Rapid Publications

Presence of Liver CGRP/Amylin Receptors in Only Nonparenchymal Cells and Absence of Direct Regulation of Rat Liver Glucose Metabolism by CGRP/Amylin

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Human calcitonin gene–related peptide (hCGRP-1) and human amylin (hA) have been reported to increase hepatic glucose output in vivo and to bind with high affinity to rat liver plasma membranes, resulting in increased cAMP production. These observations have led to the hypothesis that CGRP or amylin may be physiological regulators of liver glucose metabolism. Liver plasma membranes are derived from several cell types, including parenchymal (hepatocyte), Kupffer, endothelial, lipid storage, and smooth muscle cells. Because the parenchymal cell is responsible for the contribution of the liver to whole-body glucose homeostasis, it is important to verify the location and activity of the CGRP/amylin receptor to this cell. These studies separate liver cells prepared by collagenase digestion into parenchymal and nonparenchymal fractions by metrizamide gradient and differential centrifugation. ^{125}I-labeled [Tyr-0]hCGRP-1 bound with high affinity to nonparenchymal cell fraction and was displaced by both hCGRP-1 and hA. hCGRP-1 bound with greater affinity than hA (K_d = 2.1 ± 1.6 × 10^{-11} vs. 2.6 ± 1.2 × 10^{-8} M) in a manner similar to the binding reported for liver plasma membrane fraction. Linear regression of receptor concentration against nonparenchymal cell count per milliliter was significant (r = 0.999, P = 0.026), leading to an estimate of 3000 receptors/cell. The parenchymal cell fraction bound very little ^{125}I-[Tyr-0]hCGRP-1, and regression of receptor concentration against parenchymal cell count per milliliter was not significant (r = −0.708, P = 0.29), suggesting that binding was not due to parenchymal cells but instead to contamination by nonparenchymal cells. ^{125}I-labeled rat amylin (^{125}I-rA) binding gave similar results for the distribution of receptors but higher apparent affinity for hA (K_d = 2.0 ± 0.9 × 10^{-9} M). The following studies were then performed to determine the direct effect of CGRP and amylin on liver glucose metabolism. Of hCGRP-1, hA, human deamidated amylin, and rA, none alter glycogen synthesis (basal or insulin stimulated) or net glucose production (basal or glucagon stimulated) by isolated rat liver parenchymal cells. Glucagon, but not hCGRP-1 or hA, stimulated perfused liver glucose output. The lack of effect by hCGRP-1 and hA on isolated hepatocyte glucose metabolism is therefore not merely a result of the collagenase digestion procedure. These data indicate that CGRP/amylin receptors occur only on the nonparenchymal cells of rat liver and do not directly regulate liver glucose metabolism. *Diabetes* 40:395–400, 1991

Amylin, or islet amyloid polypeptide, is a COOH-amidated 37–amino acid pancreatic β-cell secretory peptide that is a major component of pancreatic amyloid in humans (1–4). Calcitonin gene–related peptide 1 (CGRP-1) is an alternate splicing product of the calcitonin gene and is secreted mainly by neural tissues (5). Both human CGRP-1 (hCGRP-1) and human amylin (hA) have been shown to counteract insulin-stimulated [^{14}C]glucose incorporation into rat soleus muscle glycogen (6,7). This effect led to the hypothesis that oversecretion of hA by the pancreatic β-cell or CGRP by intestinal/neural tissues might lead to an insulin-resistant state characteristic of non-insulin-dependent diabetes mellitus (NIDDM) (7). Insulin resistance is not, however, only a characteristic of muscle glucose disposal but is also a characteristic of liver glucose production in NIDDM and obesity (8,9). In vivo studies at pharmacological doses both support and refute hCGRP-1 and hA counterregulation of hepatic glucose production (10–13). Ciaraldi et al. (14) have demonstrated effects of hA on glucose and glycogen metabolism of a human liver carcinoma cell line, HepG2. However, recent studies by Redon et al. (15) with perfused rat liver have failed to demonstrate direct effects of hA on liver glucose metabolism in the presence and absence of insulin, although no

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independent verification of biological potency of their synthetic hA peptide was provided.

Recently, Morishita et al. (16) reported that both hA and hCGRP-1 displace ^{125}I-labeled [Tyr-0]hCGRP-1 from rat liver plasma membranes and increase cAMP production. Liver consists of several cell types with an estimated 40% nonparenchymal cells (17,18). Preparation of crude liver plasma membrane fraction does not discriminate between cell types, and the reported high-affinity binding remains undefined. The parenchymal cell is the only liver cell type with the ability to contribute to liver glucose production. It is not clear what impact binding of CGRP/amylin to a receptor located on a Kupffer, smooth muscle, or endothelial cell might have. It is also not certain that the insulin resistance induced by amylin is mediated by cAMP. Amylin induction of cAMP levels occurs at levels 1000-fold higher than required for insulin resistance in cultured L6 myocytes (19).

These studies were performed with liver cells separated into parenchymal and nonparenchymal fractions by metrizamide-gradient and differential centrifugation. hA and/or hCGRP-1 displacement of bound ^{125}I-[Tyr-0]hCGRP-1 and ^{125}I-labeled rat amylin (^{125}I-rA) are reported. Direct metabolic effects of CGRP and amylin were determined with primary rat liver systems. The direct acute effects of hCGRP-1, hA, human deaminated amylin (hDA), and rA on insulin-stimulated [^{14}C]glucose incorporation into glycogen- and glucagon-stimulated glucose production with isolated rat liver parenchymal cells are reported. Glucagon, hA, and hCGRP-1 effects on liver glucose production were also studied with the perfused rat liver.

RESEARCH DESIGN AND METHODS

^{125}I-[Tyr-0]hCGRP-1, hCGRP-1, and rA were purchased from Peninsula (Belmont, CA). ^{125}I-rA, labeled on NH_2-terminal lysine by Bolton and Hunter reagent, was obtained from Amersham (Arlington Heights, IL). hA and hDA were synthesized by E.W. Shirmer and G.S. Brooke (Lilly, Indianapolis, IN) via an automatic solid-phase synthesizer (430A peptide synthesizer, Applied Biosystems) and purified by reverse-phase high-performance liquid chromatography. hA was a gift from Amylin (La Jolla, CA). hA obtained from Amylin or synthesized at Lilly was equally potent (EC_{50} = 9 nM) at inhibiting insulin-stimulated (1000 μU/ml) [^{14}C]glucose into rat soleus muscle glycogen (6). Pork glucagon and pork insulin were obtained from Lilly. Collagenase (type D) was obtained from Boehringer Mannheim (Indianapolis, IN). Protease-free bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO) and dialyzed against Krebs-Henseleit buffer (KHB) before use (19).

Rat liver parenchymal cells (hepatocytes) were isolated from 200-g male ad libitum–fed Sprague-Dawley rats (Charles River, Portage, MI) by the method of Berry and Friend (20) as modified by Harris et al. (21). Collagenase was carefully selected from several lots to maximize high-affinity insulin binding to isolated cell surface receptors. In one study, freshly isolated hepatocytes were incubated 2 h in Dulbecco's minimum essential medium (DMEM) to regenerate any damaged receptors.

Combined isolation of liver nonparenchymal and parenchymal cells from rat liver was performed by a modification of the method of Seglen (17). Cells were combed out of the collagenase-perfused liver (prepared as above) and filtered through a 350-gauge nylon mesh. The mixed-cell suspension was layered on a step metrizamide gradient consisting of 8 ml 30% (wt/vol) metrizamide and 8 ml 18% (wt/vol) metrizamide in 10 mM Krebs-Ringer-HEPES (pH 7.4) and centrifuged at 3000 × *g* for 30 min (Beckman GPR centrifuge). Nonparenchymal cells were harvested from the 18% (wt/vol) metrizamide interface with a Pasteur pipette, diluted 1:4 with 0.3% BSA in KHB, and centrifuged at 200 × *g* for 2 min. The cell pellet was discarded and the supernatant spun at 3000 × *g* for 30 min. The nonparenchymal cells were resuspended in 2.5% BSA in KHB to 2 × 10^6 cells/ml. Nonparenchymal cells were determined to be free of parenchymal cells by light microscopy. Parenchymal cells were harvested from the 30% (wt/vol) metrizamide interface of the density gradient, diluted 1:4 with 0.3% BSA in KHB, and centrifuged at 200 × *g* for 2 m. The cell pellet was resuspended in 2.5% BSA in KHB to 2 × 10^6 cells/ml as the parenchymal cell fraction. Parenchymal cells were counted with a hemacytometer at ×100, allowing total discrimination for parenchymal cells. Nonparenchymal cells were counted with a Coulter cell counter (Edison, NJ).

Binding studies were performed at 15°C, a temperature where receptor-mediated endocytosis is impeded and binding of insulin reaches equilibrium within 3 h (22). Medium contained 1 mg/ml bacitracin, 20 mM glucose, 1.25% BSA, and 5 mM HEPES in Krebs-Ringer buffer. One milliliter of cells (1 × 10^6 cells/ml) was incubated with 0.05 μCi ^{125}I-[Tyr-0]hCGRP-1, hCGRP-1, or hA for 3 h at 15°C, then bound radioactivity was harvested with polyethyleneimine-treated glass-fiber filter with a Skatron cell harvester (Lier, Norway). Studies with ^{125}I-rA were performed in an analogous manner. Radioactive counts were determined by γ-scintillation counter (Micro Medic Systems Auto Gamma Counter).

Hepatocyte incubations were performed in 25-ml polypropylene flasks containing a total of 2 ml of cell suspension and additions in 2.5% protease-free BSA/KHB under 95% O_2/5% CO_2 at 37°C. For glycogen synthesis studies, hepatocytes isolated from fed rats were incubated with 20 mM glucose (0.04 μCi/μmol [U-^{14}C]glucose), 10 mM lactate, and 1 mM pyruvate for 60 min. [U-^{14}C]glucose incorporation into liver parenchymal cell glycogen was determined according to the method of Good et al. (23). For glucose production studies, hepatocytes isolated from fed rats were incubated in 10 mM lactate, 1 mM pyruvate, and 10 mM asparagine for 20 min. Incubations were stopped, and protein was precipitated by the addition of perchloric acid to 3% wt/vol. Centrifugal supernatants were neutralized, and glucose was assayed by the hexokinase/glucose-6-phosphate dehydrogenase method.

Liver perfusions were performed as a modification of the procedure used to isolate parenchymal cells. Medium (10 mM alanine and 10 mM asparagine in KHB) was gas exchanged (95% O_2/5% CO_2) and warmed to 37°C in a glass lung and perfused through a cannula placed in the portal vein of the liver in situ at constant pressure without recycling. Perfusate was collected via a cannula inserted in the superior vena cava, with the inferior vena cava ligated. Hormone was infused by a peristaltic pump into a 37°C polypropylene mixing chamber/bubble trap just before the portal cannula. Flow rate varied from 30 to 35 ml/min. The liver was equilibrated

during a 30-min period, and then hormone infusion started and continued for 45 min. Fractions were collected every 5 min and analyzed for glucose, lactate, and volume.

Statistical significance was determined by analysis of variance or linear regression (Statview, Abacus Concepts, Berkeley, CA). Binding constants were determined with Ligand (Laboratory of Theoretical and Physical Biology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD).

RESULTS

^{125}I-[Tyr-0]hCGRP-1 bound with high affinity to nonparenchymal liver cell fractions and was displaced by both hCGRP-1 and hA (K_d = 2.1 ± 0.6 × 10^{-11} and 2.6 ± 1.1 × 10^{-8} M for hCGRP-1 and hA, respectively; Fig. 1). Linear regression of receptor concentration, estimated by the binding data against cell count per milliliter, was significant (r = 0.999, P = 0.026), leading to an estimate of 3005 ± 83 receptors/cell. This estimate was based on total cell count including several types of cells (17) but not including parenchymal cells, which were not detected by light microscopy. Addition of binding parameters to the model for a second class of receptors did not significantly improve the fit (P = 0.46), and the apparent binding affinity was very low (K_d = 4.2 × 10^{-1} M for hCGRP-1). The Scatchard plot (not shown), however, was not linear and indicated the potential existence of low-affinity receptors.

Parenchymal cells bound only a small amount of ^{125}I-[Tyr-0]hCGRP-1, which was not significant in several experiments (Fig. 1). Data pooled from four studies, however, demonstrated significant high-affinity binding (P < 0.05). The negative slope obtained by linear regression of receptor concentration (estimated by the binding data) against cell count per milliliter was not significant (r = −0.708, P = 0.29), suggesting that binding was not due to parenchymal cells but was due to contamination by nonparenchymal cells. The affinity for hCGRP-1 binding was not significantly different from that found with the nonparenchymal cell fraction (K_d = 8.2 ± 14.9 × 10^{-12} M). Contamination of parenchymal cells by nonparenchymal cells was not estimated in this study but is routinely between 1 and 5% (17).

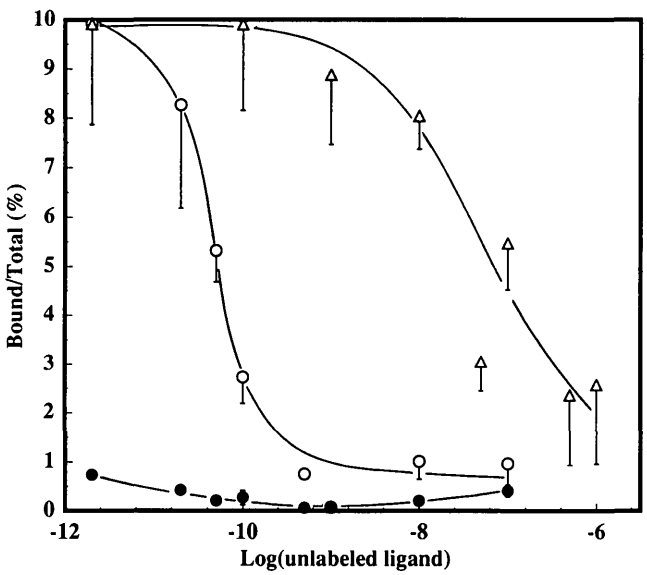

FIG. 1. Displacement of bound ^{125}I-labeled Tyr-0 human calcitonin gene–related peptide 1 (hCGRP-1) from nonparenchymal cells (○, △; 1 × 10^6 cells/ml) and parenchymal cells (●; 1 × 10^6 cells/ml). Mean specific bound/total labeled ligand (%) is plotted against free ligand concentration for hCGRP-1 (○; n = 3; ●, n = 4) or hA (△, n = 3) after correction for nonspecific binding (7.5 and 8.2% for nonparenchymal and parenchymal cell fractions, respectively).

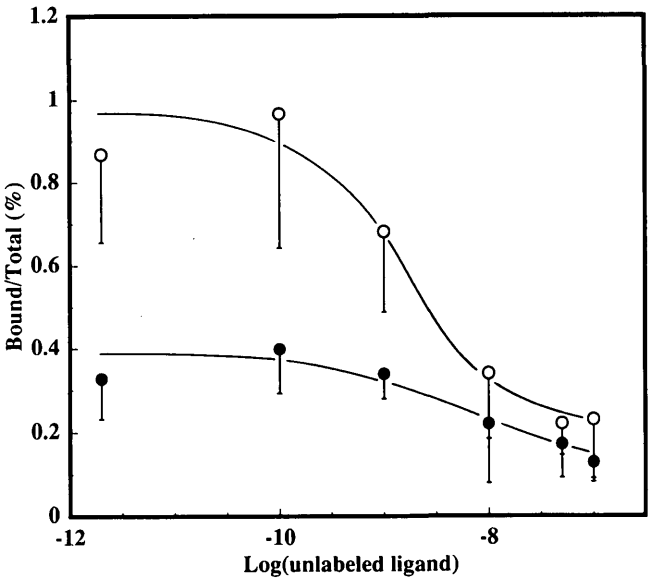

FIG. 2. Displacement of bound ^{125}I-labeled rat amylin from nonparenchymal (○; 0.94 × 10^6 cells/ml) and parenchymal (●; 1 × 10^6 cells/ml) cells. Mean specific bound/total labeled ligand (%) (2 separate preparations of cells) is plotted against free ligand concentration for human amylin after correction for nonspecific binding (1.9 and 2.3% for nonparenchymal and parenchymal cell fractions, respectively).

^{125}I-rA also bound to nonparenchymal cells with high affinity and was displaced by hA (P < 0.05; Fig. 2). Based on these data, the K_d for hA was 2.0 ± 0.9 × 10^{-9} M with 5600 ± 2400 receptors/cell. Binding of ^{125}I-rA to parenchymal cells was not significant (P < 0.5; Fig. 2). The higher apparent affinity of hA for this binding site based on these data may be due to the assumption during statistical analysis of equivalent binding for hA and ^{125}I-rA. Further studies are required to determine the relative affinities for these two ligands.

Of hCGRP-1, hA, hDA, and rA (100 nM), none affected basal or insulin-stimulated [U-^{14}C]glucose incorporation into rat parenchymal cell glycogen (P = 0.8; Fig. 3). Insulin stimulated glycogen synthesis threefold (P = 0.0001) with an EC_{50} of 8.6 nM, corresponding well with the K_d for insulin binding (1.6 nM; unpublished observations). The existence of high-affinity insulin binding and the insulin effect on glycogen synthesis suggest that cell surface receptors are not damaged by the collagenase digestion during isolation of cells. The similarity between insulin-receptor binding and insulin-receptor processing in the cultured and freshly isolated hepatocyte has been demonstrated previously (22).

Of hCGRP-1, hA, hDA, or rA (100 nM), none affected basal or glucagon-stimulated net glucose production from fed rat liver parenchymal cells supplemented with lactate, pyruvate, and asparagine (P = 0.8; Fig. 4). Glucagon readily stimu-

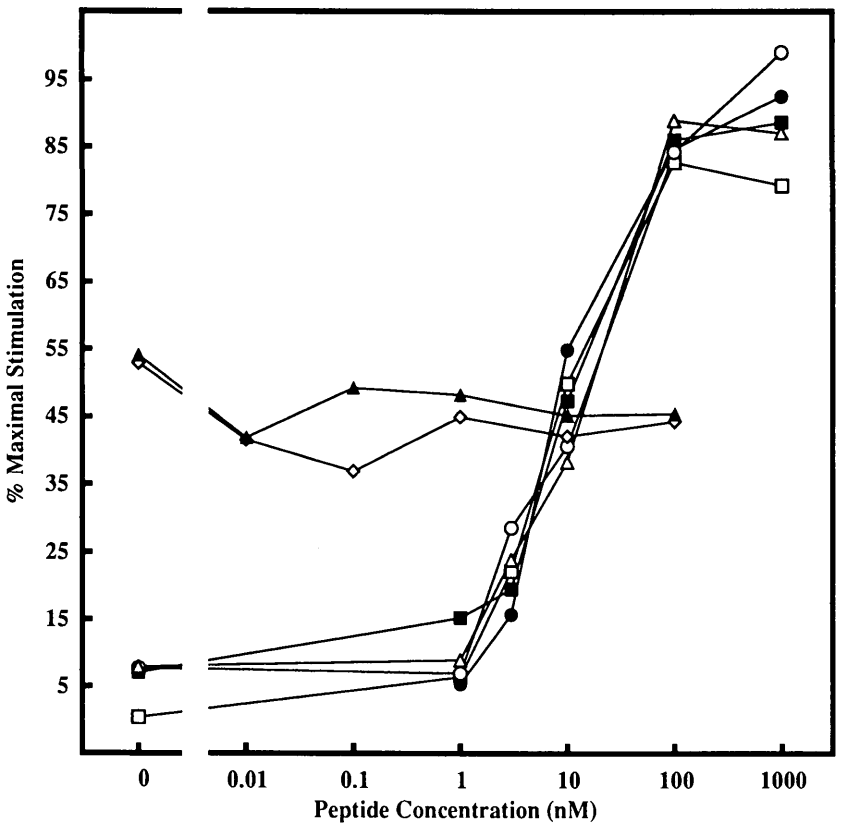

FIG. 3. Effect of human calcitonin gene–related peptide 1 (hCGRP-1), human amylin (hA), human deamidated amylin (hDA), and rat amylin (rA) on insulin-stimulated [^{14}C]glucose incorporation into fed rat parenchymal cell glycogen. [^{14}C]glucose incorporation into fed rat parenchymal cell glycogen was measured at indicated concentrations of pork insulin with no additions (□; n = 7), 100 nM hCGRP-1 (○; n = 4), 100 nM hA (△; n = 4), 100 nM hDA (■; n = 3), 100 nM rA (●; n = 3), or with insulin held constant at 10 nM and indicated concentration of hCGRP-1 (▲; n = 2) or hA (◇; n = 3). Values are mean percentage of maximal stimulation for n hepatocyte preparations.

lated net glucose production threefold (P = 0.0001) with an EC_{50} of 0.3 nM, again suggesting that the collagenase treatment had not damaged cell surface receptors. When isolated parenchymal cells were incubated at 37°C for 2 h in DMEM and then resuspended in incubation medium and treated with hA as above, hA remained ineffective at altering glucose output. This preincubation period would have allowed newly synthesized receptors to be inserted into the plasma mem-

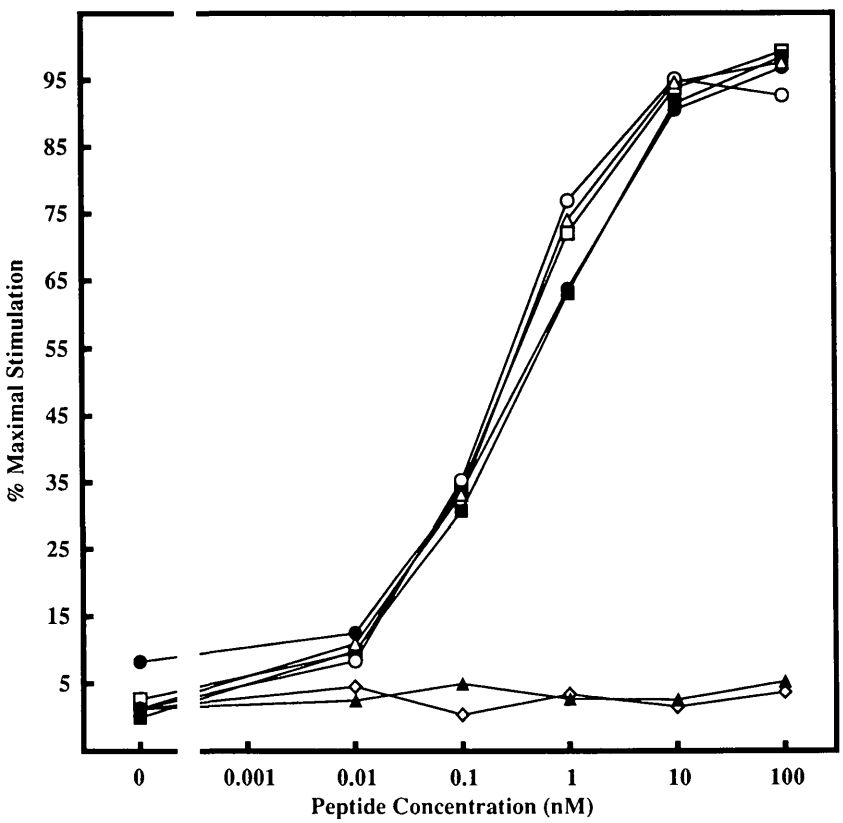

FIG. 4. Effect of human calcitonin gene–related peptide 1 (hCGRP-1), human amylin (hA), human deamidated amylin (hDA), and rat amylin (rA) on glucagon-stimulated glucose production from fed rat parenchymal cell glycogen. Glucose production by fed rat parenchymal cells was measured at indicated concentrations of glucagon with no additions (□; n = 4), 100 nM hCGRP-1 (○; n = 3), 100 nM hA (△; n = 3), 100 nM hDA (■; n = 2), 100 nM rA (●; n = 3), or in absence of glucagon and indicated concentration of hCGRP-1 (▲; n = 3) or hA (◇; n = 3). Values are mean percentage of maximal stimulation for n hepatocyte preparations.

brane and restore some biological activity, if such receptors are expressed by the hepatocyte.

hCGRP-1 and hA did not affect either insulin-stimulated [U-^{14}C]glucose incorporation into fed rat parenchymal cell glycogen (P = 0.8; Fig. 3) or net glucose production from fed rat parenchymal cells (P = 0.4; Fig. 4) through a dose range of 10^{-11}–10^{-8} M. This dose encompasses both the physiological and pharmacological levels of these potential hormones. This suggests that rat liver parenchymal cell glucose metabolism is unaffected by either hCGRP-1 or hA through the dose range tested.

Glucagon, but not hCGRP-1 or hA, stimulated perfused liver glucose output in a time- (P = 0.002; Fig. 5*A*) and dose- (P = 0.0001; Fig. 5*B*) dependent manner. Glucagon also induced a small but significant reduction in lactate output (P = 0.0001), consistent with a stimulation in neoglucogenesis. hA infusion resulted in a slight but significant reduction in glucose and lactate output (P < 0.01). Neither glucagon nor hA significantly altered flow rate (P < 0.5). Perfused liver obviously contains the full range of liver cell types undamaged by collagenase treatment and verifies that the lack of ability for CGRP/amylin to increase parenchymal cell glucose output is not the result of the preparation procedure. This study confirms the well-known regulation of liver glucose production by glucagon and refutes the hypothesis of a direct role for hA or hCGRP-1 as regulators of liver glucose production.

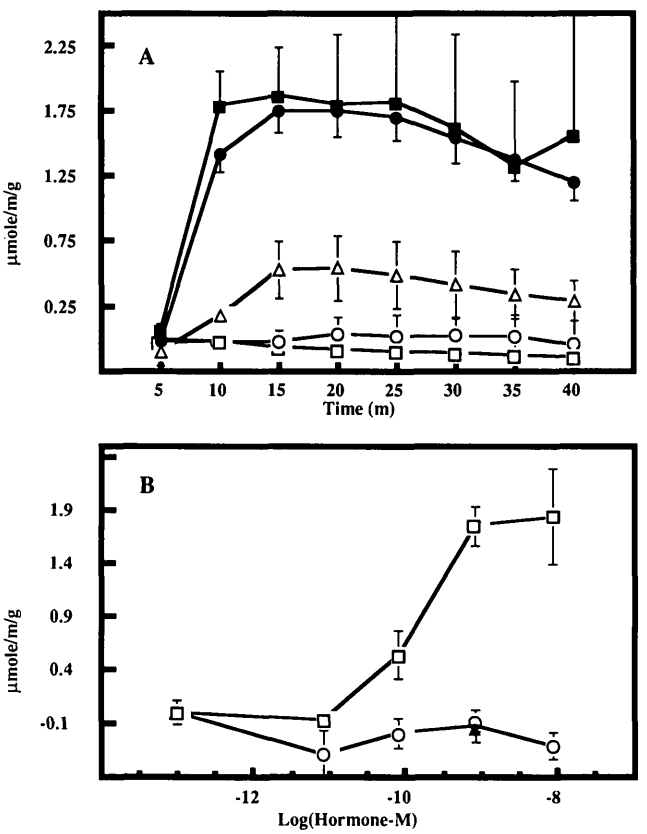

FIG. 5. *A*: effect of glucagon on glucose production from perfused liver from fed rat versus time without glucagon (○) or with 10^{-11} (□), 10^{-10} (△), 10^{-9} (●), and 10^{-8} M (■) glucagon. *B*: effect of glucagon, human amylin (hA), and human calcitonin gene–related peptide 1 (hCGRP-1) on glucose production from fed rat perfused liver versus dose at indicated concentrations of glucagon (□), hA (○), or hCGRP-1 (▲). Values are mean ± SE glucose production (µmol · min^{-1} · g^{-1}) for 3–4 separate perfusions of rat liver per dose per hormone (n = 4 for control, n = 3 for others).

DISCUSSION

hCGRP-1, hA, hDA, and rA do not affect liver (perfused or isolated parenchymal cell), glucose, or glycogen metabolism in either the presence or absence of insulin or glucagon. Glucose production by parenchymal cells and perfused liver is the combined product of glycogenolysis and neoglucogenesis. The lack of effect on glucose production therefore suggests that neither of these pathways are directly affected by these peptides or that they are affected in a compensating manner. Because glycogen synthesis was found to be unaffected by independent means, neither glycogenolysis nor neoglucogenesis are affected directly by CGRP or amylin. During the preparation of this article, an abstract appeared by Roden et al. (15) describing the lack of effect of hA (1×10^{-7} M), in the presence and absence of insulin, on liver glucose production. The biological activity of the amylin used in their study was undefined; however, their results are in agreement with current studies. The lack of effect on perfused liver glucose production also suggests that CGRP/amylin do not act indirectly by stimulating the release of factors (i.e., prostaglandins) by nonparenchymal cells that alter parenchymal cell glucose metabolism or that these factors must accumulate in a recirculating system to be effective.

The lack of a direct effect by CGRP-1 or amylin on liver glucose metabolism can readily be explained by the absence of receptor on the parenchymal cell. The significant positive correlation between high-affinity receptor concentration and nonparenchymal cell number (r = 0.999) versus the nonsignificant negative correlation between high-affinity receptor concentration and parenchymal cell number (r = −0.708) suggests that the receptor exists only on the nonparenchymal cell. The presence of receptors in the nonparenchymal cell fraction is the apparent explanation for the high-affinity liver plasma membrane binding reported by Morishita et al. (16). Isolation of rat liver plasma membranes from a crude whole-liver homogenate does not distinguish between cell types, and all the binding in the reported study could be due to plasma membranes originating from the nonparenchymal cells. The binding affinity for hCGRP-1 versus hA to the nonparenchymal cell fraction in this study was in relatively good agreement with these published results. Yamaguchi et al. (24,25) have published binding data with liver plasma membranes that indicate the presence of only one class of binding sites. Our studies found no statistical evidence for more than one class of receptors; however, the Scatchard plot is not linear and suggests the possibility of a very-low-affinity class. Because these receptors appear to be linked to cAMP production and are coupled to GTP-binding proteins, this second class could be associated with G-protein interactions (25).

This study would appear to disagree with the study of Ciaraldi et al. (14), who reported effects of hA on HepG2 [^{14}C]glycosyl-glycogen exchange and [^{14}C]lactate conversion to [^{14}C]glucose. This cell type is, however, known to be dedifferentiated, including the expression of the brain (GLUT1) rather than the liver (GLUT2) type of glucose trans-

porter (26). The presence of CGRP/amylin receptor on HepG2 cells could be due to the expression of a receptor not normally found in liver parenchymal cells resulting from transformation to a tumor cell type.

Reports of hCGRP-1 and hA effects on hepatic glucose production in vivo have been mixed, with some researchers reporting positive effects (10,11,13) and others observing no effects (12). All of the studies, however, have been performed at pharmacological levels of hCGRP-1 and hA and have not been able to distinguish between direct and indirect effects on liver glucose metabolism. It is possible that hCGRP-1 or hA, at pharmacological doses, cause the release of some factor that in turn influences liver glucose production.

The existence of CGRP/amylin receptors in the nonparenchymal cell fraction is in full agreement with other reports of activities for CGRP. CGRP-1 is a neuropeptide and is one of the most potent naturally occurring vasodilators known, suggesting that receptors may be found on smooth muscle cells of the arterial wall (27). Known enhancement by CGRP-1 of neurogenic inflammatory response may also be due to effects on extravasation (28). Branches of nerve fibers terminating on smooth muscle fibers are found in the liver, which suggests that CGRP/amylin receptors of the liver are associated with smooth muscle cells (29). The nonparenchymal cell fraction consists of several cell types, including sinusoidal cells (Kupffer, endothelial, and fat storage cells) and smooth muscle cells. It is not possible with current data to determine which of these cell types binds CGRP/amylin.

The physiological role of CGRP/amylin receptors in the liver remains in question. Our studies have demonstrated that the CGRP/amylin receptor of rat liver exists in the nonparenchymal cell fraction and does not contribute to the direct regulation of liver glucose metabolism. Further studies are required to determine which nonparenchymal cell possesses this receptor and what biological function it serves in that cell. This information will then allow in vivo studies or studies with perfused liver to evaluate more directly the importance of the CGRP/amylin receptor.

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