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# Localization of cholecystokinin-like immunoreactivity in isolated nerve terminals

(synaptosomes/neuroregulator/cerebral cortex/radioimmunoassay/gastrointestinal hormone)

MICHEL PINGET, EUGENE STRAUS, AND ROSALYN S. YALOW

Veterans Administration Hospital, Bronx, New York 10468; and The Mount Sinai School of Medicine, City University of New York, New York 10029

*Contributed by Rosalyn S. Yalow, October 2, 1978*

ABSTRACT **Subcellular fractionation of the rat cerebral cortex demonstrated the presence of immunoreactive cholecystokinin in the pellet identified by electron microscopy as containing a high proportion of synaptic vesicles. The recovery in this pellet of 40% of the total immunoreactivity in the initial cortical extract is quite comparable to the recovery of other peptides such as vasoactive intestinal polypeptide and somatostatin, which are also located in synaptosomes and for which roles as neuroregulators or transmitters have been suggested. The evidence of concentration of cholecystokinin-like peptides in the synaptosomal pellet is consistent with our earlier demonstration by immunohistochemical techniques of cholecystokinin's presence in rabbit cerebral cortical neurons. These observations and the evidence for diminished concentration of cholecystokinin-like peptides in the brains of hyperphagic mice are consistent with cholecystokinin's suggested role as a neuroregulator for appetite.**

There are several likenesses in the biologic properties of cholecystokinin (CCK) and vasoactive intestinal polypeptide (VIP). Each was originally isolated from gastrointestinal mucosa (1–4) and was shown subsequently to be present in the brain (5–8). There do appear to be some differences in that there is not uniform agreement as to the hormonal form(s) of each of these peptides in each tissue site in which it has been found. Some have reported that VIP immunoreactivity in brain and gut was accounted for by a single component resembling intact VIP (6, 7), while another group has detected a minor component of smaller molecular size in the colonic mucosa (9). However, in the case of CCK, COOH-terminal fragments appear to be quite prominent. Several laboratories have reported that the COOH-terminal fragments, including the octapeptide (CCK-8), may account for virtually all the recoverable activity in the brain (10, 11) or in the gut (11). We have found both intact CCK and CCK-8 in the brain and gut, the apparent relative concentrations depending on the site and the extraction technique employed (8, 12). Both VIP (13) and CCK-like peptides (14) have been shown to be localized in the neurons of the cerebral cortex. A possible role for VIP as a neurotransmitter or regulator has also been suggested by the observations demonstrating its concentration in synaptosomal preparations (15). In this report we investigate whether the subcellular distribution of CCK-like immunoreactivity in the rat cerebral cortex is similar to that of VIP.

## MATERIALS AND METHODS

**Preparation of Subcellular Fractions.** After decapitation the brains of 18 female Sprague Dawley rats (362 ± 9 g) were removed. Six pools of the entire cerebral cortex (400 ± 10 mg) of three rats were prepared by homogenizing the tissue gently in 10% (wt/vol) sucrose (0.32 M) by using a Teflon tissue grinder (10 strokes, 500 rpm).

Generally subcellular fractionation was carried out according to the method of Whittaker (16). Portions of the extracts were centrifuged at 4°C for 10 min at 1000 × *g*. The resulting pellet was resuspended in the same volume of 0.32 M sucrose and centrifuged again under the same conditions. The pellet ($P_1$) was saved for assay. The two supernatants were pooled ($S_1$), a portion was saved for assay, and the remainder was centrifuged in a Sorvall RC2B for 55 min at 17,000 × *g*. The supernatant ($S_2$) was saved for assay. The pellet ($P_2$) was resuspended in 3 ml of cold 0.32 M sucrose, 1 ml was saved for assay, and the remainder was applied to a discontinuous gradient. Two milliliters of the suspension was mixed with 5 ml of 1.4 M sucrose and placed at the bottom of the tube (mean concentration 1.2 M sucrose). Then 5 ml of 0.8 M and 2 ml of 0.32 M sucrose were layered above and the tube was centrifuged for 110 min at 55,000 × *g*. The fractions floating at the interface between 0.32 M and 0.8 M sucrose (A), between 0.8 M and 1.2 M (B), and below 1.2 M (C) were collected separately and the remaining sucrose was discarded. The A, B, and C fractions were each diluted in Krebs–Ringer phosphate buffer, then separated into two portions and centrifuged for 60 min in a Spinco model L ultracentrifuge fitted with a Beckman SW 50.1 swinging-bucket rotor at 100,000 × *g*. For each set one pellet was fixed in glutaraldehyde and saved for examination by electron microscopy, and the other pellet was stored at −50°C prior to extraction for assay. All supernatants and pellets to be stored for assay were first suspended in Krebs–Ringer phosphate buffer and centrifuged for 100,000 × *g* for 60 min, and the pellets were stored at −50°C prior to extraction. After pelleting the supernatants were also assayed for residual immunoreactivity.

In two experiments subcellular fractionation was carried out according to the method of Jones and Matus (17), which differs from that of Whittaker (16) in that the centrifugation leading to the $P_1$ pellet is for 20 min at 900 × *g* and the one leading to the $P_2$ pellet is for 20 min at 9000 × *g*. The $P_2$ pellet was then incubated for 30 min in 2 ml of 5 mM Tris·HCl buffer, pH 7.5, prior to fractionation on the discontinuous density gradient described above.

The protein contents of the final pellets were determined by the method of Lowry *et al.* (18).

**Radioimmunoassay.** Immunoreactive CCK was extracted from all pellets by suspension in 0.1 M HCl at a concentration of 100 mg/ml, followed by incubation in a boiling water bath for 5 min. The extracts were centrifuged for 20 min at 60,000 × *g* and the supernatants were either assayed directly or stored at −50°C prior to assay.

The assay was performed according to methods previously published (8, 12). $^{125}$I-Labeled porcine heptadecapeptide gastrin was used as tracer. The rabbit B antiserum used in this assay crossreacts identically with intact CCK and the COOH-

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Abbreviations: CCK, cholecystokinin; CCK-8, COOH-terminal cholecystokinin octapeptide; VIP, vasoactive intestinal polypeptide.

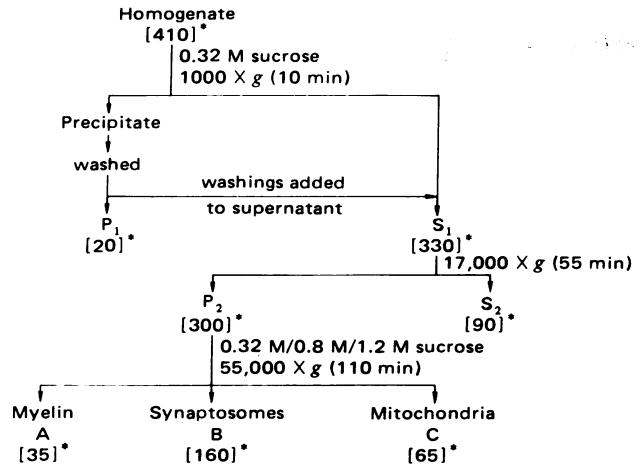


FIG. 1. Flow diagram and recovery of CCK-like immunoreactivity for subcellular fractionation of a rat cerebral cortical extract. * Numbers in brackets are ng of CCK-8 equivalents per g wet weight of tissue.

terminal peptides. CCK-8 was used as standard and all concentrations are reported as ng of CCK-8 equivalent per g wet weight of original tissue after correction for dilution and separation into the various portions.

## RESULTS

The flow diagram and the recovery of CCK-like immunoreactivity for the subcellular fractionation of a rat cerebral cortical extract according to the Whittaker method (16) are shown in Fig. 1. The electron micrographs (Fig. 2) demonstrate that, as expected, pellet A (*left*) consisted primarily of myelin, pellet B (*center*) contained a high proportion of synaptic vesicles with some mitochondria, and pellet C (*right*) contained primarily mitochondria. In the experiment shown, pellet B, the synaptosomal fraction, contained 40% of the immunoreactive content of the initial cerebral cortical extract and 64% of the immunoreactivity recoverable in the three final pellets. In four similar experiments the B pellet contained 38%, 39%, 46%, and 60% of the starting immunoreactivity and 52%, 61%, 65%, and 72% of the immunoreactivity recovered in the final pellets, respectively.

Portions of two of the original cerebral cortical extracts were fractionated according to the method of Jones and Matus (17). The immunoreactivity recovered in the B pellet (16% and 36% of the initial immunoreactivity) was somewhat less than in the Whittaker method. It accounted for 55% and 73% of the total recovered in the three final pellets. The loss occurred in part in the second centrifugation. The $P_2$ pellet generally contained only 50% of the immunoreactivity in $S_1$ compared to the 90% generally found by using the Whittaker centrifugation, which was of higher speed and longer duration. Additional losses occurred during the 30-min incubation in Tris buffer.

In one experiment using the Whittaker method the pellets A, B, and C contained 38, 54, and 15 μg of CCK-8 equivalent immunoreactivity per g of protein.

In a single study performed similarly in which the starting material was an extract of the cerebellum, there was no CCK-like immunoreactivity in the initial homogenate or in any of the subsequent fractions.

## DISCUSSION

Synaptosomes prepared according to the Whittaker method (16) or some modification thereof retain the morphologic features and, presumably, the transmitter content of the intact presynaptic terminal. In our hands the Whittaker technique (16) appeared preferable to that of Jones and Matus (17) because on the average twice as much CCK-like immunoreactive material was recovered in the synaptosomal fraction. The recovery of the initial immunoreactivity in the synaptosomal fraction in these studies (≈40%) is quite comparable to that for VIP (15, 19) and somatostatin (20), which have been suggested to function as neuroregulators. The finding of a relatively high fraction of the CCK-like immunoreactivity in cerebral cortical extracts in the synaptosomal fraction is thus consistent with its potential role as a neurotransmitter or regulator.

Further evidence for the possible role of CCK-like peptides as neuroregulators is derived from the well-accepted observations that the intact ventromedial hypothalamus serves as an appetite center and that CCK-like peptides appear to have a role in induction of satiety, particularly when administered intracranially (21, 22). That endogenous cerebral CCK may play such a role is also suggested by our recent finding that the brains of hyperphagic *ob/ob* mice are relatively deficient in this peptide (23).

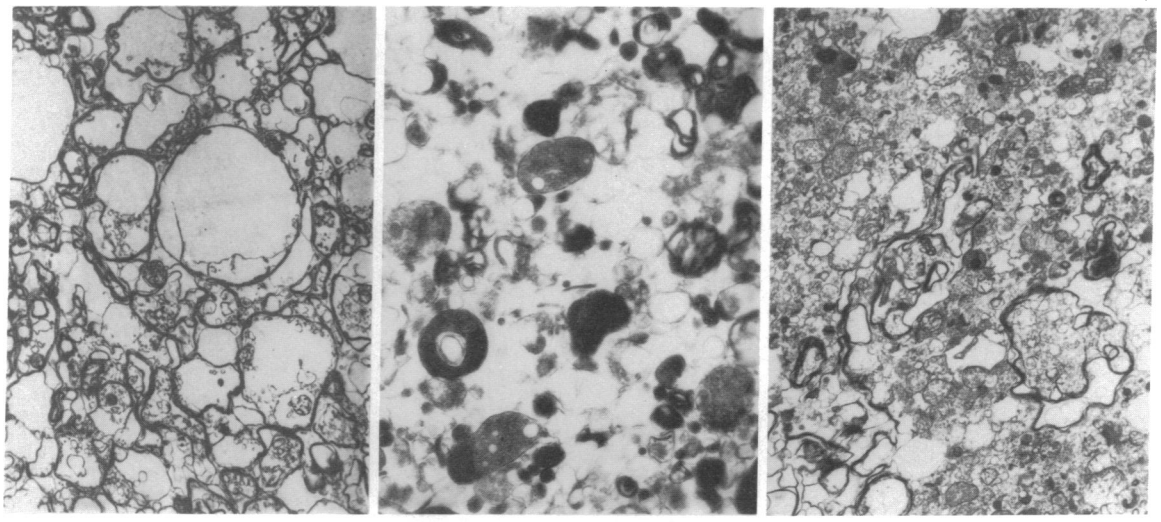


FIG. 2. Electron micrographs of final pellets after subcellular fractionation. Pellet A (*Left*) consists primarily of myelin. (×28,000.) Pellet B (*Center*) contains a high proportion of synaptic vesicles with some mitochondria. (×120,000.) Pellet C (*Right*) contains primarily mitochondria. (×28,000.)

The evidence of concentration of CCK-like peptides in rabbit cortical neurons (14) and, as shown here, in the synaptosomal pellet from extracts of the rat cerebral cortex, and the evidence for diminished concentration of CCK in the brain of hyperphagic mice, are all consistent with CCK's serving as a neurotransmitter involved in the regulation of appetite. Although similar roles as neuroregulators have been suggested for VIP or somatostatin, no evidence has as yet been presented that defines the function(s) that they are purported to regulate.

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