

Characterization of muscarinic acetylcholine receptors on the rat pancreatic gastrin-producing cell line B6 RIN

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The mechanisms of cholinergic stimulation of gastrin cells were studied in the rat pancreatic cell line B6 RIN. Carbachol induced an increase in intracellular Ca^{2+} and stimulated gastrin release in a dose-dependent manner over the range 10^{-5} – 10^{-3} M. These effects were completely abolished by atropine, suggesting the implication of muscarinic cholinergic receptors. The binding properties of these receptors were investigated. [*N*-Methyl-³H]scopolamine ([³H]NMS) binding on cell homogenates was time-dependent, saturable and consistent with a single high-affinity binding class ($K_d = 39.5$ pM, and $B_{\text{max}} = 7.9$ fmol/mg DNA). Carbachol competitively inhibited [³H]NMS binding. The potency of inhibition of [³H]NMS binding by subtype selective antagonists was hexahydrodifenidol > pirenzepine > AF-DX 116. These results suggest the M_3 muscarinic receptors may be involved in the carbachol-induced gastrin release from B6 RIN cells.

Muscarinic acetylcholine receptor; Cytosolic free calcium; Carbachol; Gastrin release; B6 RIN cell

1. INTRODUCTION

Cholinergic agonists are strong stimulants of gastrin release in the rat and dog [1–3], presumably through binding to specific receptors on gastrin G-cells. Such receptors have not been characterized so far on native G-cells because cell enrichment through the currently available methods does not reach homogeneity. To overcome this problem, we used a cell line derived from a radiation-induced insulin-producing rat tumor [4]. The B6 RIN cell line: (i) expresses the gastrin gene at high levels while the insulin, somatostatin and cholecystokinin genes are not expressed [5]; and (ii) secretes fully processed gastrin [5]. Although some reports suggest that muscarinic receptors are expressed by RIN cell lines [6], no data are available on their binding characteristics. These were investigated in the present study, as well as the functional relationship between the occupation of muscarinic receptors and gastrin release.

2. MATERIALS AND METHODS

2.1. Materials

[³H]NMS (74 Ci/mmol) was purchased from Amersham (Les Ulis, France). Atropine sulfate and carbachol were from Sigma Chemicals

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Abbreviations: [³H]NMS, [*N*-methyl-³H]scopolamine methyl chloride; AF-DX 116, 11-([12-[(diethylamino)methyl]-1-piperidinyl]-acetyl)-5,11-dihydro-6H-pyridol [2,3-b][1,4]benzodiazepine-6-one

(St. Louis, USA). Pirenzepine (Gastrozepin) and AF-DX 116 were kindly provided by Dr. Waelbroeck (Brussels, Belgium). Hexahydrodifenidol was obtained from Dr. Lambrecht (Frankfurt, FRG).

2.2. Cell culture

B6 RIN cells were grown in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (PBS Organics, Illkirch, France), 2 mM glutamine and antibiotics (100 IU/ml penicillin + 50 µg/ml streptomycin) in a 5% CO_2 /95% air incubator at 37°C. Cells were routinely transferred every 3 days in 4 vols. of fresh medium.

2.3. Gastrin release

Confluent cells (4×10^6 cells/35 mm well) were incubated in a total volume of 2 ml RPMI medium containing 0.5% bovine serum albumin (BSA) and the indicated effectors for 2 h at 37°C. The incubation medium was collected, and cells were disrupted in boiling water and centrifuged. Immunoreactive gastrin was measured in supernatants and in cell extracts with a radioimmunoassay previously described in a study on gastrin in rats [7].

2.4. Binding studies

Cells were harvested from culture flasks with trypsin/EDTA (0.05%/0.02%) in Hanks medium, lysed in hypotonic 1 mM NaHCO_3 (pH 7.0) and quickly frozen in liquid nitrogen. After thawing, the lysate was homogenized in 20 mM Tris-HCl buffer (pH 7.5) enriched with 0.25 M sucrose and directly used for binding studies. Homogenates (120–150 µg protein) were incubated for 2 h at 25°C in 1.2 ml of incubation buffer (75 mM sodium phosphate buffer, 3 mM MgCl_2 , 1.5% BSA, pH 7.4), and the indicated concentrations of [³H]NMS, carbachol or antagonists. The reaction was stopped by rapid filtration on GF/C glass-fiber filters (Whatman, Maidstone, UK), followed by 4 rinsing cycles with 2 ml of ice-cold 50 mM phosphate buffer (pH 7.4). The filters were dried and the radioactivity was counted by liquid scintillation. Non-specific binding was defined as binding in the presence of 1 µM atropine. Competition curves were analyzed by computer fitting to a model of one or two classes of binding sites using the LIGAND program [8]. The dissociation constants

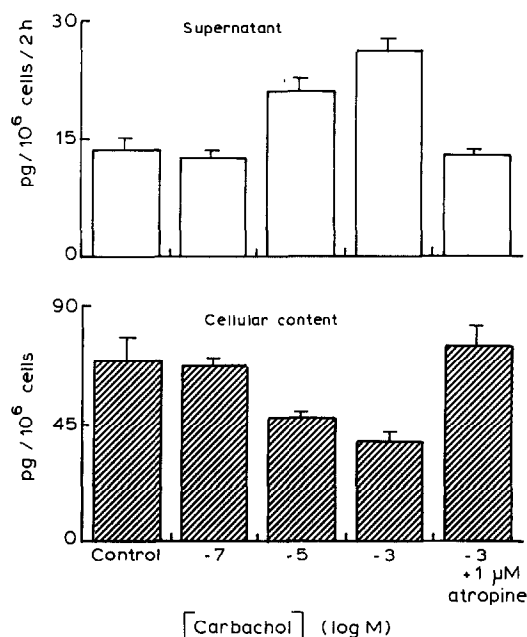


Fig. 1. Effects of carbachol on gastrin release (upper panel) and on gastrin intracellular contents (lower panel). Cells were incubated for 2 h at 37°C with the indicated concentrations of carbachol or with 1 mM carbachol plus 1 μM atropine. The results are the mean ± SEM of 8 separate experiments. Asterisks indicate statistically significant variations ($P < 0.05$) from control values.

of the analogues tested were corrected according to the method of Cheng and Prusoff [9]. DNA was determined using the method of Labarca and Paigen [10].

2.5. Determination of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured as described in [6]. Briefly, cells were incubated for 30 min at 37°C in RPMI medium containing 1% fetal calf serum, 10 mM HEPES and 1 μM fura2/AM under continuous stirring. After this loading period, cells were washed and resuspended in a modified Krebs Ringer bicarbonate buffer. The fluorescence signal of fura2 was recorded with excitation and emission at 340 and 510 nm, respectively.

3. RESULTS

Carbachol produced a dose-dependent increase in gastrin release from B6 RIN cells over the range 10^{-5} – 10^{-3} M (Fig. 1, upper panel). Maximal gastrin release was observed with 1 mM carbachol (25.7 ± 1.5 pg/10⁶ cells/2 h compared to 13.7 ± 1.3 pg/10⁶ cells/2 h basal release, $P < 0.05$, $n = 8$). Carbachol-stimulated gastrin release was linked to a decrease in intracellular gastrin content (Fig. 1, lower panel), from 70.0 ± 7.8 pg/10⁶ cells in control cells to 38.1 ± 3.3 pg/10⁶ cells in 1 mM carbachol-pretreated cells ($P < 0.05$, $n = 8$). Incubation of the cells in the presence of the muscarinic antagonist atropine did not alter basal gastrin release but completely inhibited carbachol-stimulated gastrin release.

Muscarinic cholinergic receptors on B6 RIN cell homogenates were characterized using the reversible cholinergic antagonist [³H]NMS. [³H]NMS binding was measured at 25°C for 2 h, which corresponds to equilibrium binding conditions for all tested concentrations of [³H]NMS (not shown). Fig. 2, left, shows that binding of [³H]NMS in the presence of 1 μM atropine was linearly related to tracer concentration. Specific binding, determined after subtraction of binding in the presence of atropine, was saturable, a plateau being achieved for a tracer concentration of 200 pM. Scatchard analysis of the specific binding curve (Fig. 2, right) provided a mean equilibrium dissociation constant (K_d) of 39.5 pM and a maximal binding level (B_{max}) of 7.9 fmol/mg DNA. The linear nature of the Scatchard plot ($r = 0.966$) indicates that binding of the antagonist is to a single class of high affinity sites. Cholinergic agonist interaction with B6 RIN cell muscarinic receptors was also studied using [³H]NMS binding (Fig. 3). Carbachol competitively inhibited binding of [³H]NMS. Computer analysis of the displacement curve suggested high ($K_d = 2.1$ μM) and low ($K_d = 16.2$ μM) affinity sites for carbachol, that applied to 26 and 74% of the receptor population, respectively.

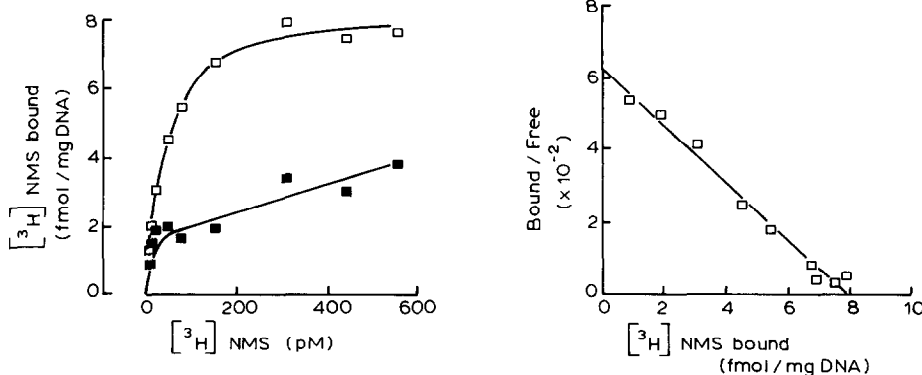


Fig. 2. Binding of [³H]NMS to B6 RIN cell homogenates. (Left) Experimental data. Homogenates were incubated for 2 h at 25°C with indicated concentrations of [³H]NMS either with or without 1 μM atropine. □, specific binding; ■, non-specific binding. The results are the means of 3 experiments performed in duplicate. (Right) Scatchard analysis of specific binding curve shown on the left.

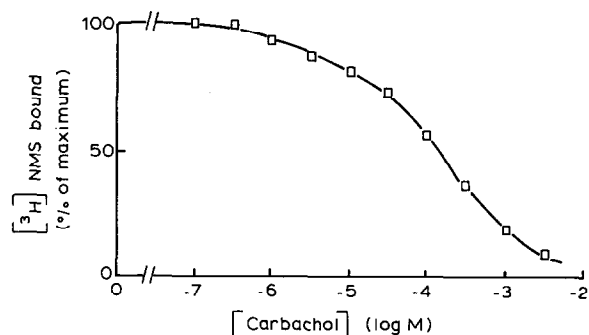


Fig. 3. Carbachol inhibition of [^3H]NMS binding to B6 RIN cell homogenates. Homogenates were incubated for 2 h at 25°C in the presence of 150 pM [^3H]NMS and of increasing concentrations of carbachol. The data are expressed as the percentage of [^3H]NMS specifically bound (means of 3 experiments performed in duplicate).

Competition studies of [^3H]NMS binding with M_1 , M_2 and M_3 muscarinic antagonists yielded the following order of potency: hexahydrodifenidol ($K_i = 23.6$ nM) > pirenzepine ($K_i = 166.3$ nM) > AF-DX 116 ($K_i = 1431$ nM) (Fig. 4), consistent with the presence of M_3 subtype receptors on B6 RIN cells.

Carbachol caused a biphasic increase in $[\text{Ca}^{2+}]_i$, which peaked promptly after addition of the secretagogue and then returned to a lower plateau value. This effect was dose-dependent over the range $10^{-5} - 10^{-3}$ M, and completely abolished by the addition of 100 μM atropine (Fig. 5).

4. DISCUSSION

Only one study, based on the isolated rat stomach preparation, bore on the muscarinic receptor subtypes involved in the cholinergic stimulation of gastrin release [11]. High affinity receptors were indirectly evidenced, and found to be sensitive to the M_1 antagonist pirenzepine, with an apparent inhibition constant of 2 nM. However, other studies have shown that

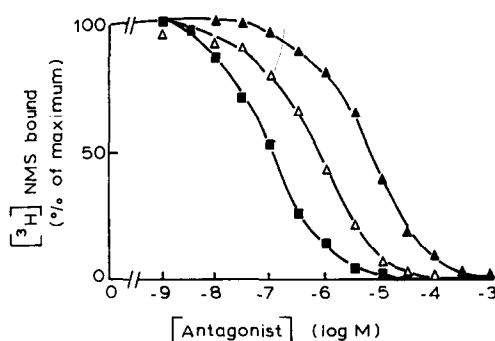


Fig. 4. Inhibition of [^3H]NMS binding to B6 RIN cell homogenates by pirenzepine (Δ), AF-DX 116 (\blacktriangle) and hexahydrodifenidol (\blacksquare). Homogenates were incubated for 2 h at 25°C in the presence of 150 pM [^3H]NMS and of increasing concentrations of antagonists. The data are expressed as in Fig. 3 and are the means of 3 experiments performed in duplicate.

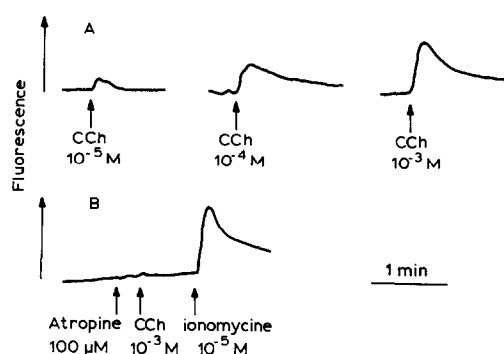


Fig. 5. Effects of carbachol on cytosolic free calcium in fura2-loaded B6 RIN cells. (A) Dose-dependent effect of carbachol. (B) Inhibition of carbachol-induced calcium increase by 100 μM atropine. Recordings are representative of 3 separate experiments.

cholinergic stimulation of gastrin release in rats may result from inhibition of the G-cell restraint by antral somatostatin cells [12]. Conclusions drawn from pharmacological experiments in the isolated rat stomach may thus fall short of specifically applying to the presumptive muscarinic receptors on G-cells.

Since (i) 5 subtypes of muscarinic receptors were recently cloned [13], and (ii) various antagonists were developed, a reappraisal of the muscarinic receptor subtype(s) mediating the response of G-cells to cholinergic agents was required. The B6 RIN cell line was used here. Carbachol increased gastrin release from these cells, and correlatively reduced the cellular gastrin contents, suggesting that it did not increase gastrin synthesis to any measurable extent in the present short term experiments. Carbachol induced a definite increase of cytosolic free calcium. Gastrin release, depletion of intracellular gastrin and rise of calcium were all abolished by atropine.

Pharmacological characterization of the muscarinic receptors with specific antagonists of receptor subtypes showed that the lowest K_i value of [^3H]NMS binding was obtained for the M_3 antagonist hexahydrodifenidol, the inhibition constant being 10 times lower than that of the M_1 antagonist pirenzepine, and 100-fold lower than that of the M_2 antagonist AF-DX 116. This order of potency is similar to that observed with the muscarinic antagonists pirenzepine, AF-DX 116 and hexahydrodifenidol (M_3 antagonist) in *Xenopus* oocytes and in NG108-15 neuroblastoma-glioma hybrid cells transfected with cDNA encoding the muscarinic M_3 subtype receptor [14,15]. Similar results were also reported using 4-DAMP as M_3 subtype receptor antagonist in SH-SY5Y neuroblastoma cells that are thought to possess M_3 muscarinic receptors [5]. Taken together, these data suggest the presence of M_3 muscarinic receptors on B6 RIN cells.

Using RNA blot hybridization analysis with specific probes, M_3 receptors have been demonstrated in

cerebral cortex [17,18], lachrymal and parotid glands [19], and intestinal smooth muscle tissue [19]. Our study demonstrates for the first time that muscarinic M₃ subtype receptors are also localized on an endocrine cell line, thus extending the distribution spectrum of M₃ subtype receptors.

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