Biochemical Pharmacology, Vol. 34, No. 7, pp. 1057–1063, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

MODULATION OF RAT PANCREATIC MUSCARINIC CHOLINERGIC RECEPTORS BY CAERULEIN

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(*Received* 12 *April* 1984; *accepted* 8 *October* 1984)

Abstract—To evaluate the modulation of pancreatic muscarinic receptors in two states of pancreatic growth, hypertrophy and hyperplasia, caerulein, a cholecystokinin analog, (1 μg/kg) was administered thrice daily for 2 and 4 days to adult rats. After 2 days of treatment, pancreatic hypertrophy was well established as evidenced by increases in pancreatic weight, cellular mass and protein content. Using an increase in DNA content as an index of hyperplasia, we demonstrated that pancreatic hyperplasia occurred only after 4 days of caerulein treatment. Caerulein increased the concentration of muscarinic receptors per DNA in pancreatic homogenate by 57% over control value after 2 days of treatment without modification of the receptor affinity for the ligand QNB. This increase involved mainly receptors in the low affinity state for carbamylcholine and their concentration returned to control levels after 4 days of treatment. The functional capacity of the acini was significantly increased after 2 days of caerulein as amylase release (U/mg DNA) was significantly increased but the sensitivity of these acini to carbamylcholine was significantly decreased. After 4 days of caerulein, the functional capacity has returned towards control values but the sensitivity to carbamylcholine remained decreased. The increase in muscarinic receptor concentration could be ascribed to a general increase in cellular proteins, as part of the hypertrophic effect of caerulein. This specific effect would also explain the increased functional secretory capacity of the caerulein-treated acini but the decreased sensitivity to carbamylcholine probably resulted in changes at a postreceptor loci since the affinities of the muscarinic receptors for carbamylcholine remained unaffected.

Over the last few years, we have demonstrated the presence of cholinergic muscarinic receptors in pancreas homogenate [1]. Using the rat dispersed pancreatic acini as a model, these receptors were characterized and demonstrated to be saturable, specific for cholinergic agonists and antagonists, and stereospecific [2]. It was also shown that these acini possess two populations of agonist binding sites. By comparison between the dissociation constant of the high affinity sites for carbamylcholine and the apparent affinity constant of the carbamylcholine effect on amylase secretion, it was proposed that the physiological response of the acini to a muscarinic agonist involved the high affinity receptors [2]. However, in a recent study [3], after the reserve receptors have been blocked by an irreversible antagonist, it was found that the real affinity constant of agonists for amylase secretion correlated well with the affinity constant for the low affinity binding sites, indicating that the secretion of amylase in response to carbamylcholine involved the occupancy of the low affinity sites.

In recent studies, we have indicated that these receptors could be modulated or regulated by premature weaning [4], fasting and refeeding [5], and by long-term exposure to a cholinergic agonist [6].

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It is now well established that pancreatic growth can be stimulated by the gastrointestinal hormone cholecystokinin [7] as well as by its decapeptide analog caerulein [8, 9]. The tissue presents a time specific development with hypertrophy taking place during the first two days of a 5 day treatment, followed by hyperplasia well established after 4 days [10].

The pancreas growth pattern during caerulein treatment has been well characterized with regard to secretory enzyme, protein and RNA contents [8, 9] and rates of protein, RNA and DNA synthesis [10, 11]. However, our knowledge about what is happening to the receptors (hormonal and cholinergic involved specifically in enzyme secretion) during pancreatic hypertrophy and hyperplasia is rather limited. This study was therefore designed to answer this question and focuses specifically on the cholinergic muscarinic receptor.

MATERIALS AND METHODS

Hormone and chemicals. Caerulein was injected in hydrolysed gelatin (calcitonin diluent B, containing 160 mg hydrolysed gelatin/ml, a gift from Armour Pharmaceutical, Kankakee, IL) to prolong its absorption [9]. Synthetic caerulein was a gift from Adria Laboratories (Wilmington, DE). [^{3}H](−)-QNB (30–43 Ci/mmole) was purchased from Amersham Searle (Toronto, Canada); Whatman GF/B glass fibre filters were obtained from Canlab Lab-

oratories (Montréal, Canada); all other drugs and chemical products were from commercial sources. Liquid scintillation cocktails (BBS-3-toluene-Ready Solv Na) were purchased from Beckman Instruments (Montréal, Canada).

Treatment schedule. Animals used in these experiments were male Sprague–Dawley rats weighing between 205 and 230 g. The rats received thrice daily, at equally spaced (8 hr) intervals, a 0.2 ml subcutaneous injection containing 1 μg/kg caerulein or saline during 2 or 4 days. Twelve rats were used in each treatment group and for each duration of treatment. All animals were fasted overnight (water allowed) and the last injection was always given 15 hr before death. Each rat was weighed at the beginning of the treatment and before sacrifice. The whole pancreas was carefully dissected out, trimmed from lymph nodes and adipose tissue and weighed.

Tissue preparation. Each pancreas was homogenized in 9 vols of cold 0.32 M sucrose at low setting on a Polytron (PT 20, Brinkman Instruments) as previously described [1]. An aliquot of the homogenate was precipitated in 2.1 N perchloric acid (PCA) for DNA determination. The homogenate was then filtered through four layers of cheese-cloth.

Assays. Protein was measured according to the method of Lowry *et al.* [12] using bovine serum albumin as the standard. DNA was extracted into 0.5 N PCA for 15 min at 90° [13] and determined by the diphenylamine method using calf thymus DNA as the standard [14].

Binding assays. The ligand binding assay was performed according to a modification of Larose *et al.* [1]. Specific [^{3}H](−)QNB binding was experimentally determined from the difference between [^{3}H](−)QNB bound in the absence and presence of 10 μM atropine sulphate. It corresponded to 40–85% of total binding in saturation experiments and around 80% in all other experiments which used 2.6×10^{-10} M[^{3}H](−)QNB. Each binding assay was performed in triplicate and carried out at 37° in 5 ml of 50 mM Na–K phosphate buffer, pH 7.4, containing [^{3}H](−)QNB and protein concentrations between 30 and 60 μg/ml. Saturation curves were performed for 180 min whereas competition (labelled antagonist/agonist) lasted 120 min. The reaction was stopped by filtration through GF/B glass fibre filter followed by four rinses with 5 ml of ice-cold buffer. The filters were shaken overnight at room temperature in a scintillation cocktail solution containing BBS-3-toluene-Ready Solv NA (1:2:5) and thereafter were counted in a Beckman LS-7000 liquid scintillation spectrometer with an efficiency of 45%.

Secretion studies. After sacrifice, suspensions of dispersed acini were prepared from pancreas of saline and caerulein treated rats as reported by Larose *et al.* [2]. Dose–response curves of amylase release in response to carbachol 10^{-8} to 10^{-3} M were performed during a 30 min incubation period. Amylase secretion was determined as previously reported [2] and was calculated relative to the DNA content of the acinar cells.

Analysis of the data. Pancreatic weight, DNA and protein contents were compared using Student's *t*-test and were considered significantly different from control if $P < 0.05$. Analyses of the binding data were performed according to the equations described by Fields *et al.* [15] and then compared using Student's *t*-test. The theoretical binding curves [^{3}H]-(−)QNB/carbamylcholine were fitted to the experimental data points by nonlinear least square regression analysis using a computer program. Individual data points were the mean of 4 observations in triplicate. The program provided estimates of the best-fit parameter values together with estimates of the S.E. and confidence limits for each parameter [16]. The K_D values for both classes of binding sites were corrected for the radioactive ligand occupation according to the equation of Cheng and Prusoff [17].

RESULTS

Pancreatic response to caerulein

Weights. Body weights averaging 215 g at the beginning of the experiment were not significantly different between control (190 g) and treated groups (192 g) after 2 days and between control (217 g) and treated groups (201 g) after 4 days of treatment. Pancreatic weight expressed per g of body weight increased by 44.8% after 2 days of caerulein and by 54.1% over the control value after 4 days (Fig. 1). The cellular mass (pancreatic weight expressed per 100 μg DNA) was increased significantly by 27.3% over the control value only after 2 days of caerulein (Table 1).

DNA. After 2 days of caerulein treatment, total pancreatic DNA was not statistically different from the control group but after 4 days of caerulein, DNA content showed a significant increase of 54.5% over the control group (Fig. 1, Table 1).

Protein. Protein content showed an evolution parallel to pancreatic weight (Fig. 1) during the treatment period. Indeed the protein contents were 87.7% and 95.5% above control respectively after 2 days and 4 days of caerulein treatment. However, the ratio between protein and DNA contents, corresponding to an index of hypertrophy, was higher

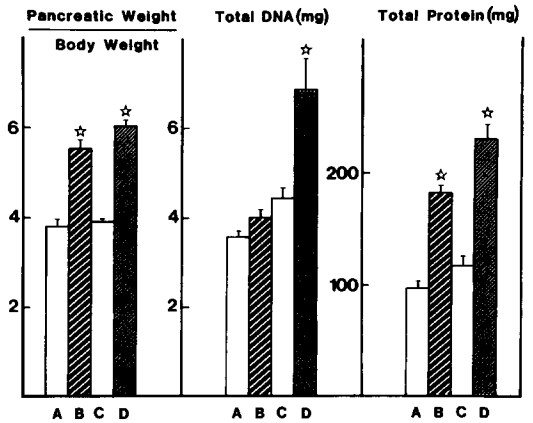

Fig. 1. Effect of caerulein treatment on total pancreatic weight (mg) per g of body weight, total pancreatic DNA and protein contents: (A) control saline 2 days; (B) caerulein 2 days; (C) control saline 4 days; (D) caerulein 4 days. Results are the means ± S.E. of 12 rats per group. ☆: different from the control group at $P < 0.005$.

Table 1. Effect of caerulein treatment on pancreatic growth

	Caerulein 2 days	Caerulein 4 days
Index of hyperplasia		
DNA	1.11	1.54*
Index of hypertrophy		
Protein/DNA	1.54*	1.38†
Pancreas wt/DNA	1.27*	1.02

Values are ratios of treatment groups to control group. Each group had 12 rats. Values significantly greater than 1.00 and therefore significantly greater than control: *$P < 0.005$; †$P < 0.01$.

after 2 days of caerulein (+ 54%) than after 4 days (+ 38%) as a result of the increase in total DNA content (Table 1).

Characterization of muscarinic receptor

Saturation curves (*Fig.* 2). When tested after 180 min (i.e. at equilibrium), specific [^{3}H](−)QNB binding reached saturation at 10^{-9} M ligand concentration.

After Scatchard transformation of the saturation curves [18], a straight line could be drawn with a good correlation coefficient suggesting the presence of a single population of QNB binding sites. Analysis of 5–6 individual saturation isotherms for each control and treated groups after 2 and 4 days of treatment gave an estimate of the receptor density by the intercept of the Scatchard plot with the abscissa (Table 2).

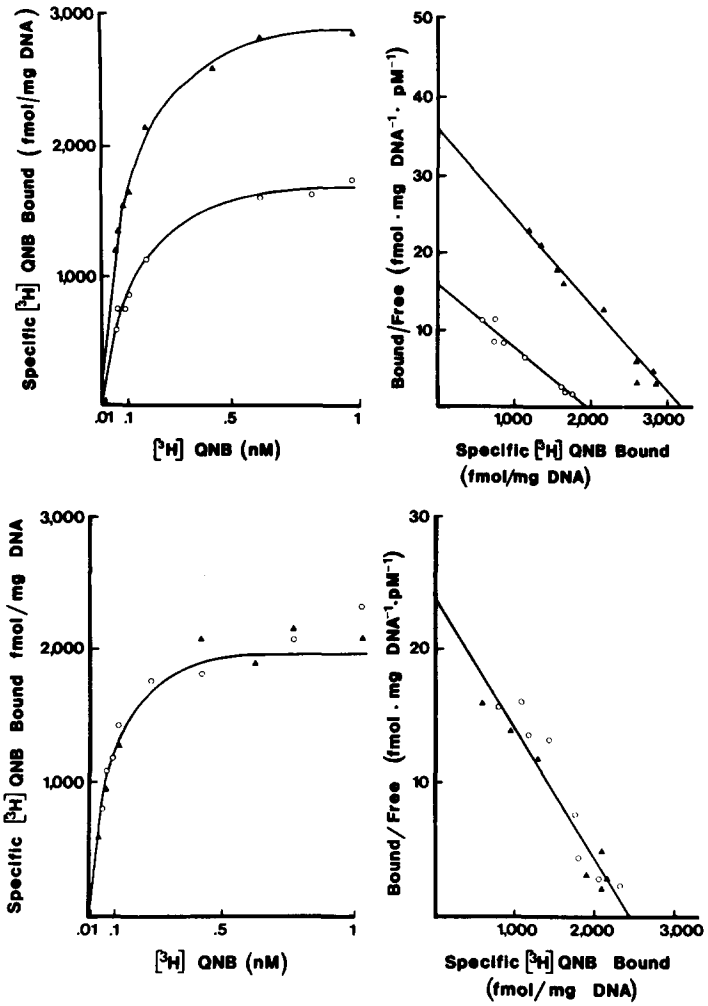

Fig. 2. Specific [^{3}H](−)QNB binding (left panel) and Scatchard plots (right panel) of this binding to pancreatic homogenates prepared from control rats (○) and from rats treated with caerulein (▲) for 2 days (upper panel) and 4 days (lower panel). [^{3}H](−)QNB 10^{-11} to 10^{-9} M was incubated for 180 min at 37°. Each point is the mean of triplicate determinations from a representative experiment.

Table 2. Specific [^{3}H](−)QNB binding sites in treated and control groups

	Specific [^{3}H](−)QNB bound† (fmoles/mg DNA)	K_D app × 10^{-10} M‡
Control saline 2 days (5)*	2236 ± 325	1.53 ± 0.45
Caerulein 2 days (5)	3509 ± 467§	1.28 ± 0.25
Control saline 4 days (6)	2235 ± 104	0.80 ± 0.05
Caerulein 4 days (5)	1989 ± 337	1.08 ± 0.14

Results are the mean ± S.E.M. Pancreatic tissue was incubated as described in Materials and Methods.

* Number of experiments.

† Specific [^{3}H](−)QNB bound determined by Scatchard plot (means ± S.E.M. of the experiments listed).

‡ K_D determined from the slope of Scatchard plot (means ± S.E.M. of the experiments listed).

§ $P < 0.01$.

The density of muscarinic receptor expressed in fmoles/mg DNA was significantly increased from 2236 ± 325 fmoles/mg DNA to 3509 ± 467 fmoles/mg DNA after 2 days of caerulein treatment: a 57% increase over the control value. After 4 days of caerulein treatment, the density of the pancreatic muscarinic receptor was not significantly different from the control group. Analysis of the saturation isotherms yielded K_D values between 0.8 and 1.53×10^{-10} M with no significant difference between them (Table 2).

Competition curves (*Fig.* 3, *Table* 3). Competition studies were performed with one antagonist, atropine and one agonist, carbamylcholine. Atropine was approximately 10,000-fold more potent in inhibiting [^{3}H](−)QNB binding than carbamylcholine. Competition curves with unlabelled atropine and the derived values of the Hill coefficient [19] which stood near unity in control and treated groups, suggested the existence of one class of antagonist binding sites (Fig. 3). When correcting for tracer concentration [17], an inhibition constant (K_i) ranging from 1 to 2 nM was obtained not significantly different between control and treated groups.

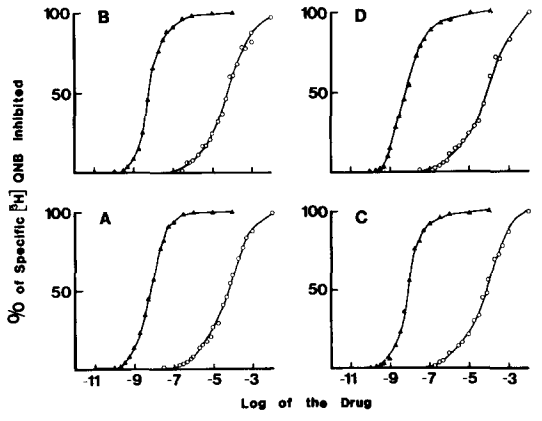

Fig. 3. Inhibition of specific [^{3}H](−)QNB binding to pancreatic homogenates by atropine (▲) and carbamycholine (○): (A) control 2 days (▲ mean of 2 experiments; ○ mean of 4 experiments); (B) caerulein 2 days (▲ mean of 3 experiments; ○ mean of 4 experiments); (C) control 4 days (▲ mean of 2 experiments; ○ mean of 4 experiments); (D) caerulein 4 days (▲ mean of 3 experiments; ○ mean of 4 experiments). The concentration of [^{3}H](−)QNB used in these experiments ranged from 0.1 to 0.3 nM.

Carbamylcholine exhibited a flatter competition curve than atropine (Fig. 3), giving Hill coefficients constantly below one (Table 3), thus suggesting the existence of more than one population of agonist muscarinic receptors, each having a different affinity constant for the agonist.

By analysing the data of these competition curves between [^{3}H](−)QNB and carbamylcholine by a nonlinear regression computer program, we have found two populations of muscarinic agonist receptors (Table 3). The percentage of high affinity sites has lightly decreased after 2 days or 4 days of caerulein treatment when compared to control values but these changes were not statistically significant (Table 3). The K_Ds of the high affinity sites ranged from 1.87 to 4.43×10^{-7} M while those of the low affinity sites stood between 1.24 and 2.19×10^{-5} M. There were no significant differences between the control and treated groups concerning the K_H and K_L, and the Hill coefficient as evaluated by analysis of variance. The increase in the total number of receptors after 2 days of caerulein treatment (Table 2) should be distributed amongst high and low affinity sites as their respective proportions were not significantly modified (Table 3). Since the percentage of the low affinity sites was greater than that of the high affinity sites, only the number of the low affinity sites expressed in terms of fmoles/mg DNA appeared significantly increased (67.4%) over the control value, while that of the high affinity sites was moderately increased (22%), which was not statistically significant. After 4 days of treatment, the number of high and low affinity sites expressed in fmoles/mg DNA came back to control values.

Secretory response to carbachol

When the amylase released from isolated acini during a 30-min incubation was studied as a function of the concentration of carbamylcholine, typical biphasic amylase dose–response curves were obtained (Fig. 4). Amylase release in units per milligram DNA was plotted against the concentration of carbamylcholine in the medium. Dose–response curves of amylase release were obtained from pancreatic acini prepared from 2 days (upper panel) and

Table 3. Inhibition of [^{3}H](−)QNB binding to the pancreatic muscarinic receptor by carbamylcholine

	$K_H \times 10^{-7}$ M†	$K_L \times 10^{-5}$ M†	% High	nb of high affinity sites (fmoles/mg DNA)	nb of low affinity sites (fmoles/mg DNA)	n_H‡
Control 2 days (4)*	4.43 ± 1.28	2.19 ± 0.26	23.19 ± 2.84	518 ± 63	1718 ± 63	0.664 ± 0.087
Caerulein 2 days (4)	2.85 ± 1.60	1.24 ± 0.19	18.02 ± 4.39	632 ± 154	2877 ± 154§	0.600 ± 0.042
Control 4 days (4)	3.66 ± 1.55	1.85 ± 0.27	22.04 ± 3.52	492 ± 78	1742 ± 78	0.526 ± 0.065
Caerulein 4 days (4)	1.87 ± 0.89	1.53 ± 0.29	18.79 ± 3.57	420 ± 79	1815 ± 79	0.519 ± 0.088

* Number of experiments.
† Value of K high and K low given by the computer program corrected by the equation presented by Fields *et al.* [15].
‡ Hill coefficient calculated from the mean of the experimental data. Each value represents the mean ± S.E.M.
§ $P < 0.005$.

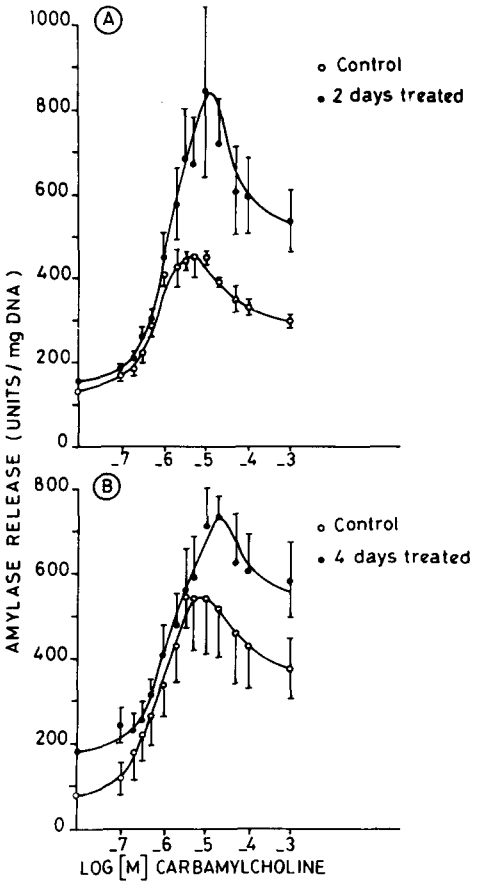

Fig. 4. Dose–response curves of amylase release stimulated by carbamylcholine. Upper panel: amylase release into the medium relative to DNA concentration after 2 days of caerulein treatment. Lower panel: amylase release into the medium relative to DNA concentration after 4 days of caerulein treatment. Results shown are means ± S.E. 2 days: control, N = 5; caerulein, N = 6.4 days: control, N = 3; caerulein, N = 6.

4 days (lower panel) control and caerulein treated rats.

After 2 days of caerulein (Fig. 4, upper panel), the maximal amylase release induced by carbamylcholine was significantly elevated from 488 units to 848 units per mg DNA. However, the sensitivity of the acini to the cholinergic agonist was significantly reduced as evidenced by a shift to the right of the dose–response curve with the EC_{50} significantly increased ($P < 0.05$) from $5.8 \pm 0.8 \times 10^{-7}$ M in the control to $1.2 \pm 0.2 \times 10^{-6}$ M in the caerulein group. In acini from control rats, maximal amylase release was obtained at 6.8×10^{-6} M carbachol, but only at 1.2×10^{-5} M in the caerulein group.

After 4 days of caerulein treatment (Fig. 4, lower panel), the maximal amylase release from the acini remained higher than in the control group but not significantly different with 564 units in the control vs 772 units per mg DNA in the caerulein group. However, the sensitivity of the acini to the cholinergic agonist remained significantly reduced as the EC_{50} of the dose–response curve of the caerulein-

treated group was significantly increased ($P < 0.05$) from $6.3 \pm 1.1 \times 10^{-7}$ M in the control to $2.2 \pm 0.4 \times 10^{-6}$ M in the caerulein group. In acini from control rats, maximal amylase release was reached at 6.0×10^{-6} M carbachol but only at 3.3×10^{-5} M in the caerulein group.

DISCUSSION

This study, like others [8, 9], confirms the growth-promoting effect of caerulein on the pancreas. It also points out that this growth process proceeds in cycle with hypertrophy of the acinar cells being established first followed two days later by the increase in cell number as evidenced by the increase in DNA content (Fig. 1).

Hypertrophy of the pancreatic tissue is characterized by increases in protein and RNA synthesis [11] and enzyme, total protein, and RNA contents [8, 9]. Our data indicate that synthesis of the muscarinic receptors probably has been stimulated also since their concentration was increased by 57% after 2 days of treatment (Table 2). These changes are likewise the result of a non specific effect as part of the whole hypertrophy phenomenon in which the stimulated cells synthesize all the needed proteins before cell division. The similar increases in receptor concentration corrected for DNA content (57%) and in the ratio of protein content to DNA content (54%) (Tables 1 and 2) plead for this possibility. The return to control values after 4 days of caerulein treatment suggests a redistribution of the newly synthesized receptors within the new cells. It is interesting to notice that 57% of the new receptors would have been evenly distributed in 54% new cells, since DNA content was increased by 54% after 4 days of caerulein (Fig. 1).

If the amount of receptors can be increased by caerulein, what are the changes observed in their basic characteristics? The affinity of the receptor for the ligand QNB was not affected by the hormone (Table 2); this observation was also confirmed with another antagonist, atropine (similar K_i in control and caerulein treated groups).

The caerulein treatment did not alter the affinity of carbamylcholine for the two classes of binding sites; indeed, the K_H and K_L are comparable in the saline and caerulein groups. The change in total receptor concentration following 2 days of caerulein is also reflected in the concentration of high and low affinity sites. As indicated in Table 3, the concentration of high affinity sites was moderately raised (22%) while that of the low affinity was increased by 67%; one can thus suggest that most of the newly synthesized receptors were in a low affinity state, but synthesis of the high affinity sites at the same time could not be excluded. In a recent study [3], it was suggested that the low affinity binding sites were involved in amylase release while a previous study [2] proposed that the physiological response of the pancreatic acini to a muscarinic agonist would involve the high affinity site population. The physiological significance of the binding data was explained in those studies by different ways using [3], or not using [2], an irreversible muscarinic antagonist in order to block an increasing number of spare receptors. Therefore different conclusions were reached.

Since all the binding studies were performed on homogenates, one cannot speculate on the location of these new receptors, in the cytoplasm or on the plasma membrane. However, it was demonstrated that the muscarinic receptors present on pancreatic homogenates [1] and on purified acini [2] had similar binding properties (receptor density and apparent K_D).

Moreover, the binding characteristics of the muscarinic receptors (B_{max}, $K_D$app, K_H, K_L, proportions and absolute number of high and low affinity binding sites) studied on pancreatic acini [6] were identical to those on pancreatic homogenates as reported in the present study, suggesting the location of the pancreatic muscarinic receptors on the plasma membrane.

It was also shown that acinar cells accounted for the great majority of cells which were synthesizing DNA after caerulein treatment [10], suggesting that the trophic effect of caerulein is mainly applied upon the acinar cells and that the observed changes in receptor density after caerulein treatment could be ascribed principally to the acinar cells allowing comparison with the amylase secretion data.

The evaluation of the amylase release results must take into account that acinar cells from the control and caerulein-treated groups are two different populations of cells because of the hypertrophied and hyperplastic pancreatic tissue in the caerulein-treated groups. We have therefore expressed our amylase release data in units/mg DNA, an indicator of the total output per cell.

When we examine total amylase release per cell, U/mg DNA, the pancreatic functional capacity of the acini was significantly increased after two days of caerulein treatment; this phenomenon was less evident after four days of treatment. These secretion data do, however, reflect what happened to the pancreatic tissue as summarized in Table 1. Indeed, the functional capacity of the acini is maximal after two days of caerulein when pancreatic hypertrophy is also optimal as evidenced by larger increases in cell mass and in protein to DNA ratio. After four days of caerulein, hyperplasia is definitely present with reduced hypertrophy.

We are evaluating functional capacity of isolated acinar cells but not of the whole tissue. Then, since after four days of caerulein the number of acinar cells has increased, we can expect that the functional capacity of the whole organ would have remained comparable to that of the hypertrophied gland after two days of treatment because of this increased number of cells.

Indeed, *in vivo* studies of pancreatic secretion following chronic treatment with CCK [20] showed that the maximal protein output in response to cholecystokinin increased proportionately to the increase in pancreatic weight.

Besides these changes in the amount of amylase released, there was also a moderate loss of acinar sensitivity to carbamylcholine after 2 or 4 days of caerulein treatment which cannot be explained by changes at the receptor level. Indeed the data presented in Table 3 indicate that the affinities of the

two receptor classes, K_H and K_L, and their relative proportions (% of high and low affinity sites) were not affected by the 2 and 4 days caerulein treatment. It would then seem that some changes did occur at a post receptor step.

In a similar study investigating the effects of a CCK-8 treatment on the pancreatic CCK receptor and on the pancreatic amylase release, Otsuki and Williams [21] obtained pancreatic hypertrophy after 7 days and hyperplasia after 14 days of CCK-8 injection. In agreement with our results, these authors demonstrated an increase in the functional capacity of the acini mainly related to pancreatic hypertrophy and a moderate decrease in the acinar sensitivity to CCK or carbamylcholine without major changes in the affinity of CCK receptors suggesting a post receptor desensitization. It would then seem that the control of CCK and muscarinic receptor synthesis and distribution in response to CCK and its analog are similar.

However, if a cholinergic agent, bethanechol, is used to stimulate pancreatic growth [22], leading to pancreatic hypertrophy associated with hyperplasia after 14 days of treatment, both the affinity of the two populations of muscarinic binding sites and the density of the low affinity sites decreased [6] as well as the sensitivity of amylase release to carbamylcholine. These results were suggesting that a specific change at the muscarinic receptor level has been induced by long term cholinergic treatment. So the pancreatic response to a cholinergic agonist affecting directly its specific receptor seems quite different.

Combination of hyperplasia and hypertrophy of secretory cells was also obtained from submandilar rat gland after chronic administration of the β-adrenergic agonist, isoproterenol [23]. A decrease in the density and the affinity of the muscarinic receptors was observed following this treatment.

Therefore modulation of the muscarinic receptors by those hormones, isoproterenol and caerulein, which both stimulated the growth of exocrine gland in the rat, maybe by increasing the cyclic AMP levels, appears to involve different cellular steps.

This study indicates that caerulein can modulate the concentration of the pancreatic muscarinic receptors without modifying their basic characteristics with regard to their interaction with cholinergic agonist and antagonist. The answer to the question as to whether this modulation is caerulein specific and part of its hypertrophic action will be clarified by further experiments with other trophic agents such as secretin and hydrocortisone.

Acknowledgements—This work was supported by the MRC of Canada, NSERC of Canada and FCAC of the Province of Québec, Grants MA-7320, A0145 and 0733. We wish to acknowledge the designing assistance of M. Martin and the secretarial assistance of C. Rancourt and B. Balot.

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