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MUSCARINIC CHOLINERGIC RECEPTORS IN PANCREATIC ACINAR CARCINOMA OF RAT

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The active enantiomer of tritiated quinuclidinyl benzilate (^{3}H(-)QNB) was used as a ligand to evaluate the muscarinic receptors. The ^{3}H(-)QNB binding characteristics of muscarinic cholinergic receptors obtained from normal and neoplastic tissues were studied to determine changes in receptor properties during neoplastic transformation. Saturable and stereospecific binding sites for ^{3}H(-)QNB are present in homogenates of rat pancreatic adenocarcinoma. The proportions of high- and low-affinity agonist binding sites are similar for neoplastic and normal tissues. The density of muscarinic receptors is higher in neoplastic (200 femtomoles/mg protein) than in normal pancreatic homogenates (80 femtomoles/mg protein). The muscarinic binding sites of the neoplastic and fetal pancreas show similar K_D values which are higher than those observed for normal pancreas.

We have recently demonstrated the existence of muscarinic receptors in the pancreas of the rat (Larose *et al.*, 1979; Ng *et al.*, 1979) and established a correlation between the occupation of these receptors and the cholinergic secretory response of isolated pancreatic acini (Larose *et al.*, 1981).

The initial step in the action of a secretagogue usually involves its reversible binding to a specific receptor on the outer surface of the plasma membrane. The occupation of this receptor initiates a sequence of biochemical changes that result in the secretion of enzymes. In normal pancreas these events can be initiated by gastrointestinal hormones such as cholecystokinin and secretion and by the neurotransmitter acetylcholine (Gardner *et al.* 1977). During neoplastic transformation of the pancreas, changes were observed in the glycoconjugate pattern associated with the cell surface (Jamieson *et al.*, 1981) and in the profile of secretory proteins (Iwanij *et al.*, 1982*b*). Such changes may be related to modification in the coupling between secretagogue stimulus and enzymatic secretion. This may represent an important stage in the neoplastic transformation of the pancreas.

In this study, we have chosen to compare normal and neoplastic tissues of the pancreas in terms of ^{3}H(-)QNB binding characteristics of their muscarinic receptors. Any differences between these tissues may indicate a role for the alteration of the receptors in the neoplastic transformation of acinar cells. The transplantable pancreatic acinar carcinoma of the rat, which is an effective model in the examination of such binding characteristics, is used in this study.

MATERIAL AND METHODS

Material

Tritium-labelled (-) quinuclidinyl benzilate (^{3}H(-)QNB) (30–43 Ci/mmol) was obtained from Amersham Searle, Toronto, Canada. Dexetimide (+ benzetimide) and levitimide (- benzetimide) were obtained from Janssen Pharmaceutica, Toronto, Canada. Whatman Glass fiber filters (GF/B) were supplied by Canlab Laboratory Supply, Montreal, Canada. All other drugs and chemicals were obtained from commercial sources. Liquid scintillation medium (BBS-3-Toluene, Ready-Solv NA) was purchased from Beckman Instruments, Montreal, Canada.

Preparation of homogenates

The tumor was an azaserine-induced acinar-cell carcinoma designated CA 20948. The neoplastic tissue has been induced in Wistar-Lewis rats and was transplanted subcutaneously in rats of the same strain, as previously outlined (Longnecker *et al.*, 1979) and the rats were examined regularly to follow tumor growth for at least 3 weeks. After the tumor reached a certain size (2-3.0 cm) normal and neoplastic pancreatic tissues from the same animal were carefully dissected. Each tissue was added to 9 vol of ice-cold 0.32 M sucrose and was homogenized for 15 sec at 4°C by 3 up-and-down movements on a Polytron (Brinkmann Instr. USA), setting No. 5. The homogenates were filtered through 4 layers of cheese-cloth. The content of DNA and protein from filtered samples was determined by the methods of Volkin and Cohn (1976) and Lowry *et al.* (1951) respectively. DNA was extracted according to the procedure of Mainz *et al.* (1973).

Ligand binding studies

A tritiated muscarinic cholinergic antagonist, quinuclidinyl benzilate (^{3}H(-)QNB) was used in the detection of the muscarinic receptors (Yamamura and Snyder, 1974). The binding assay was conducted in the presence or absence of 1 μM atropine sulfate at 37°C on aliquots of homogenized tissues which were diluted in a 0.05 M phosphate buffer (pH 7.4) containing KH_2PO_4 0.005 M $MgCl_2$ (buffer A) and tritiated ^{3}H(-)QNB at an appropriate concentration. The homogenates were diluted in buffer A at a protein concentration of between 40 and 50 μg/ml. The final volume of the assay was 5 ml. Addition of soya-bean trypsin inhibitor to the homogenization buffer or to the incubation buffer did not affect the number of binding sites or the apparent K_D of the receptor. At various times the reaction was stopped by vacuum filtration of the incubating medium through GF/B glass fiber filters

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followed by 4 rinses with 5 ml of ice-cold assay buffer. Samples were shaken overnight at room temperature in scintillation solution (BBS-3-Toluene, Ready-Solv NA) and counted in a liquid scintillation spectrometer (Beckmann LS 7000). The difference between counts bound in the absence and presence of atropine sulfate determined the specific binding of ^{3}H(-)QNB. The assays were performed in triplicate for the determination of both specific and non-specific binding.

Calculations

The affinity constants were corrected according to Cheng and Prusoff (1973). Calculation of the rate constants of association (k+1) and dissociation (k-1) was carried out essentially as described by Fields *et al.* (1978). The theoretical binding curves were fitted to the experimental data points by a non-linear square regression analysis using a computer program similar to that reported by Birdsall *et al.* (1978). Individual data points were the mean of 6 observations in triplicate and were weighted according to the inverse of the standard error of the mean. The program provides estimates of the SE and confidence limits for each parameter. Each parameter makes an independent contribution to the residual sum of square about the optimum solution. Statistical analyses were performed using Student's t-test.

Secretion studies

Two hundred and fifteen mg of neoplastic acinar cells and normal dispersed acini per flask were incubated in 5 ml of tissue culture medium for 3 periods of 60 min at 37°C under a constant O_2-CO_2 (95% - 5%) atmosphere (Larose *et al.*, 1981). *After each incubation period, the content of each flask was transferred into new flasks containing fresh oxygenated medium. Increasing concentrations of carbamylcholine ranging from 0.1 μM to 10 μM were added to the incubation medium at the beginning of the third period. The amylase in the incubation medium and associated with the cellular precipitate of the neoplastic and normal tissue was measured following the final incubation.

RESULTS

DNA and protein contents

Expressed in units of mg per *g* of tissue, both DNA and protein were measured in 33 normal and 26 neoplastic pancreatic homogenates. The DNA content was more than 3 times higher in the neoplastic (13.7 ± 0.5) than in the normal (4.3 ± 0.2) homogenates. However, the protein contents as measured in the normal (136 ± 4.1) and pancreatic adenocarcinoma (126.5 ± 3.0) were not statistically different.

Specific binding of tritiated QNB as a function of protein concentration

Figure 1 shows that a linear relationship exists between the concentration of protein and the amount of specific ^{3}H(-)QNB bound. For both normal and neoplastic tissues, the relationship between the specific binding of ^{3}H(-)QNB and the protein concentration per assay was linear between 20 and 175 μg/ml. Binding studies were performed at a protein concentration of 50 μg/ml.

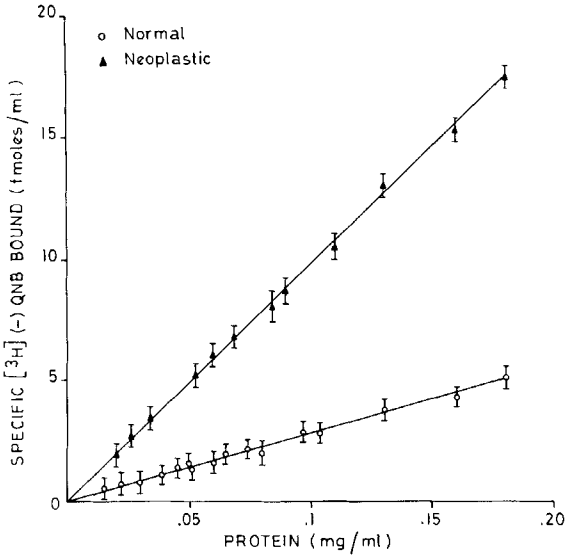

FIGURE 1 - Relationship between protein concentration and specific ^{3}H(-)QNB binding to homogenates prepared from normal and neoplastic pancreas. Various amounts of normal (○) and neoplastic (▲) pancreatic tissues were incubated for 120 min at 37°C in 5 ml of buffer (pH 7.4) containing 0.05 M KH_2PO_4, 0.05 M Na_2HPO_4, 0.005 M $MgCl_2$ with 0.25 nM ^{3}H(-)QNB. The data represent the average of 4 and 6 experiments for normal and neoplastic tissue, respectively. Correlation coefficients are 0.99 for neoplastic tissue (▲) and 0.98 for normal tissue (○).

Kinetics experiments

The kinetics properties of ^{3}H(-)QNB binding to the muscarinic receptors of both normal and neoplastic tissues are presented in Figure 2. At a concentration of

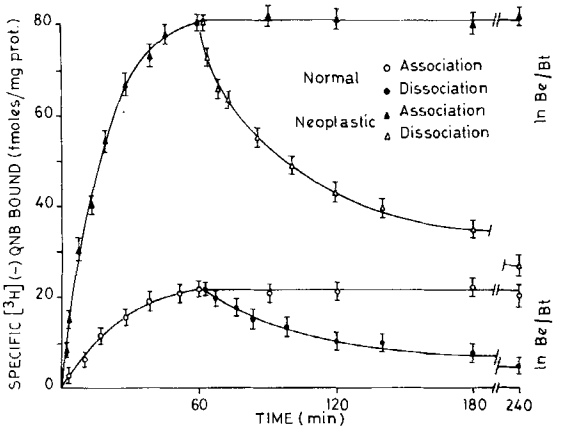

FIGURE 2 - Typical experiment showing association and dissociation of ^{3}H(-)QNB and the normal (○, ●) and neoplastic (▲, △) muscarinic receptors. The assays contained 0.2 nM of ^{3}H(-)QNB and were performed with 50 μg protein/ml. Dissociation was initiated by the addition of atropine sulfate 10^{-6}M. Each point represents the mean value of triplicate samples. ▲, ○: without atropine; △, ●: with atropine.

*The incubation buffer solution (pH 7.4) contained 24.1 mM Hepes, 98 mM NaCl, 6 mM KCl, 2.1 mM KH_2PO_4, 5mM sodium pyruvate, 5 mM sodium fluranate, 5 mM sodium glutamate, 11.4 mM glucose, 0.01% (u/v) trypsin inhibitor, 2.5% (u/v) amino acid mixture, 2% (u/v) glutamine, 1% (u/v) albumin, 0.5 mM $CaCl_2$, and .2 mM $MgCl_2$.

0.2 nM ^{3}H(-)QNB the association rate was much greater for the tumor with a t 1/2 of 8 min than for the normal pancreas with a t 1/2 of 25 min. The dissociation rate which was established by competition between the bound ^{3}H(-)QNB and an excess of atropine sulfate 10^{-6}M, was similar for both normal and neoplastic receptors as indicated by a t 1/2 of 128 min for both tissues. Analysis of the data according to Fields *et al.* (1978) shows an association rate constant (k+1) of $6.7 \pm 1.42 \times 10^8 M^{-1}min^{-1}$ for neoplastic pancreas and $0.68 \pm 0.1 \times 10^8 M^{-1}min^{-1}$ for normal pancreas. The dissociation rate constants (k-l) for normal pancreas were $5.41 \pm 0.41 \times 10^{-3}min^{-1}$ and $5.13 \pm 0.48 \times 10^{-3}min^{-1}$ for neoplastic tissue. These values represent $\bar{X} \pm$ SE for 6 normal pancreas and 7 pancreatic adenocarcinoma experiments.

Stereospecificity

The stereospecificity of the binding sites was determined by incubating homogenates of both tissues with various concentrations of levitimide (the inactive enantiomer of benzetimide) and dexetimide (the active enantiomer of benzetimide) in the presence of 0.2 nM ^{3}H(-)QNB at 37°C for 90 min. For both normal and neoplastic tissues, dexetimide 10^{-7}M displaced 90% of specific ^{3}H(-)QNB bound whereas levitimide at the same concentration displaced only 5% of the radiolabelled ligand. The IC_{50} for dexetimide is $4.0 \pm 0.8 \times 10^{-10}$M for normal and $2.0 \pm 0.6 \times 10^{-10}$M for neoplastic tissue.

Determination of the number of receptor sites

The muscarinic binding sites saturable in neoplastic and normal pancreas as indicated by the saturation isotherms (Fig. 3*a*). Analysis of non-specific binding showed a value of 25% for normal pancreas and 12% for neoplastic tissue measured at 1 nM ^{3}H(-)QNB. The linearity of Scatchard analysis of these data (Fig. 3*b*) is compatible with the concept of normal and neoplastic tissues possessing but one population of receptors.

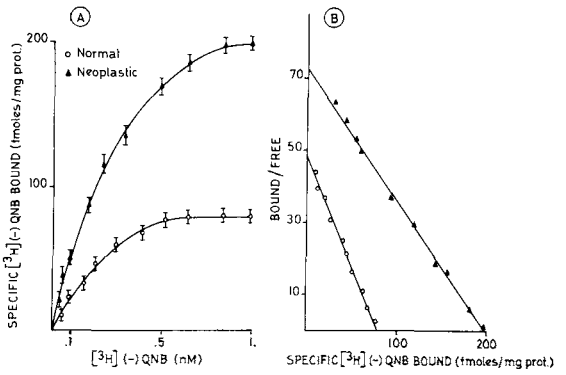

FIGURE 3 - (*a*) These saturation isotherm curves of ^{3}H(-)-QNB bindings to normal pancreas (○) and pancreatic adenocarcinoma (▲) are typical of 6 others. Samples containing 50 μg protein per ml of tissue were incubated at 37°C for 180 min with various ^{3}H(-)QNB concentrations ranging from 40 pM to 1 nM. (*b*) Scatchard analysis of the saturation isotherm curves presented in Figure 3*a*. Correlation coefficients are 0.98 for the normal (○) and 0.97 for the neoplastic tissue (▲). The receptor density was 81.87 ± 13.38 femtomole/mg protein in the normal and 200.36 ± 30.32 femtomole/mg protein for the neoplastic tissue. The apparent K_D values are $1.59 \pm 0.44 \times 10^{-10}$M for normal and $0.81 \pm 0.14 \times 10^{-10}$M for neoplastic tissue.

TABLE I - INHIBITION OF QNB BINDING BY ATROPINE AND CARBAMYLCHOLINE

	Normal pancreas	Pancreatic adenocarcinoma
A. Inhibition by atropine		
$IC_{50} \times 10^{-9}$M[1]	2.36 ± 1.27	6.15 ± 0.89
$K_i \times 10^{-9}$M[2]	0.63 ± 0.36	0.28 ± 0.03
N_H[3]	0.91	0.95
B. Inhibition by carbamylcholine		
$K_H \times 10^{-7}$M	17.2 ± 3.2	4.9 ± 0.8
$K_L \times 10^{-7}$M	9.09 ± 2.02	1.92 ± 0.45
%H	55	53
N_H	0.58	0.56

[1]The IC_{50} represents the concentration of the drug inhibiting 50% of the ^{3}H(-)QNB specifically bound at 0.2 nM.-[2]K_i represents the value of IC_{50} corrected according to the equation of Cheng and Prusoff.-[3]N_H is the Hill coefficient.

The apparent K_D calculated from the Scatchard plot were $1.59 \pm 0.44 \times 10^{-10}$M for the normal and $0.81 \pm 0.14 \times 10^{-120}$M for the neoplastic tissues. The maximum number of saturable binding sites (Bmax) was 81.8 ± 13.3 femtomoles/mg protein for the normal and 200.3 ± 30.3 femtomoles/mg protein for the neoplastic tissue.

Competition studies

Assays were performed by incubating homogenates with 0.2 nM ^{3}H(-)QNB for 90 min at 37°C in the presence of increasing concentrations of atropine sulfate (an antagonist) and carbamylcholine (an agonist). The results are the mean values of 8 experiments for normal and 10 experiments for neoplastic tissues. For the antagonist atropine (Table IA), the Hill coefficients are close to 1; similar K_i values for normal and neoplastic tissues were obtained by correcting the IC_{50}'s according to the equation of Cheng and Prusoff (1973).

Competition experiments performed with carbamylcholine (Table IB) showed Hill coefficients near 0.5. The percentage of high-affinity sites ranged in the neighborhood of 50% for both tissues. The dissociation constants for high (K_H) and low (K_L) affinity sites are higher for normal than for neoplastic tissue.

Secretion studies

As shown in Figure 4, 10^{-5}M carbamylcholine did not increase amylase release in the neoplastic tissue while the same concentration of carbamylcholine stimulated the release of 22% of the total content of normal pancreas. The basal level of amylase release from neoplastic tissue was 8.8 ± 0.1% and 4.1 ± 0.1% from normal pancreas.

DISCUSSION

Several reports indicate that acinar tumoral cells express functional receptors for several classes of pancreatic secretagogues but show quantitative and qualitative differences when they are compared with normal pancreas with regard to the content and secretory mechanisms of the secretory proteins (Warren *et al.*, 1981; Iwanij *et al.*, 1982*b*).

In this study we have analysed the functional characteristics of the muscarinic receptors in normal and neoplastic pancreas.

The number of binding sites on a mg per protein basis found in the normal tissue were similar to values that we had previously published (Dumont *et al.*,

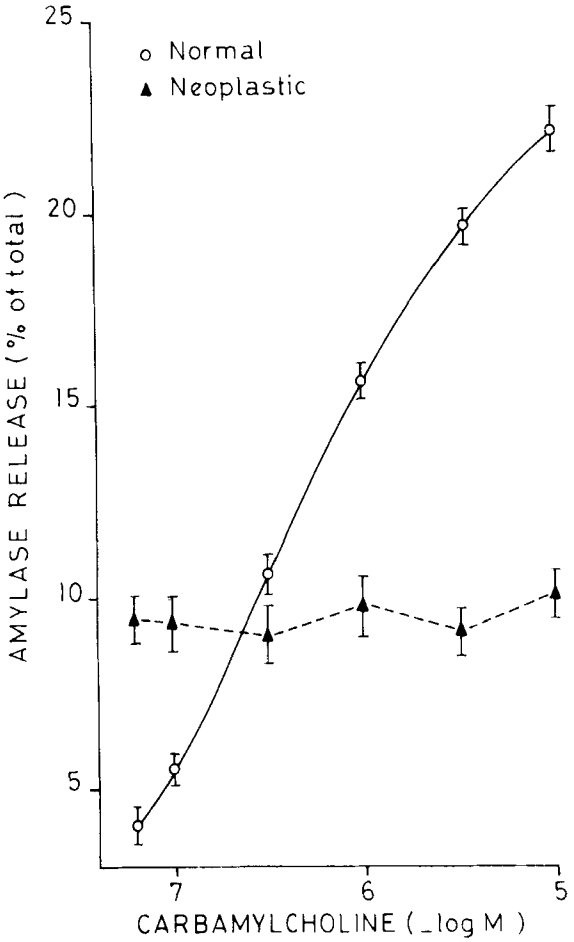

FIGURE 4 – Effect of carbamylcholine on amylase release from normal pancreatic (○) tissue and neoplastic (▲) tissue. This experiment is typical of 3 others. Normal and neoplastic tissues were incubated as indicated in "Material and Methods." In each experiment each assay was done in triplicate.

1981*a*). However, the receptor density of the neoplastic tissue is more than twice as high as in the normal pancreas. This observation could be explained by the absence of cholinergic innervation of the tumor, producing a supersensitization phenomenon (Anderson *et al.*, 1977). By performing kinetics experiments we have determined the association (k+1) and dissociation (k-1) rate constants using the equations previously described by Fields (1978). The association rate, which is 10 times higher in the neoplastic tissue, is similar to that observed for the binding of cardiac muscarinic receptors (Bernfeld *et al.*, 1955). The dissociation kinetics in normal and neoplastic tissues are closer to those of the brain (Yamamura *et al.*, 1974), bovine retina (Hruska *et al.*, 1978) and human putamen (Wastek *et al.*, 1978).

In order to evaluate the density and affinities of the different classes of agonist muscarinic binding sites,

TABLE II – COMPARISON BETWEEN FETAL AND NEOPLASTIC RAT PANCREAS

	Fetal pancreas[1] (21 days)	Pancreatic adenocarcinoma
$(k+1) \times 10^{8}M^{-1}min^{-1}$	2.01 ± 0.8	6.7 ± 1.42
$(k-1) \times 10^{-3}min^{-1}$	5.58 ± 0.26	5.13 ± 0.46
%L	50 ± 7	46 ± 5
$K_H \times 10^{-7}M$	6.6 ± 2.12	4.9 ± 0.8
$K_L \times 10^{-5}M$	1.45 ± 0.48	1.92 ± 0.45

[1]From Dumont *et al.* (1981*a*,*b*).

we have conducted competition assays using carbamylcholine as an agonist (Table IB) and atropine as an antagonist (Table IA) against the binding of ^{3}H(–)QNB. Atropine sulfate has a much higher affinity for the ^{3}H(–)QNB binding sites than has carbamylcholine. As observed in other tissues, carbamylcholine recognizes at least the presence of two sites on the receptor while the antagonist, atropine sulfate, recognizes only one. The K_i's found for atropine sulfate are similar for both tissues and are comparable to those found in many tissues (Fields *et al.*, 1978; Hruska *et al.*, 1978; Wastek *et al.*, 1978; Yamamura *et al.*, 1974). The high-affinity agonist binding sites are equally distributed with the low-affinity sites for the two tissues.

As can be seen from Figure 4, neoplastic tissue does not respond to cholinergic stimulation of carbamylcholine. The pancreas of the rat becomes responsive to cholinergic stimulation only 3 days after birth (Larose *et al.*, 1977). However, this lack of response recalls that of fetal rat pancreas which displays high levels of basal release of amylase as does the pancreatic neoplasm. Comparing the binding characteristics of muscarinic receptors from neoplastic tissue with those of fetal pancreas (Table II), we have found no significant difference either in association and dissociation rate constants or in the percentages of low- and high-affinity sites. The K_H and K_L values obtained from neoplastic tissues and from fetal pancreas of 21 days (Dumont *et al.*, 1981 *a*, *b*) did not differ. The results presented here support the hypothesis that neoplastic tissue of the rat may, in part, be related to a stage of normal pancreatic development. The similar profiles of the secretory proteins from both the developing pancreas and the exocrine neoplastic cells of the rat further substantiate this hypothesis (Iwanij *et al.*, 1982*a*).

In conclusion, our results demonstrate that neoplasms of the pancreas, as compared to normal tissue, are characterized by an increase in the number of muscarinic receptor sites and by a loss of the stimulus secretion coupling response.

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