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Stimulation of Adenylate Cyclase by Amylin in CHO-K1 Cells

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SUMMARY

The CHO-K1 cell line responds to the peptide amylin by a rapid elevation of cAMP. The related peptide calcitonin gene-related peptide (CGRP) is 100 times less potent at stimulating adenylate cyclase than is amylin. The actions of amylin at this receptor are concentration dependent and not antagonized by the CGRP antagonist CGRP-(8–37). Although these cells have receptors for calcitonin, amylin is unable to take part in any high affinity interaction with these receptors, as assessed by radioligand binding. The CHO-K1 cell line has receptors for amylin that are distinct from those for calcitonin and CGRP.

Amylin is a recently identified peptide hormone that is primarily synthesized (1) and secreted (2) by the pancreatic β cells. Gene sequencing has shown that the hormone is highly conserved across species, with variations in sequence having some correlation with the formation of amyloid deposits in diabetic pancreatic islets (3). In non-insulin-dependent, or type II, diabetes, there is an accumulation of amyloid deposit, or plaque, the major protein constituent of which is amylin (4, 5). The relationship of amylin to type II diabetes has led investigators to examine the actions of amylin on glucose metabolism (6, 7). Amylin inhibits both basal and insulin-stimulated glucose uptake in rat soleus muscle strips, with an apparent effect on glycogen synthesis rather than lactate formation (6).

The actions of amylin on skeletal muscle are shared with another peptide hormone, CGRP, which has a sequence similar to that of amylin (4, 8, 9). It is now apparent that CGRP is also a potent *in vitro* inhibitor of basal and insulin-stimulated glycogen synthesis in isolated rat soleus muscle strips (9). Both amylin and CGRPs α/β have 37 amino acids, a positionally conserved disulfide bond between the cysteines at residues 2 and 7, and an essential amidated carboxyl-terminal residue (4). More striking, the sequences of human CGRP α and β have 43% and 46% sequence identity, respectively, with human amylin (4). This degree of similarity suggests that the two hormones may work in concert, perhaps through a common receptor, to regulate glycogen metabolism in skeletal muscle, the major organ for glucose disposal. Apart from observations on glucose metabolism in isolated rat muscle, the only functional response to amylin that has been reported is the stimulation of cAMP production via CGRP receptors in rat liver and myocyte membranes (7, 10, 11). Thus, although the CGRP receptor may be poorly characterized, the receptor for amylin has not yet been identified. In view of the similarities between these hormones, the characterization of one or more receptors mediating their effects is essential to understanding physiological or pathological cross-talk between these hormones, particularly in the regulation of glucose metabolism. We have characterized in detail a CGRP-preferring receptor in the L6 skeletal muscle cell line (12). In the CHO-K1 Chinese hamster ovary cell line, we now report an adenylate cyclase response to amylin, with nanomolar potency. This is the first report of a functional biochemical response to amylin in which its potency greatly exceeds that of CGRP. These results imply a unique "amylin receptor," at which CGRP is only weakly effective.

Materials and Methods

Synthetic amylin, calcitonin, rat CGRP α/β, and hCGRP α/β were obtained from Bachem (Torrence, CA) or as a gift from Celltech (Slough, UK). The CHO-K1 cell line was obtained from American Type Culture Collection (Rockville, MD). Ham's F-12 modified medium and trypsin were obtained from Sigma. All other chemicals were of reagent grade and obtained from standard suppliers.

Cell culture. CHO-K1 cells were cultured in Ham's modified F-12 medium supplemented with 10% fetal calf serum (complete medium), and they were maintained in an atmosphere of 7% CO_2 at 37°. Subcultures were carried out by treatment with 0.05% trypsin for 2–5 min at 37°. An equal volume of complete medium was added, followed by centrifugation at 200 × *g* for 6 min and resuspension of the cell pellet in complete medium. Cells were subcultured every 4–6 days, into six-well tissue culture plates or 15-cm^2 dishes, in complete medium.

Measurement of cAMP levels in intact cells. Medium from cells

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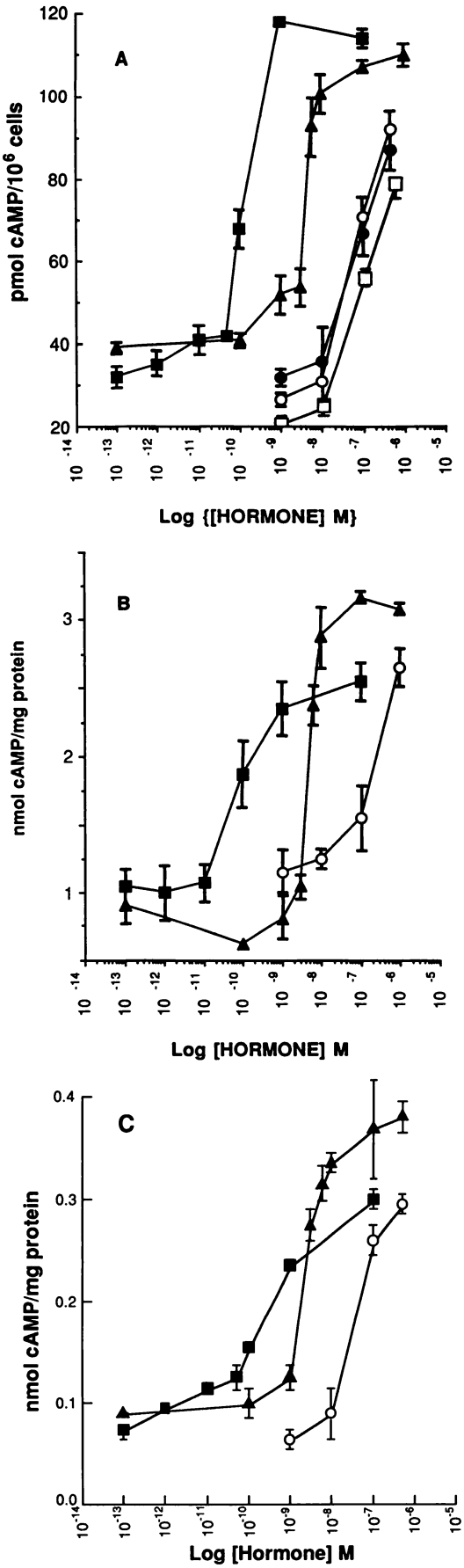
ABBREVIATIONS: CGRP, calcitonin gene-related peptide; hCGRP, human calcitonin gene-related peptide; sCT, salmon calcitonin; IBMX, isobutylmethylxanthine; BSA, bovine serum albumin.



in six-well plates was aspirated and replaced with 900 μl of serum-free Ham's medium containing 0.3% BSA, 1 mM IBMX, and 0.4 mM bacitracin. Hormones were made up to 10 times the final concentration in the aforementioned medium, and 100-μl aliquots were added to the cells. Cells were incubated for 10 min in the presence of added hormone, at 22°. After this incubation period, the medium was aspirated and the cells were washed in 1 ml of cold 20 mM Tris buffer, pH 7.5, containing 5 mM EDTA, rapidly scraped, and placed into a boiling water bath for 5 min. After centrifugation (Beckman microfuge), 50 μl of the supernatant were taken and assessed for cAMP content by the method of Gilman (13). Briefly, 50-μl samples were added to 50 μl of [^{3}H]cAMP (10 nM; amount in terms of counts), followed by the addition of 100 μl of 1 mM citrate buffer, pH 6.5, containing protein kinase A (10 μg) and 2.0 mM dithiothreitol. After at least 2 hr of incubation at 4°, 100 μl of Tris, pH 7.7, containing 5% charcoal, 0.2% BSA, and 5 mM EDTA, were added. Samples were then vortexed, allowed to stand for 10 min, and centrifuged (Beckman microfuge); 200-μl aliquots of the supernatant were taken, mixed with 4 ml of scintillant, and counted in a Beckman LS 1801 scintillation counter. A standard curve was constructed with cAMP standards varying from 10 nM to 1 μM.

Measurement of adenylate cyclase in enriched membrane fractions and cell homogenates. Homogenates were prepared according to the method of Michelangeli *et al.* (14). Briefly, confluent cells were scraped and homogenized in 25 mM Tris buffer, pH 8.2, containing 230 mM sucrose, 1 mM dithiothreitol, and 1 mM EDTA (sucrose buffer). Peptides were made up in electrolyte buffer, i.e., 25 mM Tris, pH 8.1, containing 30 mM potassium chloride, 10 mM magnesium sulfate, and 0.1% BSA. Samples containing 50 μl of cell homogenates, 25 μl of electrolyte buffer, and 25 μl of ATP-regenerating system (2 mM phosphocreatine, 50 units/ml creatine kinase, 10 mM magnesium chloride, 3 mM ATP, 2 mM IBMX) were incubated at 37° for 10 min, and the reaction was stopped by placing the samples in a boiling water bath for 5 min. The samples were centrifuged (Beckman microfuge) for 5 min, and 50-μl aliquots of the supernatant were removed for the assay of cAMP content, as described above.

Preparation of amylin(1–11) and amylin(12–37). Amylin (20 μg) was taken up in 200 μl of phosphate-buffered saline containing 0.01% trypsin. The sample was incubated at 20° for 1 hr and then applied to a Waters C_{18} μ-Bondapak reverse phase column. A linear gradient of 0 to 60% acetonitrile, in 0.1% trifluoroacetic acid, was run over a time period of 30 min. Peptide peaks were analyzed at 210 nm, and 1-ml fractions were collected. The sequence of peptide fragments was confirmed using an Applied Biosystems 477A protein sequencer.

^{125}I-sCT binding to CHO-K1 cells. ^{125}I-sCT was prepared using chloramine-T oxidation and Sep-Pak C_8 cartridges for purification (15). Specific radioactivity was 1500–2000 μCi/μg. Confluent cells were washed with fresh medium and incubated for 30 min in serum-free medium with 0.1 nM ^{125}I-sCT, in the absence or presence of increasing concentrations of hormone, at 22°. The medium was removed and cells were washed three times with cold phosphate-buffered saline. The cells were removed by solubilization with 2 volumes (0.5 ml) of 0.5 M NaOH, and the cell-bound radioactivity was counted in a Packard multiwell counter, with 75% efficiency. CHO-K1 cell numbers were obtained by trypsinization (0.05% trypsin) of confluent cells and counting by means of a hemocytometer.

Fig. 1. cAMP content of intact CHO-K1 cells (A), whole-cell homogenates (B), and membrane suspensions (C), after 10-min incubation with increasing concentrations of human amylin (▲), rat CGRP α (○), rat CGRP β (●), hCGRP (□), or sCT (■), as described in Materials and Methods. EC_{50} values are as follows: for human amylin, intact cells, 3.1 ± 0.3 nM; homogenates, 3.4 ± 0.3 nM; membranes, 2.4 ± 0.3 nM; for sCT, intact cells, 0.12 ± 0.02 nM; homogenates, 0.78 ± 0.14 nM; membranes, 0.32 ± 0.11 nM. Results are the means ± standard errors of triplicate determinations in three separate experiments, except for C, where data are from a single experiment.

Results

cAMP accumulation in CHO-K1 cells. When intact cells (Fig. 1A), cell homogenates (Fig. 1B), or membranes (Fig. 1C) were incubated with increasing concentrations of human amylin, sCT, hCGRP (intact cells only), or rat CGRP, the most potent responses were observed with sCT and human amylin. Both hormones gave a 2.5-fold increase in cAMP levels, with half-maximal stimulation at 0.12 and 3.1 nM, respectively, in intact cells. Similar values were found in homogenates (0.78 and 3.4 nM) and in membranes (0.32 and 2.4 nM). Both hCGRP and rat CGRP produced half-maximal stimulation of adenylate cyclase at concentrations greater than 50 nM. It should be noted that, after cells were frozen and thawed, their sensitivity to amylin was reduced, so that the EC_{50} was greater than 50 nM. We have not identified the mechanism underlying this phenomenon, but it does suggest that receptor expression is variable.

Time course of cAMP production. cAMP accumulation over basal was half-maximal within 2 min after addition of 100 nM amylin, and activity reached a plateau by 15 min (Fig. 2). We were unable to obtain a reproducible response to amylin in the absence of IBMX and so could not investigate directly the time course of desensitization. However, if cells were preincubated for 24 hr with 100 nM amylin and then rechallenged with fresh peptide, no increase in cAMP levels could be measured, indicating that the receptor had become desensitized over this time period. sCT was still able to increase cAMP levels under these conditions (Table 1). The response to CGRP was abolished, suggesting that its actions on this preparation were mediated via the amylin-selective receptor.

Effect of the CGRP antagonist peptide hCGRP-(8–37). When CHO-K1 cells were incubated with amylin in the absence or presence of the CGRP antagonist hCGRP-(8–37), no effect of this peptide was observed (Fig. 3). Similarly, hCGRP-(8–37) (10^{-7} M) showed no antagonistic effect on the adenylate cyclase response of CGRP. hCGRP-(8–37) on its own was unable to stimulate adenylate cyclase at concentrations of 10^{-7} and 10^{-6} M. However, the same preparation of hCGRP-(8–37) was biologically active and showed an antagonistic effect for hCGRP in L6 cells (12), consistent with its behavior as a true CGRP antagonist.

Amylin-(1–11) and amylin-(12–37) fragments. Tryptic digestion of human amylin produced two peptide fragments, human amylin-(1–11) and human amylin-(12–37). Fig. 4 shows the effect of these peptides on adenylate cyclase stimulation with human amylin. Human amylin-(1–11) and human amylin-(12–37) (10^{-6} M) had no effect on the stimulation of adenylate cyclase by human amylin (10^{-8} M). In addition, human amylin-(1–11) and human amylin-(12–37) (10^{-6} M) on their own did not stimulate increases in cAMP.

Binding. Attempts to produce a biologically active radiolabeled analogue of amylin were unsuccessful. ^{125}I-sCT (0.1 nM) bound to intact CHO-K1 cells, and competition for binding was shown by unlabeled sCT, with half-maximal inhibition of binding at 0.1 nM. hCGRP and human amylin were weak competitors at 10^{-7} M (Fig. 5), and the unrelated hormones isoprenaline and human growth hormone did not compete at 10^{-7} M (data not shown). ^{125}I-CGRP did not bind to these cells, in keeping with its low potency in functional assays.

Discussion

In the present study, we have examined the response of the Chinese hamster ovary cell line CHO-K1 to the peptide hormone amylin and its relatives CGRP and calcitonin and have demonstrated stimulation of the adenylate cyclase-cAMP system. The physiological levels of amylin in human plasma from normal and non-insulin-dependent diabetic patients are not clear, being reported as concentrations on the order of 3 pM (16) to concentrations more than 10 times higher than CGRP levels in normal individuals (5). The increase in cAMP levels at nanomolar concentrations of the peptide, which have not been observed before, suggest a functional amylin receptor. The increase in cAMP levels in membranes, cell homogenate preparations, and intact cells provides strong evidence that this response to amylin is a primary transduction event, as opposed to a nonspecific interaction or a secondary consequence, such as the activation of adenylate cyclase via calcium-calmodulin (17). Thus, it appears that receptors for amylin in CHO-K1 cells are linked to stimulation of adenylate cyclase.

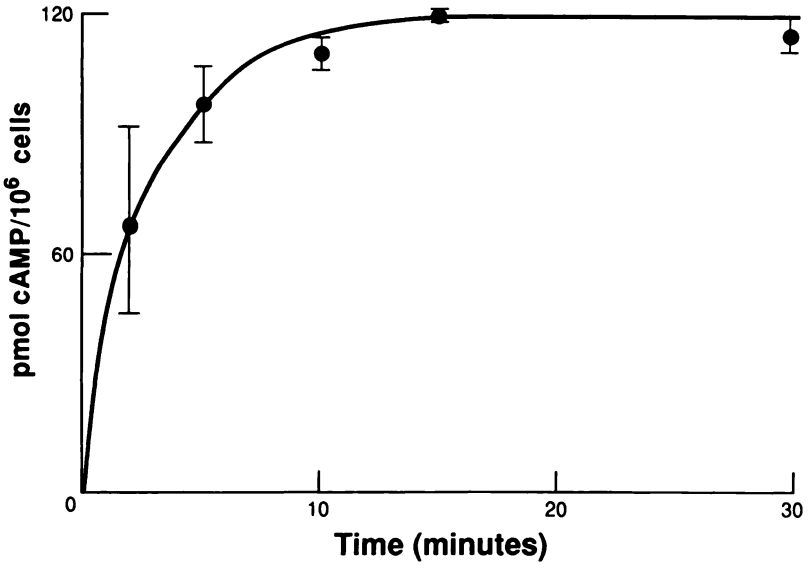


Fig. 2. Time course of cAMP production in intact CHO-K1 cells incubated with 10^{-7} M human amylin. Cells were preequilibrated with 2 mM IBMX, as described in Materials and Methods. Results are means ± standard errors of triplicate determinations in three seperate experiments.

TABLE 1

Desensitization of the amylin stimulation of adenylate cyclase

Cells were incubated overnight with or without 100 nM amylin. Thirty minutes before the start of the incubations, 2 mM IBMX was added. Incubations were started by addition of drugs as shown and were terminated as described in Materials and Methods. Values represent cAMP content of intact cells after 10-min incubations with the indicated substances and are means ± standard errors of three experiments.

Addition	cAMP	
	Control	24-hr preincubation with 100 nM amylin
	pmol/10⁶ cells	
Basal	23 ± 9	60 ± 8
10 nM Calcitonin	201 ± 31	141 ± 11
100 nM Amylin	138 ± 16	72 ± 6
100 nM CGRP	120 ± 10	58 ± 9

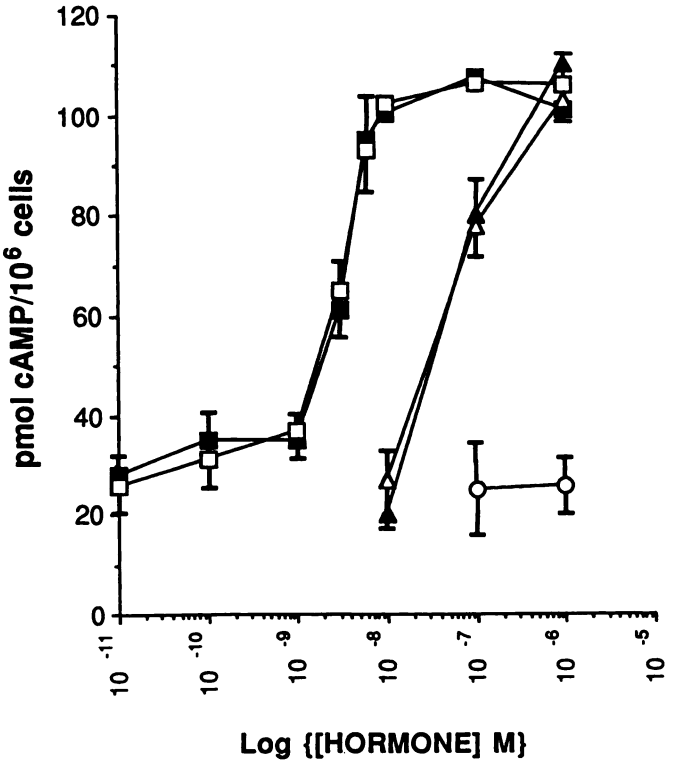


Fig. 3. cAMP content of intact CHO-K1 cells after 10-min incubation with increasing concentrations of human amylin (*squares*) or rat CGRP α (*triangles*), in the absence (*open symbols*) or presence (*closed symbols*) of hCGRP-(8–37) (10^{-7} M). The cAMP response to hCGRP-(8–37) alone was also measured (○). Results are means ± standard errors of triplicate determinations from three separate experiments. Fitted EC_{50} values are as follows: human amylin, 2.9 ± 0.2 nM; human amylin with hCGRP-(8–37), 3.1 ± 0.3 nM.

These results add to the similarities between amylin and the peptide hormone CGRP. In addition to their structural similarity (43–49% homology), CGRP also shows activity with amylin in inhibiting insulin-stimulated glucose uptake in isolated rat soleus muscle (6) and activating adenylyl cyclase.

Our findings suggest that amylin and CGRP may well act via distinct cognate receptors that, nevertheless, share a common mechanism that is also shared with a third member of this peptide family, calcitonin. The adenylate cyclase response to CGRP in CHO-K1 cells supports the idea that CGRP can cross-talk with an amylin receptor, because high concentrations of CGRP are required for cAMP responses in CHO-K1 cells. The lack of antagonistic effect of CGRP-(8–37), an established antagonist of CGRP receptors, further distinguishes amylin receptors from characterized CGRP receptors (12, 18).

Because other CGRP fragments have also been reported to have antagonist effects [for example, CGRP-(12–37) is a weak antagonist in spleen membranes (19)], analogous amylin fragments were prepared and tested for their effect on adenylate cyclase stimulation. An arginine residue at the 11-position in human amylin provided a convenient cleavage spot for tryptic digestion of the hormone, producing two fragments, amylin-(1–11) and amylin-(12–37). The absence of any agonist or antagonist activity of either of these peptides further shows that different structure-function relationships exist for amylin and its receptor, compared with CGRP. It is still possible, however, that receptor subtypes exist for amylin and that these fragments are not active at the subtype expressed in CHO-K1 cells.

Earlier reports (7, 20), as well as sequence similarity with CGRP, a hormone known to function via adenylate cyclase, have suggested that amylin may also function through the adenylate cyclase system. Although increases in cAMP levels in rat liver membranes were reported (7), those authors suggested that amylin may well have been acting through CGRP receptors, because half-maximal stimulation occured at 100 nM. In their preparation, amylin was 1000 times less potent than CGRP (EC_{50}, ~0.1 nM). A similar potency difference was found on rat L6 skeletal myocytes, where it is most likely that only a CGRP receptor is expressed (11, 12).

In vivo and on isolated rat soleus muscle, amylin produces a number of metabolic effects, for which it has EC_{50} values similar to those of CGRP (6, 20, 21) and which are antagonized by CGRP-(8–37) (22). The relationship between the amylin receptor described here and that responsible for those metabolic effects is not completely certain. However, the two may be very similar. The high potency of CGRP acting on skeletal muscle may simply reflect the fact that it is acting through its own high affinity receptors, as seen most clearly on the L6 skeletal myocytes, where the separate amylin receptors are not expressed (11, 12). On rat soleus muscle, CGRP-(8–37) has a K_b against amylin of 80 nM, apparently making it 300 times less potent than against CGRP (21). In this study, 100 nM CGRP-(8–37) did not significantly shift the amylin concentration-response curve. If the aforementioned K_b value for the amylin receptor in isolated rat soleus muscle is identical to that for this receptor in cultured Chinese hamster ovary cells, then it can be calculated that the expected shift in the amylin concentration-response curve would be 2.2-fold. This is probably within the error of our experiments, and the most striking feature of the two studies is the relative insensitivity of the amylin responses to CGRP-(8–37). Clearly, it is of interest to investigate further the relationships between the two receptors. An additional functional response described for amylin is inhibition of osteoclast bone resorption (22). Here the potency order is calcitonin > amylin ≫ CGRP, identical to that found in this study. It is possible that these cells may also express seperate calcitonin and amylin receptors, as postulated for our CHO-K1 cells (see below). However, further work is needed on osteoclasts to determine the full extent of receptor expression.

An unexpected complication in the CHO-K1 cells was the potent cAMP elevation in response to sCT. However, the adenylate cyclase response to sCT in these cells followed the

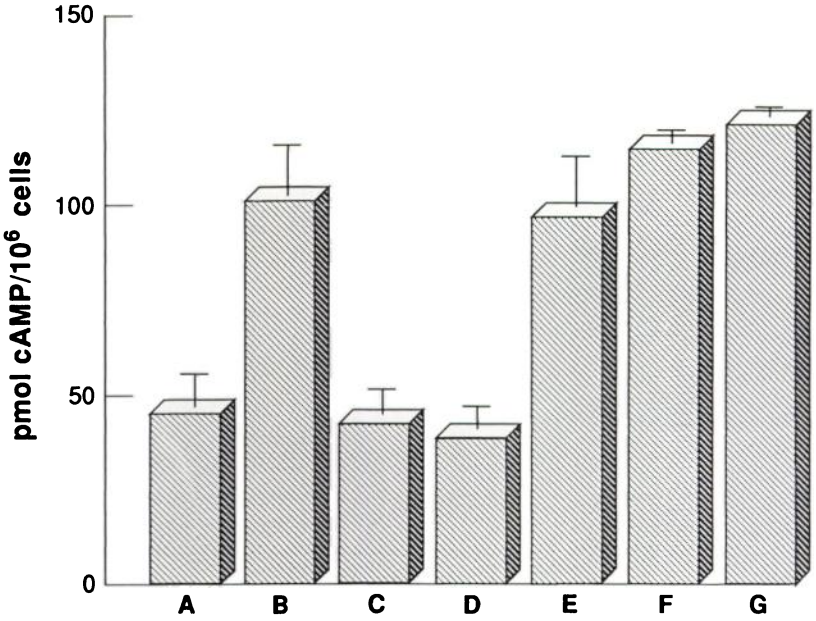


Fig. 4. cAMP content of intact CHO-K1 cells after 10-min incubation in the absence of any addition (*A*) or in the presence of 10^{-8} M human amylin (*B*), 10^{-6} M human amylin-(1–11) (*C*), 10^{-6} M human amylin-(12–37) (*D*), 10^{-8} M human amylin with 10^{-6} M human amylin-(1–11) (*E*), 10^{-8} M human amylin with 10^{-6} M human amylin-(12–37) (*F*), or 10^{-5} M forskolin (*G*).

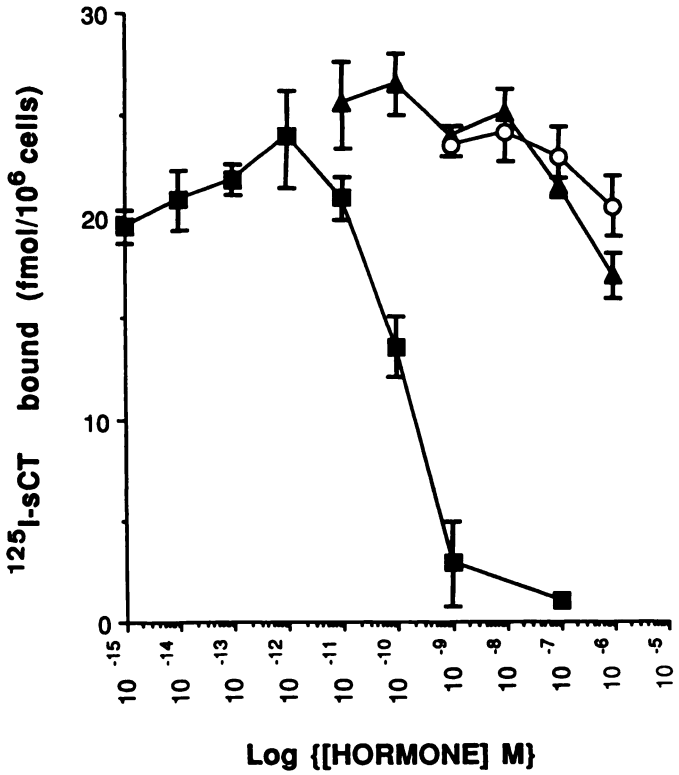


Fig. 5. Displacement of bound ^{125}I-sCT by human amylin (▲), sCT (■), and rat CGRP (○). CHO-K1 membranes were incubated with 0.1 nM ^{125}I-sCT and bound radioactivity was determined as described in Materials and Methods. The fitted IC_{50} for sCT was 145 ± 5 pM, and the Hill coefficient was 1.4 ± 0.6. Results are means ± standard errors of triplicate determinations from three separate experiments.

criteria for calcitonin receptors established in other tissues.[4] The possibility that amylin may be working via these calcitonin receptors is unlikely, given that amylin only weakly competed for ^{125}I-sCT in binding studies ($IC_{50} > 1$ μM). Indeed, competition for binding of ^{125}I-sCT by amylin paralleled that by CGRP in the same experiment. Moreover, the amylin response could be selectively desensitized, whereas that for calcitonin was not affected. The presence of high affinity calcitonin receptors makes it impossible to determine the potency of calcitonin on the amylin receptors. All that can be said is that calcitonin cannot have an EC_{50} on the amylin receptor smaller than 0.1 nM, the EC_{50} on the calcitonin receptors themselves.

In conclusion, amylin receptors are linked positively to cAMP production in CHO-K1 cells. The physiological importance of receptors for amylin in the ovary and derived CHO-K1 cells remains to be determined, but these findings may predict a reproductive function for amylin *in vivo*. Finally, CHO-K1 cells have repeatedly proven to be a convenient cell line for somatic cell genetic studies and stable expression of a number of membrane proteins, including insulin receptor and insulin receptor mutants (23), as well as the expression of glucose transporter proteins. Together with the availability of mutant cell lines of CHO-K1 that are defective in glucose transporter proteins, this cell line represents a convenient model for the study of the amylin receptor, especially with regard to its role in glycolysis and gluconeogenisis, and complements the L6 skeletal muscle cell line, which expresses authentic CGRP receptors.

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