BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1998 Nov; 19 (6): 523 – 526

Development of muscarinic m_3 and m_4 receptor antibodies with pharmacological activities[1]

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KEY WORDS muscarinic receptors; ileum; thoracic aorta; cerebral cortex; myocardium; salivary glands; radioligand assay; antibodies

AIM: To investigate the feasibility of developing subtype-selective anti-receptor antibodies with pharmacological activities for the study of subtypes of receptors. **METHODS**: New Zealand white rabbits were immunized with synthesized subtype-selective peptide segments of m_3 and m_4 receptors to develop antibodies. The effects of the antibodies on ligand-binding to muscarinic receptors were studied by competitive radioligand assay. The effects of the prepared antibodies on the contraction or relaxation activity of ACh in isolated rat ilea and aortic rings were studied. **RESULTS**: Antibodies against synthesized m_3 and m_4 receptor subtype-selective peptides were successfully prepared. Both antibodies inhibited [^{3}H]QNB binding to muscarinic receptors with different maximal inhibitions which may be the proportions of m_3 or m_4 subtypes among the total muscarinic receptors in the tissues. The maximal inhibitory rates in rat cerebral cortex, myocardium, and salivary glands were 12.1 % ± 2.1 %, 15.7 % ± 1.1 %, and 63.6 % ± 2.8 % for m_3 antibodies, whereas 28 % ± 6 %, 19.3 % ± 2.6 %, and 1.6 % ± 1.4 % for m_4 antibodies respectively. The m_3 antibodies inhibited the contraction activity of ACh in isolated rat ilea and the relaxation activity of ACh in isolated rat aortic rings. **CONCLUSION**: It is feasible to develop subtype-selective anti-receptor antibodies as new tools in the study of the functions of m_3 and m_4 subtypes of muscarinic receptors.

Most receptors are lacking in full subtype-selective ligands[1]. By synthesizing a peptide specific for one subtype of receptor and developing antibody against the peptide, we can obtain an anti-receptor antibody which subtype-selectively binds to the subtype of receptor. Anti-receptor antibodies have been used to study the distribution of receptor subtypes by immunoprecipitation, immunohistochemistry, and immunoblotting[2-4]. Compared with subtype-selective ligands, subtype-selective anti-receptor antibodies are not difficult to obtain and, therefore, becoming important in the study of subtypes of receptors.

The problem is to develop a substitute tool for subtype-selective ligands for the study of the functions of individual subtypes of receptors. Whether subtype-selective antibodies can play the role depends on whether they possess pharmacological activities like agonists or antagonists. Anti-receptor antibodies may be pharmacologically active[5,6]. In this study, we attempted to develop subtype-selective anti-m_3 or m_4 receptor antibodies with pharmacological activities.

MATERIALS AND METHODS

Materials New Zealand white rabbits (weighing 2.8 kg ± *s* 0.3 kg, Certificate № 94A37) and SD rats (weighing 235 g ± *s* 15 g, Certificate № 95A06) were purchased from Experimental Animal Center of Sun Yat-sen University of Medical Sciences. Amino acids for synthesis of peptides, Sigma. [^{3}H]QNB, 1554 TBq·mol^{-1}, Amersham Radio-chemical Center. Ultracentrifuge, RC5C, Sorvall Instruments. Polycellular harvester, XX 2702550, Millipore Corp. Liquid scintillation counter, LS3801, Beckman.

Preparation of antibodies Peptides corresponding to 18 – 36 amino acid sequence of human m_3 receptor and 4 – 21 amino acid sequence of human m_4 receptor in extracellular

[1] Project supported by the National Natural Science Foundation of China, № 39570753.
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Received 1997-06-09 Accepted 1998-03-03

amino termini were synthesized by Shanghai Institute of Tumors. The m_3 sequence was SSWIHSPSDAGLPPGTVTH, and the m_4 sequence was FTPVNGSSGNQSVRLVTS. Both sequences were subtype-specific[7]. New Zealand white rabbits were injected sc with a mixture of peptide and adjunct 3 times at intervals of 3 wk to yield antisera. Six mg of peptide was used for each rabbit. The prepared antisera were partially purified by fractional salting-out, first with 50 % $(NH_4)_2SO_4$, then with 33 % $(NH_4)_2SO_4$. The antibodies were identified by ELISA with either synthesized peptides or rat cortex membrane proteins as antigens.

Radioligand assay[8] From SD rats, 0.06 mg of cerebral cortex protein, 0.1 mg of myocardium protein, or 0.7 mg of salivary glands, submaxillary glands and parotid glands, protein was added in TE buffer (Tris-HCl 50 mmol·L^{-1}, $MgCl_2$ 10 mmol·L^{-1}, edetic acid 5 mmol·L^{-1}, pH 7.4) with a total volume of 0.4 mL. Nonspecific binding was determined in the presence of atropine 2 μmol·L^{-1}. In saturation binding assay, membrane proteins were incubated with [^{3}H]QNB 0.3 – 6 nmol·L^{-1} at 37 ℃ for 30 min. In competitive binding assay, membrane proteins were incubated with purified antisera 1 : 10 – 1 : 1280 at 30 ℃ for 60 min. Then [^{3}H]QNB was added and incubated at 37 ℃ for 30 min.

Rat ilea[9] A 2-cm rat ileum segment was bathed in oxygenated Tyrode's solution at 37 ℃. Normal contraction effects of acetylcholine (ACh) were recorded. Then ACh was washed off and purified antiserum or atropine was added. After incubating for 1 h, contraction effects of ACh were recorded again. The effects of the prepared antisera and atropine on ACh were studied.

Rat aortic rings[10,11] A 2-mm wide rat thoracic aorta ring with intact endothelium was bathed in oxygenated Krebs' solution at 37 ℃ for 2 h to balance. The relaxing effects of ACh 10 mmol·L^{-1} in aortic rings precontracted with KCl 20 mmol·L^{-1} were recorded before and after the incubation of the ring with purified antiserum 1 : 40 or atropine 10 μmol·L^{-1} for 1 h.

RESULTS

Preparation of antibodies Ten days after the second boost, rabbit sera were collected and partially purified. The titers of m_3 and m_4 antisera were about $1 : 1 \times 10^4$ by ELISA with either synthesized peptides or rat cortex membrane proteins, containing m_3 and m_4 receptor proteins, as antigen.

Radioligand assay Both m_3 and m_4 antisera inhibited specific [^{3}H]QNB binding to muscarinic receptors in rat cerebral cortex, myocardium, and salivary glands. The inhibition was concentration-dependent and reached the highest when final dilutory rates of the antibodies were 1 : 10 – 1 : 40. The maximal inhibitory rates of m_3 antibodies in rat cerebral cortex, myocardium, and salivary glands were 12.1 % ± 2.1 %, 15.7 % ± 1.1 %, and 63.6 % ± 2.8 %, respectively, whereas the maximal inhibitory rates of m_4 antibodies were 28 % ± 6 %, 19.3 % ± 2.6 %, and 1.6 % ± 1.4 %. For each tissue, the maximal inhibitory rates of the 2 antisera were different ($P < 0.01$). For each antiserum, the differences between the tissues were also significant ($P < 0.01$) (Fig 1).

Isolated rat ileum and aortic rings Neither m_3 antiserum nor m_4 antiserum had direct effect on the tone of rat ilea. The m_3 antiserum reduced the maximal contraction of ACh without affecting the EC_{50}. On the contrary, atropine increased the EC_{50} of ACh without affecting the maximal contraction (Fig 2). It suggested that m_3 antiserum acted as a m_3 receptor noncompetitive blocker and atropine acted as a competitive blocker. pA_2 of atropine was 9.76 lg[mol·L^{-1}] and pD_2' of m_3 antiserum was 1.51 lg[dilution].

ACh 0.01 mmol·L^{-1} relaxed rat aortic rings precontracted with KCl 20 mmol · L^{-1}. However, after incubation of the rings with m_3 antiserum or atropine, the relaxing activity of ACh 0.01 mmol · L^{-1} absolutely disappeared. The m_4 antiserum and control rabbit serum had no effect on the relaxing activity of ACh 0.01 mmol·L^{-1}.

DISCUSSION

Subtype-selective anti-muscarinic-receptor

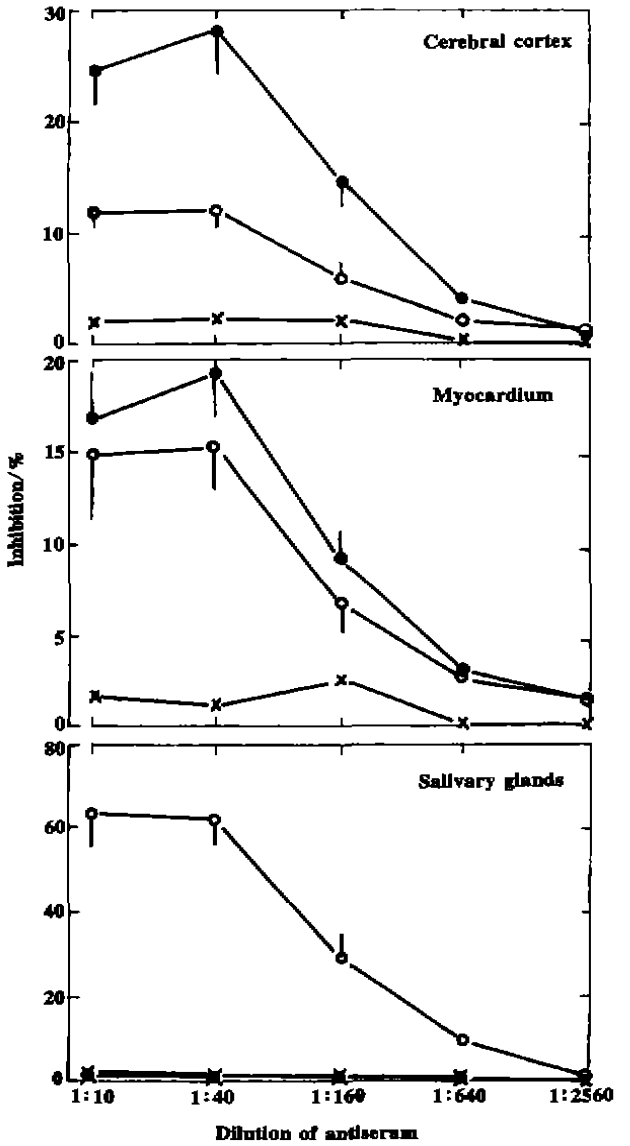

Fig 1. Inhibitory effects of m_3 and m_4 antisera on binding of [^{3}H]QNB to muscarinic receptors in rats.
○ m_3 antiserum, ● m_4 antiserum, × control rabbit serum. $n = 6$ rats, $\bar{x} \pm s$.

antibodies against intracellular parts, eg, i3 loop can not bind to receptors in intact cells and are not biologically active. With the hope of development of antibodies with pharmacological activities, we synthesized m_3 and m_4 subtype-selective peptide segments in extracellular amino termini to prepare polycolonal antibodies. The prepared m_3 and m_4 antibodies inhibited [^{3}H]QNB binding to muscarinic receptors in rat cortex, myocardium and salivary glands with various maximal inhibitions, none of which

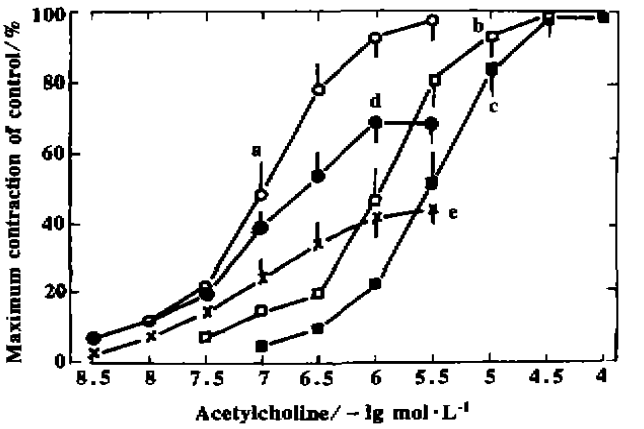

Fig 2. Effect of m_3 antiserum on the effect of ACh in isolated rat ilea. Control (a) and in presence of atropine 1 μmol·L^{-1} (b) atropine 10 μmol·L^{-1} (c), m_3 antiserum 1:100 (d) and m_3 antiserum 1:20 (e). $n = 8$ rats, $\bar{x} \pm s$.

reached 100 %, suggesting that the antibodies didn't inhibit binding of all muscarinic receptors but only some of them. Since the antibodies were against synthesized m_3 or m_4 subtype-selective peptides, so they probably acted only on m_3 or m_4 receptor. In fact, the maximal inhibition percentages of the two antibodies in rat cortex, 12.1 % ± 2.1 % and 28 % ± 6 %, are approximate to the reported proportions, 10 % and 24 %[4], of m_3 and m_4 subtypes in rat cerebral cortex. The maximal inhibitions in myocardium and salivary glands, submaxillary glands and parotid glands, also conform to the well-known conclusion that m_2 and m_3 are the major subtypes in the two tissues respectively[4,12-14].

Both contraction activity of ACh in ileum and relaxation activity of ACh in artery are mediated by m_3 receptor[9,11]. Both m_3 antibody and atropine, but not m_4 antibody and control rabbit serum, inhibited these two activities of ACh, with m_3 antibody acting noncompetitively and atropine competitively. Theoretically, antibodies can bind to receptors like ligands, they may stimulate or block receptors like ligands as well. At least, when conformation and affinity for ligands of receptors change because of binding to antibodies, or when antibodies are taking the space for ligands, antibodies may act like blockers. In fact, it was reported that anti-receptor antibodies were involved in some self-immunity diseases such as myasthenia gravis and

Chagsic's disease and might be agonist or antagonist-like[5,6]. From our study, it was confirmed that development of subtype-selective anti-receptor antibodies with pharmacological activities was feasible. We did not study the biological activity of m_4 antibody because there was no detectable biological activity mediated by m_4 receptor. And without cell lines expressing individual subtypes of receptors we could not identify the subtype-selectivity of the developed antibodies as reported[2-4]. This paper is only a preliminary one dealing with the feasibility of developing subtype-selective anti-receptor antibodies with pharmacological activities. Our ideal merits further investigation because this kind of antibodies may be new tools for the study of the functions of receptor subtypes.

REFERENCES

1 Hulme EC, Birdsall NJM, Buckley NJ. Muscarinic receptor subtypes. Annu Rev Pharmacol Toxicol 1990; 30: 633 - 73.

2 Levey AI, Kitt CA, Simonds WF, Price DL, Brann MR. Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-selective antibodies. J Neurosci 1991; 11: 3218 - 26.

3 Blake AD, Anthony NM, Chen HH, Harrison JB, Nathanson NM, Sattelle DB. Drosophila nervous system muscarinic acetylcholine receptor: transient functional expression and localization by immunocytochemistry. Mol Pharmacol 1993; 44: 716 - 24.

4 Yasuda RP, Ciesla W, Flores LR, Wall SJ, Li M, Satkus SA, *et al*. Development of antisera selective for m4 and m5 muscarinic cholinergic receptors: distribution of m4 and m5 receptors in rat brain. Mol Pharmacol 1993; 43: 149 - 57.

5 Fu ML. Agonist-like activity of anti-peptide antibodies directed against an autoimmune epitope on the heart muscarinic acetylcholine receptor. Receptors-Channels 1994; 2: 121 - 30.

6 Sterin-Borda L, Cantore M, Pascaul J, Borda E, Cossio P, Arana R, *et al*. Chagasic IgG binds and interacts with cardiac β adrenoceptor-coupled adenylate cyclase system. Int J Immunopharmacol 1986; 8: 581 - 8.

7 Bonner TI. The molecular basis of muscarinic receptor diversity. Trends Neurosci 1989; 12: 148 - 51.

8 Yamamura HI, Snyder SH. Muscarinic cholinergic binding in rat brain. Proc Natl Acad Sci USA 1974; 71: 1725 - 9.

9 Thomas EA, Baker SA, Ehlert FJ. Functional role for the m2 muscarinic receptor in smooth muscle of guinea pig ileum. Mol Pharmacol 1993; 44: 102 - 10.

10 Choo LK, Malta E, Mitchelson F. The affinity of some selective muscarinic receptor antagonists for the muscarinic receptor mediating endothelium-dependent relexation of the rabbit and rat thoracic aorta. J Pharm Pharmacol 1986; 38: 843 - 5.

11 Boulanger CM, Morrison KJ, Vanhoutte PM. Mediation by m3-muscarinic receptors of both endothelium-dependent contraction and relexation to acetylcholine in the aorta of spontaneously hypertensive rat. Br J Pharmacol 1994; 112: 519 - 24.

12 Doods HN, Maithy MJ, Davidesko D, van Charldorp KJ, de Jonge A, van Zwieten PA. Selectivity of muscarinic antagonists in radioligand and *in vivo* experiments for the putative M1, M2 and M3 receptors. J Pharmacol Exp Ther 1987; 242: 257 - 62.

13 Mitchelson F. Muscarinic receptor differentiation. Pharmacol Ther 1988; 37: 357 - 423.

14 Doods HN, Willim KD, Boddeke HWGM, Entzeroth M. Characterization of muscarinic receptors in guinea-pig uterus. Eur J Pharmacol 1993; 250: 223 - 30.

具有药理活性的毒蕈碱 m_3 与 m_4 受体抗体的制备[1]

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关键词 毒蕈碱受体；回肠；胸主动脉；大脑皮质；心肌；唾液腺；放射配位体测定；抗体

目的：探讨制备具有药理活性的亚型选择性抗受体抗体和以其研究受体功能的可行性。**方法**：合成 m_3 与 m_4 受体亚型特异的多肽免疫兔制备抗体；放射配位体测定研究抗体对 M 受体配基结合的影响。研究抗体对乙酰胆碱的回肠收缩与动脉环舒张作用的影响。**结果**：制备成抗 m_3 与 m_4 受体的抗体。m_3 抗体对[^{3}H]QNB 与大鼠大脑皮质、心肌、及唾液腺（颌下腺及腮腺）M 受体结合最大抑制率分别为 12.1 % ± 2.1 %、15.7 % ± 1.1 %与 63.6 % ± 2.8 %，m_4 抗体则分别为 28 % ± 6 %、19.3 % ± 2.6 %与 1.6 % ± 1.4 %。m_3 抗体抑制乙酰胆碱的回肠收缩与动脉环舒张。**结论**：制备具有药理活性的亚型选择性抗受体抗体并用以研究受体功能是可行的。