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Identification and localization of glucagon-related peptides in rat brain

(glucagon-like immunoreactivity/hypothalamus/magnocellular neurons/immunocytochemistry)

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ABSTRACT **Immunochemical and immunocytochemical techniques have been used to identify and characterize glucagon-related peptides of the rat central nervous system. These peptides show immunoreactivity with antiglucagon sera directed towards the central portion of the hormone, but not with antisera specific for the free COOH terminus of glucagon. Highest concentrations were found in hypothalamus (6.1 ± 1.6 ng/g wet weight) although lower amounts (approximately 2 ng/g) were found in cortex, thalamus, cerebellum, and brain stem. Gel filtration of brain extracts revealed at least two immunoreactive forms, which have molecular weights of about 12,000 and 8000. Both peptides had radioimmunoassay dilution curves parallel to the curve for glucagon and both had identical counterparts in extracts of rat intestine. Digestion of the brain and intestinal peptides with trypsin plus carboxypeptidase B released the immunoreactive COOH-terminal tryptic fragment of pancreatic glucagon from these larger forms. Immunocytochemical studies using antiglucagon serum and peroxidase–antiperoxidase staining identified glucagon-like material in neuronal cell bodies and processes in the magnocellular portion of the paraventricular nucleus, as well as in scattered cells in the supraoptic nucleus and in fibers in the median eminence. These results suggest that glucagon-containing peptides that have undergone the intestinal type of posttranslational modification are present in neuronal cells of the rat hypothalamus.**

During recent years, a number of intestinal peptides, including substance P (1, 2), vasoactive intestinal peptide (3, 4), and cholecystokinin (5–7), have been identified within the mammalian brain. Immunoreactive and biologically active pancreatic insulin has also been identified in central nervous tissue of the rat and has been localized, in part, to cells in the olfactory lobe and hypothalamus (8). Conversely, somatostatin (9) and thyrotropin-releasing hormone (10), peptides originally identified in the hypothalamus, have now been found in pancreatic islets. Although in many cases the anatomical diversity of these peptides has yet to be explained functionally, these findings emphasize a close relationship among peptides of the nervous system and those of the pancreas and gastrointestinal tract.

Peptides related to pancreatic glucagon occur in several tissues of the body and represent an unusual group of proteolytically modified forms: although gastric glucagon appears to be identical to the 29-residue hormone isolated from pancreas (11), intestinal glucagon-like peptides have higher molecular weights and contain the structure of the hormone extended from its NH_2 and COOH termini (12–16). By structural (14, 15), immunocytochemical (16), and biosynthetic (17, 18) criteria, it appears that these larger peptides represent incompletely processed forms of proglucagon. The mapping of biological tissues for these glucagon-containing peptides thus permits an assessment of selective posttranslational modification as well as an examination of specific gene expression or cellular uptake. This report describes the identification and localization of higher molecular weight forms of glucagon within the brain of the rat. These peptides, like their intestinal counterparts, appear to have both NH_2-terminal and COOH-terminal extensions on the glucagon sequence and probably result from a limited posttranslational modification of the glucagon precursor. While this work was in progress, two short reports on the subject appeared (19, 20).

MATERIALS AND METHODS

Antisera and Radioimmunoassays. Three antisera directed against pancreatic glucagon were used in the studies reported here. Antiserum NS (15, 21) is a low-titer serum prepared in a chicken by using a glucagon-albumin conjugate. It reacts well with higher molecular weight glucagon-containing peptides having COOH-terminal extensions on the glucagon sequence. Antiserum CTS (15) was prepared in a rabbit by using an albumin conjugate of the COOH-terminal tryptic fragment of glucagon; antiserum CTS reacts with the fragment itself and with glucagon-related forms not having extended COOH termini. Antiserum R-1 (21) is a high-titer serum prepared in a rabbit by using a glucagon-albumin conjugate and has 2–3% crossreactivity (relative to serum NS) for extended forms of the hormone. When used under conditions of antibody excess, this serum will immunoprecipitate both unlabeled and biosynthetically labeled high molecular weight forms of glucagon bearing COOH-terminal extensions (ref. 17 and unpublished observations). Details of the radioimmunoassay of glucagon and glucagon-related peptides using these antisera have been reported (15, 21).

Tissue Extractions and Peptide Analysis. Rats of the Sprague–Dawley strain (200–300 g, either fed ad lib or fasted for 24 hr) were killed by decapitation and the brains were quickly removed and placed on ice. Whole brains or their dissected portions were weighed and were rapidly homogenized in 4–10 vol (ml/g wet weight) of 3 M acetic acid, using hand-held ground glass homogenizers; the resulting suspensions were clarified by centrifugation. For direct measurement of immunoreactive peptides, samples of the resulting solutions were dried under reduced pressure at 21°C and the residues were extracted for 24 hr at 4°C into the buffer used for radioimmunoassay [0.1 M Tris containing 0.05 M NaCl, 0.2% crystalline bovine serum albumin, and Trasylol (Mobay Chemicals, New York) at 200 units/ml, brought to pH 7.6 with HCl].

For analysis of the heterogeneity of glucagon-related peptides, the initial tissue extracts were directly applied to a 2 × 90 cm column of Bio-Gel P-10 (Bio-Rad Laboratories) equilibrated with 3 M acetic acid. After gel filtration, samples of

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column fractions were dried and the dissolved residues were subjected to radioimmunoassay. In one study, an extract of rat intestine, prepared as described (15), was analyzed by gel filtration for comparison. Conditions for the proteolytic digestion of glucagon-containing peptides have been reported (15). Briefly, dried column fractions were dissolved in 0.1 ml of 0.1 M Tris brought to pH 7.5 with HCl and containing 10 mM $CaCl_2$ and trypsin (treated with tosylphenylalanine chloromethyl ketone, from Worthington) at 100 μg/ml. The solutions were incubated at 37°C for 1 hr and were then placed in a boiling water bath for 0.5 min. Carboxypeptidase B (2 μl of a 5 mg/ml solution, Boehringer-Mannheim) was added to the cooled solutions. After a 1-hr incubation at 37°C, the solutions were heated to boiling, cooled, and subjected to radioimmunoassay.

Immunohistochemical Staining. Fed Sprague–Dawley rats were anesthetized with pentobarbital and the animals were perfused through the aorta with 200 ml of 4% formalin in 0.14 M sodium phosphate buffer at pH 7. Brains were removed, cut into three or four blocks, and then postfixed in the above solution for 48 hr. Pancreas was removed from several animals and was fixed in a similar way. The tissue was washed in several changes of 70% (vol/vol) ethanol over a 96-hr period and was then dehydrated into toluene and vacuum embedded in paraffin. Six-micrometer sections of brain (cut in the coronal plane) or of pancreas were mounted on slides coated with gelatin/chromalum, deparaffinized in xylene, rehydrated through graded ethanol, and rinsed thoroughly in a buffer containing 0.05 M Tris and 0.9% NaCl brought to pH 7.6 with HCl.

Sections were stained according to Sternberger (22), using the peroxidase–antiperoxidase technique. Briefly, sections were overlayered for 10 min with several drops of 10% normal goat serum. The normal serum was then replaced by rabbit anti-glucagon serum R-1 diluted 1:300 in 1% normal goat serum and the sections were incubated for 40–46 hr at 4°C in a humidified chamber. The slides were reequilibrated to room temperature and washed (three times, 10 min each) with the Tris buffer described. The sections were then incubated with goat anti-rabbit immunoglobulin (Cappel Laboratories, Cochranville, PA) diluted 1:20 with 10% normal goat serum for 30 min followed by washing (three times, 10 min each) with the Tris buffer. The tissue was next incubated for 30 min with rabbit peroxidase–antiperoxidase (Accurate Chemical and Scientific, Hicksville, NY) diluted 1:40 with 1% normal goat serum and was again washed with the buffer. For localization of peroxidase, the sections were treated for 3 min with 0.025% diaminobenzidine plus 0.01% hydrogen peroxide, prepared in the Tris buffer. The sections were washed (five times, 3 min each) with water and incubated with 1% aqueous OsO_4 for 5 min. After thorough washing in water, sections were dehydrated, coverslipped with Permount, and photographed on a Zeiss photomicroscope. For some control experiments the diluted antiglucagon serum was preincubated with pancreatic glucagon (3 μg/ml) or with arginine vasopressin (0.8 μg/ml) for 24 hr at 4°C prior to use. In others, the diluted antiserum was replaced by normal rabbit serum (diluted 1:300) or by rabbit anti-vasopressin serum (diluted 1:1000, the generous gift of R. Miller of The University of Chicago).

RESULTS

Because initial experiments revealed only low amounts of glucagon-like immunoreactivity in homogenates of rat brain, tissue extracts were filtered through a gel to rule out the possibility of nonspecific interference in the radioimmunoassay. Fig. 1*A* shows the results obtained when an acetic acid extract of whole rat brain was filtered on a column of Bio-Gel P-10 in 3 M acetic acid. Application of the radioimmunoassay to samples of the column fractions, using anti-glucagon serum CTS, which requires the free COOH terminus of pancreatic glucagon, failed to identify glucagon-related material in the tissue extract. Radioimmunoassay using the centrally directed anti-glucagon serum NS, however, revealed two major peaks of immunoreactive peptides. The molecular weights of these peptides were determined to be approximately 12,000 (Fig. 1*A*, fraction 35) and 8000 (Fig. 1*A*, fraction 46) by application of peptides of known molecular weight to the same column. The sizes of these peptides and their selective glucagon-like immunoreactivities were reminiscent of intestinal glucagon-related peptides identified in other species (15). To make the comparison closer, a partially purified extract of whole rat intestine was filtered on the same column of Bio-Gel P-10. As shown in Fig. 1*B*, the elution volumes and selective immunoreactivities of the glucagon-related peptides from the two tissues were essentially the same. Thus, both tissues contain higher molecular weight forms of glucagon that are reactive only with an antiserum directed towards the central portion of glucagon.

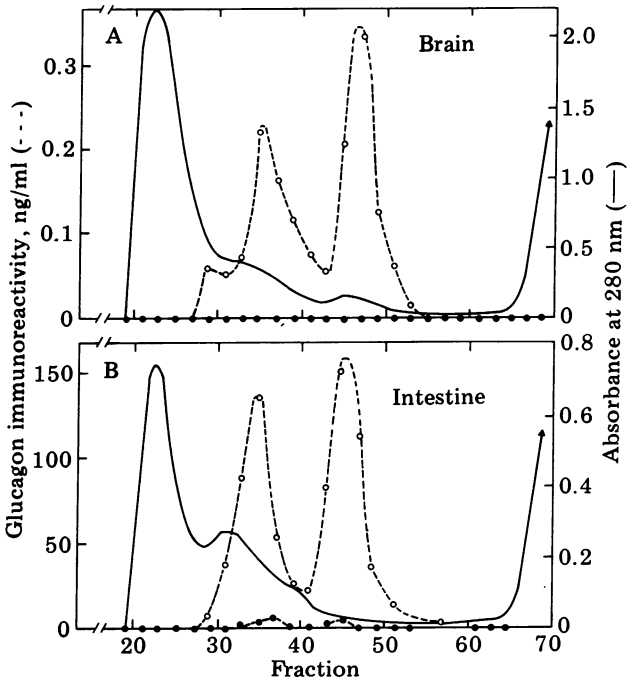

FIG. 1. Gel filtration profiles of glucagon-related peptides from rat brain and intestine. The profiles for brain (*A*, 4.5 g of tissue) and intestine (*B*, 30 g of tissue) after chromatography on a 2 × 90 cm column of Bio-Gel P-10 in 3 M acetic acid are shown. In both cases, 4.5-ml fractions were collected. The figure shows optical absorbance at 280 nm (—) and glucagon-like immunoreactivity measured by radioimmunoassay using centrally directed antiserum NS (○) or COOH-terminus-specific antiserum CTS (●). Rats used for this experiment were nonfasting males.

To quantitate tissue levels of glucagon-related peptides, the brains from five fed male rats were individually homogenized and filtered through a gel as before. These studies showed both that the tissue contains 2.9 ± 0.7 ng of glucagon-related peptide per g of fresh tissue (mean ± SD) and that the larger, 12,000-dalton, peptide represents 50 ± 6% of the total glucagon-like forms. Preliminary experiments have shown that the tissue concentration of glucagon-like material does not vary significantly between either fed or fasted or male or female animals. For studies of regional localization, individual brains were dissected and separately homogenized in 3 M acetic acid. The clarified extracts were directly assayed for glucagon-related peptides, using antiserum NS. The regional concentrations for

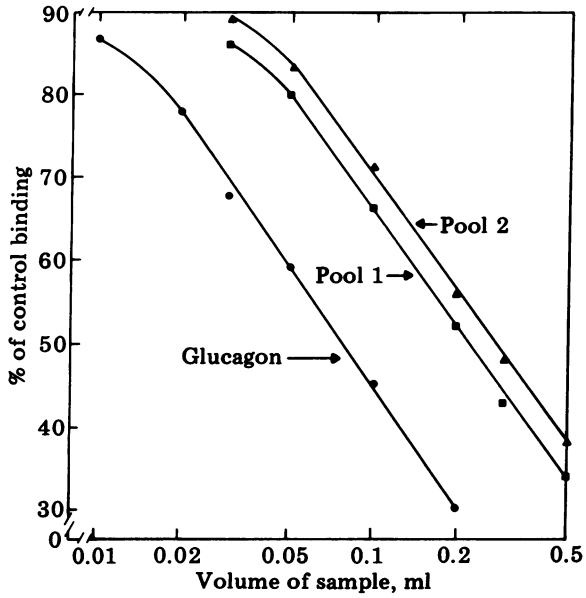

FIG. 2. Immunoreactivity of glucagon-related peptides from rat brain. Solutions of pancreatic glucagon (2 ng/ml) and of pooled, dried, and reconstituted fractions from the gel filtration shown in Fig. 1*A* were prepared and subjected to radioimmunoassay using centrally directed antiserum NS. Data are shown for glucagon (●), pooled brain fractions 34–37 (pool 1, ■), and pooled brain fractions 45–48 (pool 2, ▲). The volumes of each solution taken for radioimmunoassay are shown on the abscissa. The percent of control binding of ^{125}I-labeled glucagon to the antibody in the absence of added competitor is recorded in each case.

five brains, determined as ng of glucagon-like material per g of fresh tissue, were 2.9 ± 0.2 for cerebral cortex, 6.1 ± 1.6 for hypothalamus, 3.9 ± 1.1 for thalamus, 2.9 ± 0.2 for cerebellum, and 4.9 ± 1.9 for brain stem. Because experiments in which the separated, intestinal glucagon-like peptides were added to brain extracts showed that recoveries of immunoreactive material were only 41 ± 4% for the 12,000-dalton peptide and 83 ± 6% for the 8000-dalton peptide, the above amounts may be somewhat underestimated. However, gel filtration of separately pooled extracts of cortex, hypothalamus, and cerebellum

Table 1. Proteolytic digestion of glucagon-related peptides from rat brain and intestine

Tissue	Peptide	Antiserum	Glucagon immunoreactivity, ng/ml	
			Control	Digested
Brain	1	NS	0.19	0
		CTS	0	0.12
	2	NS	0.27	0
		CTS	0	0.13
Intestine	1	NS	0.45	0
		CTS	0.04	0.35
	2	NS	0.52	0
		CTS	0.03	0.31

Fractions from the gel filtrations whose profiles are shown in Fig. 1 were pooled as follows: brain peptide 1, fraction 34–37; brain peptide 2, fractions 45–48; intestine peptide 1, fractions 33–36; intestine peptide 2, fractions 44–47. The solutions were dried and the immunoreactive peptides were extracted into 0.1 M Tris that had been brought to pH 7.5 with HCl. Samples of the resulting solutions were removed and subjected to the radioimmunoassay, using antiglucagon sera NS and CTS without treatment (control) and after digestion with trypsin and carboxypeptidase B (digested).

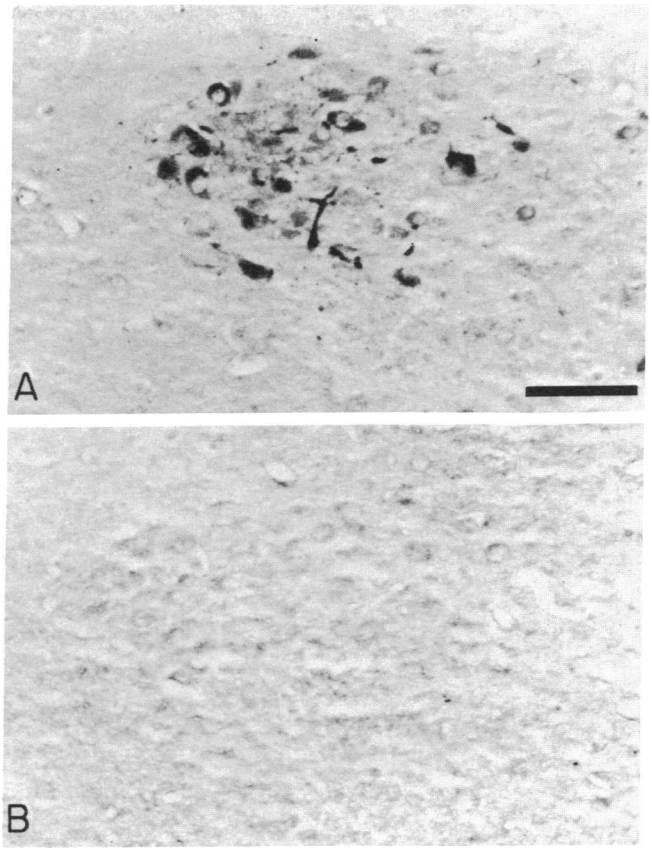

FIG. 3. Immunocytochemical localization of glucagon-related peptides in the hypothalamic paraventricular nucleus of the rat brain. Serial, frontal sections from formalin-fixed tissue were stained by the peroxidase–antiperoxidase technique. (*A*) Staining of a section through the paraventricular nucleus with antiglucagon serum R-1. (Note positive stain in cytoplasm and processes of magnocellular neurons.) (*B*) As a control, an adjacent section stained with antiserum R-1 that had been preincubated with pancreatic glucagon. Both sections were photographed under identical conditions, using a ×6.3 objective. The bar represents 100 μm.

showed that in each case the glucagon-like immunoreactivity was recovered as two components of the appropriate molecular weight and in the approximate ratio 1:1.

Further experiments were designed to probe the immunological and chemical properties of the glucagon-related peptides from brain. Fractions 34–37 and 45–48 from the gel filtration of Fig. 1*A* were separately pooled, concentrated, and diluted in parallel. Fig. 2 shows that the 12,000- and 8000-dalton peptides both competed for the binding of ^{125}I-labeled glucagon to antiserum NS in a manner indistinguishable from that for glucagon itself. These results validate the measurement of the peptides by using antiserum NS and suggest that the three peptides contain the same antigenic determinant. Neither glucagon-related peptide of the rat brain competed for the binding of ^{125}I-labeled glucagon to the COOH-terminus-specific antiserum CTS, even at the highest concentration noted in Fig. 2.

Previous studies on glucagon-related peptides have shown that their immunoreactivities can be modified by proteolytic digestion: although peptides bearing COOH-terminal extensions on the glucagon sequence are not reactive in the native state with antiserum CTS, digestion of these peptides with trypsin and carboxypeptidase B results in the formation of the immunoreactive COOH-terminal tryptic peptide of the hormone (15). Table 1 shows the application of these findings to

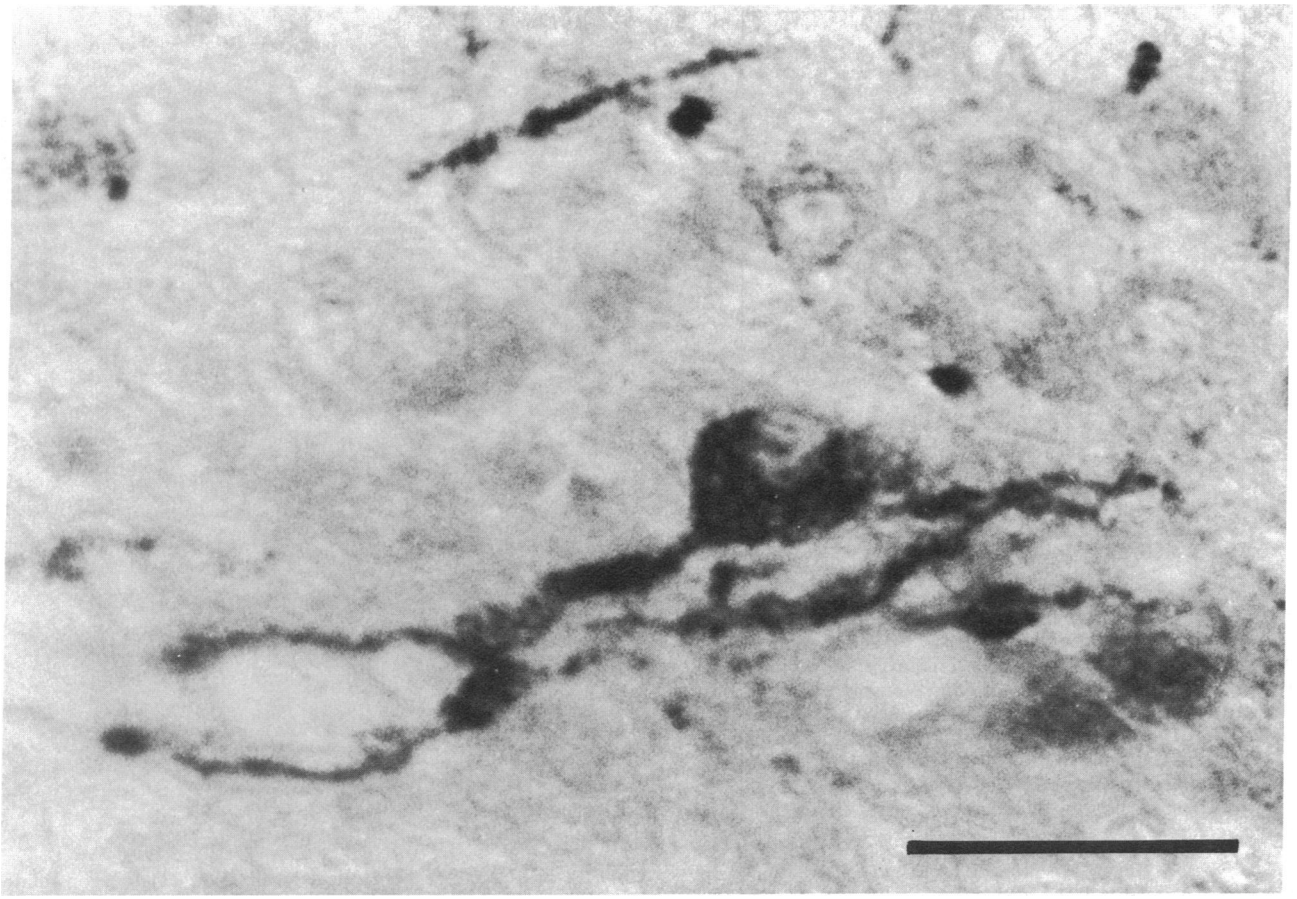

FIG. 4. Magnocellular neuron of the rat paraventricular nucleus stained for glucagon-related peptides. The figure shows a higher power photomicrograph (taken through a ×16 objective) of a frontal brain section stained as described in the legend to Fig. 3 and in *Materials and Methods*. (Note the intense cytoplasmic staining, large nucleus, and positively stained processes extending from the cell body.) The bar represents 50 μm.

an immunological comparison of the native and enzyme-digested peptides of rat brain and intestine, using both antiserum NS and antiserum CTS. As expected, enzyme digestion of any of the peptides with trypsin plus carboxypeptidase B eliminated reactivity with centrally directed antiserum NS. This treatment, however, resulted in a glucagon fragment that reacts with the COOH-terminus-specific antiserum CTS. Control studies showed that treatment of the brain or intestinal peptides with trypsin or carboxypeptidase B alone did not augment reactivity with antiserum CTS and that treatment of these peptides with carboxypeptidase A did not decrease immunoreactivity with antiserum NS, as it does for glucagon itself (15). Taken together, these results suggest that the glucagon-related peptides of rat brain, like those of intestine, contain the COOH-terminal tryptic fragment of glucagon and that this sequence is extended from its COOH terminus by a trypsin-sensitive site and by an additional short segment.

In order to ascertain if these glucagon-related forms were localized to specific neuronal cells, brain sections were examined by the techniques of immunocytochemistry. As shown in Fig. 3*A*, peroxidase–antiperoxidase staining revealed glucagon-immunoreactive cell bodies and processes in the rostral magnocellular portion of the paraventricular nucleus within the rat hypothalamus. The strongly positive staining was eliminated by prior incubation of the anti-glucagon serum with glucagon (Fig. 3*B*) or by use of normal rabbit serum in place of the antiserum (not shown). Caudal to this nucleus, scattered cells with positive stain were located dorsal or lateral to the fornix in the accessory supraoptic nucleus. Immunoreactive material was also seen in some cells of the supraoptic nucleus itself and in fibers, especially in the median eminence. All glucagon-positive cells are large (20–30 μm in diameter), with abundant cytoplasm and long processes that can often be traced through adjacent sections (Fig. 4). In all cases, immunoprecipitate appeared only in cytoplasm and processes. Because the glucagon-immunoreactive cells were near to or coincident with vasopressin-immunoreactive cells (within both the paraventricular and supraoptic nuclei), we attempted to block the reactivity of the anti-glucagon serum with arginine vasopressin. Although preincubation of vasopressin antiserum with arginine vasopressin prevented staining of hypothalamic magnocellular cells and fibers, preincubation of glucagon antiserum with vasopressin resulted in no diminution of staining intensity. In addition, A cells of the rat pancreatic islet showed no immunocytochemical reaction with anti-vasopressin serum although they stained strongly with anti-glucagon serum.

DISCUSSION

Our identification of glucagon-like peptide in brain extends the list of pancreatic and gastrointestinal hormones found in the mammalian central nervous system. Interestingly, our studies on the rat and those of Conlon *et al.* on the dog (19) show that the brain peptides are physically and immunochemically more closely related to the major glucagon-containing peptides of intestine than they are to pancreatic glucagon; that is, these peptides appear to contain the glucagon structure extended from its COOH terminus by both a trypsin-sensitive site and by an additional short sequence. It is likely that the two gluca-

gon-related peptides of brain differ by the lengths of their NH_2-terminal extensions, as do the related peptides of intestine (15). Although the amounts of glucagon-like material in brain are low, in all cases, and especially in hypothalamus, the concentrations of these peptides exceed those found in the peripheral circulation (approximately 1 ng/ml of plasma). The localization of glucagon-related peptides to well-defined cells and fibers of the hypothalamus further indicates that the radioimmunochemical detection of the pepides results from neither contamination by extracellular fluid nor nonspecific diffusion of the peptide through neural tissue. Although the cellular concentrations of these peptides are undoubtedly much greater than those suggested by direct immunoassays of tissue extracts, the sensitivity of the peroxidase–antiperoxidase technique was probably crucial to our identification of glucagon-related material in neuronal cell bodies: using indirect immunofluorescence, Loren *et al.* identified glucagon-like peptides only in fibers of the rat hypothalamus and thalamus (20).

In the absence of direct studies, we have little cause to presume that the glucagon-related peptides of the brain are synthesized locally or that their occurrence represents a selective uptake and sequestration of the material from plasma or cerebral spinal fluid. In this regard, it should be noted that the biological activity of these peptides, even in the periphery, is a matter of some controversy (23): the purified, 12,000-dalton component of porcine intestine [called glicentin (13, 14)] does not appear to combine with plasma membrane glucagon receptors or to stimulate the usual cellular responses associated with pancreatic glucagon. Because (*i*) extracts of pancreas contain low amounts of the intestinal forms of glucagon (15), (*ii*) these peptides have structures consistent with that of a previously identified fragment of pancreatic proglucagon (13–15, 24), (*iii*) the 12,000-dalton intestinal peptide has been localized to the pancreatic A cell by specific immunohistochemical techniques (16), and (*iv*) peptides of similar size have been identified as intermediates in the biosynthetic conversion of proglucagon to glucagon (17, 18), it is probable that intestinal and pancreatic forms of glucagon are derived from a single precursor, which undergoes tissue-specific processing. To the extent that the glucagon-related peptides of brain are synthesized locally, it appears that hypothalamic proglucagon undergoes an intestinal rather than pancreatic type of post-translational modification in its conversion to final products.

The morphological and functional associations among glucagon-immunoreactive cells and others of the hypothalamic neurosecretory system remain to be examined in detail and are important matters for further investigation. Although our results indicate an association between glucagon-containing and vasopressin-containing magnocellular neurons of the hypothalamus, the lack of cysteine incorporation into biosynthetically labeled proglucagon (25) and the absence of identifiable vasopressin-like material in A cells of the pancreatic islet (this report) fail to suggest any compulsory relationship between these two hormones. Nevertheless, the specific localization of a variety of peptides of the gastrointestinal tract and pancreas to defined cells of the brain indicates that their occurrence is not the result of a haphazard process. Although their physiological and biochemical roles remain to be defined, the identification of glucagon-like, insulin-like, and cholecystokinin-like peptides in central nervous tissues of animals as diverse as insects (26–28) and mammals (refs. 5–8, 19, 20 and this report) further suggests that the anatomical distribution of many of these forms, as well as the peptides themselves, arose remarkably early during the evolution of higher animals.

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