Photoaffinity labeling reveals two muscarinic receptor macromolecules associated with the presence of calcium in rat adenohypophysis

Sofia Avissar, Ethy Moscona-Amir and Mordechai Sokolovsky*

Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv 69 976, Israel

Received 30 September 1982; revision received 5 November 1982

1. INTRODUCTION

Recently, we have demonstrated the presence of muscarinic receptors in the rat adenohypophysis and described their biochemical characteristics [1]. Our results showed that in contrast to other brain regions [2] antagonist binding in this area is heterogeneous with the existence of at least two subclasses of sites [1].

Physiological data indicate that muscarinic receptors may be coupled to Ca^{2+}-channels in certain systems (3–5) and that calcium may then act as a second messenger. Extracellular calcium is required for the release of several adenohypophysis hormones [6,7].

Here, we examined whether Ca^{2+} affects the biochemical characteristics of muscarinic binding in the adenohypophysis, using the highly specific, tritiated muscarinic antagonist, *N*-methyl-4-piperidyl benzilate ([^{3}H]4NMPB). We also examined whether the heterogeneity of the binding sites in this area originates from different components of the muscarinic receptor. For this purpose we used the specific photolabile muscarinic antagonist, *N*-methyl-4-piperidyl azidobenzilate (azido-[^{3}H]4NMPB), which has been shown to provide irreversible labeling of a M_r 86 000 polypeptide in rat cortex [8].

2. EXPERIMENTAL

2.1. *Materials*

[^{3}H]4NMPB (65 Ci/mmol) and azido-[^{3}H]4NMPB (9.2 Ci/mmol) were prepared as in [2,8]. The purity of the compounds was > 97%. *Methoxyverapamil* (known also as 'D-600') was a gift from Dr T. Bartfai. Adult female rats of the CD strain were supplied by Levinstein's farm (Yokneam, Israel) and maintained in an air-conditioned room at 24 ± 2°C for 14 h fluorescent illumination and 10 h darkness daily. After an adjustment period of at least 4 weeks, daily vaginal smears were taken and only those females having a regular 4-day estrous cycle were used. Since in [1] we found differences in the biochemical characteristics of muscarinic receptors in the adenohypophysis at various stages of the estrous cycle, we chose a well-defined physiological stage – the estrous day – to investigate the effect of calcium.

2.2. *Binding assay*

Direct binding of [^{3}H]4NMPB to muscarinic receptors in homogenates of rat adenohypophysis (25°C, pH 7.4) was measured in modified Krebs–Hensleit buffer containing 25 mM Tris–HCl, 118 mM NaCl, 4.69 mM KCl, 1.9 mM $CaCl_2$, 0.54 mM $MgCl_2$, 1.0 mM NaH_2PO_4 and 11.1 mM glucose as in [1]. When the effect of removal of Ca^{2+} from the buffer was investigated, it contained 0.1 mM EGTA and no Ca^{2+} was added. Experiments with methoxyverapamil (4×10^{-7} M) were carried out in buffer containing 1.9 mM Ca^{2+}, as used in the control assay.

2.3. *Photolabeling*

P_2 fraction was prepared as in [8] and incubated in modified Krebs–Hensleit solution (pH 7.4) [1]

* To whom correspondence should be addressed

Published by Elsevier Biomedical Press
00145793/82/0000—0000/$2.75

containing 5 nM azido-[^{3}H]4NMPB at room temperature in the dark for 10 min. The reaction mixture was then photolyzed with a long-wave ultraviolet spot light lamp (Thomas Scientific Apparatus, model B-100A) at a distance of 7.5 cm with continuous stirring for 4 min.

2.4. *Gel electrophoresis*

Polyacrylamide slab gel electrophoresis (7.5% acrylamide in 0.1% $NaDodSO_4$) was used to resolve membrane proteins. The gels were cut manually into 1 mm slices, each was incubated overnight at 37°C with 4.5 cc of 3% Protosol (New England Nuclear) in scintillation medium (Hydroluma). Radioactivity was then measured in a scintillation spectrometer. Recovery of radioactivity from the gels was 80–90%, counting efficiency was 35%. Clathrin (M_r 180 000), RNA polymerase (M_r 155 000), phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), and carbonic anhydrase (M_r 30 000) (Sigma) were used as M_r markers.

3. RESULTS AND DISCUSSION

In previous work we have examined the binding of muscarinic antagonists to homogenates of rat adenohypophysis. Assays were carried out in modified Krebs–Hensleit solution containing 1.9 mM Ca^{2+}, and yielded curvilinear Scatchard plots indicating heterogeneity of binding with the existence of at least two subclasses of sites. It should be noted that the existence of site–site interactions among the muscarinic sites could not be excluded ([1] and fig. 1A).

As depicted in fig. 1B, after the removal of Ca^{2+}, a linear Scatchard plot is obtained, indicating the conversion of the heterogeneous population (fig. 1A) into a homogeneous population of binding sites, with a dissociation constant value which is an intermediate between the dissociation constant values of the α and β subclasses, obtained when Ca^{2+} is present in the reaction mixture (table 1).

Since methoxyverapamil is known to block calcium uptake, we chose this compound as an additional mean to investigate the specificity of the Ca^{2+} effect on the binding. In agreement with the above findings, binding of [^{3}H]4NMPB in the presence of this blocker (at 4×10^{-7} M) presents the same characteristics as those obtained after the removal of Ca^{2+} (fig. 1C, table 1). These results indicate that calcium affects the heterogeneity of muscarinic antagonist binding sites.

The question arises whether the heterogeneity of binding sites in the adenohypophysis originates from different components of the muscarinic receptor, and how Ca^{2+} affects these components.

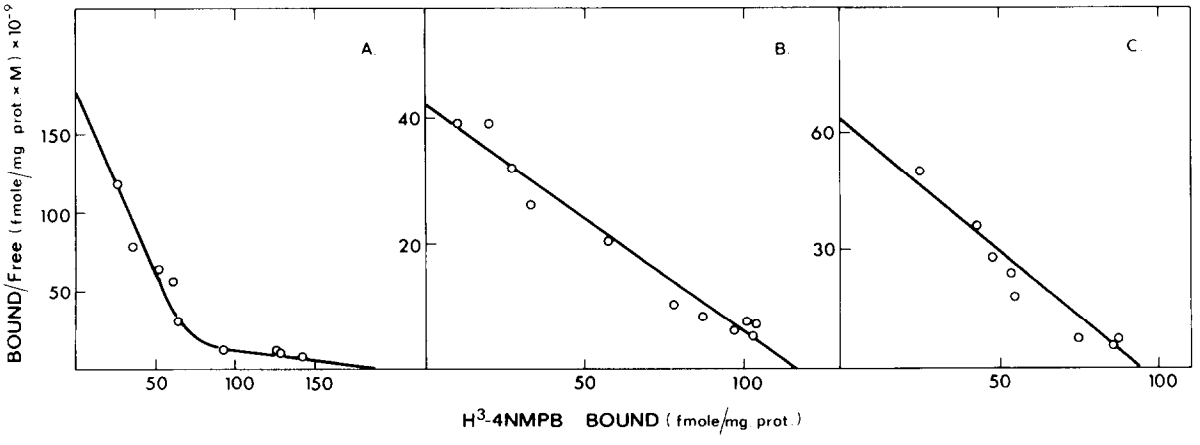

Fig. 1. Scatchard plots of [^{3}H]4NMPB binding to homogenates of adenohypophysis: in buffer containing 1.9 mM Ca^{2+} (A); in the absence of Ca^{2+} and in the presence of 0.1 mM EGTA in the reaction mixture (B); in the presence of 1.9 mM Ca^{2+} and 4×10^{-7} M methoxyverapamil in the reaction solution.

Table 1

Binding characteristics of the muscarinic antagonist [^{3}H]4NMPB, determined by direct binding in rat adenohypophysis[a]

	+ Ca^{2+}[b]	− Ca^{2+}[c]	+ Ca^{2+} + D-600[d]
Dissociation constant (nM)	K = 0.64 ± 0.06 K = 11.2 ± 0.25	K_d = 2.45 ± 0.04	K_d = 1.4 ± 0.13
Maximal binding capacity (fmol/mg protein)	B^{α}_{max} = 65 ± 5 B^{β}_{max} = 100 ± 10	B_{max} = 119 ± 9	B_{max} = 107 ± 17

[a]The dissociation constants and the binding capacities presented in this table are taken from analysis of the Scatchard plots shown in fig. 1

[b]Expts were carried out in modified Krebs–Hensleit solution (see section 2) in the presence of 1.9 mM Ca^{2+}. α and β designate the higher and lower affinity antagonist binding sites, respectively

[c]Ca^{2+} was omitted from the reaction mixture, and 0.1 mM EGTA was added

[d]The binding reaction contained 1.9 mM Ca^{2+} and 4 × 10^{-7} M D-600

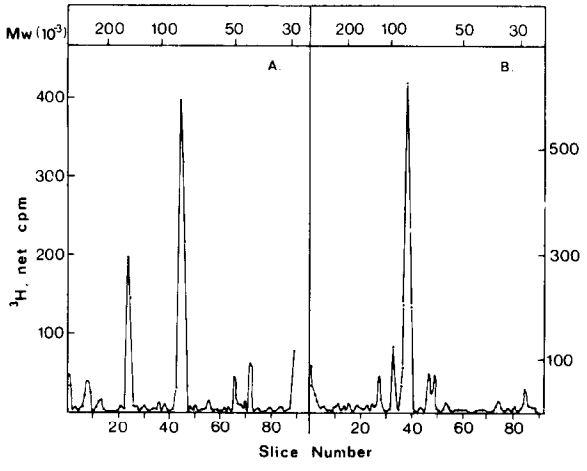

Fig. 2. NaDodSO$_4$/polyacrylamide gel-radioactivity pattern of proteins from adenohypophysis membranes specifically labeled with 5 nM azido-[^{3}H]4NMPB. After electrophoresis, gels were sliced into 1 mm pieces and the amount of radioactivity of each piece was determined (see section 2).

To clarify this we used the photolabile antagonist azido-[^{3}H]4NMPB. This agent provides an irreversible labeling of the receptor sites, thus enabling the detection of specifically labeled macromolecules by gel electrophoresis [8]. When experiments were carried out in the presence of Ca^{2+} (fig. 2) the radioactivity profile of the gel revealed two major labeled macromolecules with app. M_r of ~86 000 and 160 000. Upon removal of Ca^{2+} from the incubation buffer, labeling from the M_r160000 macromolecule disappeared, whereas the labeling of the M_r 86 000 polypeptide remained unchanged. Interestingly, the proportions of the populations in the binding experiments (table 1) are similar to the proportions of the specific peaks found in gel electrophoresis after covalent attachment of azido-[^{3}H]4NMPB to membranes from adenohypophysis in the presence (fig. 2A), or absence (fig. 2B) of Ca^{2+}.

A possible explanation for the above phenomena is that in the adenohypophysis there are two independent polypeptides, M_r 86 000 and 160 000, which carry the muscarinic binding sites. The binding to the M_r 160 000 polypeptide requires calcium. Another possibility is that calcium induced polymerization of the M_r 86 000 polypeptide to form the M_r 160 000 macromolecule, concomitantly with an increase in the binding capacity. The second explanation is more reasonable in view of [9]. We presented evidence that in the cortex and hippocampus a single specifically labeled protein macromolecule with an app. M_r of 86 000 was detected by gel electrophoresis, while in the medulla-pons, cerebellum and cardiac atria, there was also a M_r 160 000 band in addition to the M_r 86 000 polypeptide. Under certain conditions, alkali or hydroxylamine treatment caused a dissociation of both larger macromolecules into a

single M_r 40 000 polypeptide. These results strongly suggest that the muscarinic receptor exists in oligomeric forms and that both a dimer and tetramer of a basic M_r 40 000 peptide may exist as interconvertible species. Based on this data, we have proposed a model to explain the biological architecture of the muscarinic receptors. This model, and the results described therein, are currently under investigation.

In conclusion, our findings indicate that in vitro, calcium affects the muscarinic receptor subunits and supports a possible physiological role for calcium in the adenohypophysis. Experiments are being carried out in our laboratory to assess this hypothesis and to evaluate the physiological significance of such calcium effect.

ACKNOWLEDGEMENT

This work was supported in part by the Recanati Endowment Fund for Medical Research (Israel).

REFERENCES

[1] Avissar, S., Egozi, Y. and Sokolovsky, M. (1981) Neuroendocrinology 32, 303.
[2] Kloog, Y., Egozi, Y. and Sokolovsky, M. (1979) Mol. Pharmacol. 15, 545.
[3] Young, P.W., Bicknell, R.J. and Schofield, J.G. (1979) J. Endocrinology 80, 203.
[4] Prokopczuk, A., Pytkowsski, B. and Lewartowski, B. (1981) Eur. J. Pharmacol. 70, 1.
[5] Keryer, G. and Rossignol, B. (1978) Eur. J. Biochem. 85, 77.
[6] Conn, P.M., Rogers, D.C. and Sandher, F.S. (1979) Endocrinology 105, 112.
[7] Naor, A., Leifer, A.M. and Catt, K.J. (1980) Endocrinology 107, 1438.
[8] Amitai, G., Avissar, S., Balderman, D. and Sokolovsky, M. (1982) Proc. Natl. Acad. Sci. USA 79, 243.
[9] Avissar, S., Amitai, G. and Sokolovsky, M. (1983) Proc. Natl. Acad. Sci. USA, in press.