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PARALLEL MATURATION OF THE PANCREATIC SECRETORY RESPONSE TO CHOLINERGIC STIMULATION AND THE MUSCARINIC RECEPTOR POPULATION

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1 The appearance of pancreatic muscarinic receptors during development has been measured by use of the specific ligand [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB).

2 QNB binding sites are present in foetal pancreas; their maximal concentration is attained at the age of 30 days and a significant decrease is observed in one year old animals.

3 Affinity of [^{3}H]-QNB for the muscarinic receptor does not change with age.

4 An evaluation of the pancreatic secretory response to a cholinoceptor agonist as a function of age indicates that the development of this response parallels that of the receptor population.

5 It is suggested that, at all ages from 3 days after birth onwards, the maximal secretory response of the exocrine pancreas to a cholinoceptor agonist mobilizes the same proportion of the total population of QNB binding sites.

Introduction

In recent years, the availability of tritiated specific muscarinic antagonists, such as quinuclidinyl benzilate (QNB), has allowed the characterization of muscarinic receptors in various tissues such as the heart (Fields, Roeske, Morkin & Yamamura, 1978), the ileum (Yamamura & Snyder, 1974b) and the brain (Yamamura & Snyder, 1974a).

The development of muscarinic receptor populations has been investigated in the avian heart (Sastre, Gray & Lane, 1977), chicken retina (Sugiyama, Daniels & Nirenberg, 1977) and rabbit brain (Yavin & Harel, 1979). To our knowledge, no one has yet established any relationship between the maturation of these receptor populations and a physiological response typical of the tissue under investigation.

In previous studies, we have examined the pre- and postnatal development of the pancreatic acinar cell responsiveness to a cholinoceptor agonist (Larose & Morisset, 1977) and defined the characteristics of the pancreatic muscarinic receptors (Larose, Lanoe, Morisset, Geoffrion, Dumont, Lord & Poirier, 1979). In this paper, we describe the maturation of the pancreatic muscarinic receptor population and establish a correlation between it and the secretory response of the tissue in foetal, growing and adult rats.

Methods

Male Sprague-Dawley rats were used throughout these studies. In order to obtain 21 days foetal pancreas and those from rats aged 1 to 365 days, the procedure of Larose & Morisset (1977) was followed. All animals were fasted overnight and killed by decapitation; their pancreas was then removed, trimmed of fat tissue and lymph nodes and pooled in ice-cold 0.32 M sucrose until 1–2 g of tissue was obtained. The tissue was then homogenized in 0.32 M sucrose at low setting on a Polytron (PT 20, Brinkmann Instruments) and the homogenate was filtered through 4 layers of cheese-cloth. Protein was measured according to Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as the standard. DNA was extracted according to Mainz, Black & Webster (1973) and determined according to Volkin & Cohn (1954) with calf thymus DNA used as the standard. Amylase activity was assayed according to Bernfeld (1955); a unit of amylase activity is that liberating 1.0 μmol of reducing groups calculated as maltose, per minute at 37°C. Data were analysed by Student's *t* test.

Binding assay

The ligand binding assay was performed according to a modification of Yamamura & Snyder's method (1974a) as reported by Larose *et al.* (1979). Specific [^{3}H]-QNB binding was experimentally determined from the difference between counts bound in the absence, and presence of 1 μM atropine sulphate. Incubations were carried out in 10 or 25 ml of a Na-K phosphate buffer 50 mM, pH 7.4 containing [^{3}H]-QNB at concentrations ranging between 10^{-11} and

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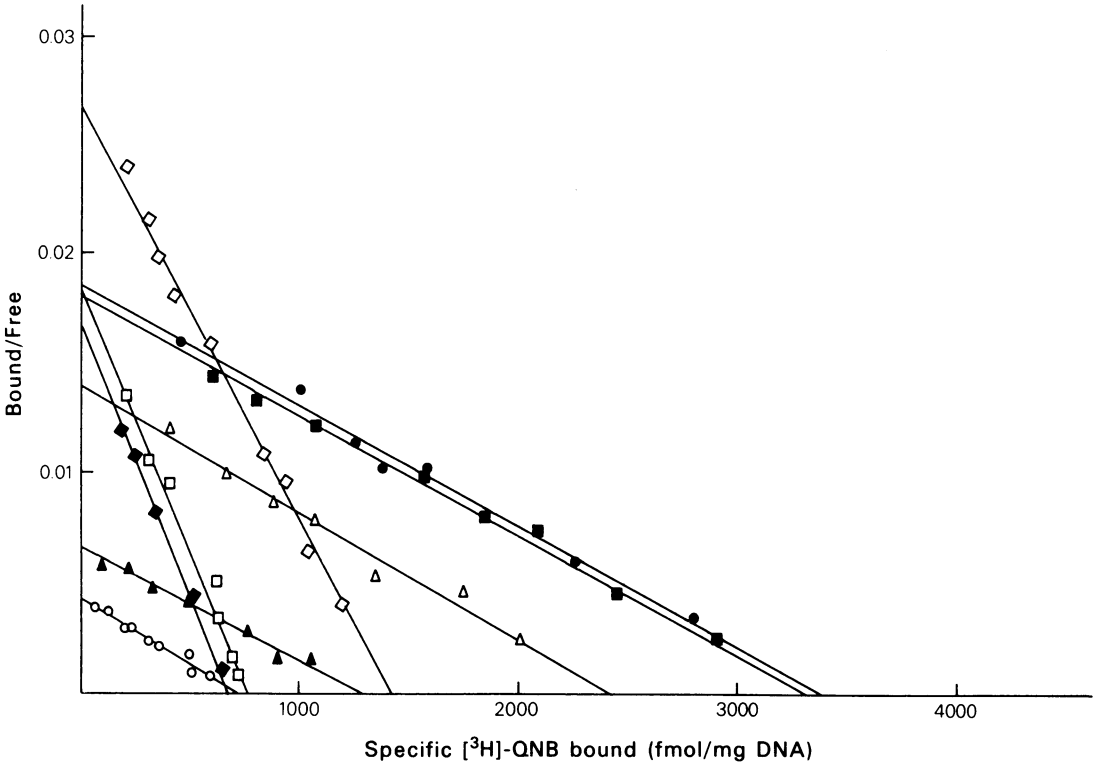

Figure 1 Scatchard analysis of the binding of [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) to pancreas homogenates from rats of different ages. Specific [^{3}H]-QNB binding is expressed in fmol/mg DNA. Each line represents a typical Scatchard plot. Symbols: (♦) foetal pancreas; (□) 5 days; (○) 15 days; (▲) 19 days; (△) 25 days; (●) 30 days; (■) 60 days; (◇) 365 days.

10^{-9} M, with protein concentrations between 30 and 75 μg/ml, for 150 min. The reaction was stopped by filtering the incubation medium through GF/B glass fibre filter followed by 4 rinses with 5 ml of ice-cold buffer. Samples were shaken overnight at room temperature in a scintillation solution (BBS-3-Toluene-Ready Solv NA:1:2:5) and counted in a Beckman LS-7000 liquid scintillation spectrometer with an efficiency of 43%. Analysis of the data was performed assuming a bimolecular process, by a Scatchard plot.

In vitro *secretion studies*

Incubation of pancreatic tissue for secretion studies was performed as previously described by Larose & Morisset (1977). Amylase secretion was used to monitor pancreatic enzyme release with 10^{-5} M bethanechol stimulation.

Drugs

(±)-[^{3}H]-QNB (29.4 Ci/mmol) was purchased from New England Nuclear and (−)-[^{3}H]-QNB (43 Ci/mmol) from Amersham. GF/B glass fibre filters were obtained from Whatman. All other drugs and chemicals were from commercial sources.

Results

Figure 1 shows typical Scatchard transformations of the binding data obtained from pancreas of rats of different ages. In each case, a straight line was obtained with correlation coefficients between −0.92 and −0.98. These data thus suggest that, at each age studied, a single population of QNB binding sites is present. All apparent K_D calculated from these individual plots (Table 1) vary between 112 and 289 pM for (±)-[^{3}H]-QNB and 38 to 68 pM for (−)-[^{3}H]-QNB indicating that they are comparable.

Table 1 also presents the concentration versus age of the pancreatic muscarinic binding sites expressed in fmol/mg protein or DNA. Presentation of the receptor population calculated on a cellular basis (fmol/mg DNA) indicates that the 21 days foetal

Table 1 Ontogenesis of muscarinic cholinoceptors in the pancreas

Age (days)	n[a]	*Exp*[b]	*Specific* [^{3}H]-*QNB bound*[c] (fmol/mg protein)	(fmol/mg DNA)	(K_D) pm[d]
Foetal					
21	210	3	30 ± 3	652 ± 46	38 ± 16*
After birth					
1	312	3	26 ± 1	1194 ± 117	68 ± 23*
5	280	4	45 ± 3	670 ± 100	55 ± 7*
11	140	3	31 ± 1	584 ± 18	112 ± 20
13	90	3	37 ± 11	598 ± 159	289 ± 54
15	60	3	44 ± 5	637 ± 17	182 ± 4
17	60	3	66 ± 15	848 ± 111	242 ± 12
19	80	5	71 ± 10	1288 ± 95	149 ± 49
21	60	6	78 ± 5	1578 ± 53	137 ± 20
23	49	5	125 ± 3	2277 ± 67	158 ± 27
25	30	3	130 ± 3	2302 ± 61	184 ± 7
27	56	6	89 ± 4	2413 ± 153	138 ± 37
30	38	4	165 ± 13	3605 ± 218	224 ± 22
60	9	3	58 ± 3	3280 ± 25	174 ± 6
365	8	4	37 ± 3	1564 ± 213	63 ± 21*

Results are the mean ± s.e. Pancreatic tissue was incubated as described in Methods.
[a] Number of rats used at each age; [b]number of experiments; [c]specific [^{3}H]-QNB bound determined by Scatchard plot (means ± s.e. of the experiments listed in b; [d]K_D determined from the slope of Scatchard plot (means ± s.e. of the experiments listed in b).
*Affinity determined with (−)-[^{3}H]-QNB. All the others were done with (±)-[^{3}H]-QNB.

pancreas already possesses a number of sites comparable to those present in pancreas from animals aged between 3 and 15 days after birth. Two weeks after birth, the number of receptors increases regularly to reach a maximum at 30 days; the population remains stable until 60 days but is significantly lower in one year old animals. The evolution of the pancreatic muscarinic receptors as a function of age is presented in Figure 2.

Table 2 shows that there is no change with age in the potencies of atropine and QNB in reducing [^{3}H]-QNB binding to the receptors as the I_{50} remained similar. Also, at the ages listed in Table 2, the muscarinic receptors were found to be stereospecific with (+)-benzetimide 10^{-8} M displacing 85% of QNB binding whereas (−)-benzetimide 10^{-8} M displaced only 10%.

In parallel to this receptor population evaluation, *in vitro* pancreatic amylase secretion in response to 10^{-5} M bethanechol has also been studied. Figure 3 shows that the secretory response of the exocrine pancreas to the cholinoceptor agonist developed quite similarly to that of the muscarinic receptor population. Indeed, from 5 to 15 days after birth, the secretory response remained constant but began to increase from day 15 to reach its peak at 30 days. The enzyme output remained stable until 60 days and, as for the receptors, a sharp decrease was observed in one year old rats.

The correlation existing between maximal amylase output in response to 10^{-5} M bethanechol and the QNB binding sites from animals aged 5 days to a year is presented in Figure 4. As observed, a correlation coefficient of 0.96 was obtained indicating that stimulated enzyme secretion can be related to the total number of muscarinic binding sites per cell. In calculating this correlation, receptors from foetal and one day old rats were deleted because it has previously been shown that under our experimental conditions, the pancreas from such animals is unresponsive to the cholinoceptor agonist (Larose & Morisset, 1977).

Discussion

This study describes for the first time data on the development of the muscarinic cholinoceptor population in the rat pancreas. Moreover, it is also demonstrated that the ontogeny of these receptors parallels the maturation of the secretory response of the pancreatic tissue to a cholinoceptor agonist and a good correlation has been established between the maximal pancreatic secretory response to bethanechol and the number of receptors.

In order to obtain a very accurate estimation of the receptor population during development, the data should be presented on a DNA basis because this is

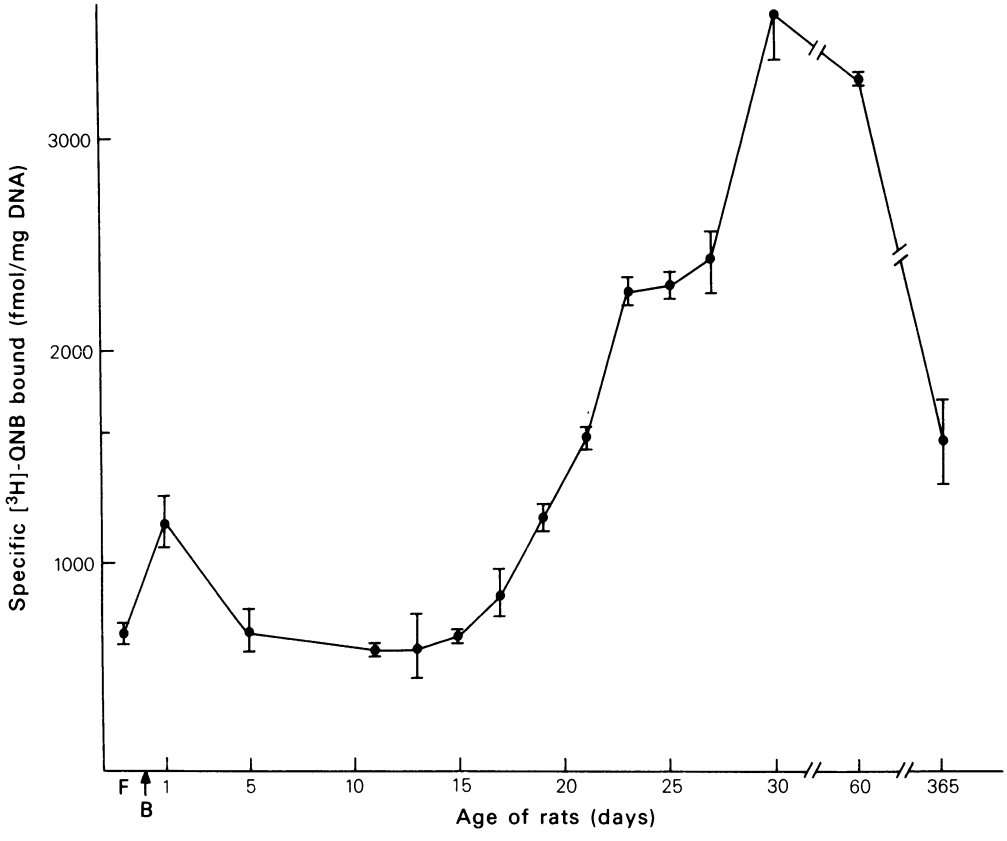

Figure 2 Evolution of the muscarinic cholinoceptor population as a function of age. Each point represents the mean of B_{max} of [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) (fmol/mg DNA); vertical lines show s.e.

Table 2 Relative potencies of atropine and quinuclidinyl benzilate (QNB) in reducing [^{3}H]-QNB binding in pancreas homogenates from rats of different ages

	[^{3}H]-QNB binding (I_{50} nM)	
Age (days)	*Atropine*	*QNB*
Foetus	3.6	0.33
1	3.2	0.35
11	3.4	0.31
19	2.9	0.32
60	3.7	0.35

The I_{50} represents the drug concentration inhibiting 50% of total specific [^{3}H]-QNB bound. The competition curves were done at 10^{-10} M [^{3}H]-QNB and non specific binding determined in the presence of atropine 10^{-6} M. These are the data of one experiment.

the only parameter that remains constant from cell to cell. By using DNA, we have a true estimation of the receptor concentration per cell which indicates that the maximal concentration was reached on day 30 (Table 1).

Scatchard plot analysis of all the binding data obtained from rats of different ages indicates that the ligand interacts with a single population of binding sites with identical affinity. This property of the pancreatic receptors indicates that they are compar-

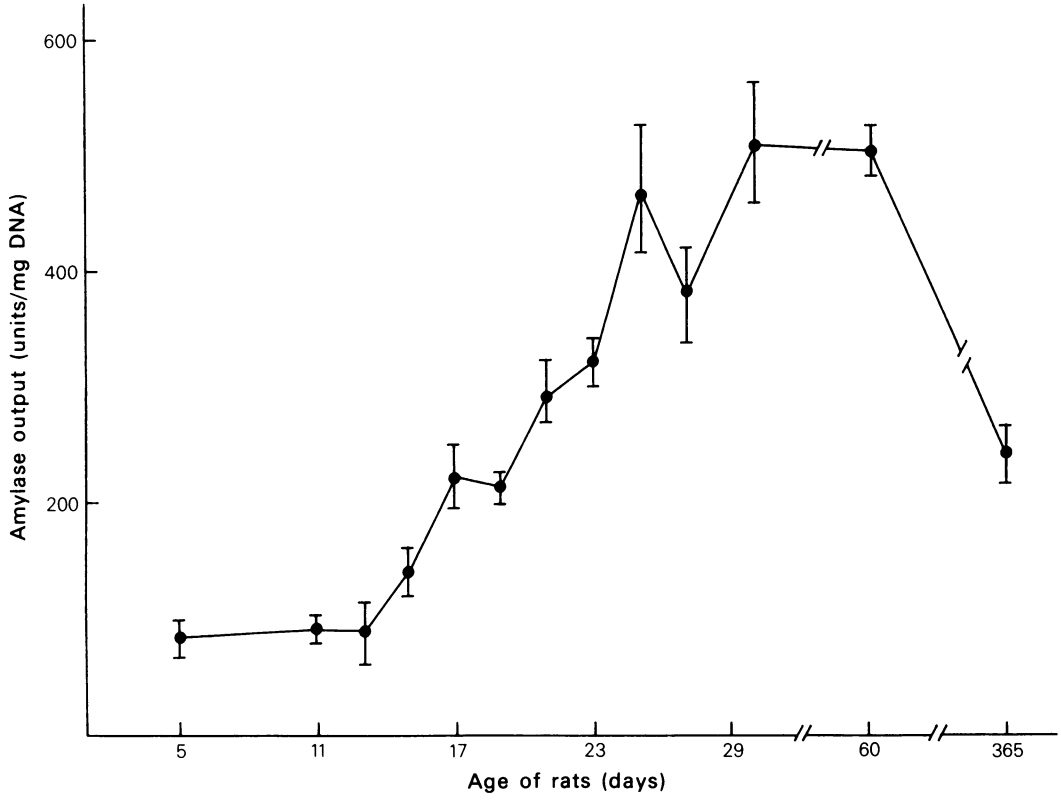

Figure 3 Evolution of the secretory response of the exocrine pancreas in response to bethanechol as a function of age. Amylase release (units/mg DNA) was measured *in vitro* for 90 min under 10^{-5} M bethanechol stimulation. Each point represents the mean of 10 pancreas; vertical lines show s.e.mean.

able to those of the mouse heart (Roeske & Yamamura, 1978), the chick heart (Galper, Klein & Catterall, 1977) and the mouse brain (Aronstam, Kellog & Abood, 1979).

Development of the muscarinic receptor population seems to depend on the organ and the species. Indeed, maximal concentrations in chick embryo brain (Enna, Yamamura & Snyder, 1976), chick retina (Sugiyama *et al.*, 1977), avian heart (Sastre *et al.*, 1977) and rabbit brain (Yavin & Harel, 1979) are observed during embryonic life while those in rat cerebral cells (Dudai & Yavin, 1979), mouse heart and brain (Roeske & Yamamura, 1978; Aronstam *et al.*, 1979), parotid glands (Ludford & Talamo, 1980) and rat pancreas reach their full development after birth.

According to our data, the 21 day foetal pancreas and that from the newborn rat carry a muscarinic receptor population comparable to that of older rats; however, previous results (Larose & Morisset, 1977) indicate that these tissues did not secrete enzyme in response to bethanechol before 3 days after birth. This delay in the physiological response to a cholinoceptor stimulation in the presence of muscarinic receptors was also observed in chick heart (Galper *et al.*, 1977) where significant increases in the receptor population occurred 2 days before any chronotropic response to a cholinoceptor agonist could be detected.

This lack of early response to cholinoceptor stimulation does not seem to be related to the biochemical characteristics of the receptors because those of the embryonic and newborn pancreas are similar to those of the older animals. Indeed, they are all stereospecific and they all present identical ([^{3}H]-QNB) competition curves against atropine and QNB. It is then possible that the message transmission between the receptor and the intracellular reactions involved in the cell response are not yet coupled. Presumably some reactions of the stimulus-secretion coupling system have not yet matured. These may involve the activation of the adenylate or guanylate cyclase system following receptor coupling or the dynamics of calcium metabolism which is very active in exocytosis (Poulsen & Williams, 1977; Gardner, 1979). In the adrenals, Albano, Joseph, Jacks, Gould, Nathaniels

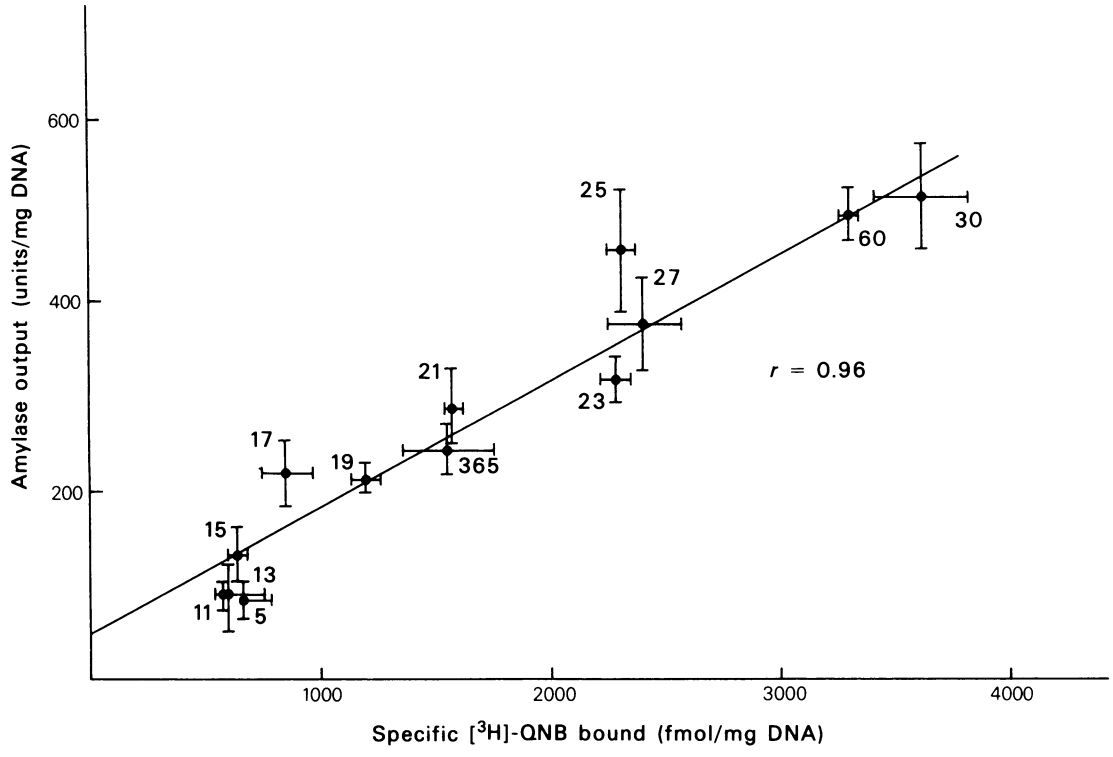

Figure 4 Correlation between amylase output and the concentration of muscarinic receptors. Data represent amylase (units/mg DNA) and receptor (fmol/mg DNA); vertical and horizontal bars show s.e. Numbers beside points indicate the age of the animals.

& Brown (1975) have observed that the adenylate cyclase system was responsive to sodium fluoride as early as day 11 of foetal life while the response to ACTH appeared only 13 days later. In support of a limiting step somewhere after the hormone-receptor coupling is the observation that exogenous dibutyryl cyclic AMP, 8-Br-cyclic GMP or the calcium ionophore A-23187 can initiate enzyme secretion from foetal pancreas (Doyle & Jamieson, 1978; Werlin & Grand, 1979).

In aging rats, a substantial decrease in the pancreas receptor population has been observed which can explain the reduced response to bethanechol. This reduction in the receptor concentration is not peculiar to the muscarinic receptors since similar decreases were observed for the β-adrenoceptor (Greenberg & Weiss, 1978), the oestradiol receptor (Kanungo, Patnaik & Koul, 1975), and the glucocorticoid receptor (Roth, 1974); it was also observed for the brain muscarinic receptor (James & Kanungo, 1976).

Our results show that a clear relationship exists between the pancreatic secretory response to cholinoceptor agonists and the concentration of muscarinic receptors. The correlation established is so striking that it could suggest that all the QNB binding sites need to be occupied for maximal enzyme secretion. However, caution is required before assuming that such is the case since muscarinic antagonists react with one class of receptor with Hill coefficients near 1.0 while the binding of agonists occurs on more than one class of receptor as shown by Hill coefficients below 1.0 (Hulme, Burgen & Birdsall, 1975; Birdsall, Burgen & Hulme, 1978; Larose *et al.*, 1979). It would seem then that the correlation between secretion and binding is good mainly because the ratio of the receptors directly involved in the secretion process remains constant in percentage terms with regard to the total population of sites.

To confirm the existence of these different classes of pancreatic muscarinic receptors, studies are in progress examining competition curves of specific [^{3}H]-QNB bound by carbachol.

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