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AMYLIN OR CGRP (8-37) FRAGMENTS REVERSE AMYLIN-INDUCED INHIBITION OF ^{14}C-GLYCOGEN ACCUMULATION

Rhonda Oetting Deems, Francis Cardinaux, Richard W. Deacon, and Douglas A. Young

Sandoz Research Institute, East Hanover, NJ 07936

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The peptides amylin and calcitonin-gene related peptide (CGRP) have been shown to have similar effects on glycogen metabolism in vivo and in vitro. However, it is not clear whether they act via separate receptors. Peptide fragments based on the amino acid sequence of amylin or CGRP were evaluated for their ability to inhibit the action of the peptides in vitro. Insulin-stimulated glycogen turnover, as measured by ^{14}C-glycogen accumulation, was inhibited about 70% by amylin (10nM) and 85% by CGRP (10nM). In the absence of exogenous peptide, peptide fragments based on the 8-37 and 10-37 amino acid sequences of rat amylin (10 uM) had no affect on ^{14}C-glycogen accumulation. In the presence of amylin (10nM), the 8-37 and 10-37 fragments blocked amylin-induced inhibition of ^{14}C-glycogen accumulation 100% and 11.4%, respectively. The 8-37 and 10-37 amylin fragments blocked CGRP inhibition of ^{14}C-glycogen accumulation by 23.2% or 28.6%, respectively. The CGRP 8-37 fragment was equally effective as the amylin 8-37 fragment at blocking amylin. The CGRP 8-37 fragment was more effective at reversing the effects of amylin than at reversing the effects of CGRP. These results demonstrate that amylin (8-37) completely antagonizes the effects of amylin with limited ability to block CGRP. Removing the eighth and ninth amino acids reduced the effectiveness of the inhibitor by about 90%.

Islet amyloid polypeptide (IAPP) or amylin is a 37 amino acid peptide originally isolated from amyloid plaques in the pancreases of patients with non-insulin dependent diabetes mellitus (NIDDM; 1,2). Amylin shares about 50% homology with calcitonin gene-related peptide (CGRP), a neuropeptide that is concentrated in small sensory nerve fibers and is thought to be involved in transmission of gut reflexes (see 2,3). Amylin and CGRP have similar effects on glycogen metabolism in vivo and in vitro (4-9). Because of the similarities in function, it has been suggested that the peptides act via the same receptors. Receptors have been identified for CGRP on rat liver plasma membranes (10), and amylin has been shown to activate adenylate cyclase via interactions with these

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CGRP receptors (11). Whereas CGRP stimulates adenylate cyclase as determined by an elevation in cAMP levels (12,13) amylin appears to act via a cAMP-independent mechanism (9), at least at concentrations known to affect glycogen levels in soleus muscles (i.e., 100 nM). We have recently shown that amylin induces glycogenolysis by inhibiting glycogen synthase and stimulating glycogen phosphorylase in vitro, but does not affect cAMP levels (9). Alternatively, in L6 myocytes, Zhu and colleagues (14) have found that amylin stimulates cAMP levels at concentrations above 100 nM, while CGRP increases cAMP levels at concentrations below 0.1 nM.

The existence of separate amylin and CGRP receptors would provide greater support for a possible physiological role of amylin in glycogen metabolism. The identification of selective antagonists for amylin and CGRP would aid in the evaluation of individual metabolic roles of the peptides. In an attempt to identify inhibitors of the peptides, the present study determined the ability of peptide fragments based on the sequences of amylin or CGRP to antagonize the actions of the native peptides.

METHODS

Subjects. Male Sprague-Dawley rats weighing 50-60 g were used. Animals were fed Purina Rat Chow ad libitum, except when fasted overnight.

Isolated Muscle Procedure. Soleus muscles excised from 18 hr fasted rats were immediately placed in 25 ml erlenmeyer flasks for a 15 min pre-incubation rinse at 37°C. The flasks contained 2 ml of Krebs-Henseleit buffer in addition to 3.5% (w/v) essentially fatty-acid free bovine serum albumin, 5 mM glucose and insulin (200uU/ml). The gas phase in all conditions was 95% 0_2/ 5% CO_2. Where appropriate the rinse media also contained the peptide fragments. The rinse was followed by a 60 min incubation period. In addition to the rinse media, the incubation media contained ^{14}C-glucose (4 ul/ml). Peptide fragments (1 or 10 uM) and amylin or CGRP (10 nM), were included where appropriate. At the end of the incubation, muscles were immediately frozen in liquid nitrogen and stored at -70°C until assayed.

Rat amylin, rat CGRP, and human CGRP (8-37) were obtained from Peninsula Laboratories (Belmont, CA). ^{14}C-Glucose was obtained from New England Nuclear. Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Peptide Fragment Synthesis. Rat amylin 8-37 and 10-37 were prepared by the solid-phase method on a p-methylbenzhydrilamine-polystyrene support (15) using side-chain protection and coupling procedures and an automated apparatus (model 430A, Applied Biosystems, Foster City, CA), as described previously (16). An acetyl group was attached to the N-terminus of the completed sequence. Peptide resins were subsequently treated with liquid HF at 0°C according to the 'low-high' procedure (17) and the crude peptides purified by preparative HPLC on a C18 reversed-phase column. The purified peptides showed correct amino acid ratios after hydrolysis in 6N HCl and the expected molecular ions in FAB-MS. Both N-acetylated peptide amides were soluble to more than 1% in water.

N-Acetyl-amylin-(8-37) amide (human) was prepared similarly. This compound, during purification, tended to precipitate to a very insoluble form. From the soluble fraction the compound was isolated in small yield. The purified product was first dissolved in a few drops of 95% acetic acid and diluted with water to a final concentration of 1% for testing.

^{14}C-Glycogen Accumulation. Muscles were trimmed and weighed over dry ice. ^{14}C-Glucose incorporation into ^{14}C-glycogen was determined by dissolving the sample in 30% KOH, precipitating the glycogen with ethanol, and redissolving the precipitate in water.

Statistics. Differences between groups were determined by analysis of variance and t-tests (RS1, BBN Software).

RESULTS AND DISCUSSION

Amylin and CGRP (10 nM) decreased ^{14}C-glycogen accumulation to 30% and 16% of control, respectively (see Figure 1). This concentration produced maximum inhibition of ^{14}C-glycogen accumulation by amylin (data not shown). Amylin-induced inhibition of ^{14}C-glycogen accumulation was reversed by all peptide fragments tested. Amylin 8-37 proved to be the most effective antagonist of amylin as it fully reversed amylin's effects. There was no difference between rat or human amylin 8-37 in ability to block amylin (data not shown). However, the human fragment was less soluble than the rat fragment. Therefore, in all other studies, the rat amylin 8-37 was employed.

It is clear that a difference of two amino acids can account for the majority of the effectiveness of the antagonist; amylin 8-37 produced a 100% reversal of amylin, whereas amylin 10-37 reversed approximately 11%. The eighth amino acid is different between amylin and CGRP, whereas the ninth is identical. Dennis and colleagues (18,19) have demonstrated that the 9-37 fragment is important for receptor recognition of CGRP. This might suggest the interaction of amylin with CGRP receptors. There was no difference in ability of the amylin peptide fragments to reverse CGRP-induced inhibition of ^{14}C-glycogen accumulation; amylin 8-37 or 10-37 fragments produced approximately 25% reversal of CGRP's effects.

A dose response of amylin (8-37) indicated that 1 uM produced a 57% reversal and 0.1 uM a 15% non-significant reversal of amylin's effects (data not shown). To reduce the required amount of compound, in studies with the CGRP 8-37, the concentration of antagonist was decreased to 1 uM. The CGRP 8-37 (1 uM) was equally effective as the amylin 8-37 (1 uM) at blocking amylin-induced inhibition of ^{14}C-glycogen accumulation (62% vs 57% reversal, respectively). Between these two fragments there are 43% homology, with identical amino acids at positions 9, 11-13, 16, 18, 19, and 29-34. These results further indicate the importance of the ninth amino acid. CGRP 8-37 produced an approximate 24%

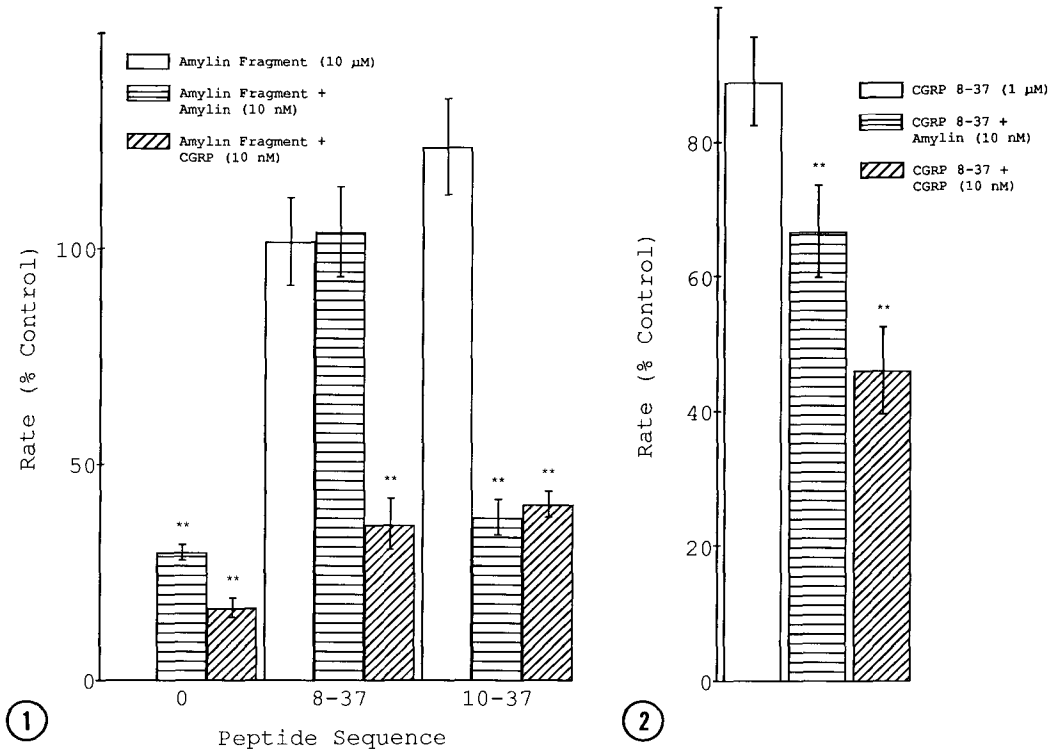

Figure 1. Ability of amylin fragments to block amylin- or CGRP-induced inhibition of ^{14}C-glycogen accumulation in isolated soleus muscles from 18 hr fasted rats. All media contained insulin (200 μU/ml). Control muscles were incubated in the absence of added peptide. All groups contain at least 5 muscles. **p<0.001, compared to control.

Figure 2. Ability of CGRP 8-37 to block amylin- or CGRP-induced inhibition of ^{14}C-glycogen accumulation in isolated soleus muscles from 18 hr fasted rats. **p<0.001, compared to insulin control.

reversal of CGRP-induced inhibition (see Figure 2). Human CGRP 8-37 has previously been shown to competitively antagonize native CGRP's central nervous system-mediated inhibition of food intake (19). Additionally, a recent abstract suggested that human CGRP 8-37 acts as an antagonist of the in vivo effects of amylin on heart rate and blood pressure (20).

In summary, rat or human amylin 8-37 completely antagonizes the in vitro effects of amylin on glycogen accumulation. Amylin 8-37 may be considered selective for amylin since only partial reversal of the effects of CGRP occurs in the presence of the peptide fragment. Amylin 8-37 may therefore serve as a useful tool to distinguish between actions of amylin and CGRP, both in vitro and in vivo.

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