Vol. 94, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1980 Pages 704-709

ALKALINE EXTRACTION OF CHOLECYSTOKININ-IMMUNOREACTIVITY FROM RAT BRAIN

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Received April 21,1980

SUMMARY: Alkaline aqueous extractants remove from rat brain 2 to 4 times the CCK-immunoreactivity that is removed by acidic or neutral aqueous extractants. The distribution among the various hormonal forms appears to be independent of the extractant: about 1/10 in the larger basic forms (CCK-33 and CCK-39); about 1/4 as the C-terminal dodecapeptide (CCK-12) and the remainder as the octapeptide (CCK-8). In contrast, alkaline and acidic aqueous solutions are equally efficient in extraction of enkephalin-immunoreactivity from the same tissues. We are presently unable to account for the very different efficiencies of the various extractants in removing CCK-immunoreactivity from brain.

INTRODUCTION

Prior investigations of cholecystokinin (CCK) peptides in brain have employed neutral or acidic aqueous solutions as the extractant medium (1-10). During studies of the adsorption of CCK to QUSO (microfine precipitated silica) (11), it was noted that alkaline solutions were capable of eluting CCK from the adsorbent. This study was performed to determine the extractability of CCK-immunoreactivity from rat brain by acidic, neutral and alkaline aqueous solutions and to compare the relative efficiencies of these extractants for removing another neuropeptide, leucine-enkephalin.

MATERIALS AND METHODS

Preparation of tissue extracts: Thirteen 200-400 gram male Sprague-Dawley rats were decapitated and the brains were removed quickly. The rhombencephalon, consisting of the pons, medulla oblongata and cerebellum, was separated from the remainder of the brain by a transverse section through the midbrain and then discarded. The entire remainder of the whole brain was immediately placed on dry ice. While still frozen, the whole brains were pulverized to form a coarse powder and then mixed together. Either 0.5M acetic acid, 0.1N HCl, distilled water, 100 mM Tris buffer (pH 10) or 0.1N NaOH was added to a 200-400 mg aliquot of the frozen pulverized mixture to a concentration of 0.2 gm wet weight of tissue per ml. The extraction solutions were placed in a boiling water bath for

Abbreviations used are: CCK, cholecystokinin; CCK-39, cholecystokinin variant; CCK-33, intact cholecystokinin; CCK-12, C-terminal dodecapeptide of cholecystokinin; CCK-8, C-terminal octapeptide of cholecystokinin..

0006-291X/80/100704-06$01.00/0

5 min and the tissue then homogenized with a Teflon tissue grinder. In one series of experiments, homogenates using acidic, neutral or alkaline extractants were prepared without boiling. Tissue extracts were centrifuged at 10,000xg for 30 min, the supernatants removed and frozen at -20°C until assayed. The pellets from unboiled and boiled water extracts were additionally extracted with boiling water and/or NaOH, centrifuged and the supernatants frozen until assayed. Prior to assay, the extracts were neutralized by dilution (1:8) in 0.25M phosphate buffer (pH 7.5) containing 2% fetal bovine serum (FBS). Sets of acetic acid, HCl, water, Tris-buffer and NaOH extracts were prepared concomitantly and assayed within 5 days of preparation.

Fractionaction of CCK-immunoreactivity: Fractionation of the CCK-immunoreactivity contained in brain extracts was accomplished by adsorption to QUSO (11) which binds the larger, basic forms of CCK, and by starch gel electrophoresis, which permits distinction among the C-terminal fragments of CCK (12).

0.2 ml of tissue extract was added to 5 mg of QUSO G32 (Philadelphia Quartz Co., Phila., Pa.) suspended in 1.4 ml of 0.25M phosphate buffer (pH 7.5) containing 2% FBS. The mixture was vortexed for 30 sec, centrifuged at 2000xg for 15 min at 4°C, decanted and the supernatants assayed for CCK. Adsorbed CCK was eluted from QUSO by addition of 0.5 ml of 0.1N NaOH to the residual pellet. The mixture was vortexed, centrifuged as above and the supernatant decanted. Ten to 50 µl portions of the NaOH QUSO eluates were assayed directly for CCK.

In order to determine the relative amounts of the C-terminal fragments of CCK, 0.2 ml of the tissue extracts were subjected to starch gel electrophoresis, as previously described (12). To establish reference markers for interpreting the starch gel electrophoretic patterns, synthetic sulphated CCK-8 (Squibb, Princeton, N.J.) and synthetic sulphated CCK-12 (prepared by Dr. M. A. Ondetti and received through the courtesy of Dr. Victor Mutt, Karolinska Institute, Stockholm) were subjected to similar starch gel electrophoresis.

Radioimmunoassay: Tissue extracts, supernatants of tissue extracts following QUSO adsorption, NaOH eluates of the QUSO pellets and starch gel eluates were assayed for CCK peptides using published methods (1,2). CCK-8 (Squibb) was employed as standard and ^{125}I-gastrin-17 (porcine heptadecapeptide gastrin, a gift from Dr. R. A. Gregory through the courtesy of Dr. Morton I. Grossman, Wadsworth VAMC) was used as tracer. Rabbit B antiserum cross-reacts identically on a molar basis with CCK-39, CCK-33, CCK-12 and CCK-8. The minimal detectable concentration of CCK in this system is 5 pg/ml.

The enkephalin content of tissue extracts was measured using a leucine-enkephalin radioimmunoassay developed according to the technique of Miller et al (13). Leucine-enkephalin (Calbiochem) was employed as standard and ^{125}I-leu-enkephalin as tracer. The rabbit anti-leu-enkephalin serum (1:3000) displayed a 2% cross-reactivity with methionine-enkephalin. The minimal detectable concentration of leu-enkephalin with this assay is 40 fmoles/ml.

RESULTS

Serial dilutions (10 to 50 µl) of all tissue extracts were superposable on the CCK-8 and leu-enkephalin standard curves (Fig. 1).

Extraction of CCK-immunoreactivity: Table 1 records the levels of CCK-immunoreactivity contained in extracts of rat brain prepared in boiling solutions.

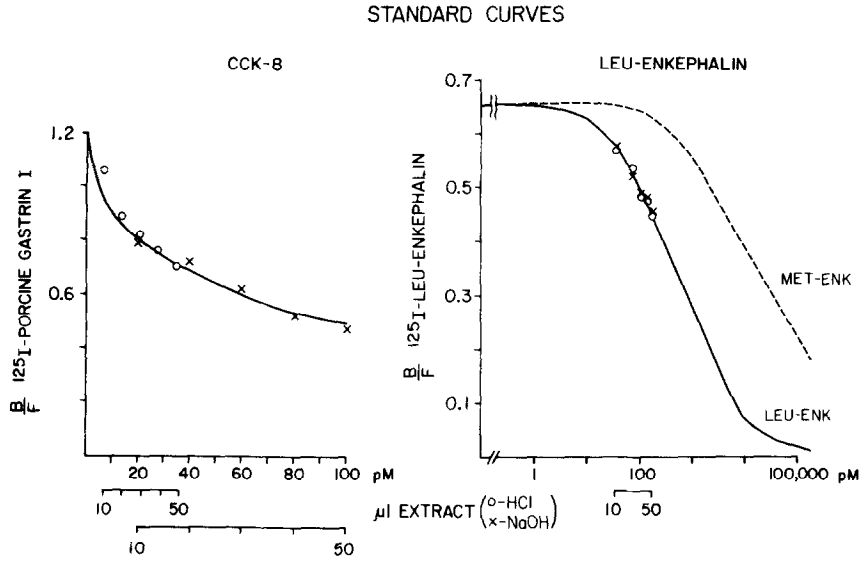

Fig. 1 - Standard curves for CCK-8 and leu-enkephalin assays. The immunoreactivities in 10 to 50 µl portions of HCl (0) and NaOH (X) extracts superpose along the standard curves. Note the increased CCK-8 immunoreactivity and similar leu-enkephalin immunoreactivity in the alkaline as compared to the acid extracts.

Both Tris-buffer and NaOH extracted more CCK-immunoreactivity from rat brain than did acetic acid, HCl or water. Compared to the amounts removed by acetic acid, HCl and water, NaOH extracted about 2 to 4 times both total ($p<.001$) and QUSO adsorbable ($p<.01$) CCK-immunoreactivities. Neither the percentage of CCK-immunoreactivity adsorbing to QUSO nor the starch gel patterns of the CCK-immunoreactivity varied with the different extractants. Generally more than 2/3 of

TABLE 1 - Effect of extractant on recovery of CCK and enkephalin immunoreactivity from rat brain.

	CCK-8 Equivalents (ng/g)		Leu-Enkephalin (pmoles/g)
Extractant	Mean ± SEM (number of extractions) Total	QUSO Eluate	
0.5M Acetic Acid	57±15 (2)***	---	---
0.1N HCl	97±10 (9)***	11±2 (6)**	76±13 (5)
H_2O	129±14 (8)***	11±2 (4)**	---
Tris (100mM, pH 10)	213±14 (6)**	14±3 (6)**	---
0.1N NaOH	273± 8 (13)	49±8 (7)	69±11 (5)

** $p<.01$
*** $p<.001$ level of significance compared to NaOH

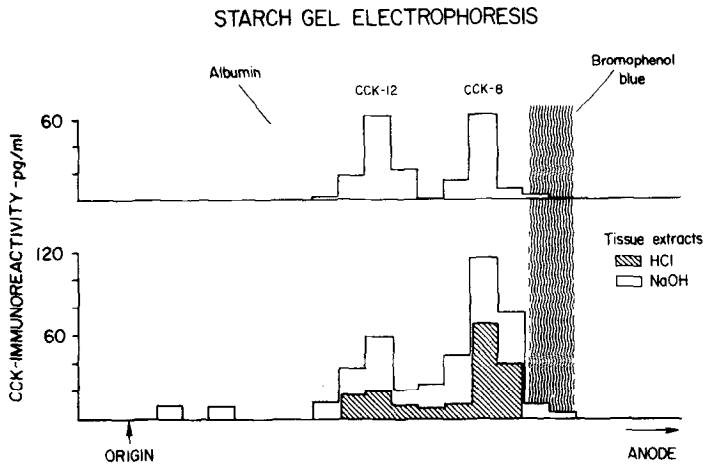

Fig. 2 - Starch gel electrophoretic patterns of CCK-immunoreactivity in HCl and NaOH extracts of brain (bottom). The patterns of authentic CCK-12 and CCK-8 are also shown (top).

the immunoreactivity recovered from starch gel had an electrophoretic mobility resembling CCK-8 and most of the remainder corresponded to CCK-12 (Fig. 2). CCK-33 is poorly recovered in the starch gel eluates.

It is of interest that, in another set of 4 extractions, 0.1N NaOH at room temperature extracted about the same amount of CCK-immunoreactivity (222±26 ng/g) as did the boiling alkaline extraction (227±31 ng/g). Similarly 0.1N HCl at room temperature was equally efficient in extraction (92±8 ng/g) as was the boiling acid (84±6 ng/g). However distilled water at room temperature was only about 10% as efficient in extracting CCK-immunoreactivity as is boiling water (Table 2).

Extraction of enkephalin-immunoreactivity from brain: In contrast to the marked differences in extractable CCK-immunoreactivity which depended on the method employed, virtually identical amounts of enkephalin were recovered with boiling HCl or boiling NaOH extractants (Table 1). The amounts recovered are comparable to those previously reported by Gros et al (16) and Miller et al (13).

Extraction of CCK from tissue pellet: Since CCK-immunoreactivity depended so strikingly on the extraction method, the question was addressed as to whether

TABLE 2 - Re-extraction of CCK-immunoreactivity from residual pellets

	CCK-8 equivalents (ng/g) mean ± SEM (number of extractions)	
Room Temp. H_2O	13±3 (6)***	
Boiling H_2O Extraction of Tissue Pellet		102± 5 (2)
Total CCK Extracted		115±13 (2)
Boiling NaOH Extraction of Tissue Pellet		222±48 (2)
Total CCK Extracted		235±48 (2)
Boiling H_2O	111±3 (2)	
NaOH Extraction of Tissue Pellet		133± 8 (2)
Total CCK Extracted		244± 9 (2)

***p<.001 level of significance, unboiled vs. boiled extractant.

it was due simply to failure to remove the immunoreactivity or whether there was a loss because of proteolytic degradation or other factors. The residual pellets from the room temperature and boiling water extractions were then re-extracted. Whereas room temperature water extraction had removed only 13 ng/g, re-extraction of the remaining pellet with boiling NaOH resulted in a total recovery (235 ng/g) (Table 2) comparable to that with an initial alkaline extraction. Re-extraction of the room temperature water pellet with boiling water extracted only about 1/2 the residual immunoreactivity (Table 2). Re-extraction of the pellet remaining after the boiling water extraction had removed 111 ng/g with boiling NaOH resulted in a total recovery (244 ng/g) also comparable to an initial alkaline extraction. Thus the decreased extraction in acid or neutral solution was not attributable to destruction of the CCK-immunoreactivity but simply to inefficiency of extraction. Re-extraction of the pellet after NaOH extraction removed only about 20±2% additional immunoreactivity so that the original alkaline extractions appear to be quite efficient.

DISCUSSION

The demonstration that CCK, originally thought to be only a gut peptide, is found in the brain in concentrations comparable to those found in the gut (1-10) and that it is localized in cortical neurons (14, 15) has provoked considerable

interest in its function. However, proper interpretation of the role of CCK and its changes in response to physiologic or pathophysiologic manipulations require quantitative recovery of total immunoreactivity and knowledge of the distribution of hormonal forms in brain tissue. Extraction in alkaline solutions appears to give optimal recovery of total immunoreactivity. The fraction in the form of the C-terminal fragments was about 90% and was independent of the pH of the extractant. The lower efficiency of acid or neutral extractants cannot be attributed to destruction of CCK-immunoreactivity since total recovery following re-extraction of the pellets with alkaline solutions results in quantitative recovery independent of the efficiency of the original extraction. We are unable to account for the rather similar recoveries of enkephalin-immunoreactivities in acid or alkaline extractions and the very different recoveries of CCK, consisting primarily of the C-terminal peptides, depending on the pH of the extractant.

ACKNOWLEDGEMENT

This work was supported in part by the Medical Research Program of the Veterans Administration.

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