCK than binding in the cerebral cortex, a pattern similar to that seen with ^{125}I -CCK-binding to pancreatic membranes (Innis and Snyder, 1980). However, subclassification of peptide hormone receptors according to differences in the affinity of peptides with fairly limited degrees of selectivity is problematic, especially in view of the susceptibility of peptides to endogenous metabolic enzymes. The use of nonpeptide antagonists having high degrees of selectivity provides a much better basis upon which to classify receptor subtypes, both within and without the CNS. The close agreement between the affinity of L-365,031 for CCK receptors in the pancreas and those in the AP-NTS complex, together with the direct labeling of the brain receptors with ^3H -L-364,718, is reconcilable only with the fact that the receptors in these areas are truly of the peripheral type, termed CCK-A or alimentary receptors by Moran and colleagues. In fact, the pharmacological profile of compounds inhibiting ^3H -L-364,718 binding to membranes prepared from the discrete brain areas is very similar to that reported using pancreatic membranes and ^{125}I -CCK (Sankaran et al., 1979; Innis and Snyder, 1980; Steigerwalt and Williams, 1981; Williams et al., 1986) or ^3H -L-364,718 (Chang et al., 1986a).

The localization of L-365,031-sensitive ^{125}I -CCK binding and of ^3H -L-364,718 itself were identical in the areas examined. ^3H -

Table 1. pIC_{50} values for inhibition of ^3H -L-364,718 binding to homogenates of AP, NTS, and IPN

Compound	$\text{pIC}_{50} \pm \text{SEM}$
sCCK-8	7.8 ± 0.08
L-365,031	7.9 ± 0.30
dCCK-8	5.7 ± 0.06
Cerulein	7.4 ± 0.20
(S)Tifluadom	7.3 ± 0.26
(R)Tifluadom	6.6 ± 0.30
CCK-6	<4.0
CCK-4	<4.0
Gastrin	<4.0

Membrane fractions were prepared from freshly dissected area postrema (AP), nucleus tractus solitarius (NTS), and interpeduncular nucleus (IPN). These were incubated with 0.2–0.3 nM ^3H -L-364,718 either alone or in the presence of a range of test compound. Nonspecific binding was measured using 1 μM L-365,031 and was subtracted from total binding to give the specific portion. Values are geometric means $\pm$ SEM of the negative logarithm of the molar concentration producing 50% inhibition of specific binding from between 3 and 6 separate experiments.

L-364,718 may therefore be considered the ligand of choice for imaging these receptor sites, especially in view of the good definition given by tritium in autoradiographic experiments. However, by using both ^{125}I -CCK and displacing with “cold” antagonist, as well as labeling directly with ^3H -L-364,718, complementary information concerning the relative distribution of “central” and “peripheral” sites was obtained, even within a narrowly defined anatomical region such as the AP-NTS complex. Thus, the occlusion of “A” sites in certain parts of the NTS allowed detection of a binding that was of the type usually found within the CNS—high affinity for dCCK and low affinity for L-364,718 or L-365,031 (CCK-B receptor). Conversely, direct labeling with ^3H -L-364,718 allowed the localization of “A” sites to be made in an uncompromised fashion, and the results strongly suggest that regional densities of binding within the NTS vary from the medial to lateral portions of the nucleus. Such variations in binding density within the nucleus may be associated with afferent input via the vagus from a particular anatomical region (Berger, 1979; Kalia and Mesulam, 1980; Higgins and Schwaber, 1981; Leslie et al., 1982). Nonetheless, the highest density of CCK-A sites was found in regions of the area postrema. Perhaps this is not totally surprising in view of the afferent vagal input to the area from peripheral organs such as the stomach (Leslie et al., 1982) and the high CCK content of the vagus itself (Dockray et al., 1980; Palkovitz et al., 1982). Visualization of the projections of the vagus into the AP and NTS using HRP (Kalia and Sullivan, 1982) parallels the distribution of ^3H -L-364,718 binding in this area, suggesting the involvement of CCK-A receptors in the processing of afferent information within this nucleus. Indeed, sulfated CCK-8 administered peripherally or directly to the NTS has been shown to have profound effects on both cardiovascular and respiratory function in the cat, effects not mimicked by compounds with low affinity for the CCK-A sites, for example, CCK-4 and desulfated CCK (Denavit-Saubie et al., 1985). Furthermore, sCCK-8 (but not dCCK) has been shown to inhibit respiratory neurons in the NTS (Morin et al., 1983), which again suggests the involvement of CCK-A receptors.

Why peripheral type receptors should be present in other areas such as the IPN is unclear, as, in contrast to the AP, the IPN is located well within the blood–brain barrier. CCK-like im-

munoreactivity is present in cell bodies located on the rostral margins of the nucleus, and dense networks of fiber plexi, which also show CCK-like immunoreactivity, can be detected surrounding the IPN (Kubota et al., 1983; Hamill et al., 1984). However, the density of these is much less within the nucleus itself. Despite that the exact function of the IPN remains unclear, it is probable that if released from fibers around the IPN, CCK would act at A-type CCK receptors.

Finally, the sensitivity to guanyl nucleotide shown by CCK-A receptors in AP, NTS, and IPN is another feature shared with their peripheral counterparts (Innis and Snyder, 1980). This modulation of agonist, but not of antagonist, binding by GppNHP reflects the linkage of the receptor to one of a series of GTP-binding proteins (see Rodbell, 1980) and the ability to form a ternary complex when activated. Pancreatic receptors stimulate phosphoinositol (PI) turnover (Hokin-Neaverson and Sadeghian, 1984; Lin et al., 1986) and have also been shown to stimulate adenylate cyclase (Svoboda et al., 1976), although PI metabolism is more likely to be linked to enzyme secretion. CCK-B receptors may also be linked to a GTP-binding protein in a similar way (Innis and Snyder, 1980; but see Van Dijk et al., 1984), yet they do not appear to be linked to a second messenger, as neither PI turnover nor adenylate cyclase is stimulated. Clearly, one intriguing question for the future is whether brain CCK-A receptors are linked to PI turnover or cyclase or whether, like their more ubiquitous CNS counterparts, they are linked to a G-protein other than Gs, Gi, or Gp.

In conclusion, the present work demonstrates the existence of subtypes of CCK receptors within the rat CNS that are sensitive to nonpeptide compounds previously shown to have high affinity and selectivity for pancreatic CCK receptors. These receptors have been further characterized by binding studies using membranes prepared from the AP-NTS complex and IPN using ³H-L-364,718; the pharmacological specificity of brain sites labeled with this radioligand is similar to that seen in the pancreas. The determination of the function of such receptors within the brain awaits further study, but this should be facilitated by the use of nonpeptide antagonists that are selective for CCK-A receptors present in periphery and CNS.

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