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Identification of glucagon receptors in rat brain

(anatomical localization/subcellular distribution/limbic system/pituitary/adenylate cyclase)

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ABSTRACT **The binding of radiolabeled glucagon to rat brain membranes was investigated. Regional distribution studies indicate higher specific binding of ^{125}I-labeled monoiodoglucagon to olfactory tubercule, hippocampus, anterior pituitary, and amygdala membranes, with somewhat lower binding to membranes from septum, medulla, thalamus, olfactory bulb, and hypothalamus. ^{125}I-labeled glucagon bound to rat brain synaptic plasma membrane fractions with high affinity (K_D = 2.24 nM). Specific binding was greater to synaptosomal membrane fractions relative to myelin, mitochondrial nuclear, or microsomal fractions. Inclusion of 0.1 mM GTP in the binding assay reduced the glucagon binding affinity (K_D = 44.5 nM). Several neuropeptides and other neuroactive substances tested did not affect binding of labeled glucagon to brain membranes. Three different glucagon analogs inhibited labeled glucagon binding. Synthetic human pancreatic growth hormone-releasing factor, hpGRF-44, also inhibited binding, although the concentration required for half-maximal displacement was 100-fold higher than for native glucagon. Addition of glucagon to brain membranes resulted in ≈3-fold maximal activation of adenylate cyclase over basal levels. Glucagon at a concentration of 4.74 nM was required for half-maximal activation of pituitary membrane adenylate cyclase. These findings provide evidence for rat brain binding sites that respond to the pancreatic form of glucagon and can transduce this binding into the activation of adenylate cyclase.**

Glucagon, a 29-amino acid peptide product of the "A" cells of the endocrine pancreas, regulates plasma fuel concentrations, acting as a counter-regulatory hormone to insulin (1). The most widely studied target cell for glucagon is the hepatocyte to whose plasma membrane receptors it binds to bring about activation of adenylate cyclase (2). Glucagon is a member of a family of evolutionarily related hormones (3). Higher molecular weight forms of glucagon isolated from the gastrointestinal tract (1, 4, 5) and pancreatic glucagon may be derived from a common precursor by tissue-specific processing (6).

Peptide hormones earlier thought to be secretions specific to the gastro-entero-pancreatic system are increasingly being found in the mammalian brain (7, 8). There have been several reports of glucagon and/or glucagon-like immunoreactivities in the central nervous system of rats (9–12) and other mammals, including humans (13–17), and in the retina of goldfish, frog, and pigeon (18). We are unaware of any study identifying glucagon receptors in nervous tissue. We have, therefore, sought to determine the existence of such receptors as well as their regional and subcellular distribution and to partially characterize them in the rat brain.

MATERIALS AND METHODS

Porcine glucagon was a gift from Eli Lilly. Synthetic human pancreatic growth hormone-releasing factor, hpGRF-44, was graciously provided by A. Felix (Hoffmann–La Roche). Glucagon analogs, N^{ϵ}-acetimidoglucagon (19) and des-His^{1}-N^{ϵ}-acetimidoglucagon were prepared as described by Flanders *et al.* (19); [*S*-methyl-Met^{27}]glucagon was prepared as described by England *et al.* (20). 1,3,4,6-Tetrachloro-3,6-diphenylglycouril (IODO-GEN) was purchased from Pierce, and cellulose acetate oxoid membrane filters were from Oxoid (Columbia, MD). Ultrapure-grade sucrose came from Schwarz/Mann. Theophylline and dithiothreitol were from Sigma, and ATP and GTP were from Calbiochem. Creatine phosphate and creatine phosphokinase came from Boehringer Mannheim.

Preparation of ^{125}I-Labeled Monoiodoglucagon (^{125}I-Monoiodoglucagon). Purified porcine glucagon was iodinated by using IODO-GEN and ^{125}I-monoiodoglucagon was purified as described by England *et al.* (20) to provide a homogeneous and stable derivative with a specific activity of ≈8 × 10^5 cpm/pmol.

Preparation of Brain Membranes. Male Sprague–Dawley rats weighing 300–350 g were decapitated, and their brains were rapidly removed and placed on ice. Separate identified regions were macroscopically dissected. Routinely, individual or pooled regions (as described in figure legends) were homogenized in 10 vol of a solution containing 1 mM dithiothreitol, 0.1% bovine serum albumin, 30 mM Tris·HCl (pH 7.5) (buffer A) at 4°C in a Teflon glass homogenizer. For subcellular distribution studies, pooled regions were homogenized in 10 vol of 0.32 M sucrose and fractions were prepared according to the method of Gray and Whittaker (21). All fractions were kept on ice and assayed immediately. Membrane protein concentration was determined by the method of Markwell *et al.* (22).

Preparation of Liver Plasma Membranes. Partially purified liver plasma membranes were prepared according to the method of Neville (23) through step 11 as described by Pohl (24). The final pellet was resuspended in 1 mM $NaHCO_3$ and divided into aliquots (≈6 mg of protein) that were stored in liquid nitrogen and used within 2 months.

Glucagon Binding Assay. The binding assay was essentially that of England *et al.* (20). The incubation buffer contained 1 mg of bovine serum albumin per ml, 1 mM dithiothreitol, 30 mM Tris·HCl (pH 7.5). The incubation mixture, of total volume 1 ml, contained 200 μg of brain membranes or thawed liver plasma membranes, as indicated, and 0.13 pmol (≈1 × 10^5 cpm) of ^{125}I-monoiodoglucagon. Other additions are given in figure legends. Reaction mixtures were incubated at 4°C for 30 min and then rapidly (<5 s) vacuum filtered through Oxoid membrane filters (0.45 μm) previously soaked for at least 30 min in bovine serum albumin at 100 mg/ml. Each filter was washed twice with 3 ml of ice-cold incubation buffer, dried, and assayed for radioactivity in a Beckmann Biogamma Counter.

Nonspecific binding measured in the presence of 1 μM un-

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Abbreviations: IC_{50}, concentration required for half-maximal inhibition of binding; hpGRF, human pancreatic growth hormone-releasing factor.

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labeled peptide was 25–30% of total and was subtracted from the total to give specific binding. Results are analyzed by curve fitting the data to a four-parameter equation describing a sigmoidal dose–response curve (25). Competition assays were performed at least twice. The standard deviation of each assay point performed in duplicate was <5%.

Adenylate Cyclase Assay. The adenylate cyclase assay medium contained the following in a final volume of 0.1 ml: 2 mM ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM GTP, 1 mM theophylline, 2 mM EDTA, 30 mM Tris·HCl (pH 7.5), and an ATP regenerating system consisting of 5 mM creatine phosphate and creatine phosphokinase at 0.3 mg/ml (9.3 units/ml). Peptides diluted in 0.1% bovine serum albumin/Tris·HCl, pH 7.5, were added in 30-μl aliquots and the reaction was initiated by the addition of 60 μg of pituitary or brain homogenates or thawed liver membranes in 30 μl of buffer A. Assay samples were incubated at 30°C for 10 min and the reaction was terminated by heating at 110°C for 2 min. After centrifugation, 50 μl of supernatant from each tube was assayed for cAMP by using the Amersham cAMP radioimmunoassay kit. The standard deviation of each assay point performed in triplicate was <5%. Basal activity in olfactory homogenate and liver plasma membranes was about 0.95 nmol and for pituitary homogenate was 1.88 nmol of cAMP formed per mg of protein in 10 min. This activity was increased ≈3-fold for each membrane preparation at maximal hormonal stimulation. Results are expressed as the percentage of maximal activation.

RESULTS

Regional Distribution of Glucagon Binding Sites. In preliminary studies (data not shown) binding of ^{125}I-monoiodoglucagon to brain membranes at 4°C attained steady-state levels within 30 min and remained constant through 60 min. The optimum pH was 7.5. Association of ^{125}I-monoiodoglucagon that was more rapid at 30°C than at 4°C and abolished at 56°C was not enhanced by the inclusion of Bacitracin at 1 mg/ml. Less than 2% of the ^{125}I-monoiodoglucagon was degraded during a 30-min incubation at 4°C, as determined by trichloroacetic acid precipitation of the labeled peptide. Binding was linear with membrane protein concentrations between 100 and 600 μg/ml. Table 1 indicates that the distribution of specific binding of ^{125}I-monoiodoglucagon to lysed P_2 (crude mitochondrial-synaptosomal) fractions prepared from various regions of brain differs from that seen for insulin binding to similar preparations. Areas rich in glucagon binding sites were olfactory tubercule, hippocampus, anterior pituitary, and amygdala, whereas other areas provided intermediate or negligible values for specific binding. Although the actual amount of specific binding differed from one experiment to another, in four experiments the rank order of binding to the various regions was uniformly maintained. Whereas ^{125}I-labeled insulin bound to all regions reported, with olfactory bulb displaying the highest and pituitary the lowest binding, ^{125}I-monoiodoglucagon did show negligible binding to some regions. Pituitary membranes exhibited relatively high binding, which was confined to the anterior lobe.

Binding to Subcellular Fractions. Regions that displayed high specific binding were pooled and subjected to subcellular fractionation procedures (21, 27) that yield fractions enriched in morphologically identifiable components (Table 2). As shown in Table 2, specific binding of labeled glucagon to the various fractions differed significantly. In three experiments the specific binding of ^{125}I-monoiodoglucagon was higher in the P_2 fraction than in the P_1 or P_3 fraction and was higher in the P_2B fraction than in the P_2A or P_2C fraction.

To characterize the binding affinity, subcellular fractions were used in competition assays. As illustrated in Fig. 1, the half-maximal concentrations required for the binding of glucagon were 2.24 nM for the P_2B (synaptosomal plasma membrane) fraction, 5.01 nM for the homogenate, and 2.24 μM for the P_2A or myelin membrane fraction. The P_2 fraction, which contains myelin, synaptosomal, and mitochondrial membranes (21), bound glucagon with two different affinities. The lower affinity binding is in the micromolar range (K_D = 4.51 μM), similar to that of the myelin fraction, whereas the higher affinity binding is in the nanomolar range (K_D = 5.71 nM), more characteristic of the binding to synaptosomal membrane fractions. Furthermore, the glucagon binding affinities of synaptosomal membranes and homogenates are in the nanomolar range, similar to that of liver plasma membranes (Fig. 2 and ref. 20).

Effect of GTP. Since GTP is known to reduce the affinity of hepatocyte receptors for glucagon (28), competition assays were performed with liver plasma membranes and pooled homogenates in the presence and absence of GTP.

Table 1. Regional distribution of glucagon and insulin binding sites of the rat brain

Region	Specific binding, % of total added: ^{125}I-Monoiodo-glucagon	^{125}I-labeled insulin*
Olfactory bulb	1.3	4.5
Olfactory tubercule	5.0	ND
Hippocampus	4.6	2.8
Anterior pituitary	4.3	0.2
Posterior pituitary	<0.2	
Anterior hypothalamus	0.8	2.6
Posterior hypothalamus		2.0
Thalamus	1.6	1.6
Medulla	2.0	1.0
Cerebral cortex	<0.2	2.8
Cerebellum	<0.2	2
Colliculli	<0.2	ND
Striatum	<0.2	1.9
Midbrain	<0.2	ND
Septum	2.2	2
Amygdala	3.2	2

Lysed P_2 (crude synaptosomal) fractions were prepared from discrete rat brain regions and used in binding assays. Numbers indicate specific binding of ^{125}I-monoiodoglucagon to 500 μg of membrane protein and are representative of four separate experiments. Values shown for specific binding of ^{125}I-labeled insulin to rat brain regions, given for comparison, represent specific binding in the presence of ≈4 μM unlabeled insulin; 500 μg of membrane protein per ml was used. ND, not determined.
*From Havrankova *et al.* (26).

Table 2. Specific binding of ^{125}I-monoiodoglucagon to subcellular fractions prepared from rat brains

Fraction	Specific binding, cpm/mg of membrane protein
Pooled rat brain regions	
Homogenate	4050 ± 240
Subcellular fraction	
Crude nuclear (P_1)	668 ± 45
Microsomal (P_3)	1527 ± 89
Crude synaptosomal (P_2)	3100 ± 200
Myelin (P_2A)	1344 ± 130
Synaptosomal (P_2B)	5063 ± 239
Mitochondrial (P_2C)	2875 ± 197

Pituitary, hypothalamus, hippocampus, olfactory lobes, and tubercules were pooled from 20 rat brains to obtain ≈5 g of tissue. Pooled regions were homogenized in 10% (wt/vol) 0.32 M sucrose. Values are means ± SD of triplicate determinations and are representative of three separate experiments.

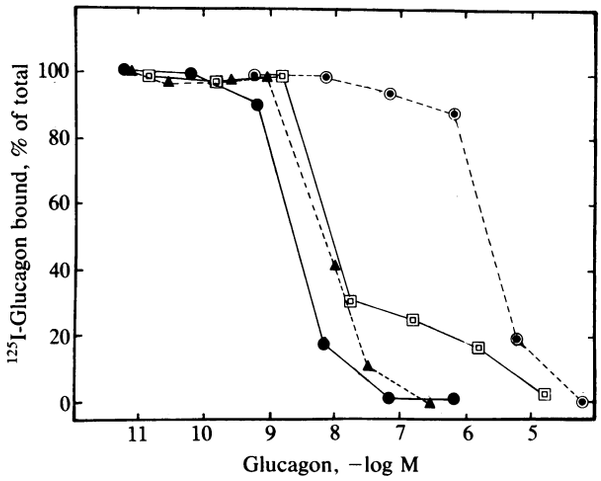

FIG. 1. Displacement of ^{125}I-monoiodoglucagon bound to rat brain membrane preparations. Percent of the total ^{125}I-monoiodoglucagon bound specifically is plotted as a function of unlabeled glucagon concentration for lysed P_2B (●), homogenate (▲), lysed P_2 (□), and P_2A (○).

Inclusion of 0.1 mM GTP shifted the concentration of unlabeled glucagon required for the half-maximal displacement of ^{125}I-monoiodoglucagon from 1.01 nM to 28.7 nM for liver plasma membranes and from 5.01 nM to 44.5 nM for brain membranes (Fig. 2), illustrating that the same type of altered affinity is apparent for the rat brain glucagon receptor as recognized for hepatocyte receptors.

Specificity of ^{125}I-Monoiodoglucagon Binding. The abilities of several neuropeptides, neurotransmitters, and glucagon analogs to inhibit ^{125}I-monoiodoglucagon binding to pooled brain homogenates were investigated. At the concentrations indicated, none of the following neuropeptides were able to compete with ^{125}I-monoiodoglucagon: insulin (5 μM), vasoactive intestinal peptide (5 μM), vasopressin (5 μM), and somatostatin (10 μM). Other neuroactive compounds at a concentration of 100 μM were also unable to displace bound ^{125}I-monoiodoglucagon as follows: isoproterenol, chlorpromazine, atropine, dopamine, and ascorbic acid. Synthetic hpGRF-44 was the only neuropeptide tested that was able to compete with ^{125}I-monoiodoglucagon binding to both brain and liver plasma membrane preparations (data not shown). However, a 100 times higher concentration of hpGRF-44 was required for half-maximal displacement (IC_{50} = 447 nM with brain homogenate and IC_{50} = 158 nM with liver plasma membranes). Three glucagon analogs tested each inhibited ^{125}I-monoiodoglucagon binding but required concentrations higher than native glucagon to bring about half-maximal displacement (Fig. 3). N^{ϵ}-acetimidoglucagon, a glucagon derivative having an acetimidyl group at the ε-amino group of lysine 12 (19) was 1/10th as effective as native glucagon in competing for ^{125}I-monoiodoglucagon binding (IC_{50} = 31.6 nM). With des-His^1-N^{ϵ}-acetimidoglucagon, prepared by manual Edman degradation of N^{ϵ}-acetimidoglucagon (19), and [*S*-methyl-Met^{27}]glucagon (20) half-maximal displacement occurred at 398 nM and 296 nM, respectively.

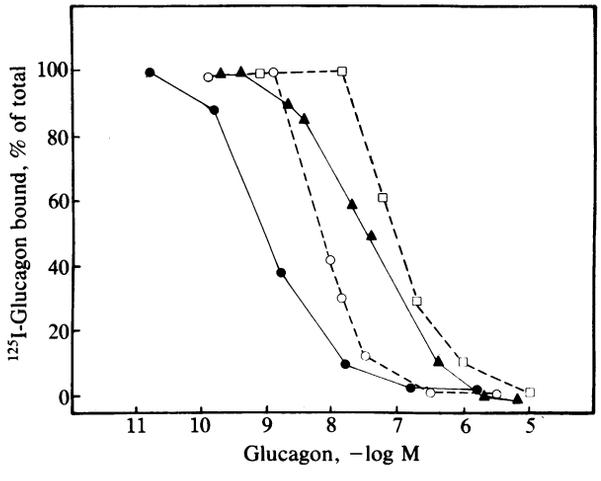

FIG. 2. Effect of GTP on ^{125}I-monoiodoglucagon binding to liver plasma membranes and brain homogenates. Percent of the total ^{125}I-monoiodoglucagon bound is plotted as a function of unlabeled glucagon concentration in the absence (●) and presence (▲) of 0.1 mM GTP for liver plasma membranes; similar plots are shown for pooled brain homogenates in the absence (○) and presence (□) of 0.1 mM GTP.

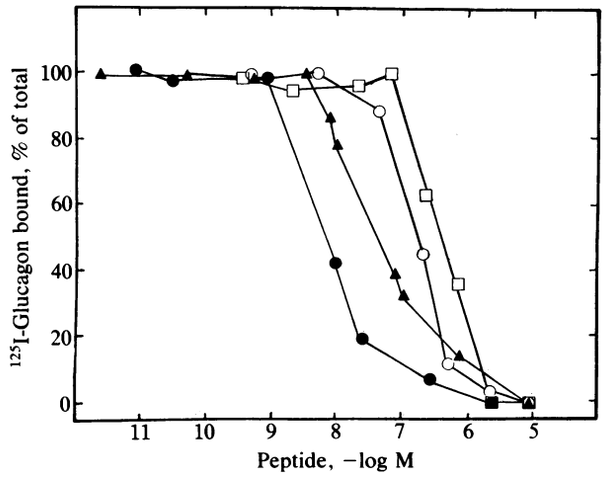

FIG. 3. Competition curves with glucagon analogs. Competition of ^{125}I-monoiodoglucagon binding to rat brain pooled homogenates by native glucagon (●), N^{ϵ}-acetimidoglucagon (▲), [*S*-methyl-Met^{27}]glucagon (○), and des-His^1-N^{ϵ}-acetimidoglucagon (□).

Adenylate Cyclase Activation. Dose–response curves for the activation of adenylate cyclase by glucagon in different tissues are shown in Fig. 4. The concentration of glucagon required for half-maximal activation was 3.65 nM with partially purified liver plasma membranes as compared with 5.63 nM with rat olfactory and pituitary homogenates.

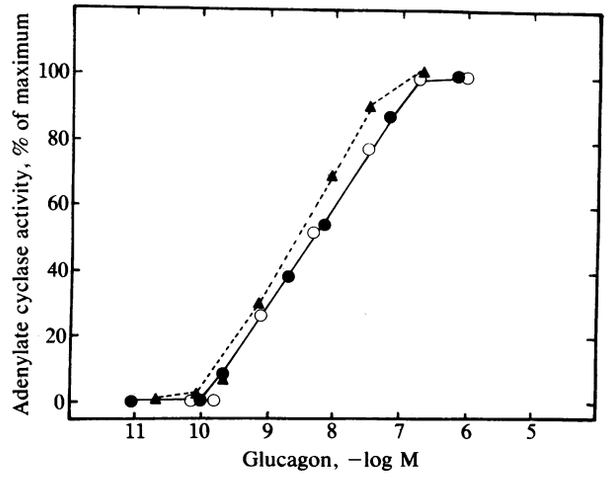

FIG. 4. Activation of adenylate cyclase. Dose–response curves for the activation of adenylate cyclase by glucagon in liver plasma membranes (▲), pituitary homogenates (●), and olfactory tissue homogenates (○).

DISCUSSION

Although the presence of glucagon and glucagon-like peptides in brain has been reported (9–17), to our knowledge glucagon receptor identification and localization in the central nervous system has not been reported previously.

Elements within the rat brain limbic system (olfactory tubercule and bulb, hippocampus, amygdala, and septum), as well as hypothalamus, thalamus, medulla, and anterior pituitary membranes displayed significant glucagon binding (Table 1). A neuronal role for glucagon is suggested by these data and the higher binding of glucagon to subcellular fractions enriched in synaptic plasma membranes (Table 2). Differential specific binding of radiolabeled insulin to blood vessels in different brain regions has been reported (29). However, in our study it is an unlikely possibility that some contamination of the synaptosomal-enriched (P_2B) fraction by blood vessels or cells could account for the high glucagon binding to this fraction inasmuch as the P_1 fraction (containing cellular debris) displayed little binding (Table 2). Ultrastructural demonstration of the binding of glucagon to recognizable synaptic elements is desirable. Glucagon bound to liver and synaptic plasma membranes with similar affinity ($K_D \simeq 2$ nM) and binding affinity for both membranes was diminished in the presence of 0.1 mM GTP (Figs. 1 and 2).

Insulin, somatostatin, vasopressin, vasoactive intestinal polypeptide, and several neurotransmitters examined did not inhibit binding of glucagon to rat brain homogenates. The regional distributions in mammalian brains of glucagon-related peptides such as vasoactive intestinal polypeptide (30), secretin (31), gastrin (32), and PHI-27 (33) differ from that for glucagon (9–17). hpGRF-44, which is also a member of the glucagon-secretin family of evolutionarily related peptides, did inhibit glucagon binding. However, 100 times higher concentration of hpGRF-44 compared to glucagon was required to bring about half-maximal displacement of ^{125}I-monoiodoglucagon from brain and liver membranes, ruling out the possibility of hpGRF exerting its physiological effects through these receptors, which exhibit a high affinity for glucagon. Synthetic hpGRF-44 is also known to bind with low affinity to vasoactive intestinal polypeptide receptors in rat and human intestinal epithelial membranes (34).

In competition assays the amino-terminal, carboxyl-terminal, and centrally modified glucagon derivatives displayed lower affinities for brain homogenates compared to native glucagon. This is consistent with the observation that virtually the whole glucagon molecule participates in the hepatic receptor regulation process (20). The rank order of potency of glucagon analogs examined for brain membranes (native glucagon > N^{ϵ}-acetimidoglucagon > [*S*-methyl-Met^{27}]glucagon > des-His^1-N^{ϵ}-acetimidoglucagon) (Fig. 3) differed from that for liver plasma membranes (native glucagon = N^{ϵ}-acetimidoglucagon > des-His^1-N^{ϵ}-acetimidoglucagon > [*S*-methyl-Met^{27}]glucagon) (unpublished data), suggesting that the central nervous system glucagon receptor may differ in molecular structure from the hepatic receptor.

Glucagon binding to receptors generally results in the activation of adenylate cyclase to produce the second messenger, cAMP. Although we have not yet examined other possible mechanisms for glucagon action, the addition of native glucagon to rat brain and pituitary homogenates also results in activation of adenylate cyclase (Fig. 4). The concentration of glucagon required for half-maximal activation was nanomolar for both olfactory and pituitary homogenates, similar to that required for the half-maximal binding of glucagon to brain and liver membranes and activation of adenylate cyclase in liver membranes (refs. 20 and 28; Figs. 1 and 4).

There is good correlation between the distribution of glucagon receptors in the rat brain in our study and the distribution of glucagon-like peptides in mammalian brains reported in the literature. Several investigators (9–17) have found a high content of glucagon-like peptides in the hypothalamic area. One of the prime functions of the hypothalamus is control of the pituitary. In the present study anterior pituitary membranes did display high specific binding of ^{125}I-monoiodoglucagon and activation of adenylate cyclase by native glucagon, suggesting that some hypothalamic neurons communicating with the pituitary may contain glucagon. The physiological significance of such an association is an important matter for further investigation. Other brain regions that have also been reported to contain glucagon-like peptides are amygdala (10, 12–17), thalamus (10–15), septum (10), and medulla (11, 15, 16), which in our study also have glucagon receptors.

To date, local synthesis or uptake of glucagon in brain has not been demonstrated. Tager *et al.* (11) have suggested that glucagon-containing peptides found in rat brain have undergone intestinal rather than pancreatic type of processing (6). Dorn *et al.* (12) were unable to distinguish between pancreatic and gut-type glucagon, and Tominaga *et al.* (9) and Conlon et al. (13) found pancreatic glucagon in addition to gut-type glucagon in mammalian brains. However, the central nervous system binding sites that we have identified in this study fulfill the criteria of defined localization, high affinity, high specificity, and biological response to the pancreatic form of glucagon.

Several recent studies have demonstrated pancreatic glucagon-induced release of somatostatin from perifused hypothalamus (35, 36) and cAMP formation in neuroblastoma × glioma hybrid cells (37). Other studies have shown somatostatin release from the median eminence into the hypophyseal portal vessel by pancreatic glucagon (38). Somatostatin release has also been shown to be cAMP dependent (39, 40). The above cited reports and our finding of glucagon-mediated cAMP formation suggest that glucagon in the hypothalamus may be involved in the release of somatostatin through cAMP-dependent mechanisms.

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