J.Sci.Soc.Thailand, 16 (1990) 177–185

COLLAGENASE DIMINSHES MUSCARINIC RECEPTOR BINDING IN PARTIALLY PURIFIED BOVINE ADRENAL CHROMAFFIN CELLS

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(Received 12 December 1990)

ABSTRACT

The presence of muscarinic receptors in partially purified intact bovine chromaffin cells was investigated by binding assay using the antagonistic-specific high affinity ligand, [^{3}H] -quinuclidinyl benzilate [^{3}H)-QNB]. The kinetics of [^{3}H]-QNB binding to mechanically isolated intact bovine chromaffin cells was examined. The specific [^{3}H]–QNB binding to mechanically isolated intact bovine chromaffin cells that were treated with 0.1% or higher collagenase concentrations was decreased. Scatchard analysis combined with computer program "LIGAND" was used for characterization of the specific [^{3}H]–QNB binding. It was found that partially purified, mechanically and enzymatically (0.15% callagenase) isolated bovine chromaffin cells each had a single high affinity class of binding sites with a K_d of 0.12 ± 0.02 nM and 0.14 ± 0.06 nM and a B_{max} of 3.1 ± 0.6 fmol/10^3 cells and 0.021 ± 0.0007 fmol/10^3 cells, respectively. These data demonstrated that 0.15% collagenase treatment decreases the receptor density (B_{max}) of muscarinic receptors in partially purified bovine chromaffin cells while the K_d remains unchanged.

INTRODUCTION

In 1974 Livett and his colleagues[1, 2] explored the possibility of using collagenase to disperse the adrenal gland, as this technique had been successfully used by Berry and Friend[3] to obtain a high yield of viable hepatocytes for functional studies. It was observed that within 20 min of retrograde perfusion with collagenase the adrenal gland became flaccid and subsequently could be easily dissociated into single cells by dicing and incubation in collagenase. A yield of 100 million cells was obtained from 3 bovine adrenal medulla.[1, 2, 4] Perlman and his group reported similar success using collagenase to digest the medulla from guinea-pig adrenal gland.[5]

Collagenase treated slices of bovine superior cervical ganglion, rat cortex, and rat corpus striatal released significantly less acetycholine (ACh) in response to potassium-induced depolarization than did normal tissue slices.[6-8] Almazan *et al.*[9] showed that the nicotinic cholinoceptor-mediated catecholamine release in 3 day-old cultured bovine chromaffin cells prepared by collagenase dispersion was significantly reduced with respect to control release.

Recently, Tokuno and Tomita failed to demonstrate a clean electrical response to catecholamines in smooth muscle cells dispersed from the guinea pig taenia caeci. They considered the possibility that collagenase enzyme treatments used for cell dispersion might have deleterious effects on the receptors.[10] We have performed a study on the effect of collagenase on muscarinic receptor binding in intact bovine adrenomedullary chromaffin cells.

MATERIALS AND METHODS

Preparation of cells

Bovine adrenomedullary chromaffin cells were prepared according to the method described by Livett[11] with the following modifications. After the fat was removed from the gland the adrenal lumbar vein was canulated and perfused with warm (37°C) buffer, then perfused with warm 0.15% collagenase (Sigma) solution. Further incubation of the glands were carried on at 37°C in a waterbath. At the end of the incubation the medulla could be very readily stripped off from the cortical tissue. The medulla tissue was finely minced with scissors. The minced tissue was then further incubated with 0.15% collagenase solution. Cell suspension was filtered successively through a sandwich of filters (Spectrum, 74 and 41 μm). The suspension was washed twice by centrifugation in buffer. Living cell number was counted by using the trypan blue exclusion method and was found to be greater than 95%. The purity of cells was determined by the neutral red inclusion method. More than 90% of living cells were found to be chromaffin cells. Mechanically isolated chromaffin cells were prepared as described above except that the enzymatic step was omitted.

Collagenase treatments

Chromaffin cells prepared by the mechanical method were incubated with 6 different collagenase concentrations (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3%) for 60 min at 37°C in a waterbath. The collagenase treated chromaffin cells were washed twice by centrifugation in buffer prior subject to radioreceptor binding assay.

Muscarinic radioreceptor binding assay

The specific binding of muscarinic receptors was determined by a modification of the glass fiber filter binding assay of Yamamura and Snyder[12] using [^{3}H]-QNB at a saturating concentration. Isolated chromaffin cells prepared either by enzymatic or mechanic methods were incubated with 0.0046 to 2.4 nM [^{3}H]-QNB (Amersham, specific activity 42 Ci/mmol) in 1.0 ml of ice cold 0.05 M Na/K phosphate buffer, pH 7.4 for 60 min at 37°C. Nonspecific binding was characterized by using 10 μM atropine sulfate (Sigma). The incubation was stopped by rapid filtering onto Whatman GF/C glass fiber filters (prewashed with ice-cold buffer) positioned over a vacuum. The filters were washed three times under vacuum with 3 to 5 ml of ice-cold buffer. The filters were then placed into scintillation vials, and 3 ml of scintillation fluid was added and the samples were counted at least 8-12 hours later. The radioactivity was determined by liquid scintillation counter (Beckman LS 1901). In the logarithmic

transformation of kinetic data, the linear least squares regression was used to estimate K_{ob} (observed association rate constant) and k_{-1} (dissociation rate constant). Saturation curves were initially analyzed by the method of Scatchard[13] and then analyzed by using a nonlinear least-squares regression analysis program, known as LIGAND, described by Munson and Rodbard.[14]

RESULTS

Kinetics studies of [^{3}H]-QNB binding

The kinetics of [^{3}H]-QNB binding to partially purified, mechanically isolated intact bovine adrenal chromaffin cells was examined. The association rate showed that specific [^{3}H]-QNB binding to the intact cells was slow, reaching equilibrium by 60 min incubation at 37°C and remaining stable for more than 2 hours (Fig. 1). Replotting of association data as $\ln (B_e/B_e - B)$ (B_e = [^{3}H]-QNB bound at equilibrium, B = [^{3}H]-QNB bound at time) vs time, the K_{ob} (an observed association rate constant) of 0.04 min^{-1} was obtained (Fig. 1-inset). Then, by the relationship $k_{ob} - k_{-1} = k_{+1}.L_T$ (k_{+1}=an association rate constant; L_T=the total ligand concentration), the k_{+1} of intact bovine chromaffin cells was calculated to be 1.2×10^8 M^{-1} min^{-1}. The dissociation experiment was also performed and the data were calculated by plotting $\ln (B/B_o)$ (B = bound at time t; B_o = bound at 0 min) vs time (Fig. 2). The dissociation rate constant (k_{-1}) was 1.28×10^{-2} min^{-1}. The dissociation of [^{3}H]-QNB receptor complex at 37°C occurred slowly with a half time (t 1/2) of 54 min. In addition, the dissociation equilibrium constant (K_d) can be derived from dissociation and association rate constant (k_{-1}/k_{+1}). The K_d value was calculated to be 0.106 nM.

Effects of collagenase on specific [^{3}H]-QNB binding

The specific [^{3}H]-QNB binding of mechanically isolated bovine chromaffin cells that were pretreated with 0.1%, 0.15%, 0.2%, 0.25% and 0.3% collagenase was decreased when compared to the specific binding of the cells that were exposed to 0.05% collagenase (Fig. 3).

The data analyzed by Scatchard plot indicated that partially purified, mechanically and enzymatically (0.15% collagenase) isolated cells had a single class of binding sites. The weighted best-fit curves of the saturation isotherm of [^{3}H]-QNB binding to mechanically and enzymatically (0.15% collagenase) isolated bovine chromaffin cells from one experiment conducted in triplicate determinations are shown in Figs. 4 and 5. The K_d and B_{max} values for [^{3}H]-QNB in the saturation studies gathered from three individual experiments were 0.12 ± 0.02 nM and 3.1 ± 0.6 fmol/10^3 cells for mechanically isolated cells and 0.14 ± 0.06 nM and 0.02 ± 0.0007 fmol/10^3 cells for enzymatically (0.15% collagenase) isolated cells, respectively.

The B_{max} of the [^{3}H]-QNB bound muscarinic receptor obtained from the partially purified, enzymatically isolated group was significantly decreased ($p < 0.005$) when compared to that of the partially purified, mechanically isolated group. However, there was no significant difference in the K_d values of both groups.

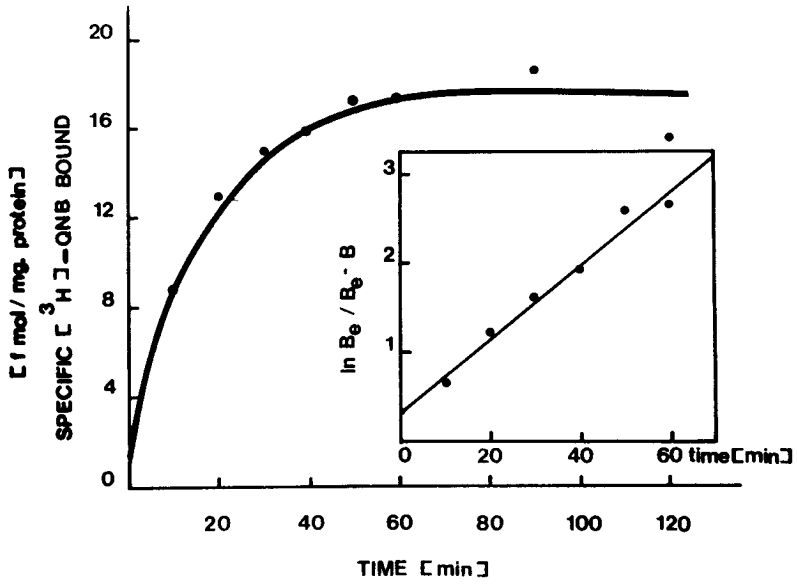

Fig. 1. Time course of specific [^{3}H]–QNB binding to partially purified, mechanically isolated intact bovine adrenal chromaffin cells. Data are mean of three individual experiments conducted in triplicated determinations. Inset shows the analysis of initial time points. B, amount of [^{3}H]–QNB bound at indicated time; Be, amount of [^{3}H]–QNB bound at equilibrium.

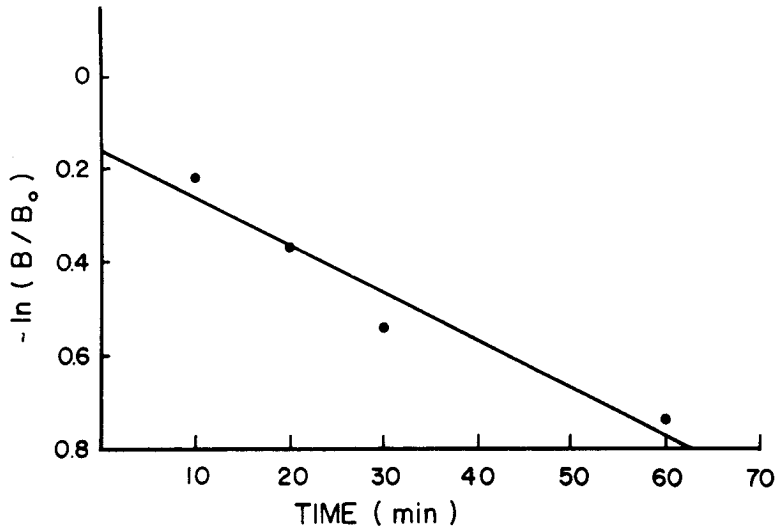

Fig. 2. Dissociation of specific [^{3}H]–QNB binding to partially purified, mechanically isolated intact bovine adrenal chromaffin cells. Data are a mean of three individual experiments conducted in triplicated determinations. B, amount of [^{3}H]–QNB bound at the indicated time; Bo, amount bound at t=0.

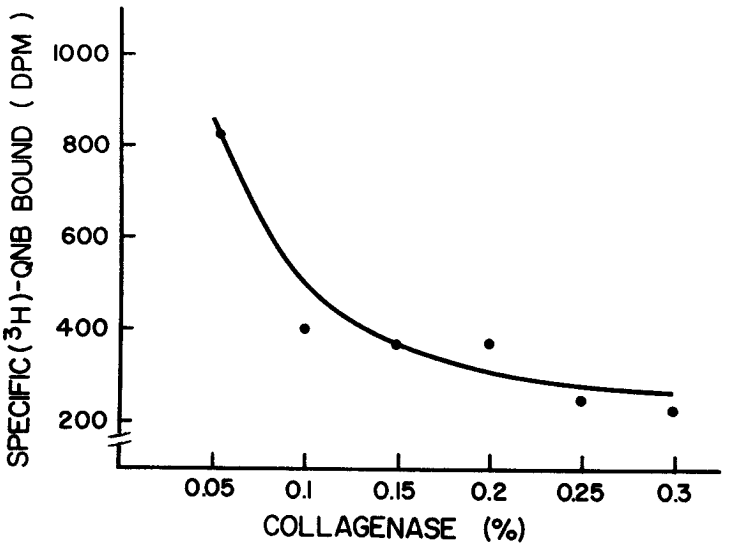

Fig. 3. Effect of various collagenase concentrations treatment on specific [^{3}H]–QNB binding in mechanically isolated intact bovine adrenal chromaffin cells. Each point is a mean of two independent experiments conducted in triplicate determinations.

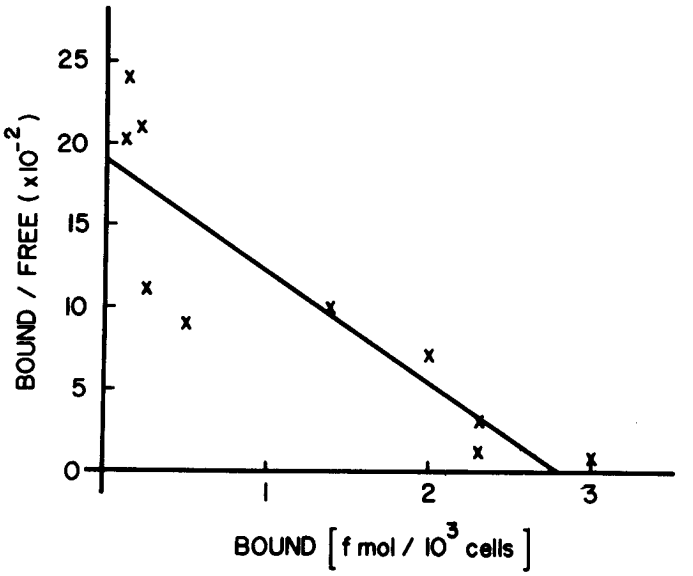

Fig. 4. Scatchard analysis of [^{3}H]–QNB binding to partially purified, mechanically isolated intact bovine adrenal chromaffin cells. Each point which has been obtained from triplicate determinations represent the specific binding of [^{3}H]–QNB. The apparent dissociation equilibrium constant (K_d) and the maximal number of binding sites (B_{max}) values were 0.09 nM and 2.7 f mol/10^3 cells, respectively.

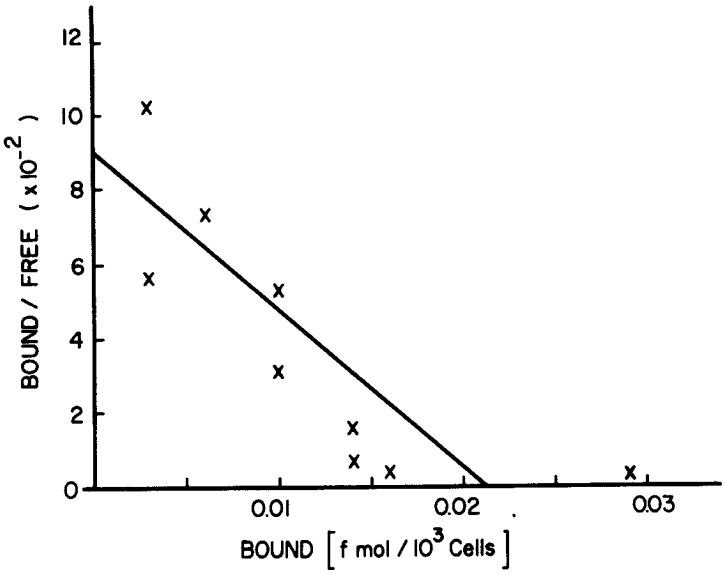

Fig. 5. Scatchard analysis of [^{3}H]–QNB binding to partially purified, enzymatically (0.15% collagenase) isolated intact bovine adrenal chromaffin cells. Each point which has been obtained from triplicate determinations represent the specific binding of [^{3}H]–QNB. The apparent dissociation equilibrium constant (K_d) and the maximal number of binding sites (B_{max} values were 0.08 nM and 0.021 f mol/10^3 cells, respectively.

DISCUSSION

The present study provides evidence that suggests the existence of the muscarinic cholinergic receptor in intact bovine adrenal chromaffin cells. The [^{3}H]-QNB binding to chromaffin cells satisfied the established criteria for receptor characterization. The specific binding of [^{3}H]-QNB continued to increase linearly with increasing amounts of intact cells (data not shown). This demonstrated that the kinetic condition[3] for binding analysis are suitable in this study. The bimolecular rate constant for [^{3}H]-QNB receptor association, K_{+1}, was 1.2×10^8 $M^{-1}min^{-1}$. The rate constant for dissociation at 37°C, K_{-1}, was 1.28×10^{-2} min^{-1}. The dissociation of [^{3}H]-QNB receptor complex at 37°C occurred slowly with a half time (t1/2) of 54 min. This extremely slow dissociation constitutes a major advantage[15] of using [^{3}H]−QNB in the muscarinic cholinergic receptor binding assay. The kinetic dissociation constant (K_d) for [^{3}H]-QNB (K_{-1}/K_{+1}) was calculated to be 0.106 nM. Binding studies of muscarinic antagonists to intact cells are well described by the binding to a single class of sites with affinities similar to those seen in homogenates chick cardiac cells.[15] The binding characteristics of [^{3}H]-QNB to intact bovine adrenal chromaffin cell in the present study were quite similar to those of the muscarinic receptors in homogenates of rat brain[16] and of longitudinal muscle of the guinea pig ileum.[12]

Importantly, the present study clearly showed that collagenase at 0.1% or higher concentrations decreased the specific binding of [^{3}H]-QNB to muscarinic receptors in intact bovine adrenal chromaffin cells. Scatchard analysis has been performed to determine whether this reduction in [^{3}H]-QNB binding to intact bovine chromaffin cells was the result of a decrease in the number of muscarinic receptor sites or a change in the affinity of these receptors for the ligand. It is evident from present data that collagenase digestion results in significant decrease in the number of muscarinic receptor sites in intact bovine adrenal chromaffin cells. This decrease suggests an actual lose of muscarinic receptor sites during the collagenase digestion step. However, the affinity ($1/K_d$) of muscarinic receptors in intact bovine chromaffin cells was not changed. This suggested that collagenase may not cause conformational change in the muscarinic receptors in intact bovine chromaffin cells. Consistent with our finding, Sharma and Banerjee[17] reported that administration of 6-hydroxydopamine to the particulate fractions obtained from heart decreased the specific binding of [^{3}H]-QNB to muscarinic cholinergic receptors to about 70% of control. The presence of muscarinic receptors in 5-day dissociated cell cultures of rat anterior pituitary glands was detected by atropine-sensitive binding of [^{3}H]-QNB. The low concentrations of receptors in 5-day cultures compared to the initial minced and undispersed tissue remaining on the cheese cloth may be due to cell surface damage during enzymic dispersion.[18]

Sgaragli *et al.*[8] preincubated intact striatal, cortical P_2 fractions and membranes derived of these tissues with 1 mg purified collagenase and subsequently subjected to gel electrophoresis. In all preparations the enzyme consistently caused the disappearance of two distinct high molecular weight protein bands. The two proteins that are destroyed by collagenase are involved in the inhibition of calcium uptake. Recently, the effects of collagenase on the membrane response to catecholamines were studied with intracellular microelectrodes in the guinea-pig

taenia caeci and the main pulmonary artery by Tokuno and Tomita.[10] They showed that in the taenia, the inhibitory actions of adrenaline mediated through α and β -adrenoceptors were both nearly abolished by two 5-min treatments with 0.005% collagenase. In the pulmonary artery, the depolarizing action of noradrenaline mediated through β -adrenoceptors was markedly reduced by three 5-min treatments with 0.01% collagenase.

In summary, the present work indicates that the concentration of 0.15% collagenase which was routinely used in the bovine adrenal medulla isolation process diminished the receptor density (B_{max}) of muscarinic receptors in intact bovine adrenal chromaffin cells. The enzyme treatment had no significant effect on the dissociation equilibrium constant (K_d). The results of this study may explain why autologous transplants of the adrenal medulla are not uniformly effective as a treatment for Parkinson's disease.[19]

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บทคัดย่อ

ได้ทำการศึกษา muscarinic receptor บน bovine chromaffin cells ด้วยวิธี receptor binding assay โดยใช้ [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) เป็น radioligand ทำการทดลองหาค่า kinetics ของ specific [^{3}H]-QNB binding ที่มีต่อ bovine chromaffin cells ที่แยกโดยวิธี mechanic และเพื่อดูผลของเอนไซม์ collagenase ต่อการจับของ [^{3}H]-QNB กับ muscarinic receptors ผลจากการทดลอง พบว่าเอนไซม์ collagenase ที่ความเข้มข้นมากกว่า 0.1% ขึ้นไปมีผลทำให้ specific[^{3}H]-QNB binding ของเซลล์ที่แยกด้วยวิธี mechanic มีค่าลดลง และเมื่อใช้วิธี Scatchard ร่วมกับการใช้คอมพิวเตอร์โปรแกรม "LIGAND" ในการศึกษาถึง specific [^{3}H]-QNB binding พบว่า muscarinic receptor ของ bovine chromaffin cells ที่แยกโดยวิธี mechanic และที่ใช้เอนไซม์ collagenase (0.15%) มีค่า K_d = 0.12 ± 0.02 nM และ 0.14 ± 0.06 nM และค่า B_{max} = 3.1 ± 0.6 mol/10^3 cells และ 0.021 ± 0.0007 f mol/10^3 cells ตามลำดับ จากผลการทดลองสรุปได้ว่าเอนไซม์ collagenase ที่ความเข้มข้น 0.15% มีผลลดปริมาณ muscarinic receptor (B_{max}) แต่ไม่มีผลต่อค่า affinity ($1/K_d$) ของ muscarinic receptor ใน bovine chromaffin cells