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STUDIES OF CHOLECYSTOKININ IN THE RAT BED NUCLEUS OF STRIA TERMINALIS

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Abstract—The release of cholecystokinin from the dorsal and ventral region of the rat bed nucleus of stria terminalis was studied. Minislices from both regions were superfused with Krebs–Ringer-phosphate, and the cholecystokinin released into the physiological medium was concentrated previous to radioimmunoassay determination. For this purpose, cholecystokinin was adsorbed onto a C18 reverse-phase column and eluted with acetonitrile. Cholecystokinin standards (10–50 pg) were subjected to the above procedure, which allowed a 20- to 50-fold concentration of the peptide with an 80% recovery. Potassium-induced release of cholecystokinin from minislices of dorsal and ventral regions of the bed nucleus of stria terminalis was measured successfully using the above procedure to concentrate the peptide. Lesion of the stria terminalis, a fiber tract originating in the amygdala, provoked a significant decrease in cholecystokinin levels in the ventral region of the bed nucleus of stria terminalis. Thus, cholecystokinin released from minislices of the ventral region of the stria terminalis may be of amygdaloid origin.

Cholecystokinin (CCK†), a hormone of the digestive tract, is also present in the brain with a discrete localization. There exists good evidence to propose a neurotransmitter-like action for this peptide [for review see Ref. 1]. In this regard it is of interest to study its release and regulation.

Long incubation periods or large amounts of tissue are necessary to obtain enough peptide for reliable quantification [2, 3]. However, it has been reported that long incubation periods may allow interactions between the different neuroactive substances released into the incubation medium complicating the analysis of the results [4]. The approach of using large amounts of tissue is a difficult task when there is interest in very small regions of the brain. It seems appropriate to concentrate the superfusates from the release experiments. The traditional method of lyophilization may be employed, but it also concentrates the salts present in physiological solutions hindering the subsequent radioimmunoassay of the peptide.

The bed nucleus of stria terminalis (BNST), a small nucleus of the CNS that has a high concentration of catecholamines [5, 6] and peptides such as CCK [7–9], has not been studied in detail due to technical difficulties. The BNST is a nucleus of the limbic system with a critical position in the limbic circuitry [10–12]. Electrophysiological studies [13] have shown a high degree of convergence of inputs from the amygdala and the hypothalamus into the same neurons of the ventral BNST. However, the dorsal region of this nucleus displays very little convergence even though it is also innervated by the amygdala and the hypothalamus. Thus, there are anatomical and functional differences between dorsal and ventral regions of the BNST, illustrating the need for delicate neurochemical studies.

The purpose of this work was to study the release of CCK from the dorsal and ventral region of the BNST. Chromatography on C18 reverse-phase columns has been used successfully to separate the different forms of CCK present in tissue extracts [14]. In fact, it is possible to use a disposable C18 cartridge to extract peptides; however, this procedure is very expensive when dealing with many samples as in release experiments. Thus, it was decided to adapt the method to concentrate peptide using Sep-pak disposable cartridges to an HPLC setting. The system developed allows one to separate the peptide from the Ringer solution and concentrate it at the same time.

To further characterize the CCK system in the BNST, the effect of lesions of the stria terminalis upon the content of CCK in the dorsal and ventral BNST was investigated.

METHODS

HPLC concentration of CCK

An HPLC system with the following conditions was used to concentrate CCK standards or unknown samples: an injector (Rheodyne) with a 2-mL loop, a one-piston HPLC pump (LDC), a Biophase ODS C18 column (250 × 4.6 mm, 5 μm, BAS), and distilled water as the mobile phase at a flow rate of 1 mL/min. Samples were injected in 2-mL aliquots with 3-min intervals. Ten minutes after finishing the injection of the total sample volume, 1 mL of 100%, filtered (0.45 μm, Millipore) acetonitrile (HPLC

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† Abbreviations: CCK, cholecystokinin; BNST, bed nucleus of the stria terminalis; dBNST, dorsal region of the BNST; vBNST, ventral region of the BNST; KRP, Krebs–Ringer-phosphate; RIA, radioimmunoassay.

grade, Merck) was injected through the injector valve into the column. Two minutes later a 2-min fraction was collected into a radioimmunoassay (RIA) tube. This sample was dried completely under a stream of nitrogen, and CCK was determined by RIA. At the end of the day the column was washed with 50 mL of 70% acetonitrile.

CCK radioimmunoassay

CCK was determined using R5, a CCK antiserum prepared by Bienfeld *et al.* [15]. The assay was performed using about 10,000 cpm/tube of ^{125}I-gastrin and R5 CCK antibody at a final dilution of 1:100,000 in 50 mM Tris buffer, pH 7.8, containing 0.1% bovine serum albumin and 0.02% sodium azide. The final volume of the assay was 1 mL. After 24–48 hr at 4°, the counts bound to the antibody were separated from the free counts by precipitation with polyethylene glycol. CCK-8 sulfate (donated by Squibb) was used to prepare a standard curve. The ED_{50} of the assay under these conditions was 10–15 pg/mL.

Release experiments

Preparation of BNST minislices. BNST minislices were obtained from 250–300 g male Sprague–Dawley rats as follows: the animals were decapitated and the brains quickly removed and placed in ice-cold saline. A piece of the brain containing the BNST was mounted in a vibratome stage (CAMPDEM INST.) and sectioned in 300 μm coronal slices. The three coronal sections shown in Fig. 1 were placed in a cold glass, and the dorsal (dBNST) and ventral (vBNST) regions of the BNST were dissected with a small blade (1 mm) following the limits as indicated by Brownstein and Palkovits [6] and in the stereotaxic atlas of Paxinos and Watson [16] (Fig. 1).

Superfusion system. Minislices from dBNST and vBNST were placed in superfusion chambers and superfused with Krebs–Ringer-phosphate (KRP), saturated with 100% O_2, at a flow rate of 2.0 mL/min [17]. The composition of the KRP solution was (in mM): KCl, 4.85; $CaCl_2$ 0.75; $MgSO_4$, 1.2; NaCl, 128; Na_2HPO_4, 16; glucose, 16 pH 7.4. For high K^+-KRP, equimolar amounts of NaCl were replaced by KCl to maintain an iso-osmotic condition. After a superfusion period of 60 min, a basal sample of 2 min was collected. Release was evoked by a 2-min exposure to 55 mM K^+. The basal and stimulated samples were quickly removed and left at 4° until subjected to the concentration protocol and subsequent CCK quantification. At the end of the experiment, the slices were recovered and sonicated in 90% methanol to determine CCK content by RIA and proteins by the method of Lowry *et al.* [18].

Lesions of the stria terminalis. Animals were anesthetized with chloral hydrate (400 mg/kg) and mounted in a stereotaxic apparatus. Transection of the stria terminalis was made with a glass microknife [19]. The glass microknife (width 1.5 mm) was positioned at 2.5 mm posterior to the bregma and a cut was made from 3.0 to 4.5 mm lateral to the midline and to a depth of 6.0 mm from the surface of the brain. Sham control rats were treated in the same way, except that the microknife was not introduced into the brain. CCK levels in the dBNST and vBNST were analyzed 7 days after the surgery. The brain block containing the lesioned area was fixed in 4% formaldehyde for posterior verification of the extension of the lesion.

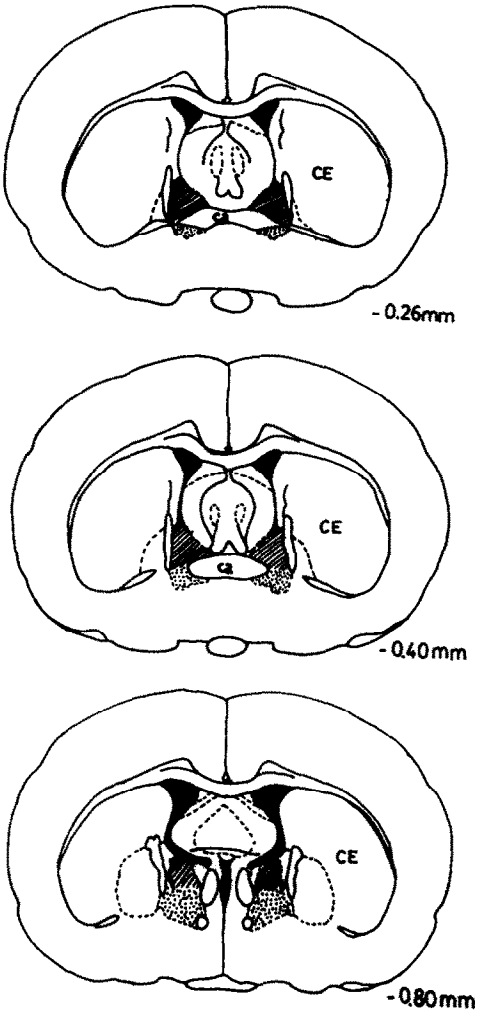

Fig. 1. Diagram of the dissection of the dorsal (hatched areas) and ventral (dotted areas) BNST. The dBNST and vBNST were dissected with the aid of a small blade from these three coronal sections following the limits marked.

RESULTS

Figure 2 shows the elution profile of a standard sample (25 pg CCK in 4 mL KRP) after following the concentration protocol. CCK eluted between 2.0 and 3.5 min after the injection of 1 mL acetonitrile, with a 77.6% recovery. In initial experiments, 100% methanol was used to elute the peptide, but it gave a much broader elution peak and CCK recovery was under 60% (data not shown). The RIA for CCK used in this work allows a maximum of 200 μL of physiological solution, as more salts in the assay interfere with the detection. Then, in the case of

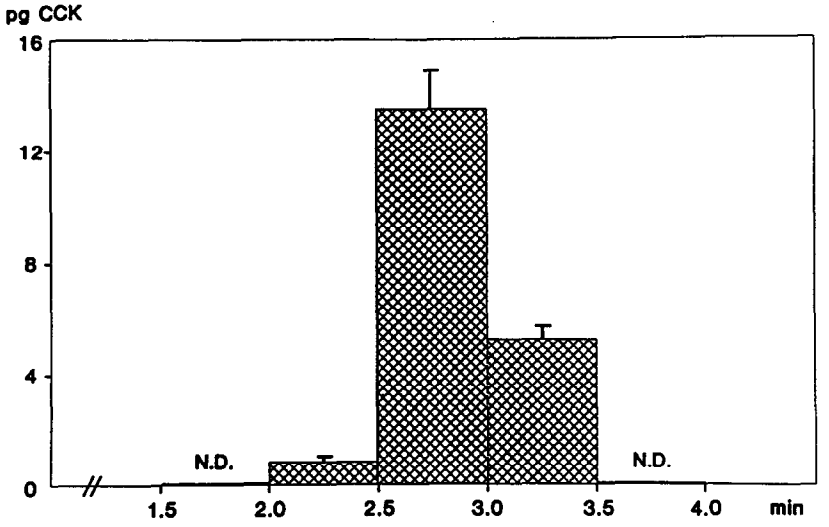

Fig. 2. Elution profile of CCK from a C18 column after injection of 1 mL of 100% acetonitrile. Samples containing 25 pg CCK in 4 mL KRP were injected into the HPLC setting as described in Methods. Aliquots were collected every 0.5 min after the acetonitrile injection and analyzed by RIA. Values are means ± SEM of 4 independent samples. ND = CCK not detectable by RIA.

Table 1. Recovery of CCK standards subjected to the concentration protocol

CCK standard (pg)	CCK recovered (pg)	Recovery (%)
10	8.7 ± 0.8	87
20	16.4 ± 1.2	82
50	40.5 ± 5.1	81

CCK samples were prepared in 4 mL of KRP and processed as described in Methods. Values are means ± SEM (N = 8).

these standard samples that were originally in 4 mL, the actual concentration was 20 times.

It was decided to collect samples from 2.0 to 4.0 min after acetonitrile injection to have a safe margin for possible pressure variations due to the injection of the sample. The system which was tested with standards in the range of 10–50 pg (Table 1) exhibited similar recoveries at these different CCK concentrations. A blank sample of 4 mL of KRP was run between each standard, and no detectable CCK was observed.

Minislices of dBNST or vBNST from three rats were pooled and subjected to the release protocol with two stimulation periods. The superfusates of basal and stimulated samples were concentrated to measure CCK release. Under basal conditions, the release of CCK was below the detection limit of the assay. K^+ (55 mM) induced a significant release of CCK from minislices of dBNST (Fig. 3A) and vBNST (Fig. 3B). The CCK release induced by the second stimulation period was lower than in the first stimulation period in both areas studied. When Ca^{2+} was omitted from the superfusion medium in the first stimulus, very little or no CCK release was observed, indicating that CCK release in the conditions studied was dependent on the presence of calcium in the extracellular medium (Fig. 3, A and B).

Seven days after transection of the stria terminalis, a marked decrease in CCK content in vBNST was observed (Table 2). In contrast, there was no significant change in CCK level in dBNST.

DISCUSSION

In the present study the release and possible origin of CCK in the dorsal and ventral regions of the BNST were studied. For this purpose, it was necessary first to work out conditions to concentrate the peptide. An adaptation of the concentration through Sep-Pack disposable minicolumns to an HPLC system was developed.

A reliable, simple and inexpensive method to simultaneously concentrate CCK and eliminate the salts present in physiological solutions was developed. This new system is based on the same principles of the disposable C18 minicolumns. However, it is very economical because it is possible to use one column for hundreds of samples. This is especially practical when the experiments generate many samples. Another advantage is the use of HPLC setting that permits a reliable injection of the sample into the column without loss, even when dealing with large volumes. The concentrating method described in this study has high recovery and facilitates the quantification of the peptide. These characteristics make it useful for the concentration of other neuropeptides.

The release experiments illustrate the applicability

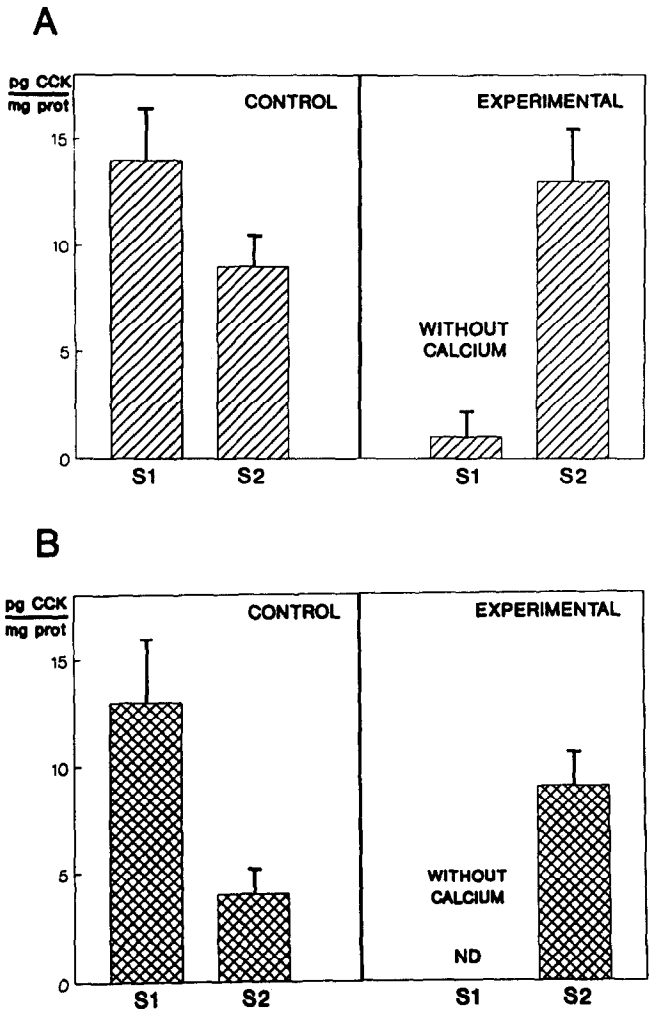

Fig. 3. Potassium-induced release of CCK from dorsal (A) and ventral (B) BNST minislices. The mean tissue content of CCK per experiment in ng/mg protein was 3.5 ± 0.2 for dorsal and 2.5 ± 0.3 for ventral BNST. Minislices were superfused as described in Methods. Fifty-five millimolar K^+-KRP evoked release, S1 and S2, is depicted as pg CCK/mg protein. In the experimental group, Ca^{2+} was omitted from the KRP used to superfuse the minislices during the beginning of the experiment and was introduced during the wash period previous to the second stimulation. The results are means ± SEM of 3–5 independent experiments.

Table 2. Effect of the lesion of the stria terminalis on CCK levels in dorsal and ventral BNST

BNST region	N	CCK (ng/mg protein)		
		Sham	Ipsilateral	Contralateral
Dorsal	7	2.22 ± 0.33	2.21 ± 0.30	2.62 ± 0.27
Ventral	9	2.40 ± 0.34	1.33 ± 0.13*	2.39 ± 0.30

The stria terminalis was lesioned by means of a glass microknife (1.5 mm). Coordinates were: AP: −2.56 mm from bregma, Lat: −3.0 mm, and DV: 6.0 mm. The sham group was anesthetized and manipulated in the same way as lesioned animals, but the microknife was not inserted in the brain. Values are means ± SEM.

* $P < 0.01$ compared to the contralateral side or to the sham group by the two-tailed Student's *t*-test.

of the method. It allowed us to study for the first time the release of CCK induced by depolarizing stimulus, from dorsal and ventral BNST. Potassium-induced release of CCK from minislices of dorsal or ventral BNST was demonstrated, and this release was shown to be calcium dependent. The basal release of CCK was below the detection limit of the CCK RIA. This was not surprising as it has been difficult to demonstrate basal release of endogenous neurotransmitters when using *in vitro* systems [20–22]. The lack of basal CCK concentration poses limitations to the quantitative interpretation of the results. However, this does not prevent the study of neurotransmitter release regulation when using this system. The S2/S1 ratio was lower than one, as it has been described previously for other peptides [22, 23]. Due to the small size of these brain areas it is not possible to study CCK release from these brain nuclei without concentrating the

superfusates, unless long incubation periods are used.

At the present time there is no information regarding the origin of the CCK in the BNST. There has been some controversy about the existence of CCK containing cell bodies in the BNST. It has been possible to visualize CCK-like immunoreactivity associated with cell bodies only after injection of high doses of colchicine into the area [24, 25]. In fact, the levels of messenger RNA coding for CCK measured by *in situ* hybridization in the BNST of naive rats is very low, indicating that most of the CCK in the BNST is present in nerve fibers and terminals originated outside the BNST [26, 27].

A good candidate for the CCK innervation to the BNST is the amygdala, an area which has a high density of CCK-containing cells [24]. The BNST receives innervation from several brain regions [11]. However, anatomical studies have clearly demonstrated that the most dense projection to the BNST is of amygdaloid origin. The afferent arrives to the BNST mainly, if not exclusively, via the stria terminalis [10–12]. This fiber tract has a topographical organization such that the medial aspects of the tract project to the ventral BNST and the lateral part to the dorsal BNST [28]. Other brain nuclei project to BNST through the medial forebrain bundle [11]. The available anatomical information plus the lesion studies of the present work suggest that the ventral, but not the dorsal BNST, receives a CCK-containing projection from the amygdala through the stria terminalis.

In conclusion, the findings reported here indicate that the dorsal and ventral area of the BNST have CCK present in a Ca^{2+}-dependent releasable compartment and that a significant portion of the CCK in the ventral BNST is of amygdaloid origin. It would be of interest to study whether CCK has a role in the stimulation of ventral BNST neurons induced by electrical stimulation of the amygdala [13].

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