FEBS Letters 352 (1994) 95–97

FEBS 14539

Two-step binding of green mamba toxin to muscarinic acetylcholine receptor

T. Toomela[a], M. Jolkkonen[b], A. Rinken[a], J. Järv[a,*], E. Karlsson[b]

[a]*Institute of Chemical Physics, University of Tartu, EE 2400 Tartu, Estonia*
[b]*Department of Biochemistry, Biomedical Centre, Box 576, S-751 23 Uppsala, Sweden*

Received 20 July 1994

Abstract The mechanism of binding of toxin MT2 from venom of green mamba *Dendroaspis angusticeps* to muscarinic acetylcholine receptors from rat cerebral cortex was investigated by studying the kinetics of the toxin–receptor interaction. The muscarinic antagonist *N*-methyl-[^{3}H]scopolamine was used as a 'reporter' ligand. Evidence for a mechanism of toxin–receptor interaction comprising at least two steps was obtained. Such a mechanism increases the potency of the toxin. The first step was fast with no competition between the toxin and the antagonist. The second step was slow with formation of a more stable toxin–receptor complex and inhibition of the antagonist binding. It is proposed that the snake toxin is a muscarinic agonist of slow action.

Key words: Mamba toxin; Muscarinic acetylcholine receptor; Toxin binding mechanism

1. Introduction

Snake venom toxins have played an important role in the investigation of nicotinic acetylcholine receptors and much is known about the structure and mechanism of interaction of a-and k-neurotoxins with these receptors (see [1] for reviews). Snake toxins against muscarinic acetylcholine receptors are less well known. The first toxins were isolated in 1988 by Adem et al. [2] and they have been detected so far only in the venom of mambas, elapid snakes of the genus *Dendroaspis*. They are polypeptides of 64–66 amino acids and four disulphides and are homologous to a-neurotoxins [3–5]. Recently, five new muscarinic toxins have been isolated from *D. angusticep* (green mamba), two from *D. polylepis* (black mamba) (to be published) and one from *D. viridis* (Western green mamba) (Cerveñansky, personal communication).

Some of these toxins are highly selective for subtypes of muscarinic receptors which should make them to very valuable biochemical and pharmacological tools. For instance, m1-toxin from green mamba is selective for subtype m1 [5,6] and toxin MT3 from the same snake is selective for subtype m4 (to be published).

Here we report the mechanism of the binding of toxin MT2 to muscarinic receptors in rat brain cortex. This toxin (66 amino acids, four disulphides) was purified from green mamba venom [3] and it appears to have a high selectivity for m2-receptors (to be published). The method of kinetic analysis used in this study was proposed previously for investigation of the mechanism of binding of non-radioactive muscarinic ligands with the receptor by using *N*-methyl-[^{3}H]scopolamine ([^{3}H]NMS) as a radioactive 'reporter' ligand [7,8].

2. Materials and methods

2.1. The toxin

Toxin MT2 was isolated from *Dendroaspis angusticeps* venom by gel filtration on Sephadex G-50 and ion-exchange chromatography on SP-Sephadex C-25 (SP = sulphopropyl) as described earlier [2]. The final purification step was HPLC ion-exchange chromatography, replacing the step of reversed-phase chromatography. The cation-exchanger BioGel TSK SP-5-PW was used and the column was eluted with a gradient of ammonium acetate (pH 6.7). Because of the limited stability of toxin MT2, an assay confirming the toxin's ability to displace radioligand from receptor was performed in parallel with each kinetic experiment.

2.2. Kinetic studies

A membrane preparation of muscarinic receptors [9] was obtained by homogenisation of rat brain cortex in ice-cold 100 mM potassium phosphate buffer (pH 7.4) and centrifugation for 10 min at 1,000 × *g*. The supernatant was centrifuged for 45 min at 30,000 × *g* and the pellet was recovered. Before use the pellet was rehomogenized in 50 mM potassium phosphate buffer (pH 7.4). The kinetic experiments were carried out at 25°C in this buffer.

The amount of the receptor sites was determined from the specific binding of L-[^{3}H]QNB (specific radioactivity 50 Ci/mmol, Amersham). For that purpose 1 nM QNB was incubated with membrane suspension for 45 min, filtered on Whatman GF/B glass-fibre filters followed by immediate washing with buffer to remove free radioligand. Bound radioactivity was determined by scintillation counting. The non-specific binding was determined in the presence of 40 mM atropine and was 2–4% of the total binding.

Kinetics [7] of the competition between the toxin and a radioligand, the muscarinic antagonist *N*-methyl-[^{3}H]scopolamine ([^{3}H]NMS) (specific radioactivity 74 Ci/mmol, Amersham) was studied. Two series of experiments were carried out. In the first one, a membrane suspension in 50 mM potassium phosphate buffer (pH 7.4) containing MT2 (concentration 1–50 mg/ml) and [^{3}H]NMS (concentration 1 nM) was used. For each toxin concentration the time course of the formation of the toxin–radioligand complex was followed by taking aliquots at different times for filtration assay. A similar experiment was then carried out with [^{3}H]NMS (1 nM) in the absence of toxin. From the plot of the concentration of bound [^{3}H]NMS versus time, the first-order rate constants k_{obs} for binding of the radioligand to the receptor were calculated by non-linear least-square fit of the kinetic curves to the equation:

$$B_t = B_{nonsp} + B_{sp}\,(1 - \exp\,(-k_{obs}t)) \qquad (1)$$

where B_t = concentration of bound radioligand at time t, B_{sp}(B_{nonsp}) = concentration of specifically (non-specifically) bound radioligand at equilibrium. The non-specific binding reached equilibrium quickly and was considered as a constant.

In the second series of experiments, the kinetics of receptor inactivation by the toxin, i.e. blocking of the [^{3}H]NMS binding, was studied. Membranes were mixed with varying concentrations (1–50 mg/ml) toxin. Aliquots were taken after different times, incubated with 1 nM

*Corresponding author. Keemilise Füüsika Instituut, Tartu Ülikool, Jakobi 2, EE-2400 Tartu, Eesti/Estonia. Fax: (372) 7 432 884.

SSDI 0014-5793(94)00926-0

[^{3}H]NMS for 10 min (as determined from first series of experiments) to ensure full binding of the radioligand and the bound radioactivity was determined by filtration. The rate constants for inactivation of the receptor (k_{in}) were calculated from the equation:

$$B_t = B_{ni} + B_{in} \exp(-k_{in}t) - B_{ni}kt \quad (2)$$

where B_t has the same meaning as in equation (1), $B_{ni}(B_{in})$ = concentration of the receptor not inactivated (inactivated) by the toxin, k = rate constant for spontaneous inactivation of the receptor.

Data processing was made on an IBM PC by using the Enzfitter program package (Elsevier).

3. Results and discussion

3.1 Simultaneous addition of toxin and [^{3}H]NMS to the receptor

The binding of [^{3}H]NMS to muscarinic receptor was complete within 10 min (Fig. 1) and during this time the toxin MT2 did not interfere with the process. The experimentally determined points could be fitted well into the first-order rate equation (1) and the rate constants k_{obs} were calculated. They showed no significant variation with toxin concentration up to 50 mg/ml (Table 1). The average value of k_{obs} was (0.64 ± 0.08) min^{-1}.

This fact can be understood if:

(a) the toxin and radioligand bind to different sites on the receptor (non-competitive mechanism),

(b) the affinity (and/or concentration) of the toxin are too low to compete with [^{3}H]NMS for the binding site, or

(c) the toxin binding is slow if compared to radioligand binding.

The fact that after half an hour a slow displacement of the bound radioligand was observed (Fig. 1) shows that the toxin–receptor interaction is a complex phenomenon. The nature of this process was studied separately as explained below.

3.2. Preincubation of receptor with toxin

Incubation of the receptor with different toxin concentrations before assay with [^{3}H]NMS revealed the time-dependent decrease of the receptor sites available for the radioligand. The maximum effect for 50 mg/ml toxin was obtained after one hour (Fig. 2). Further decrease in [^{3}H]NMS binding was due to spontaneous inactivation of the receptor as the same rate was observed as in the absence of the toxin (Fig. 2). The rate constant of the spontaneous receptor inactivation was (7.0 ± 0.5) × 10^{-4} min^{-1}.

Table 1
Interaction of toxin MT2 with muscarinic receptors

Toxin concentration		k_{obs}	k_{in}
(mg/ml)	(mM)	(min^{-1})	(min^{-1})
0	0	0.65 ± 0.05	–
1.0	0.14	0.53 ± 0.03	0.0010 ± 0.0005
2.0	0.28	0.60 ± 0.09	0.0035 ± 0.0009
5.0	0.71	0.70 ± 0.08	0.008 ± 0.003
10	1.4	0.72 ± 0.09	0.014 ± 0.007
20	2.8	0.61 ± 0.06	0.027 ± 0.009
50	7.1	0.68 ± 0.06	0.030 ± 0.016

k_{obs}, rate constant for binding of [^{3}H]NMS in presence of simultaneously added toxin. k_{in}, rate constant for receptor inactivation by toxin in the absence of [^{3}H]NMS. In each experiment 10 samples were taken at different times. The rate constants and their standard errors were determined by non-linear least square fits to equations (1) and (2) in text.

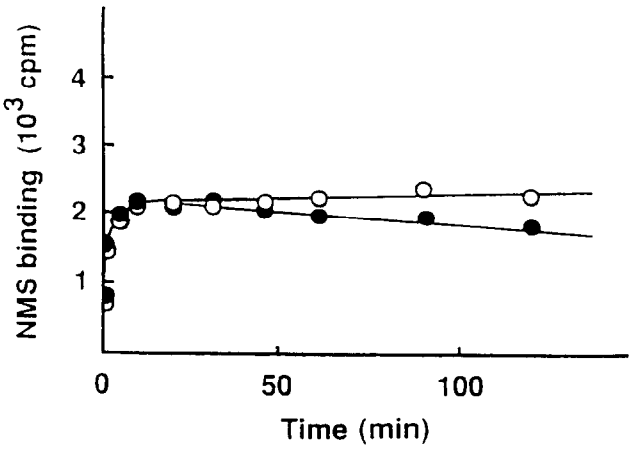

Fig. 1. Time course of *N*-methyl-[^{3}H]scopolamine binding to muscarinic receptors from rat cerebral cortex in competition with simultaneously added toxin MT2 (10 mg/ml) (closed circles) and in absence of toxin (open circles).

About 40% of the receptor sites were blocked by 50 mg/ml MT2. The inhibition of the [^{3}H]NMS binding was not significantly increased when the toxin concentration was raised up to 1 mg/ml. This is in agreement with the earlier conclusion from binding studies with [^{3}H]QNB [2].

In another experiment receptors were incubated for 5 h with toxin (20 mg/ml) and then the kinetics of [^{3}H]NMS binding to the receptor sites which were not inactivated was measured. The rate constant of the radioligand binding to receptors exposed to the toxin (k_{obs} = 0.61 ± 0.06 min^{-1}) was the same as to receptors incubated without toxin (0.57 ± 0.03 min^{-1}) and in agreement the earlier determined value in Table 1. This indicates that the toxin leaves some part of the receptor population unaffected.

The rate constants k_{in} for receptor inactivation by MT2 were calculated from equation (5). With increasing toxin concentration the k_{in} values first increase, but then level off (Table 1 and Fig. 3), indicating that the interaction of toxin with receptor is not a single-step equilibrium, but a more complex process. Strickland et al. [10] have shown that a plot of a shape as in Fig. 3 corresponds to the following reaction scheme:

$$R + T \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RT \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (RT) \quad (3)$$

For this reaction scheme the relation between k_{in} and toxin concentration can be expressed as follows:

$$k_{in} = \frac{k_2[T]}{K_1 + [T]} + k_{-2} \quad (4)$$

where $K_1 = k_{-1}/k_1$, R = receptor, T = toxin, RT and (RT) = toxin–receptor complexes, and k_1, k_{-1}, k_2 and k_{-2} = rate constants.

Two kinetic parameters were calculated from equation (4) by non-linear regression: K_1 = (1.4 ± 0.3) × 10^{-6} M and k_2 = (5.0 ± 0.4) × 10^{-2} min^{-1}.

The value of k_{-2} was not obtained from equation (4) because it was too small if compared to the experimental errors of determination of k_{in}. This means that the isomerization step of the two-step binding mechanism is practically an irreversible process.

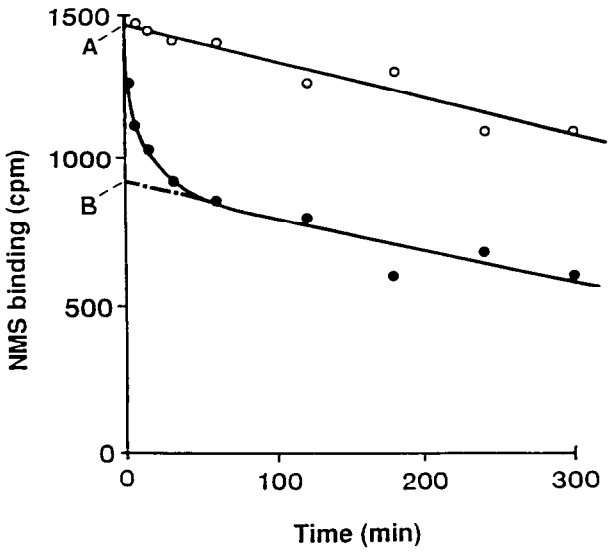

Fig. 2. Time course of decrease of [^{3}H]NMS binding capacity of muscarinic receptors during incubation in the presence of 50 mg/ml toxin (closed circles) and in the absence of the toxin (open circles) because of spontaneous inactivation of the receptor). Maximum binding of [^{3}H]NMS was obtained by extrapolation to time zero (points A and B).

3.3. Two steps of toxin binding

The hyperbolic relationship between the observed rate constants of ligand binding and ligand concentration (equation (4)) is valid when $k_{-1} > k_2$ (Strickland et al. [10]). On the other hand, as seen from the value of K_1, $k_1 \gg k_{-1}$, and hence $k_1 > k_2$. Thus the first step of the toxin–receptor interaction, the formation of RT in equation (3), is fast if compared to the following isomerization step.

The half-life for isomerization of RT to (RT) is 18 min as calculated from the rate constant k_2. Evidently, this slow isomerization process involves some conformational transition of the toxin–receptor complex. As a similar kinetic behaviour, including the slow isomerization step, was observed in separate experiments with digitonin solubilised receptors (unpublished), the slow phase of the receptor–toxin interaction does not seem to depend on any effect of the toxin on the biomembrane structure surrounding the receptor protein.

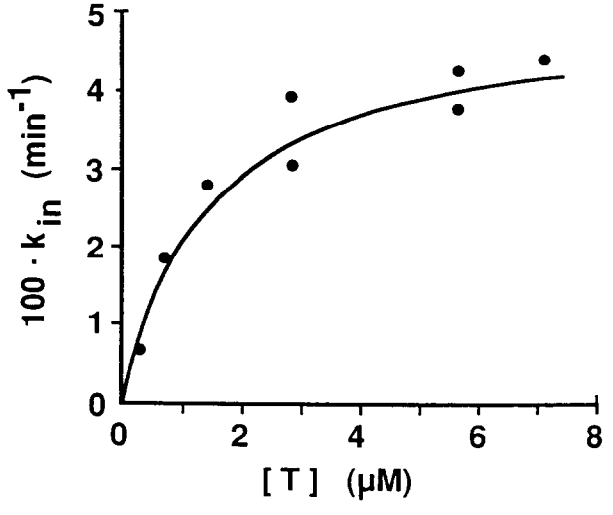

Fig. 3. The receptor inactivation rate constants k_{in} as function of toxin concentration [T].

The true affinity of the toxin for the receptor is characterized by the dissociation constant K_1 = 1.4 mM. This means that the highest toxin concentrations used in measurements of the k_{obs} values (Table 1) were sufficient to saturate the receptor. In spite of that the toxin had no effect on the kinetics of radioligand binding. Thus, the toxin and [^{3}H]NMS do not compete for the same binding site on the receptor. From this point of view the interaction of MT2 with muscarinic receptor is analogous with the action of the classical agonist carbachol [8,11]. Since the effect of toxin was observed only after some time (Fig. 1), isomerization of the toxin–receptor complex is evidently needed for displacement of the radioligand through an allosteric mechanism.

3.4. Significance of isomerization of toxin–receptor complex

The dissociation constant K_d is a combination of the two equilibrium constants K_1 and K_2:

$$K_d = K_1 K_2 \quad (5)$$

where $K_2 = k_{-2}/k_2$. A K_d of about 10 nM was obtained from binding of [^{125}I]MT2 to rat brain cortex [12]. Since this value is much smaller than K_1 = 1.4 mM, it follows that $K_2 < 1$. This means that the isomerization increases the potency of the toxin. But in the two-step binding the effect is developed slowly because of the isomerization.

The two-step binding is also characteristic of classical muscarinic antagonists like quinuclidinyl benzilate and *N*-methylscopolamine (for review see [8]). Since MT2 does not compete with an antagonist for its binding site, it may be an agonist. But in contrast to classical muscarinic agonists as carbachol it has a slow action.

Acknowledgements: This work was supported by the Swedish Natural Science Research Council, the Estonian Research Council and by Uppsala University for travel expenses.

References

[1] Harvey, A.L. (Ed.) (1991) Snake Toxins, Pergamon Press.
[2] Adem, A., Åsblom, A., Johansson, G., Mbugua, P.M. and Karlsson, E. (1988) Biochim. Biophys. Acta 968, 340–345.
[3] Karlsson, E., Risinger, C., Jolkkonen, M., Wernstedt, C. and Adem, A. (1991) Toxicon 29, 521–525.
[4] Karlsson, E., Jolkkonen, M., Satyapan, N., Adem, A., Kumlin, E., Hellman, U. and Wernstedt, C. (1994) Ann. NY Acad. Sci. 170, 153–181.
[5] Max, S.I., Liang, J.S. and Potter, L.T. (1993) J. Neurosci. 13, 4293–4300.
[6] Potter, L.T., Hanchett-Valentine, H., Liang, J.S., Max, S.I., Purkerson, S., Silberberg, H.S. and Strauss, W.L. (1993) Life Sci. 52, 433–440.
[7] Eller, M., Järv, J. and Palumaa, P. (1988) Organic Reactivity (Tartu) 25, 372–386.
[8] Järv, J. (1992) in: Selective Neurotoxicity, Handbook of Exp. Pharmacol, Vol. 102 (Herken, H. and Hucho, F., Eds.) pp. 660–680, Springer-Verlag.
[9] Langel, U., Rinken, A., Tähepõld, L. and Järv, J. (1982) Neurokhimiya 1, 343–351.
[10] Strickland, S., Palmer, G. and Massey, V. (1975) J. Biol. Chem. 250, 4048–4052.
[11] Järv, J., Hedlund, B. and Bartfai, T. (1980) J. Biol. Chem. 255, 2649–2651.
[12] Jerusalinsky, D., Cervenansky, C., Pena, C., Raskovsky, S. and Dajas, F. (1990) Neurochem. Int. 20, 237–246.