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ACCELERATED COMMUNICATION

High Affinity Amylin Binding Sites in Rat Brain

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SUMMARY

Amylin, a 37-amino acid peptide structurally related to calcitonin gene-related peptide, is synthesized in and released along with insulin from pancreatic β-cells. Amylin is proposed to act as an endocrine partner to insulin, in part through actions upon skeletal muscle that promote cycling of gluconeogenic precursors to liver. We report here that binding sites with high affinity (K_d = 27 pM) for radioiodinated rat amylin are present in the nucleus accumbens region of rat brain. Competition experiments show that sites measured in nucleus accumbens membranes have high affinity for rat amylin, lower affinity for rat calcitonin gene-related peptides, and very low affinity for rat calcitonin. In contrast to rat calcitonin, salmon calcitonin has a high affinity for these sites, indicating that it shares critical binding determinants with amylin. We further tested whether salmon calcitonin shares with amylin the ability to regulate glycogen metabolism in rat skeletal muscle. Salmon calcitonin potently inhibits insulin-stimulated glucose incorporation into rat soleus muscle glycogen, suggesting that rat skeletal muscle may also contain receptor populations that have high affinity for both amylin and salmon calcitonin.

Amylin is a peptide hormone that is secreted along with insulin from pancreatic β-cells in response to nutrient intake (1–3). Exogenous amylin alters glucose metabolism *in vivo*, producing hyperlactemia, hyperglycemia, and insulin resistance (4, 5). Amylin stimulates glycogen phosphorylase activity and inhibits glycogen synthase activity in skeletal muscle and antagonizes insulin-stimulated incorporation of glucose into muscle glycogen *in vitro* (6–8). Amylin circulates at concentrations measured in normal human plasma in the range of 3–20 pM (9, 10), with somewhat higher concentrations being reported for insulin-resistant and obese people and for rodent plasma.

Amylin has sequence identity of 43% to α-CGRP and of 46% to β-CGRP in humans. Low affinity interactions of amylin with CGRP receptors in liver, skeletal muscle, brain, and myoblasts have been described (11–14), leading some to suggest that the actions of amylin may be mediated through these receptors. However, amylin is 100–1000-fold less potent than CGRP both in its affinity for CGRP receptors and in producing CGRP receptor-associated actions such as vasodilation (15). In contrast, it is approximately equipotent with CGRP in altering skeletal muscle glycogen metabolism (16, 17). Therefore, the profile of the metabolic response to amylin does not match the reported CGRP receptor binding profile.

Through radioligand binding and autoradiographic studies with BH-radioiodinated rat amylin, we have found binding sites with high affinity for amylin in rat brain. To see whether the metabolic effects of amylin may be mediated by receptors with the selectivity profile of these sites, we tested peptides with high binding affinity for their effects on soleus muscle glycogen metabolism.

Materials and Methods

Membrane preparation. Brains from male Sprague-Dawley rats (200–250 g) were dissected into regions and homogenized in ice-cold 20 mM HEPES buffer (20 mM HEPES acid, pH adjusted to 7.4 with NaOH at 23°). Membranes were collected by centrifugation at 48,000 × *g* for 15 min and were then washed twice by resuspension in fresh buffer and centrifugation. The membrane pellet from the third centrifugation was stored at −70° until use.

Receptor binding. Membranes from 4 mg original wet weight of tissue were incubated with ^{125}I-BH-amylin (rat amylin, BH-labeled at the amino-terminal lysine; Amersham Corporation, Arlington Heights, IL) in 20 mM HEPES buffer, containing 0.5 mg/ml bacitracin, 0.5 mg/ml bovine serum albumin, and 0.2 mM phenylmethylsulfonyl fluoride, for 60 min at 23°. Incubations were carried out in duplicate tubes and were started by addition of membranes. Incubations were terminated by filtration through glass fiber filters that had been presoaked in 0.3% polyethyleneimine, followed by washing with 15 ml of cold phosphate-buffered saline. Competition curves were generated by measuring binding of 13 pM ^{125}I-BH-amylin in the presence of 10^{-11} to 10^{-6} M unlabeled peptide. Data were fitted to a four-parameter logistic equation (Inplot; GraphPAD Software, San Diego, CA) to derive half-maximal inhibitory concentrations (IC_{50} values) and slope factors ("Hill coefficients"). To generate saturation isotherms, binding of ^{125}I-BH-amylin was measured

ABBREVIATIONS: CGRP, calcitonin gene-related peptide; BH, Bolton-Hunter; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

at concentrations varying from 1 to 150 pM, in the absence (to obtain total binding) or in the presence (to obtain nonspecific binding) of 100 nM unlabeled salmon calcitonin. Protein was measured by the method of Bradford, with ovalbumin as standard.

Autoradiography. Brains from male Sprague-Dawley rats were frozen in isopentane, and 12-μm sections were cut in a cryostat set at −15°. Sections were dried at ambient temperature and stored dessicated at −20°. Sections were thawed and preincubated for 3 × 5 min at ambient temperature in 20 mM HEPES, pH 7.4, containing 100 mM NaCl, 1 mg/ml bovine serum albumin, and 0.5 mg/ml bacitracin. Sections were then incubated for 60 min in the same buffer containing 74 pM ^{125}I-BH-amylin. Nonspecific binding was measured in the presence of 1 μM salmon calcitonin. After incubation, slides were washed for 3 × 4 min in ice-cold buffer, dipped in deionized water to remove buffer salts, and rapidly dried under a stream of air. Hyperfilm-^{3}H (Amersham) was exposed to the sections in an autoradiography cassette for 4–7 days before development.

Soleus muscle glycogen metabolism. The rate of incorporation of [^{14}C]glucose into glycogen in isolated rat soleus muscle was measured and data were analyzed as described by Young *et al.* (18). Insulin-stimulated incorporation of [^{14}C]glucose into soleus muscle glycogen was inhibited by coincubation with rat amylin or related peptide for 30 min before and 60 min after addition of [^{14}C]glucose. As described by Young *et al.* (18), data from the indicated number of soleus muscle strips for each data point were fit to a four-parameter logistic equation to derive half-maximally effective concentrations (EC_{50} values) and standard errors.

Results

^{125}I-BH-amylin binding. Specific ^{125}I-BH-amylin binding (displaceable by 1 μM rat amylin) was measured using a low concentration (17–20 pM) of radioligand in membranes prepared from several peripheral tissues and from rat brains dissected into 15 regions. Specific binding was observed in several brain regions, with nucleus accumbens and surrounding tissue containing more than twice as much specific binding as any other region. ^{125}I-BH-amylin binding was further characterized using nucleus accumbens membranes. Specific binding measured at 23° reached a steady state by 60 min, which was used as the incubation interval for additional experiments.

Specific and saturable binding of ^{125}I-BH-amylin to nucleus accumbens membranes was observed at concentrations of 0.3–190 pM (Fig. 1A). Scatchard analysis of saturation data (Fig. 1B) yielded a dissociation constant (K_d) of 27.1 ± 2.1 pM and binding site density (B_{max}) of 23.8 ± 2.3 fmol/mg of protein (mean ± standard error, three experiments). The high affinity of rat amylin in competing for binding (IC_{50} = 42 pM) indicated that the ^{125}I-BH group positioned at the amino-terminal lysine does not significantly affect affinity for these sites. Through competition experiments (Fig. 2), these binding sites were found to have a unique selectivity profile for a series of structurally related peptides, with potency for rat amylin ≥ salmon calcitonin > rat β-CGRP > rat α-CGRP ≫ rat calcitonin (Table 1). Salmon calcitonin had a greater slope factor than did other peptides (Table 1), suggesting a possible complex interaction with ^{125}I-BH-amylin binding sites. Binding of ^{125}I-BH-amylin was not significantly inhibited by various unrelated bioactive peptides tested at 1 μM, including insulin, glucagon, bradykinin, substance P, and neurokinin B.

Autoradiography. The localization of ^{125}I-BH-amylin binding sites in the rat forebrain was determined by autoradiography. In coronal sections through the region used for membrane binding assays, binding sites were concentrated in the

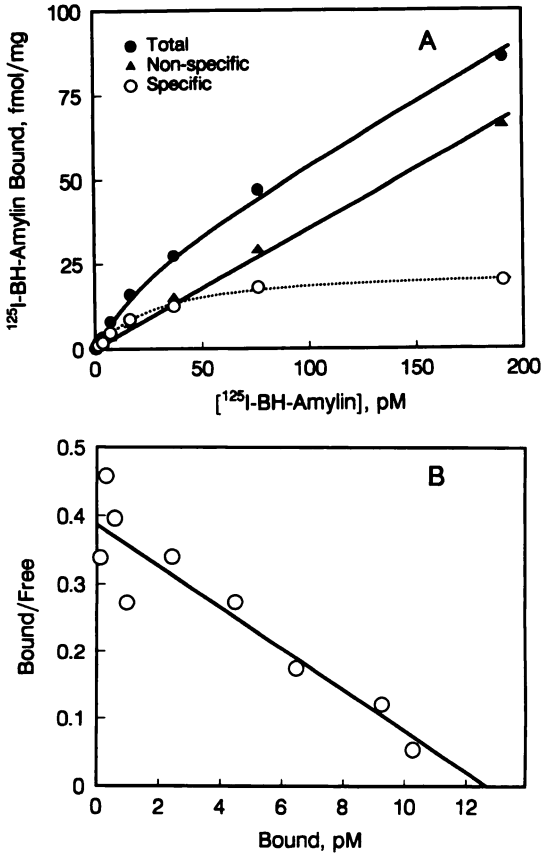

Fig. 1. Saturation binding of ^{125}I-BH-amylin to rat nucleus accumbens membranes. A, Binding of 0.3–190 pM ^{125}I-BH-amylin to rat nucleus accumbens membranes was measured in the absence (●) or presence (▲) of 1 μM rat amylin. ○, Specific binding. B, Scatchard plot of specific ^{125}I-BH-amylin binding data. Results are from one of three experiments performed in duplicate (K_d = 27.1 ± 2.1 pM, B_{max} = 23.8 ± 2.3 fmol/mg of protein; mean ± standard error, three experiments).

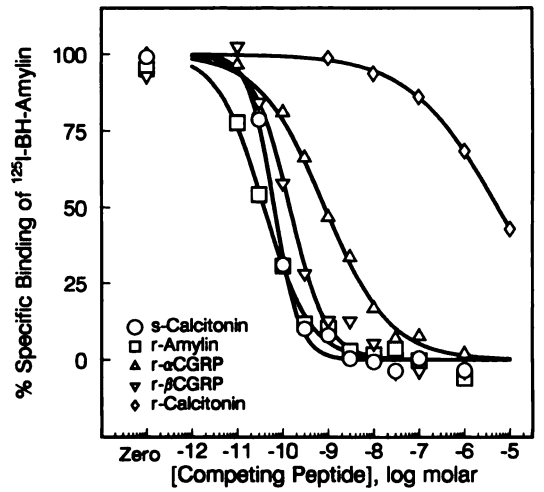

Fig. 2. Competition of peptides for ^{125}I-BH-amylin binding. Specific binding of 13 pM ^{125}I-BH-amylin to rat nucleus accumbens membranes was measured in the presence of the indicated concentrations of rat amylin, salmon calcitonin, rat β-CGRP, rat α-CGRP, or rat calcitonin. Data represent means of duplicate determinations. IC_{50} values derived from results of three to five separate experiments are listed in Table 1.

TABLE 1

Competition of peptides for ^{125}I-BH-amylin binding to rat nucleus accumbens membranes

Binding of ^{125}I-BH-amylin was measured in the presence of 10 concentrations of each peptide varying from 10^{-11} to 10^{-6} M, to obtain IC_{50} values and slope factors. Results are means ± standard errors of three to five separate experiments for each peptide.

Peptide	IC_{50}	Slope factor
	pM	
Rat amylin	42 ± 5	−0.84 ± 0.08
Salmon calcitonin	48 ± 9	−1.58 ± 0.27
Eel calcitonin	52 ± 5	−1.14 ± 0.10
Rat β-CGRP	124 ± 23	−1.11 ± 0.06
Human β-CGRP	214 ± 38	−0.86 ± 0.04
Human α-CGRP	271 ± 26	−0.80 ± 0.03
Rat α-CGRP	579 ± 90	−0.75 ± 0.07
Rat calcitonin	>1,000,000	

nucleus accumbens (Fig. 3A). In sagittal brain sections (Fig. 3B), ^{125}I-BH-amylin binding sites were also found in the area postrema, nucleus of the solitary tract, dorsal raphé, and hypothalamus. Nonspecific binding to sections, measured in the presence of 1 μM salmon calcitonin, was evenly distributed at a level slightly above film background.

Soleus muscle glycogen metabolism. In previously reported studies (18), rat amylin inhibited [^{14}C]glucose incorporation into isolated soleus muscle glycogen with an apparent EC_{50} of 3.1 nM. We determined whether salmon calcitonin was also able to inhibit insulin-stimulated incorporation of [^{14}C]glucose into glycogen in isolated rat soleus muscle (18, 19). Salmon calcitonin reduced insulin-stimulated incorporation of [^{14}C]glucose into rat soleus muscle glycogen (Fig. 4) with an apparent EC_{50} of 390 pM (standard error, ±0.084 log units; eight muscle strips). Rat calcitonin was markedly less potent than salmon calcitonin or rat amylin, with an EC_{50} of 74 nM (standard error, ±0.31 log units; four muscle strips).

Discussion

Binding sites with high affinity (27 pM) for amylin are concentrated in the nucleus accumbens and are present in a limited number of additional brain regions, including some areas not separated from plasma by the blood-brain barrier. High affinity amylin binding is similar, in both distribution and affinity for salmon calcitonin, to a calcitonin/CGRP ("C3") binding site first described by Sexton *et al.* (20). Amylin is nearly 3-fold more potent than β-CGRP, 14-fold more potent than α-CGRP, and at least 300-fold more potent than rat calcitonin in affinity for these sites. This selectivity profile differs markedly from the profile of either the $CGRP_1$ or $CGRP_2$ receptor subtype. Salmon calcitonin has very low affinity for CGRP receptors (21, 22) and is virtually inactive in producing CGRP-like cardiovascular actions (23). Amylin is much less potent than CGRP in producing vasodilation (15), in competing for CGRP receptor binding in muscle (12) and liver (11), and in activating the CGRP receptor subtype ($CGRP_2$) present in vas deferens (21, 24).

To begin examining the relationship of these binding sites to amylin actions in peripheral tissues, we studied both ^{125}I-BH-amylin binding to skeletal muscle membranes and functional effects in isolated skeletal muscle. In preliminary experiments, specific ^{125}I-BH-amylin binding to whole-rat muscle membranes was not consistently observed. However, receptor

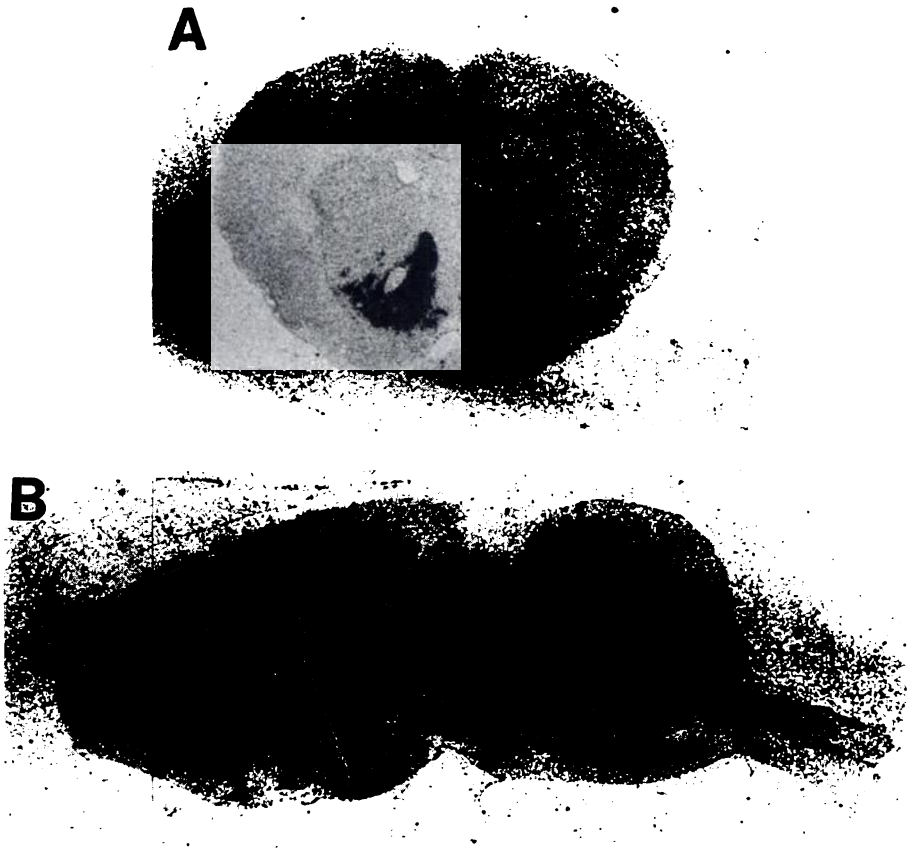

Fig. 3. Distribution of ^{125}I-BH-amylin binding in rat brain. A, ^{125}I-BH-amylin binding to a coronal brain section approximately 10 mm anterior to the interaural line is concentrated in the nucleus accumbens, with only background labeling present in cortex and other areas. B, Sagittal section shows the limited distribution of ^{125}I-BH-amylin binding in brain. In addition to the nucleus accumbens, regions demonstrating ^{125}I-BH-amylin binding included the area postrema, nucleus of the solitary tract, dorsal raphé, and hypothalamus.

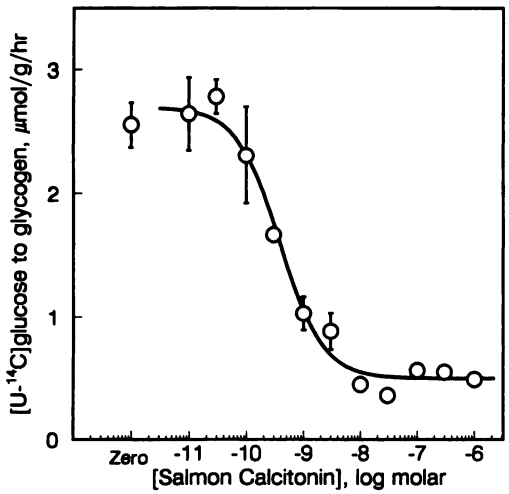

Fig. 4. Inhibition by salmon calcitonin of [^{14}C]glucose incorporation into soleus muscle glycogen. Isolated soleus muscles were incubated with 7 nM insulin to stimulate [^{14}C]glucose incorporation into glycogen in the presence of the indicated concentrations of salmon calcitonin. Results represent means ± standard errors of data from eight to 16 soleus muscles.

densities measured in peripheral tissues are often very low, in comparison with the high densities that may be present in discrete brain regions. In addition, receptor or ligand stability may be compromised in muscle homogenates.

To study functional actions, we used a key assay for amylin metabolic effects, i.e., inhibition of insulin-stimulated [^{14}C] glucose incorporation into glycogen in isolated rat soleus muscle. Amylin and CGRP are approximately equipotent in their actions upon glycogen metabolism in both isolated soleus muscle (16) and perfused rat hindlimb muscle (17). Fig. 4 shows the unexpected finding that salmon calcitonin potently produces the amylin-like action of inhibiting insulin-stimulated glucose incorporation into skeletal muscle glycogen. The high potency of salmon calcitonin, along with the similar potencies of amylin and CGRP, argue against the hypothesis that the observed alterations in skeletal muscle glycogen metabolism are mediated by CGRP receptors, which have a quite different pharmacological profile. Salmon calcitonin administration produces hyperglycemia in rats (25) and humans (26, 27), through a mechanism that has not been fully characterized. The potent amylin-like effects of salmon calcitonin described here suggest the possibility that alterations in skeletal muscle glycogen metabolism may contribute to the hyperglycemia produced by this peptide. Furthermore, these studies suggest that skeletal muscle, like brain, may contain receptor populations with high affinity for both amylin and salmon calcitonin. However, it is not clear from the present data whether the receptors mediating responses in muscle are the same as the binding sites in brain.

The presence of high affinity amylin binding sites in the brain raises the questions of whether amylin is present centrally and whether amylin has any physiological function in the central nervous system. In studies of amylin distribution, brain has not generally been reported to contain amylin (28), although amylin-like immunoreactivity has been reported in rat hypothalamus homogenates (29) and amylin-encoding mRNA is present in dorsal root ganglia (30). Additional experiments are required to determine whether amylin is synthesized and expressed in a regionally specific manner within the brain. Intracerebral administration of amylin reduces food intake in rats (31, 32) and mice (29). Central or peripheral administration of salmon calcitonin also has anorectic effects (33). In addition to causing anorexia, infusion of salmon calcitonin into the rat central nervous system is analgesic (34), modulates growth hormone release (35), and antagonizes amphetamine-induced motor hyperactivity (36, 37). Study of the effects elicited by centrally administered amylin could help in identifying which of these actions are mediated by amylin receptors and which are mediated by central calcitonin receptors (36) or CGRP receptors (22).

In conclusion, binding sites with picomolar affinity for amylin have been identified in rat brain. Salmon calcitonin also has high affinity for these sites and, unexpectedly, potently inhibits the insulin-stimulated incorporation of glucose into soleus muscle glycogen. The available selectivity data indicate that effects of amylin and salmon calcitonin upon carbohydrate metabolism are mediated mainly by receptors other than CGRP receptors. Selective amylin receptor antagonists will be important tools both for further characterizing amylin-responsive receptors and for exploring the role of amylin in normal (37, 38) and disordered metabolism.

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