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Demonstration of biological activity of brain gastrin-like peptidic material in the human: Its relationship with the COOH-terminal octapeptide of cholecystokinin

(neuropeptides/gastrointestinal peptides/pancreatic amylase and adenylate cyclase/caerulein binding/size and charge discrimination)

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ABSTRACT **The previously described peptidic material that reacts with antibodies to gastrin and is found in the central nervous system of various vertebrates is present in only the 100,000 × *g* pellet of postmortem human cerebral cortical grey matter. This immunoreactive material, extractable in boiling water, is biologically active on rat pancreatic preparations. On the basis of size, charge, immunological specificity, and patterns of biological activity, most of this material is closely related to the COOH-terminal octapeptide of cholecystokinin in its complete, sulfated biologically active form.**

A number of hypothalamic peptides, such as thyrotropin-releasing hormone (1), luteinizing hormone-releasing hormone (2), somatostatin (3), and substance P (4), have been shown to be present in the cerebral cortex. It has been hypothesized that the extrahypothalamic localization of these peptides may result from the existence of peptidergic hypothalamocortical pathways (5). Some of these hypothalamic peptides, such as substance P and somatostatin (6, 7), have also been detected in the digestive tract.

These observations, and Pearse's theory (8) of an embryological relationship between the nervous system and gut endocrine cells, have led Vanderhaeghen *et al.* (9) to search for and find immunologically active gastrin-like peptidic material (BGP) in the central nervous system (CNS) of various vertebrates. BGP was found at concentrations as high as 30 pmol of gastrin equivalent per g of wet weight. This raises the possibility of synthesis of BGP within the CNS. Because concentrations of BGP were maximal in the cerebral cortex and striatum, a hypothalamic origin of BGP is unlikely. Elution patterns of BGP on Sephadex G-50 show a lower molecular weight than gastrin-(2–17)-hexadecapeptide, and the analysis of radioimmunoassay dose–response curves shows immunological differences between gastrin-(2–17)-hexadecapeptide and BGP.

The search for digestive peptides in the CNS is currently expanding rapidly. Vasoactive intestinal polypeptide (VIP) has been detected in the brain (10–12), pancreatic polypeptide (PP) has been found in earthworm neurons and processes (13), and bombesin may be present in the brain (14). Conversely, neurotensin (15) and opioids (16) have been discovered in the digestive system. From the above considerations emerges the concept of the existence of a large number of peptides common to the nervous and digestive systems.

The existence of BGP has been confirmed by several authors. The differences reported between gastrin and BGP (9) have been explained by the close immunological relationship of BGP to the cholecystokinin family (17–20). The cholecystokinin peptides indeed possess the same COOH-terminal pentapeptide as gastrin. More precisely, the presence in brain of intact cholecystokinin (CCK) (17–19) and the COOH-terminal octapeptide of cholecystokinin (CCK-8) (17, 19) has also been suggested on the basis of immunological studies and fractionation procedures involving both size (17, 19) and charge (19) of the molecules.

The biological activity of BGP has not been investigated; coupled with fractionation procedures, it can be a valuable complement to immunological studies, because structures involved in immunological reactions are not necessarily identical to those responsible for biological activity. The present report demonstrates biological activity on rat pancreatic preparations of BGP from postmortem water-boiled extracts of human grey matter. These biological studies, coupled with immunological techniques and fractionation procedures involving size and charge, show that this material is closely related to biologically active CCK-8.

MATERIAL AND METHODS

Preparation of Brain Extracts. All specimens were inspected under the microscope for normality. Human cerebral cortical grey matter and human cerebellar cortex were dissected during autopsies 8–12 hr after death. Tissues were placed in distilled water at 4° to produce a concentration of 0.1 g wet weight of tissue per ml and then homogenized with a glass homogenizer and a motor-driven Teflon pestle. Three different extracts were prepared: First, the homogenate was boiled for 15 min and then centrifuged for 30 min at 100,000 × *g*. The supernatant was called "cerebral or cerebellar boiled homogenate." The second extract was made by centrifuging the homogenate for 30 min at 100,000 × *g*; the supernatant was called "cerebral or cerebellar supernatant." Finally, the pellet from the second preparation was resuspended in distilled water at 4° to produce a concentration of 0.1 g wet weight of initial tissue per ml, boiled and then centrifuged for 30 min at 100,000 × *g*. This third supernatant was called "cerebral or cerebellar boiled pellet."

Fractionation Procedures: Dextran Gel Filtration. Three milliliters of cerebral boiled pellet was applied to a 75- × 1.5-cm column of Sephadex G-50 (Pharmacia Fine Chemicals) equilibrated in 50 mM ammonium acetate buffer, pH 5.3. Flow rate was 10 ml/hr and 2-ml fractions were collected.

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Abbreviations: gastrin-(2–17), hexadecapeptide of synthetic human desulfated gastrin; CCK, cholecystokinin (CCK-8, CCK-10, CCK-12, respectively, COOH-terminal octapeptide, decapeptide, dodecapeptide of cholecystokinin); BGP, immunologically active brain gastrin-like peptidic material; CNS, central nervous system; VIP, vasoactive intestinal polypeptide.

Ion Exchange Chromatography. Three milliliters of cerebral boiled pellet was applied to a 5- × 0.5-cm column of diethylaminoethyl-cellulose (DE-11, Whatman) equilibrated in 50 mM ammonium acetate buffer, pH 5.3, and eluted with a linear gradient of ammonium acetate, pH 5.3, from 50–1000 mM (50 ml/50 ml).

Radioimmunoassay. Radioimmunoassay was performed as described (21). However, serum with high affinity for gastrin was replaced by serum of lower specificity that recognizes both gastrin and CCK-8. This serum does not recognize hormones of the secretin family. Dose–response curves were studied with gastrin-(2–17)-hexadecapeptide [gastrin-(2–17)] (Imperial Chemical Industries), CCK-8, CCK-12 (Squibb Institute for Medical Research, Princeton, NJ), and different brain extracts diluted in 0.1 M potassium phosphate buffer, pH 7.4, with 1% (wt/vol) bovine serum albumin and 500 kallikrein inhibition units of Trasylol (Bayer) per ml.

Amylase Release from Rat Pancreatic Fragments. The detailed procedure has been described (22). Rat pancreatic fragments were preincubated in Krebs–Ringer bicarbonate buffer with 10 mM glucose, 5 mM theophyllin, and 500 kallikrein inhibition units of Trasylol per ml. After 10 min the medium was replaced by a fresh one containing the material to be tested. The amylase output was determined after 30-min incubation at 37 °

Adenylate Cyclase Activity of Rat Pancreatic Plasma Membranes. A 10% (vol/vol) homogenate of rat pancreatic fragments in a 20 mM Tris·HCl buffer, pH 7.4, containing 0.3 M sucrose, 5 mM 2-mercaptoethanol, 1 mM EDTA, 2 mM MgCl, 500 kallikrein inhibition units of Trasylol per ml, and 1 mg of hepatic phospholipids per ml was centrifuged at 15,000 × *g* for 10 min. The resulting pellet, rehomogenized in the starting buffer, was layered on a sucrose gradient and centrifuged at 30,000 × *g* for 3 hr. Characteristics of the plasma membrane fraction found at the 27–35% interface have been described (23). Adenylate cyclase activity was studied according to Salomon *et al.* (24).

Inhibition of [^{3}H]Caerulein Binding to Rat Pancreatic Plasma Membranes. The detailed procedure of this radioreceptor assay has been described (25). Briefly, pancreatic plasma membranes, 25 nM [^{3}H]caerulein, and various concentrations of the material to be tested were incubated for 15 min in a 0.2% bovine serum albumin/20 mM Tris·HCl buffer, pH 7.4 at 37°. Bound and free ligand were separated by the addition of 2 ml of ice-cold 20 mM Tris·HCl buffer, pH 7.4/0.2% bovine serum albumin and immediate filtration of the medium on a 0.2-μm pore diameter cellulose acetate Millipore filter. For each assay, the nonspecific binding measured after addition of 10 μM unlabeled caerulein to the assay medium was subtracted.

RESULTS

Radioimmunoassay. Dose–response curves were parallel for gastrin-(2–17), CCK-8, CCK-12, or successive dilutions of cerebral boiled homogenate or cerebral boiled pellet. The serum discriminated poorly between gastrin-(2–17), CCK-8, and CCK-12, because a bound-to-free ratio of 0.5 was achieved with, respectively, 0.4. 1.4, and 1.6 nM peptide concentrations. Expressed in CCK-8 equivalent pmol/g ± SEM wet weight, concentrations were respectively 227 + 27 for cerebral boiled homogenate (n = 12) and 230 ± 14 for cerebral boiled pellet (n = 6). No activity was found in cerebral supernatant or in cerebellar extracts although 4 pmol/g of wet weight can be detected by this assay. Recovery of added CCK-8 to cerebral boiled pellet was complete.

Amylase Release. (Fig. 1) CCK-8 was the most active agent in this assay system. Dose–response curves for CCK-8, nonsulfated CCK-8 gastrin-(2–17), and successive dilutions of cerebral boiled homogenate or cerebral boiled pellet were parallel. For secretin and VIP a lower maximal effect was obtained. No activity was found in cerebral supernatant or cerebellar extracts.

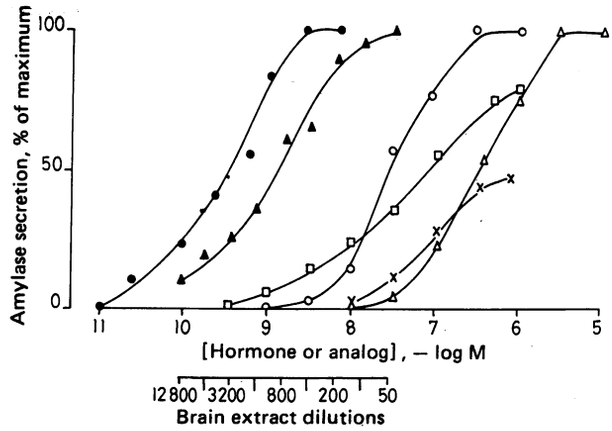

FIG. 1. Hormone stimulation of amylase secretion by rat pancreatic fragments. The data are plotted as percentages of the maximum value observed in the presence of 3 nM CCK-8 against increasing concentrations of CCK-8 (●), nonsulfated CCK-8 (○), gastrin-(2–17) (△), natural porcine secretin (□), VIP (×), and successive dilutions of cerebral boiled pellet (▲). The values are the mean of at least three determinations.

Adenylate Cyclase Activity. (Fig. 2) CCK-8 was again the most active agent in this assay system. Dose–response curves for CCK-8 and successive dilutions of cerebral boiled homogenate or cerebral boiled pellet were parallel. For secretin, VIP, gastrin-(2–17), or nonsulfated CCK-8 a lower maximum effect was obtained. No activity was found in cerebral supernatant or in cerebellar extracts. Incubation of cerebral boiled pellet with anti-gastrin serum for 48 hr at 4° resulted in inhibition of adenylate cyclase stimulation. This was not the case after incubation with serum of the same animal prior to immunization (Fig. 3). Porcine CCK, CCK-12, or CCK-10 and caerulein behave similarly to CCK-8 for adenylate cyclase stimulation.

Competition with [^{3}H]Caerulein Binding to Rat Pancreatic Plasma Membranes. (Fig. 4) CCK-8 was the most powerful

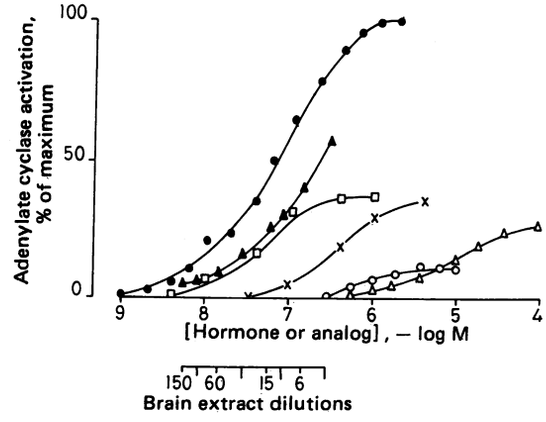

FIG. 2. Stimulation of adenylate cyclase activity of rat pancreatic plasma membranes. The data are plotted as percentages of the maximum value observed in the presence of 1 μM CCK-8, against increasing concentrations of CCK-8 (●), nonsulfated CCK-8 (○), gastrin-(2–17) (△), natural porcine secretin (□), VIP (×), and successive dilutions of cerebral boiled pellet (▲). The values are the mean of four determinations.

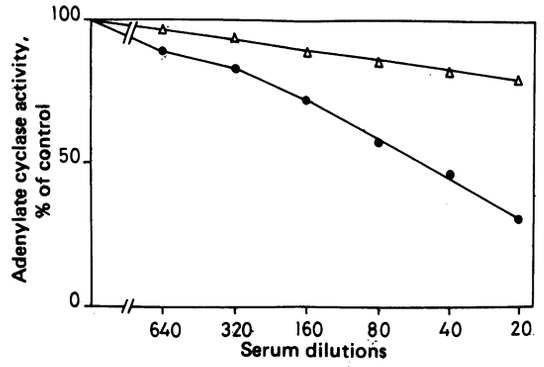

FIG. 3. Anti-gastrin serum inhibition of brain extract activity on rat pancreatic plasma membrane adenylate cyclase. Cerebral boiled pellet was incubated 48 hr at 4° with an equal volume of successive dilutions of rabbit anti-gastrin serum (●), or normal rabbit serum (△) prior to adenylate cyclase assay. The data are expressed as percentages of the value observed in the presence of the same cerebral boiled pellet incubated for 48 hr at 4° in an equal volume of 0.1 M potassium phosphate buffer, pH 7.4, with 1% (wt/vol) bovine serum albumin.

competitor. Dose–response curves for CCK-8, nonsulfated CCK-8, gastrin-(2–17), and successive dilutions of cerebral boiled homogenate" or cerebral boiled pellet were parallel. No competition was obtained with secretin or VIP. Cerebral supernatant or cerebellar extracts were devoid of any activity. In these three bioassays, substance P, neurotensin, and pancreatic polypeptide were inactive (data not shown). Bombesin, however, stimulated amylase secretion (26).

Chromatographic Procedures. On Sephadex G-50 columns (Fig. 5) the biological activity of different fractions was measured by the stimulation of adenylate cyclase activity. This biological activity eluted in the same fractions as did CCK-8, as well as most of the immunoactivity. Human gastrin-(2–17) and CCK eluted in different positions. A small portion of the biological and immunological activity eluted near the void volume, but the same thing occurred when CCK-8 was filtered in the presence of cerebellar extracts devoid of activity. On DEAR-cellulose columns (Fig. 6) most of the immunological activity and most of the biological activity assayed by stimulation of adenylate cyclase eluted in the same position as CCK-8.

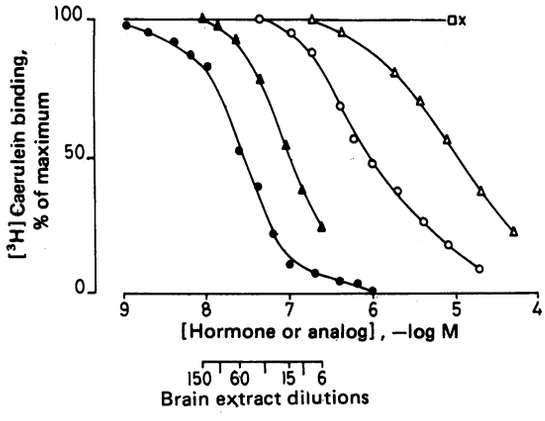

FIG. 4. Inhibition of specific binding of [³H]caerulein in rat pancreatic plasma membranes. The data are plotted as percentages of maximal binding observed in the presence of tracer only (25 nM [³H]caerulein) against increasing concentrations of CCK-8 (●), nonsulfated CCK-8 (○), gastrin-(2–17) (△), natural porcine secretin (□), VIP (×), and successive dilutions of cerebral boiled pellet (▲). Results are the mean of three determinations.

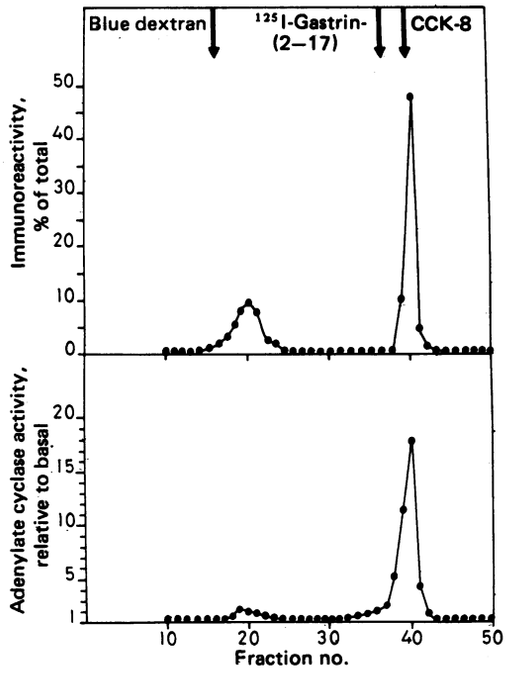

FIG. 5. Fractionation of 3 ml of cerebral boiled pellet on Sephadex G-50 (1.5 × 7.5 cm). Two-milliliter fractions were collected every 12 min. The column was eluted with 50 mM ammonium acetate buffer, pH 5.3, at 4°, and had previously been calibrated with CCK-8, ^{125}I-labeled gastrin-(2–17), and blue dextran as indicated by arrows. Rat pancreatic adenylate cyclase activity as a fraction of unstimulated value (*Lower*) and immunoreactivity as percentage of total activity (*Upper*) are given for each fraction.

gastrin(2–17) eluted at a much higher ionic strength and porcine CCK was not retained by the column. CCK-12 and CCK-10 eluted just before CCK-8.

Comparison of Figures Obtained by Measuring Immunological or Biological Activity of Brain Active Material. Recovery of CCK-8 added to cerebral boiled pellet was complete as measured with the three bioassays. However, 20 times more material was estimated to be present when bioassays were used instead of radioimmunoassays in cerebral boiled homogenate or in cerebral boiled pellet. The same discrepancy was also found when bioassays and radioimmunoassays were performed on purified chromatographic fractions.

DISCUSSION

The less specific anti-gastrin serum of high affinity for CCK-8 used in this study has greater affinity for BGP than the more specific serum used previously (9). This is in agreement with immunological studies indicating that BGP is more closely related to the cholecystokinin family than to the gastrins (17–20).

Water-boiled extracts of human cortical grey matter that contain BGP are biologically active on rat pancreatic preparations. Similar figures, expressed in CCK-8, are obtained with three different bioassays. This biologically active material consists merely of BGP, because adenylate cyclase stimulation by this compound is specifically reduced if BGP is preincubated with anti-gastrin serum.

Analysis of the three dose–response bioassays permits differentiation between biologically active material from secretin and from VIP. The most discriminative test using adenylate cyclase stimulation demonstrates that biologically active material is related to CCK-8 in its sulfated form and not to gastrin or nonsulfated CCK-8. Other substances that behave similarly

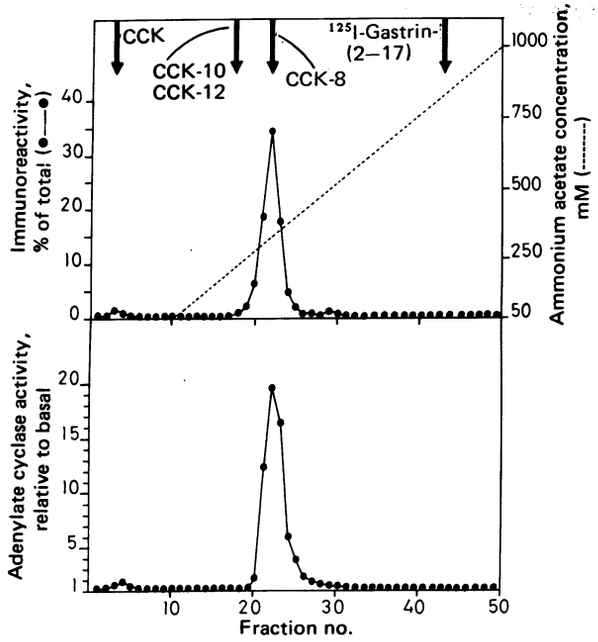

FIG. 6. Fractionation of 3 ml of cerebral boiled pellet on a DEAE-cellulose column (0.5 × 5.0 cm). Two-milliliter fractions were collected. The column was equilibrated in 50 mM ammonium acetate buffer, pH 5.3, and was eluted with a 100-ml linear gradient of ammonium acetate, pH 5.3, from 50 to 1000 mM. The column had previously been calibrated with CCK-8, CCK-10, CCK-12, ^{125}I-labeled gastrin-(2–17), and CCK as indicated by arrows. Rat pancreatic adenylate cyclase activity as a fraction of unstimulated value (*Lower*) and immunoreactivity as percentages of total activity (*Upper*) are given for each fraction.

for pancreatic adenylate cyclase stimulation were also tested (25): they are porcine CCK, amphibian skin decapeptide caerulein, CCK-10, and CCK-12. All these substances have the same COOH-terminal pentapeptide as gastrin and a sulfated tyrosine in the seventh position from the COOH terminal (27).

Most of BGP or biologically active material elutes as a single peak with CCK-8 on both Sephadex G-50 and DEAE-cellulose columns. Therefore, most of the BGP and biologically active material are closely related to CCK-8. It is clear, however, that the two chromatographic methods would not be able to distinguish between CCK-8 and a peptide having the same charge, but a difference in one or two amino acids.

The almost complete absence of molecules larger than CCK-8 in our material is not surprising. It could be related to postmortem degradation and to our extraction procedure. Further experiments by our group and another laboratory (17) suggest that multiple forms of BGP are present in rat brain. It has recently been shown that acidic instead of water extraction followed by boiling favors isolation of intact CCK-like substance in the pig brain (19). In this respect, it is interesting to note that antiserum specific for the NH_2-terminal part of CCK with no crossreactivity for CCK-8 has been used for radioimmunoassays of brain extracts. With this serum, various figures, expressed in CCK pg/mg of wet weight, were obtained in cerebral cortical grey matter ranging from 0.7 in the rat to 33.0 in the cat (18).

A discrepancy between figures obtained by bioassays and immunoassays is not uncommon. In the present study, this discrepancy is not due to the presence of an hypothetical inhibitor or activator in brain extracts. It persists in purified material. The addition of brain extracts does not alter the recovery of CCK-8 standard. The discrepancy therefore can be explained only by a difference between purified BGP and the CCK-8 standard. The serum used in this study, although specific for hormones having the same COOH-terminal pentapeptide, poorly discriminates between gastrin, CCK-12 and CCK-8. It is therefore unlikely that a small difference between BGP and CCK-8 can induce a 25-fold underestimation of BGP by radioimmunoassay. The bioassays used are much more sensitive to slight differences between BGP and CCK-8. In order to obtain the same adenylate cyclase stimulation, one must use a hundred times more of the nonsulfated CCK-8 than the sulfated form. Incomplete sulfation of the CCK-8 standard will undoubtedly result in overestimation of BGP in bioassays. Nonsulfated CCK-8 would hardly be detectable by our chromatographic procedures.

Figures around 250 pmol/g wet weight of CCK-8 obtained by radioimmunoassay in our material, even though slightly overestimated because of possible denaturation of CCK-8 standard, are still in good agreement with the figures of 50–150 pmol/g found in the rat (17) and 170 pmol/g found in the pig (19) using boiling water brain extracts.

In conclusion, most of the BGP is present in the particulate fraction of water-boiled brain extracts, is active on rat pancreatic preparations, and is closely related to biologically active CCK-8. Final identification of BGP will ultimately depend on chemical characterization after purification. The functional significance of BGP in the CNS is still unknown, as is the case for several other peptides present in both brain and digestive tract. Knowledge of BGP biological activity on peripheral tissues such as pancreatic preparations in rat may be used as a model for similar experiments in the CNS.

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