Rapid Publications

Induction of Insulin Resistance In Vivo by Amylin and Calcitonin Gene–Related Peptide

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During hyperinsulinemic glucose-clamp studies, intravenous infusion of calcitonin gene–related peptide (CGRP) in rats antagonized the ability of insulin to stimulate peripheral glucose disposal by 52% (196 ± 7.2 vs. 105 ± 10.5 μmol · kg^{-1} · min^{-1}, $P < 0.05$) and to inhibit hepatic glucose output by 54% ($P < 0.01$). CGRP also inhibited the in vitro effects of insulin to stimulate hexose uptake in cultured BC_3H1 myocytes at all insulin concentrations studied. Amylin is a peptide isolated from amyloid deposits in pancreatic islets of type II (non-insulin-dependent) diabetic subjects, is present in normal β-cells, and bears a striking homology to CGRP. When synethetic human amylin was infused during clamp studies, it inhibited the ability of insulin to stimulate glucose disposal by 56% (96.9 ± 9.4 vs. 42.4 ± 5.0 μmol · kg^{-1} · min^{-1}, $P < 0.05$) and to suppress hepatic glucose output by 64%. Therefore, amylin and CGRP can cause insulin resistance in vivo and may be implicated in insulin-resistant states such as type II diabetes mellitus.
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Type II (non-insulin-dependent) diabetes mellitus is a common human disease characterized by various abnormalities in carbohydrate metabolism leading to hyperglycemia, which is the clinical hallmark of this disorder. Although several metabolic and biochemical defects have been described, peripheral insulin resistance, increased hepatic glucose production, and impaired insulin secretion are the three characteristic features that underly the deranged carbohydrate metabolism in type II diabetes (1,2). Recently, a great deal of attention has been focused on insulin resistance and increased hepatic glucose production as pathogenic features of type II diabetes, and several physiological and cellular defects have been reported (3,4). However, despite a large body of information, the basic mechanisms underlying the metabolic abnormalities of type II diabetes remain unknown.

Pancreatic islets of type II diabetic patients contain interstitial amyloid deposits that are relatively specific for type II diabetes (5–8). In recent years, considerable progress has been made in identifying the nature of these amyloid deposits. A peptide from amyloid deposits obtained from a human insulinoma was partially sequenced and named *insulinoma amyloid polypeptide* (9,10). A nearly identical peptide was isolated directly from amyloid deposits in pancreases from type II diabetic patients (11). This material was purified and found to be a 37–amino acid peptide that has been named *amylin* (12). Analysis of this new islet-derived peptide demonstrated that the sequence is 46% identical to calcitonin gene–related peptide (CGRP; 10,11) and has more distant similarity with the A chain of insulin over a 16-residue segment (11). The cDNA for human (13,14) and rat (15) amylin has been cloned, confirming the sequence of the peptide and suggesting that the COOH-terminal is amidated.

Immunocytochemical studies have demonstrated colocalization of amylin and insulin within β-cell secretory granules from islets obtained from normal human pancreases (16) and islets of type II diabetic subjects (17). Tissue-specific expression of the rat amylin gene has been shown to occur only in pancreatic β-cells (15). CGRP has been shown to inhibit insulin secretion in vitro (18). High concentrations of amylin have also been shown to inhibit insulin secretion from isolated pancreatic islets (19).

In view of the potential relationship between a peptide present in islet amyloid of patients with type II diabetes and the pathogenesis of this syndrome, studies of the biological activity of this peptide have been undertaken. Purified amylin extracted from diabetic pancreases, synthetic amylin, and

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synthetic CGRP have been assessed for their ability to inhibit the in vitro actions of insulin (12,20). These peptides inhibit the ability of insulin to stimulate glucose incorporation into skeletal muscle glycogen at physiological insulin concentrations in an isolated rat soleus muscle preparation (20). Interestingly, these compounds did not inhibit insulin action in adipose tissue (12).

In considering any potential relationship between a pancreatic islet–derived peptide and a pathophysiological state of insulin resistance such as type II diabetes, it is important to know whether this material can induce a state of insulin resistance when administered to whole animals in vivo.

RESEARCH DESIGN AND METHODS

Hyperinsulinemic glucose-clamp studies were performed in male Sprague-Dawley rats (240–300 g) after the method of Ziel et al. (21). Rats were anesthetized with Inactin (100 mg/kg i.p., Byk-Gulden, Konstanz, FRG). A midline ventral incision was made in the neck, and the left carotid artery was cannulated to allow sampling of arterial blood. The right jugular vein was cannulated for infusion of glucose and hormones. Tracheostomy was performed on all animals. Hypothermia was prevented by conducting the procedures on a heated operating surface. Insulin was infused at a rate of 13 pmol · kg^{-1} · min^{-1}, CGRP at 5 nmol · kg^{-1} · min^{-1}, and amylin at either 5 or 0.5 nmol · kg^{-1} · min^{-1}. Rat CGRP-1 (Peninsula, Belmont, CA) and human amylin (Peninsula; Bachem, Torrance, CA) were used in the amidated form. Arterial blood was sampled at 5- to 10-min intervals, and plasma glucose was measured by the glucose oxidase method with an automated glucose analyzer (YSI 23A, Yellow Springs, OH). Euglycemia was maintained by infusion of 20% D-glucose. D-[3-^{3}H]glucose (Du Pont-NEN, Boston, MA) was infused throughout the experiments to determine overall rates of glucose appearance. To evaluate the possible contribution of the sympathetic nervous system to amylin-induced insulin resistance, we induced adrenergic blockade in a subset of three animals by infusing the β-blocker propranolol (32 μg · kg^{-1} · min^{-1}) and the α-blocker phentolamine (22 μg · mg^{-1} · min^{-1}) at doses shown to completely prevent epinephrine-induced changes in glucose turnover in rats (22) during clamp studies in which amylin was infused at 0.5 nmol · kg^{-1} · min^{-1} and insulin at 13 pmol · kg^{-1} · min^{-1}. Animals were killed on completion of the experiments. All animal studies were approved by the Animal Studies Subcommittee of the San Diego Veterans Administration Medical Center.

Hexose uptake. Rat adipocytes were isolated by collagenase digestion of epididymal fat pads, and 2-deoxyglucose (2-DG) uptake was measured as previously described (23). BC_3H1 myocytes were cultured in Dulbecco's modified Eagle's medium with 25 mM glucose, 4 mM L-glutamine, 1 mM pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. Cells were plated on 35-mm plastic culture dishes at 4 × 10^3 cells/ml, and uptake was measured 10 days later. Confluent monolayers were washed free of culture medium with a physiological salts/10 mM HEPES glucose-free buffer, pH 7.4, containing 10 g/L bovine serum albumin (fraction V, Sigma, St. Louis, MO) then preincubated for 1 h at 37°C in the same buffer with or without 10^4 pM CGRP. After preincubation, insulin was added to a final concentration of 0, 460, 18,400, or 184,000 pM, and the incubation was carried out for 1 h. 2-DG uptake was measured by exposing cells to [1-^{3}H]-2-DG (0.1 mM final concn, 0.4 μCi/dish) for 3 min. Uptake was terminated by aspiration of the incubation medium and washing the cells four times with phosphate-buffered saline, pH 7.4, containing 0.1 mM phloretin. All assays were performed in triplicate and corrected for nonspecific glucose uptake by subtracting L-glucose uptake.

Analytical procedures. Serum insulin levels were measured by a double-antibody radioimmunoassay (24). Serum CGRP levels during the rat clamps were measured by radioimmunoassay with a kit (Peninsula) according to the manufacturer's specifications. Plasma glucose specific activity was determined after deproteinating the samples with perchloric acid (25). Steady-state glucose turnover was determined as the quotient of the tracer infusion rate divided by the plasma specific activity (26).

All data are given as means ± SE unless otherwise noted. Statistical analysis was performed with Student's *t* test.

RESULTS

Figure 1 demonstrates the results of hyperinsulinemic glucose-clamp studies in rats infused with insulin and CGRP. Figure 1*A* depicts a typical study in which the protocol design was to infuse insulin at a constant rate for 210 min with administration of CGRP from 80 to 150 min. Insulin infusion led to a prompt increase in the glucose infusion rate necessary to maintain euglycemia to an insulin-stimulated rate of 147.0 ± 0.6 μmol · kg^{-1} · min^{-1}. Steady-state effects of insulin on glucose disposal were reached by 40–60 min. At 80 min, the CGRP infusion was started, which led to a prompt and marked decrease in the glucose infusion rate to a new steady state at a rate of 99.2 ± 0.6 μmol · kg^{-1} · min^{-1}. After the CGRP effect was fully established and reached steady state, the CGRP infusion was discontinued (at 150 min), and the insulin infusion was maintained. The glucose infusion rate promptly rose to the original stimulated value. In an alternate protocol, CGRP and insulin were infused concomitantly at the outset for 75 min followed by insulin alone for 130 min (Fig. 1*B*). In these studies, the mean steady-state glucose infusion rate was 54.5 ± 1.7 μmol · kg^{-1} · min^{-1} during the CGRP plus insulin infusion; during the last 60 min of the study, when only insulin was administered, the mean peak glucose infusion rate rose to an insulin-stimulated value of 178.0 ± 1.7 μmol · kg^{-1} · min^{-1} ($P < 0.01$, CGRP + insulin vs. insulin alone). From these studies, it is evident that CGRP rapidly induced a marked state of in vivo insulin resistance regardless of whether CGRP was given concomitantly with insulin or after the insulin effect was fully established. Furthermore, this effect is rapidly reversible on withdrawal of CGRP. In these studies, the overall glucose disposal rate (GDR) equals the sum of the glucose infusion rate and hepatic glucose output (HGO). [^{3}H]glucose was infused during the studies depicted in Fig. 1, and GDR was measured in the basal state and over the last 20 min of infusions of CGRP plus insulin and insulin alone. Combining all studies (n = 5), the basal GDR was 63.6 ± 5.1 μmol · kg^{-1} · min^{-1} and rose by an increment of 132.5 ± 4.7 μmol · kg^{-1} · min^{-1} to

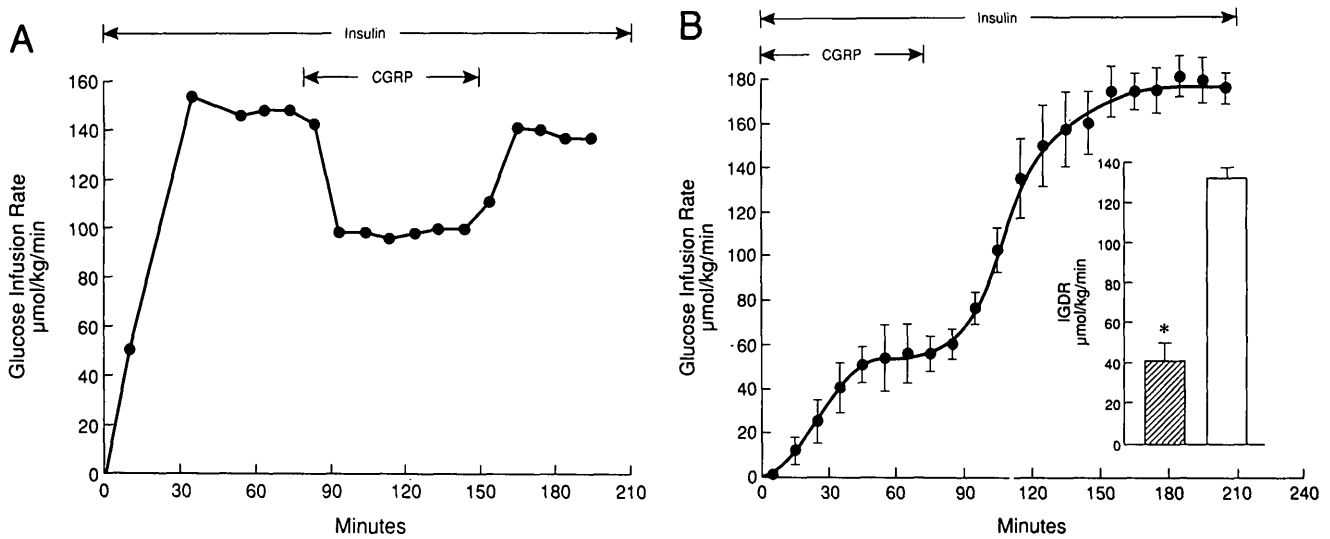

FIG. 1. Effect of calcitonin gene–related peptide (CGRP) on insulin action in vivo. *A*: representative study in which insulin (13 pmol · kg^{-1} · min^{-1}) was infused for 210 min with CGRP (5 nmol · kg^{-1} · min^{-1}) infused from 80 to 150 min, demonstrating CGRP-induced insulin resistance. Because time to reach steady state varied from rat to rat, length of peptide infusion varied also, making it impossible to calculate mean values for time course in this type of study. *B*: alternate protocol in which insulin was infused for 210 min, and CGRP was infused at outset for 75 min with insulin (n = 5). *Inset*, isotopically determined steady-state incremental glucose disposal above basal rates (IGDR) in presence (*left*) or absence (*right*) of CGRP (n = 6). *P < 0.05.

an overall GDR of 196.1 ± 7.2 μmol · kg^{-1} · min^{-1} with insulin alone; however, with CGRP and insulin, GDR only rose by an increment of 40.9 ± 8.8 μmol · kg^{-1} · min^{-1} to an overall GDR of 104.5 ± 10.5 μmol · kg^{-1} · min^{-1} (n = 6, P < 0.05). The mean steady-state plasma insulin level was 4990 ± 754 pM in the absence and 4243 ± 617 pM in the presence of the CGRP infusion (NS). Mean ± SE serum CGRP value during the final 40 min of the CGRP infusions was 4600 ± 1300 pM. Dose-response studies with CGRP showed no inhibition of insulin-stimulated GDR at a CGRP infusion rate of 1250 pmol · kg^{-1} · min^{-1} and an intermediate effect at an infusion rate of 2500 pmol · kg^{-1} · min^{-1}.

Figure 2 demonstrates the results of similar experiments performed with synthetic human amylin and depicts a typical study in which the protocol was analogous to that described for Fig. 1*A*. Insulin was infused at a constant rate for 220 min with infusion of amylin from 70 to 140 min. Infusion of insulin led to a prompt increase in the glucose infusion rate needed to maintain euglycemia at ~130 μmol · kg^{-1} · min^{-1}. Once the amylin infusion was begun, there was a prompt and marked decrease in the glucose infusion rate to ~68 μmol · kg^{-1} · min^{-1}, a 52% decrease in the glucose infusion rate needed to maintain euglycemia. Once the amylin infusion was stopped, the glucose infusion rate promptly rose to the original insulin-stimulated rate. The incremental GDR in five experiments in which amylin was infused shows that amylin depressed insulin-stimulated glucose disposal from 96.9 ± 9.4 to 42.4 ± 5.0 μmol · kg^{-1} · min^{-1} (P < 0.05), a 56% inhibition of insulin action (Fig. 2, *inset*). To assess the possibility that these in vivo effects of amylin were mediated through a secondary mechanism such as endogenous catecholamines, clamp studies with simultaneous α- and β-adrenergic blockade were performed. With a more highly purified preparation of synthetic human amylin in three rats, we found that amylin infused at 500 pmol · kg^{-1} · min^{-1} (1/10 the dose used in Fig. 2) induced marked insulin resistance even in the presence of pharmacologically induced adrenergic blockade. Amylin depressed insulin-stimulated GDR from 99.0 ± 25.3 to 53.2 ± 14.2 μmol · kg^{-1} · min^{-1} (P < 0.05), a 46% inhibition of insulin action.

Figure 3 depicts the effects of CGRP and amylin on the ability of insulin to suppress HGO. Insulin alone suppressed HGO by 66% from 63.6 ± 5.1 μmol · kg^{-1} · min^{-1} in the basal state to 21.5 ± 14.9 μmol · kg^{-1} · min^{-1}, but in the presence of CGRP, insulin suppressed HGO by only 30% to 44.1 ± 10.5 μmol · kg^{-1} · min^{-1} (n = 5, P < 0.01 insulin alone vs. insulin + CGRP). Amylin had similar effects on insulin-induced suppression of HGO. In the amylin experiments, insulin suppressed HGO by 83% from 55.1 ± 7.2 to 9.4 ± 8.3 μmol · kg^{-1} · min^{-1} (n = 5, P < 0.01), but in the presence of amylin, HGO was only suppressed to 38.6 ± 6.6 μmol · kg^{-1} · min^{-1} (n = 5, P < 0.05). Although insulin suppressed HGO significantly in the presence of amylin, amylin attenuated this effect of insulin.

Hexose uptake in isolated adipocytes and BC_3H1 myocytes. CGRP had no effect on either basal or insulin-stimulated 2-DG uptake in rat adipocytes (data not shown). The results of 10^4 pM CGRP are shown in Fig. 4. At this concentration of CGRP, which is roughly double that achieved in the clamp studies, CGRP significantly inhibited insulin-stimulated hexose uptake in cultured myocytes at all insulin concentrations studied. The EC_{50} for insulin was unchanged by CGRP, implying that the decrease in hexose uptake was due to some change in hexose uptake unrelated to insulin sensitivity.

DISCUSSION

Recent in vitro studies of CGRP and amylin have shown that these peptides may impair insulin secretion (18,19) and insulin action (12,20). Since Opie (5,6) first noted hyaline material in pancreatic islets from diabetic subjects, investigators

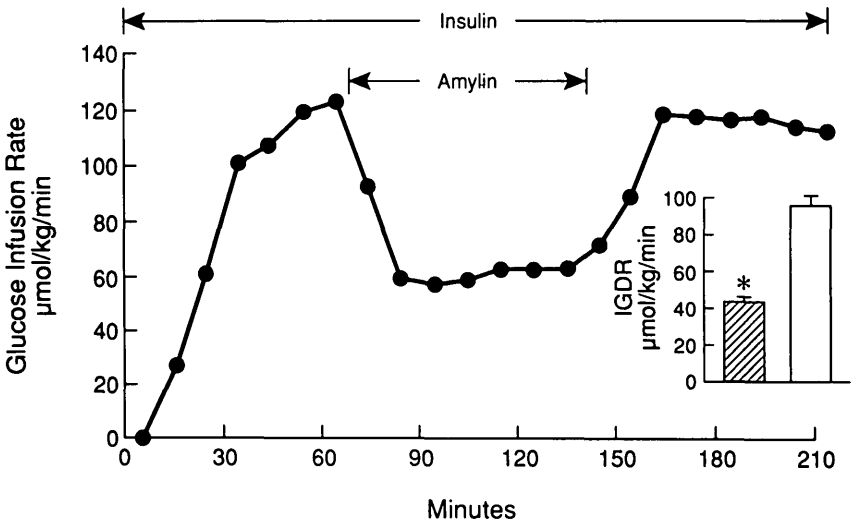

FIG. 2. Effect of amylin on insulin action in vivo. Representative study in which insulin (13 pmol · kg^{-1} · min^{-1}) was infused for 220 min with amylin (5 nmol · kg^{-1} · min^{-1}) infused from 70 to 140 min. *Inset*, isotopically determined steady-state incremental glucose disposal above basal rates (IGDR) in presence or absence of amylin (n = 5). *P < 0.05.

have sought to link these pathologic lesions of the pancreas to the pathogenesis of diabetes.

Our results show that synthetic CGRP or amylin can produce a marked state of insulin resistance when infused in vivo into rats. This insulin resistance is characterized by inhibition of the effects of insulin to stimulate peripheral glucose disposal, which primarily occurs in skeletal muscle (27), and inhibition of the normal effect of insulin to suppress HGO. Thus, both of the major glucoregulatory actions of insulin were inhibited by CGRP and amylin. These experiments do not reveal the mechanism by which these peptides induce insulin resistance. CGRP is a potent vasodilator (28), and these peptides may induce insulin resistance by changes in regional blood flow. Another possible mechanism is increasing circulating catecholamine levels via neurotransmitterlike action (29). However, the fact that amylin still decreased insulin-stimulated glucose disposal, even in the presence of adrenergic blockade, suggests that this effect of amylin is not mediated by elevation of serum catecholamine levels.

Because amylin can be identified in β-cell secretory granules (16) and in perfusates from isolated pancreases in situ (K. Luskey, R. Unger, unpublished observations), amylin is probably secreted from β-cells in vivo. Because we cannot yet measure circulating amylin levels, we have no knowledge what the possible circulating physiological amylin concentrations might be. In addition, we do not know the amylin concentrations achieved during our intravenous-infusion studies. However, infusion of CGRP (5 nmol · kg^{-1} · min^{-1}) led to a measured circulating CGRP concentration of 4.6 × 10^{-9} M; if the distribution and clearance of amylin follows similar kinetics, then the amylin infusion rates (5 or 0.5 nmol · kg^{-1} · min^{-1}) used would lead to levels in the 10^{-10}–10^{-9} M range. Until some of these issues are resolved with further experimentation, the relationship between amylin levels achieved during our infusion studies and physiologically relevant circulating amylin levels remains to be defined.

Several conclusions and implications can be derived from these studies. Relatively little is known concerning the role of CGRP, and virtually nothing is known about the role of amylin in human physiology. CGRP is expressed in several tissues, most prominantly in brain (30), spinal cord (31), thyroidal C cells (32), and pancreatic islets (18), but amylin expression is limited to the islets (15). CGRP has been shown

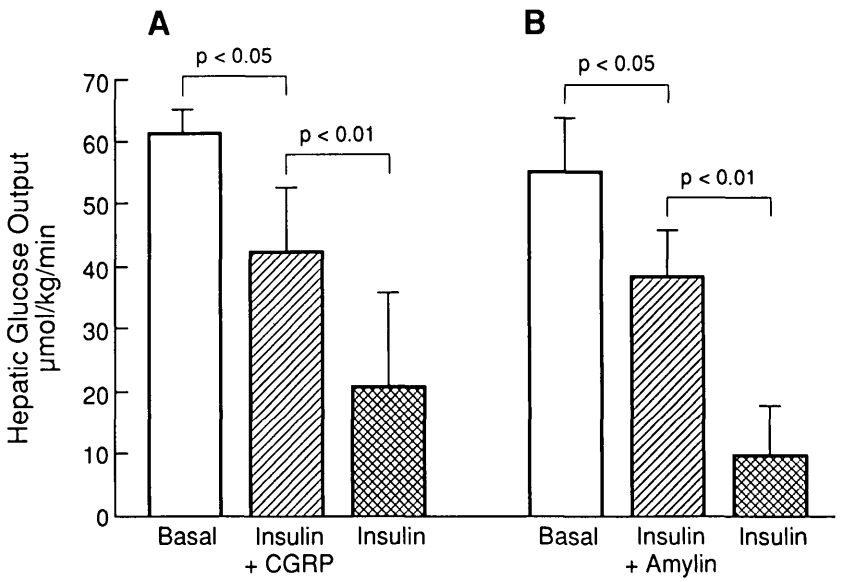

FIG. 3. Effects of calcitonin gene–related peptide (CGRP) and amylin on isotopically determined steady-state rates of hepatic glucose output (HGO). *A*: HGO in basal state, with insulin plus CGRP, and with insulin alone. *B*: HGO in basal state, with insulin plus amylin, and with insulin alone.

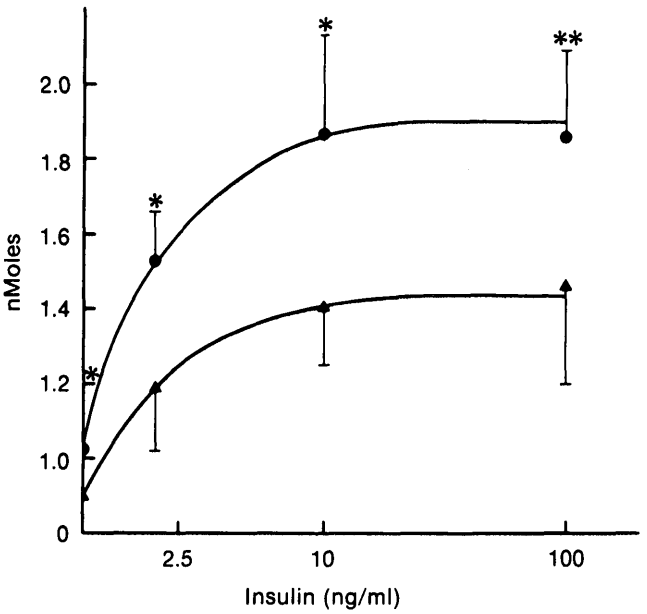

FIG. 4. Dose-response studies of hexose uptake in absence of insulin and at insulin concentrations of 460, 18,400, and 184,800 pM with (▲) and without (●) 10^{-8} M calcitonin gene-related peptide. Data are means ± SE of 5 experiments. *$P < 0.05$. **$P < 0.01$.

to inhibit insulin (18), gastric acid (33), and amylase secretion (34); is a vasodilator (28); and may function as a neurotransmitter (30). Our study clearly shows that this compound can also induce an in vivo state of insulin resistance. Although precise mechanisms are not identified in this study, the facts that CGRP can inhibit the effects of insulin in cultured muscle cells (Fig. 4) and that amylin and CGRP inhibit insulin action in isolated rat soleus muscle (20) raise the possibility that the site of the anti-insulin effect may involve inhibition of the cellular actions of insulin. Clearly, further study is needed to determine the mechanism of CGRP or amylin-induced insulin resistance. In the meantime, it is tempting to speculate that amylin is elaborated excessively in islets of individuals with type II diabetes, leading to amyloid deposits of this material within islets and elevated circulating levels of this peptide; this in turn could lead to peripheral insulin resistance and increased hepatic glucose production and could perhaps even adversely influence β-cell insulin secretion.

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