IDENTIFICATION OF MUSCARINIC RECEPTORS IN THE *TORPEDO* ELECTRIC ORGAN

Evidence for their presynaptic localization

Yoel KLOOG, Daniel M. MICHAELSON and Mordechai SOKOLOVSKY
The Department of Biochemistry, The G.S. Wise Faculty of Life Sciences, Tel Aviv University, Tel-Aviv, Israel

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1. Introduction

Synapses which employ biogenic amines as neurotransmitters are believed to contain both pre- and postsynaptic receptors. The classical postsynaptic receptors recognize the transmitter and mediate the response of the postsynaptic cell whereas the presynaptic receptors are presumed to control and modulate the extent of neurosecretion per stimulus (reviewed [1]). It has also been suggested that some cholinergic synapses contain presynaptic muscarinic receptors which regulate the extent of Ach release by feedback inhibition [2–4].

It is well established that the *Torpedo* electric organ is innervated by cholinergic synapses whose postsynaptic receptors are nicotinic cholinergic [5,6]. The observation that muscarinic ligands bind to the homogenate of the electric organ of *Torpedo* [7,8], suggests that this tissue contains muscarinic receptors. Hence, we have decided to investigate and characterize the *Torpedo* electric organ muscarinic receptors, and to determine their subcellular localization.

2. Experimental

The electric organs were homogenized and fractionated as in [9,10]. The homogenized material (H) was centrifuged for 10 min at 1000 × *g*. The resulting supernatant (S_1) was subjected to further centrifugation at 17 500 × *g* for 1 h. The P_2 pellet was resuspended and loaded onto the sucrose density gradient which was composed of 6 layers (a_1–a_6) all of which contained 1 mM EGTA and one of the following concentrations of sucrose: 0, 0.15, 0.3, 0.55, 0.8, 1.6 M. Layers a_1–a_4 also contained glycine so that their osmolarity was equal to that of the homogenization buffer. After centrifugation in a SW40 rotor spun at 110 000 × *g* for 1 h, the material was carefully collected starting at the upper interfaces. In this study, fractions a_2 and a_3, which are the most enriched in synaptosomes [10] were pooled together. Similarly fractions a_5 and a_6 which contain most of the α-bungarotoxin (α-Bgt) binding were also combined.

N-[^{3}H]Methyl-4-piperidyl benzilate (4-N[^{3}H]MPB) and 3-[^{3}H]quinuclidinyl benzilate (3-[^{3}H]QNB) binding were performed as in [11]. The nicotinic acetylcholine receptor was assayed with ^{125}I-labelled α-Bgt using the method in [12].

3. Results and discussion

The electric organ of *Torpedo ocellata* is amply endowed with the nicotinic cholinergic receptor (10.6 nmol/g electroplax tissue). As shown in table 1, the electric organ homogenate also specifically binds the muscarinic antagonists 3-[^{3}H]QNB and 4-N[^{3}H]-MPB. Within experimental error these muscarinic ligands bind to the same number of sites, which, in the homogenate, are at ~400 fmol/g electroplax tissue. Thus, the homogenate contains ~1 muscarinic antagonist site/25 000 α-Bgt binding sites. These findings are similar to those recently reported for 3-[^{3}H]QNB binding to the electric organ of *Torpedo marmorata* [8]. Following differential centrifugation,

Table 1
Distribution of ^{125}I-labelled α-Bgt, 3-[^{3}H]QNB and 4-N[^{3}H]MPB binding sites in *Torpedo* subcellular fractions

Subcellular fraction	^{125}I-labelled α-Bgt binding[a]		4-N[^{3}H]MPB binding		3-[^{3}H]QNB binding		Muscarinic binding ——— × 10^4
	(nmol/g organ)	(nmol/mg protein)	(fmol/g organ)	(fmol/mg protein)	(fmol/g organ)	(fmol/mg protein)	nicotinic binding
H	10.6 ± 1.0	0.54 ± 0.05	360 ± 40	18 ± 2	410 ± 10	20.6 ± 1	0.36
S	9.2 ± 1.0	0.64 ± 0.06	235 ± 77	16 ± 5	263 ± 15	18.1 ± 1	0.27
P_1	2.5 ± 0.5	0.62 ± 0.12	161 ± 57	39 ± 14	120 ± 20	29.2 ± 4.8	–
S_2	3.1 ± 2.0	0.37 ± 0.24	–	–	25 ± 20	3.0 ± 2.4	–
P_2	7.1 ± 1.5	1.46 ± 0.31	210 ± 90	53 ± 19	247 ± 80	51.5 ± 16	0.35
$a_2 + a_3$	0.5 ± 0.13	0.81 ± 0.20	91 ± 7	154 ± 20	101 ± 8	166.0 ± 16	1.80
$a_5 + a_6$	7.0 ± 1.40	2.02 ± 0.40	67 ± 16	20 ± 5	76 ± 15	22.0 ± 4.3	0.10

[a] Data taken from [10]

Binding presented are average ± SD of 3 separate experiments for ^{125}I-labelled α-Bgt, 3 separate experiments for 4-N[^{3}H]MPB determined at 10 nM and 3 separate experiments for 3-[^{3}H]QNB determined at 5 nM

most of the α-Bgt, 3-[^{3}H]QNB and 4-N[^{3}H]MPB binding capacities are found in the P_2 pellet (table 1).

Figure 1 depicts the distribution of ligand binding sites in the density centrifugation fractions. Most of the α-Bgt binding is found in fractions $a_5 + a_6$, whereas fractions $a_2 + a_3$ contain only ~7% of the parent fraction (P_2) binding. By contrast, fraction $a_2 + a_3$ contain about 40% of the 3-[^{3}H]QNB and 4-N[^{3}H]MPB binding. As expected, both ligands have identical binding capacities [13]. The ratio of muscarinic to nicotinic binding in fraction $a_2 + a_3$ is about 5-fold larger than in the homogenate (table 1).

The high specific binding (~160 fmol/mg protein) of muscarinic antagonists to fraction $a_2 + a_3$ suggests that this fraction is enriched in muscarinic receptors. This assertion was fortified by the following observations: 3-[^{3}H]QNB binds to the synaptosomal fraction with K_d 0.3 ± 0.1 nM and 4-N[^{3}H]MPB with K_d 1.0 ± 0.2 nM (fig.2). Competition binding experiments of atropine/3-[^{3}H]QNB and atropine/4-N[^{3}H]MPB yielded in both cases K_d 3.3 ± 0.2 nM. In all cases a Hill coefficient of 1 was obtained. Competition experiments using the agonist oxotremorine and 3-[^{3}H]QNB and 4-N[^{3}H]MPB yielded I_{50} values of

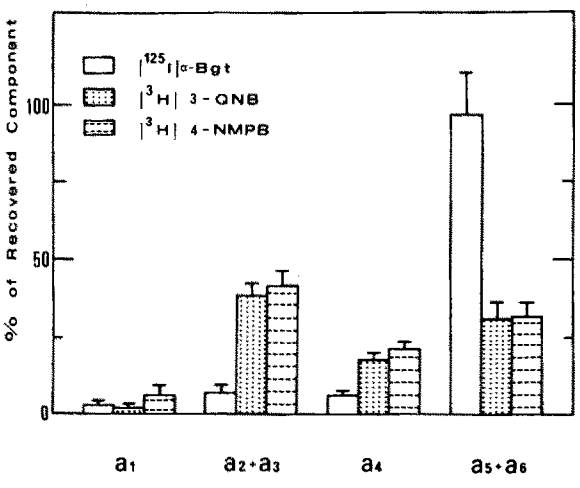

Fig.1. Distribution of ^{125}I-labelled α-Bgt, 3-[^{3}H]QNB and 4-N[^{3}H]MPB binding in the sucrose density gradient fractions. Absolute values for the parent fraction (P_2) loaded onto the gradient were as follows: 42.9 mg protein; 33 nmol α-Bgt binding sites; 1.2 pmol of 3-[^{3}H]QNB and of 4-N[^{3}H]MPB binding sites. Recoveries were as follows: protein 102 ± 10%; ^{125}I-labelled α-Bgt binding, 105 ± 10%; 3-[^{3}H]QNB 88 ± 15%; 4-N[^{3}H]MPB 102 ± 15%. Data presented are the mean of 4 separate experiments. Vertical bars represent SD.

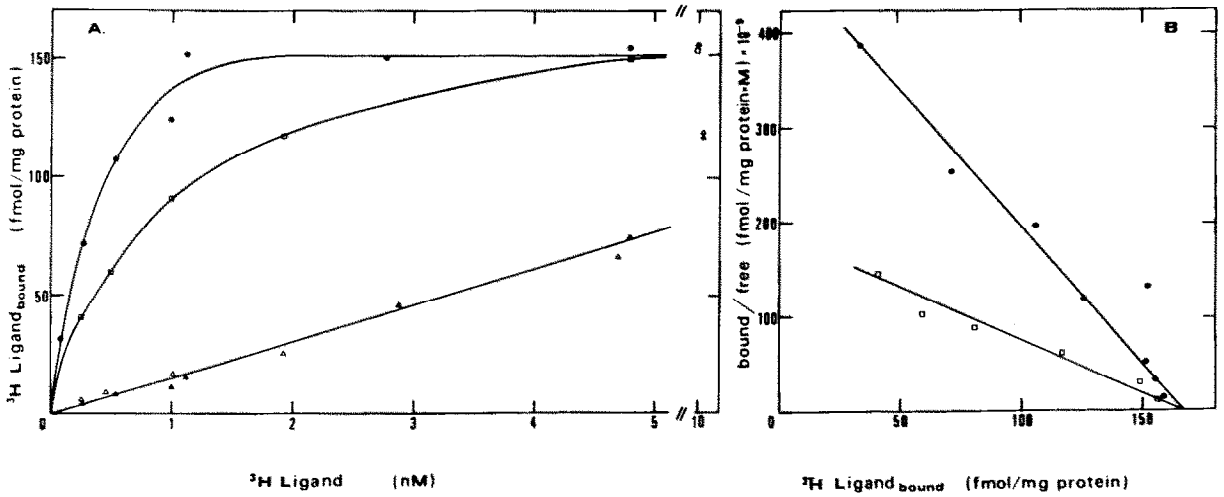

Fig.2. Binding of 4-N[^{3}H]MPB and 3-[^{3}H]QNB to fraction $a_2 + a_3$. (A) Samples at 0.2–0.4 ml (containing 0.3–0.6 mg protein) were incubated for 60 min with varying concentrations of 3-[^{3}H]QNB and for 30 min with varying concentrations of 4-N[^{3}H]MPB. Experiments were performed at 25°C in 2 ml modified Krebs solution (pH 7.4). (• – •) Specific and (▲ – ▲) non-specific binding (in the presence of 25 μM atropine) of 3-[^{3}H]QNB, and (○ – ○) specific and (△ – △) non-specific binding of 4-N[^{3}H]MPB. (B) Same data replotted according to Scatchard [14] for 3-[^{3}H]QNB (•–•) and for 4-N[^{3}H]MPB (○–○) binding.

0.085–0.1 μM and 0.1–0.2 μM, respectively and a Hill coefficient of ~0.6. The affinity of agonists is presented in I_{50} rather than in K_d values since, due to the low Hill coefficient, the assignment of K_d values depends on their mode of binding. The present findings are similar to those obtained with mammalian muscarinic receptors [11,13,15–19]. The nicotinic ligands d-tubocurarine ($\leqslant 10^{-5}$ M) and α-Bgt ($\leqslant 10^{-5}$ M) had no effect on 3-[^{3}H]QNB and 4-N[^{3}H]MPB binding. Thus we may conclude that the synaptosomal fraction $a_2 + a_3$ [10], is enriched in muscarinic receptors. The muscarinic and nicotinic receptors found in fraction $a_5 + a_6$ probably arise from postsynaptic and presynaptic membranes which under our homogenization conditions did not detach from the larger postsynaptic electroplaque membrane. A muscarinic binding capacity of about 80 fmol/mg protein in purified postsynaptic membranes was found [8] which is about 2-fold lower than that presently obtained with isolated intact synaptosomes (table 1). Synaptosomal membranes prepared by osmotic lysis and recentrifugation of $a_2 + a_3$, contain even higher specific binding, i.e., 350 fmol/mg protein.

In conclusion, the findings presented demonstrate that the *Torpedo* electric organ contains muscarinic receptors which can be separated from the classical nicotinic receptor. The high enrichment of the muscarinic receptors in the synaptosomal fraction, which contains only traces of the postsynaptic membranes, implies that in the *Torpedo* the muscarinic receptors may be located in the presynaptic membrane. As yet the physiological role of the muscarinic receptor in *Torpedo* is not known. Preliminary results in our laboratory indicate that muscarinic ligands interfere with Ca^{2+}-induced synaptosomal acetylcholine secretion, thus suggesting that these receptors may function in the control and modulation of acetylcholine release.

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