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α-Bungarotoxin interacts with the rat brain tachykinin receptors

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α-Bungarotoxin (αBgt) was shown to inhibit the binding of the ^{125}I-labeled substance P (SP) and eledoisin (EL) to the rat brain membranes with K_I values of $8.0 \pm 5.0 \times 10^{-8}$ and $1.1 \pm 0.5 \times 10^{-6}$ M, respectively. Lower inhibitory activity was manifested by several other postsynaptically acting snake venom neurotoxins. The αBgt inhibition of SP binding with a K_I value of $8.5 \pm 5.5 \times 10^{-8}$ M to solubilized preparations of the rat brain membranes was demonstrated. The capacity to displace SP was found for d-tubocurarine and phencyclidine, although at concentrations considerably higher than those affecting the nicotinic acetylcholine receptors (AChRs). The results obtained suggest that some of the αBgt-binding polypeptides, distinct from neuronal AChRs, may be functionally associated with the tachykinin receptors (TchR).

α-Bungarotoxin; Substance P; Tachykinin receptor; (Rat brain membrane)

1. INTRODUCTION

Substance P, a peptide neurotransmitter, belongs to the family of tachykinins that manifest a multitude of neuro- and immunomodulatory activities [1]. One of the reasons for this versatility may be their ability to interact not only with several types of TChRs, but also with the receptors of classical neuromediators. For example, the effects of SP on AChRs were demonstrated in several laboratories [2-5]. Taking these data into account, especially the SP inhibition of αBgt binding to AChR [5], we addressed another problem: whether αBgt and other AChR ligands can act on TChR.

2. MATERIALS AND METHODS

[^{125}I]BH-SP and [^{125}I]BH-EL were synthesized as in [6,7]. Their binding to rat brain membranes was analyzed as described in [6] for the SP derivative, for the radioactive EL a longer incubation time (50 min) was used.

When studying inhibitory activity, αBgt (Boehringer Mannheim) as well as other neurotoxins and cytotoxins were preincubated with rat brain membranes at 20°C for 60 min followed by addition of radioactive tachykinins. All other low molecular weight ligands were added simultaneously with [^{125}I]BH-SP and incubated for 45 min.

To analyze the binding to the solubilized TChR, rat brain membranes were treated with CHAPS essentially as in [8]. [^{125}I]BH-SP was incubated at 0°C for 2 h with 200 μl of solubilized membranes (3 mg protein/ml) in 50 mM Tris-HCl buffer, pH 7.4, containing 0.5% CHAPS, 50 mM NaCl, 3 mM $MgCl_2$, 1% bovine serum albumin, leupeptin (4 μg/ml), bacitracin (40 μg/ml) and phenylmethylsulfonyl fluoride (8.5 μg/ml) in the presence or absence of αBgt. Nonspecific binding was determined in the presence of 1 μM SP. Unbound radioactive ligands were removed from the solubilized preparations using Whatman GF/B filters as in [8]. The sources of ligands, as well as the references to the synthesis and purification of peptides and neurotoxins are given in more detail in [7].

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Abbreviations: AChR, acetylcholine receptor; αBgt, α-bungarotoxin; BH-EL and BH-SP, eledoisin and substance P modified with Bolton-Hunter reagent; CHAPS, (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; EL, eledoisin; SP, substance P; TChR, tachykinin receptor

3. RESULTS AND DISCUSSION

As can be seen from fig.1, preincubation of rat brain membranes with αBgt inhibits the specific binding of [^{125}I]BH-SP, a ligand with the highest affinity for an NK-1 type TChR (see [9] for receptor classification). The K_I value of $8.05 \pm 5.0 \times$

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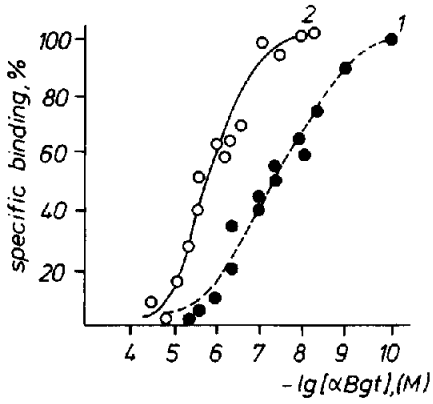

Fig.1. αBgt inhibition of [^{125}I]BH-SP (0.2 nM, curve 1) and [^{125}I]BH-EL (0.25 nM, curve 2) binding to rat brain membranes (~ 2.5 mg protein /ml). Each point in the plot is an average of duplicate or triplicate measurements. K_I values and error limits, $8.05 \pm 5.0 \times 10^{-8}$ M and $1.1 \pm 0.5 \times 10^{-6}$ M for SP and EL derivatives, respectively, were calculated from IC_{50} values. The latter were determined with different batches of radioactive ligands and membranes using for SP and EL derivatives the K_d values of 0.7 and 0.4 nM, respectively, found from the Scatchard plots (not shown).

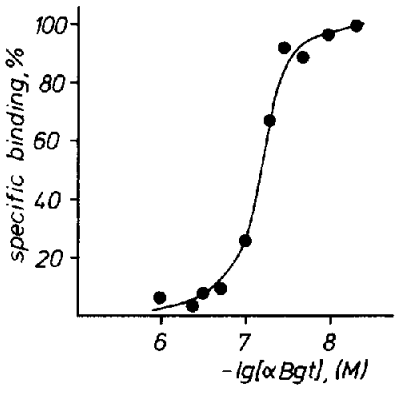

Fig.2. αBgt inhibition of [^{125}I]BH-SP (0.7 nM) interaction with the CHAPS-solubilized rat brain membranes, $K_I = 8.5 \pm 5.5 \times 10^{-8}$ M.

10^{-8} M differs by almost 3 orders of magnitude from the K_I value of 1.2×10^{-5} M characterizing the effect of SP on the interaction of radioactive αBgt with the AChR from the *Torpedo* electric organ [5]. In other words, the αBgt affinity for the NK-1 TChR is much higher than that of SP for AChR.

The effect of αBgt is virtually irreversible, since after preincubating the membranes with αBgt followed by repeated washings and 24–72 h incubation in an appropriate buffer at 0°C no [^{125}I]BH-SP binding could be observed. In control experiments, the membranes that underwent the same treatment, except for the addition of αBgt, efficiently bound the radioactive SP.

We also examined the αBgt effect on an NK-3 TChR whose preferable ligands are neurokinin B and BH-EL [9,10]. The higher value of K_I ($1.1 \pm 0.5 \times 10^{-6}$ M (see curve 2 in fig.1)) indicates that the αBgt affinity for the NK-3 receptor is lower than for the NK-1 type. This finding correlates with the less pronounced effect of EL, as compared with SP, on the AChR [4].

Treatment with CHAPS diminished the capacity to bind [^{125}I]BH-SP: for solubilized preparations the binding parameters were $K_d = 9.0 \pm 6.0$ nM and $B_{max} = 18 \pm 12$ fmol/mg protein (the binding curve is not shown), as compared with 0.7 ± 0.05 nM and 60 ± 10 fmol/mg protein for the intact rat brain membranes [6]. However, the potency of αBgt to compete with [^{125}I]BH-SP was largely preserved on solubilization (fig.2), the K_I value being $8.5 \pm 5.5 \times 10^{-8}$ M. This result suggests that αBgt either directly interacts with the rat brain TChR or binds to a component tightly associated with it. Noteworthy, SP competed with radioactive αBgt for the binding centers on the solubilized *Torpedo* AChR [5].

In order to characterize in more detail the SP/αBgt-binding sites in the rat brain, we studied the inhibitory activity of different snake venom neurotoxins of the so-called long-chain and short-chain types (see classification in [11]). Complete inhibition of [^{125}I]BH-SP binding could be achieved with the long-type toxin 3 *Naja naja siamensis* (table 1, fig.3). However, its K_I ($1.1 \pm 0.4 \times 10^{-6}$ M) characterizes a lower potency than that of αBgt. (Interestingly, αBgt and toxin 3 are virtually equipotent when interacting with the αBgt-binding sites of the rat brain [12].)

Among a series of short-chain neurotoxins, only one (*Naja mossambica mossambica*) exerted a marked effect at 10^{-5} M. As shown in table 1 for neurotoxin II *Naja naja oxiana*, the inhibition becomes quite efficient at 10^{-4} M. However, at such a concentration the effect might be, to a large extent, associated with perturbations of membrane structure, since the snake venom cytotoxins, membrane active polypeptides [11], are even more effi-

Table 1

Inhibition of [^{125}I]BH-SP binding to the rat brain membranes by different AChR ligands

Ligand	Concentration (μM)	Inhibition (%)
Long-chain neurotoxins		
αBgt	5.0	100[a]
toxin 3 *Naja naja siamensis*	28.0	100
neurotoxin I *Naja naja oxiana*	56.0	0
Short-chain neurotoxins		
toxin 3 *Naja mossambic mossambica*	22.0	20
α-toxin *Naja nigricollis*	17.0	$\leqslant$5
erabutoxin a *Laticauda semifasciata*	25.0	$\leqslant$5
neurotoxin II *Naja naja oxiana*	12.0	$\leqslant$5
	100.0	75
conotoxin G1	250	0
thymopentin	250	0
Carbamoylcholine	1000	0
Nicotine	1000	$\leqslant$5
Cytisine	1000	$\leqslant$5
d-Tubocurarine	20.0	15
	300	50
Phencyclidine	10.0	15
	500	50
Cytotoxins I and II *Naja naja oxiana*[b]	100	100

[a]Concentration of radioactive ligand was 0.2 nM in all experiments, inhibition by 1 μM SP taken as being 100%

[b]given for comparison (these compounds are not AChR ligands)

cient at similar concentrations (table 1, fig.3). Therefore, the interaction of snake venom neurotoxins with the rat brain NK-1 TChR is specific and strongly dependent on the structural features of a particular neurotoxin.

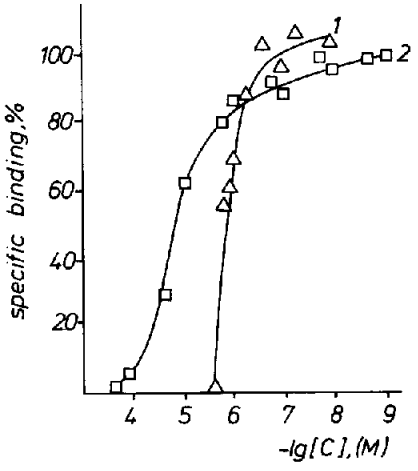

Fig.3. Inhibition of [^{125}I]BH-SP binding (0.25 nM) to rat brain membranes by toxin 3 *Naja naja siamensis* (curve 1), $K_I = 1.1 \pm 0.4 \times 10^{-6}$ M, and cytotoxin II *Naja naja oxiana* (curve 2), $K_I = 1.0 \pm 0.3 \times 10^{-6}$ M. [C], molar concentration of toxins.

Besides neurotoxins, we have also examined the influence of other AChR ligands on the SP binding (table 1). Carbamoylcholine at 1 mM failed to compete with [^{125}I]BH-SP. The presence of this agonist did not abolish the inhibitory effect of αBgt on the TChR. Nicotine, known to act on different AChRs [13] exerted practically no effect on the SP binding. Cytisine, one of the neuronal AChR ligands, was also ineffective. On the other hand, d-tubocurarine, an antagonist, and a non-competitive blocker, phencyclidine, displaced [^{125}I]BH-SP in a concentration-dependent manner. However, in both cases the effective concentrations were at least 2 orders of magnitude higher than those affecting different kinds of AChRs [14,15].

It has been demonstrated that α-conotoxins from fish-hunting cone snails [16] and thymopoietin [17] compete with αBgt for the *Torpedo* AChR. With this in mind, we tested the capacity of conotoxin G1 and thymopentin, the active fragment of thymopoietin, to inhibit association of [^{125}I]BH-SP with the rat brain membranes. The two peptides at 250 μM proved inactive. Summarizing the data of table 1, it can be concluded that the SP/αBgt-binding sites appear to differ

from the functional AChRs and display the preference towards distinct snake venom neurotoxins as compared to other tested AChR ligands.

It should be mentioned that binding of αBgt is not a property inherent in all types of nicotinic AChRs. For example, such an activity is manifested by receptors form *Torpedo* electroplaque [11,13], mammalian muscles [18], chick optic lobe [19], as well as by neuronal AChRs from *Drosophila* [15], locust [20] and goldfish brain [21]. In contrast, the neuronal AChRs from the clonal cell line PC12 [22], human and bovine [23] or rat brain [18] do not interact with αBgt.

Mammalian and avian brain also contain proteins that differ in their affinity for αBgt from 10^{-7} to 10^{-9} M, are not functional AChRs and have an obscure role [12,24]. Our data on the αBgt inhibition of [^{125}I]BH-SP binding with a K_I value of the order of 10^{-7} - 10^{-8} indicate that at least some of these proteins are identical to the SP/αBgt-binding sites described in the present communication and therefore might be implicated in mediating physiological responses to SP.

The sites involved in αBgt binding have been identified in the amino acid sequence of the *Torpedo* AChR α-subunit (see, for example, [25]). We could not find sequences homologous to those sites in the substance K receptor from bovine stomach [26], the only sequenced receptor of the TChR family. The computer analysis failed to detect extended homologies between his receptor and *Torpedo*, muscle, or neuronal AChRs (See [7] for details).

The primary structures of acetylcholine-insensitive αBgt-binding proteins from the rat brain are not yet known. However, one of them was recently demonstrated to consist of subunits similar to the α, β, γ and δ subunits of the *Torpedo* AChR [27]. There are many reasons to believe that these αBgt-binding polypeptides should be related to other AChR-gene derived proteins.

At present it is not clear whether αBgt binding is due to a polypeptide that is attached to TChR so tightly that it cannot dissociate in detergent, or αBgt interacts with a site on the TChR itself. The latter possibility seems more attractive and implies that, in contrast to the lack of homology in the primary structures, the spatial organization of the ligand-binding sites should be similar in different proteins interacting with αBgt. This feature may be a link between two types of receptor families: ligand-gated ion channels represented by AChR, and G-protein-dependent systems comprising TChR. The same situation can be anticipated for the acetylcholine-binding sites in the sequentially unrelated nicotinic AChRs and muscarinic AChRs, the latter belonging to the same superfamily as TChR [26].

To summarize, our experimental results demonstrate that, firstly, neuronal TChRs may be responsive both to their own and AChR ligands, and, secondly, the functional role of some brain acetylcholine-insensitive αBgt-binding polypeptides is associated with that of TChR.

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