Tetrodotoxin Reverses Brevetoxin Allosteric Inhibition of Scorpion α-Toxin Binding on Rat Brain Sodium Channels

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Voltage-sensitive sodium channels are responsible for the initiation of action potentials in many excitable cells. Several neurotoxins bind to distinct receptor sites on sodium channels and reveal strong allosteric interactions among them. Scorpion α-toxins, which inhibit sodium channel inactivation by binding to receptor site 3, have been very important tools to study sodium channel structure and function. Recently, we have shown that brevetoxin induce a strong negative allosteric modulation on scorpion α-toxin binding on rat brain sodium channels, in contrast to previously published studies. In this report we have examined the reasons for this discrepancy and found new, unexpected allosteric interactions between the tetrodotoxin and brevetoxin receptor sites, using scorpion α-toxin as sensitive probe for subtle conformational changes on sodium channels. Tetrodotoxin reverses the negative modulation induced by brevetoxin on scorpion α-toxin binding, revealing new dynamic interactions in sodium channel structure.

The high toxicity of brevetoxins (PbTx-n), lipid-soluble polye ther marine toxins produced by the “red tides” dinoflagellate Ptychodiscus brevis, to human and fish was found to be due to their high affinity binding to specific receptor site (site 5) on voltage-sensitive sodium channels (1). Brevetoxin binding results in membrane depolarization due to a shift of the channel activation to more negative membrane potentials and inhibition of normal inactivation (2, 3). Sodium channels are composed of about 2000 amino acids organized in four repeated homologous domains (I–IV) each consisting of six putative transmembrane segments (S1–S6) (4). Partial localization of receptor site 5 has been suggested recently, using labeled derivative of PbTx-3 and site-directed antibody mapping, to be in the region of interaction of transmembrane segments S6 and S5 of homology domains I and IV, respectively, of rat brain sodium channels (5).

Since 1981, the binding of different brevetoxins and the allosteric interaction between receptor site 5 and other neurotoxins’ receptors on sodium channels have been extensively studied using neuroblastoma cells and rat brain synaptosomes (6–13). Scorpion α-toxins were shown to inhibit inactivation of sodium channels, and their binding was demonstrated to be allosterically enhanced by alkaloid toxins (such as batrachotoxin and veratridine) (4) but not by brevetoxins. PbTx-1 and PbTx-2 were shown not to affect the binding of the scorpion α-toxin Lqq V (6, 7, 9).

In contrast to the previous results, we have recently demonstrated that PbTx-1, the most active brevetoxin analog (1), induces a strong negative allosteric modulation on the binding of another scorpion α-toxin, AaH II, on rat brain sodium channels (14). The binding affinity of AaH II to rat brain synaptosomes was shown to be the highest among all scorpion α-toxins ($K_d = 0.2–0.3$ nM) (14, 15), about 10-fold higher than that of Lqq V ($K_d = 2.2–8$ nM) (16). The structural differences between these two pharmacologically similar scorpion α-toxins, both inhibiting sodium channel inactivation and binding to receptor site 3 in a voltage-dependent manner on mammalian sodium channels (15–17) may account for the significant difference in binding affinity. In addition, the various brevetoxins were shown to have different toxicity and differentially affect the binding of [3H]batrachotoxin derivative (1, 11), related to their carbon backbone structure (1, 12).

These considerations led us to attribute the differences between ours and preceding results concerning the previously undetectable strong allosteric modulation by PbTx-1 on the scorpion α-toxin binding to: 1) structural differences between the two scorpion α-toxins used, AaH II in our case and Lqq V in the others, suggesting that interaction between brevetoxin and scorpion α-toxin site may be, at least in part, toxin specific; and 2) the inherent structural differences in the brevetoxin analogs (1, 12) used in ours and previous studies (14). In the present work we examined these possibilities and found that none can account for the observed discrepancy, suggesting that still other, unexpected reasons should exist. We found that the routine presence of TTX may account for that discrepancy.

EXPERIMENTAL PROCEDURES

Materials—Scorpion toxins AaH II and Lqq V were purified as described previously (27). Brevetoxins (PbTx-1, -2, -3, and -9) and tetrodotoxin were from Latoxan (Rosans, France). Carrier-free Na$^{125}$I was from Amersham Corp. All other chemicals were of analytical grade. Filters for binding assays were glass fiber GF/C (Whatman, United Kingdom) preincubated in 0.3% polyethyleneimine (Sigma).

Membrane Preparation and Binding Assays—Rat brain synaptosomes were prepared from adult albino Wistar rats (about 300 g, laboratory bred), according to Ref. 26. All buffers contained a mixture of protease inhibitors composed of 50 μg/ml phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 μM iodoacetamide, and 1 μM 1,10-phenanthroline. Membrane protein concentration was determined using a BioRad protein assay, with bovine serum albumin as standard.

AaH II and Lqq V were radioiodinated by lactoperoxidase and monoiodotyrosine were purified as described previously (14). The concentration of the radioiodinated toxins was determined according to the specific activity of the [125]I corresponding to 2424 dpm/mmol monoidotyrosine. Equilibrium competition and saturation assays were performed using increasing concentrations of the unlabeled toxin in the presence of a constant low concentration of the radioactive toxin. Standard binding medium composition was: in mM: chloride chloride 140, CaCl$_2$ 1.8, KCl...
TTX Allosteric Effects on Scorpion α-Toxin Binding

**RESULTS**

We have shown previously that brevetoxin (PbTx-1) inhibition on AaH II binding affinity (with no significant effect on receptor capacitance), due to increasing the dissociation rate constant, consistent with a negative allosteric interaction between brevetoxin receptor site 5 and scorpion α-toxin receptor site 3 (14). Fig. 1 demonstrates that both Lqq V and AaH II binding are significantly inhibited by PbTx-1. Lqq V-specific binding is similarly inhibited, but at apparent higher PbTx-1 concentration (Fig. 1). This result excluded the explanation in possibility 1 (see Introduction), since both scorpion α-toxins binding is shown to be negatively modulated by brevetoxin, although with some quantitative differences.

Different brevetoxin analogs, PbTx-1, PbTx-2, PbTx-3 and PbTx-9, are shown in Fig. 2 to inhibit both scorpion α-toxins binding; PbTx-1 being the most active one. As suggested in Fig. 1, the binding inhibition of the two toxins revealed significant quantitative differences (Fig. 2). To reveal possible structural basis for the above quantitative differences, we compared structural models of the two scorpion α-toxins.

AaH II and Lqq V share 63% of amino acid identity (72% similarity). Computer modeling discloses that the positively charged residues, previously suggested to participate in the receptor binding interaction (18, 19), are shown to inhibit both scorpion α-toxin binding modulation. TTX is most commonly used as specific blocker of sodium current in many studies and has been shown to compete with similar affinities on the same receptor site (8) but reveal different ability to allosterically inhibit the binding of scorpion α-toxins. The binding of AaH II has been inhibited more effectively by all the brevetoxins tested. The data are presented as percentage of inhibition of the α-toxin specific binding (mean ± S.E. of three to five experiments).

Careful inspection of the former binding conditions revealed that TTX was routinely added to binding media used for scorpion α-toxin binding modulation. TTX is most commonly used as specific blocker of sodium current in many studies and has been shown to compete with similar affinities on the same receptor site (8) but reveal different ability to allosterically inhibit the binding of scorpion α-toxins. The binding of AaH II has been inhibited more effectively by all the brevetoxins tested. The data are presented as percentage of inhibition of the α-toxin specific binding (mean ± S.E. of three to five experiments).

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TTX Allosteric Effects on Scorpion α-Toxin Binding

Fig. 3. Tetrodotoxin reverses the inhibition of scorpion α-toxin AaH II binding caused by brevetoxin PbTx-1. Rat brain synaptosomes were incubated for 30 min at 37 °C with 0.1 nM 125I-AaH II (as described in Fig. 1) in the presence or absence of brevetoxin PbTx-1 (at 30 or 100 nM) and increasing concentrations of TTX. The data points are mean ± S.E. of three to six experiments (A). A, the data are presented as percentage of 125I-AaH II bound from the control with no additions (marked as 100% binding by the dashed line). TTX increased the binding of AaH II by about 20–40%, as described previously (15, 16). The binding of 125I-AaH II in the presence of 30 and 100 nM PbTx-1 (without TTX) was 52.5 ± 14.7% and 46.6 ± 4.5%, respectively (14). Increasing concentrations of TTX progressively reversed the inhibitory effect of brevetoxin, by increasing AaH II binding to control levels and, at maximal TTX concentration, to the level with TTX alone, indicating that TTX is able to enhance AaH II binding even in the presence of saturating concentration of brevetoxin. B, Scatchard analysis of AaH II-specific binding to rat brain synaptosomes in the presence of 30 nM brevetoxin PbTx-1 with or without 1 μM TTX. Membranes (0.13 mg of protein/ml) were incubated with 0.1 nM 125I-AaH II in the presence of 30 nM brevetoxin PbTx-1 and increasing concentrations of unlabeled AaH II, in the presence or absence of 1 μM TTX at 37 °C for 30 min, and specific binding was determined as described in Fig. 1. Scatchard plots were analyzed with the program LIGAND (Cold Saturation). Equilibrium binding constants determined were as follows (mean ± S.E., n = 3): PbTx-1 30 nM, Kd = 0.52 ± 0.09 nM, Bmax = 0.20 ± 0.03 pmol/mg; TTX 1 μM and + 30 nM PbTx-1, Kd = 0.24 ± 0.06 nM, Bmax = 0.19 ± 0.03 pmol/mg protein.

DISCUSSION

Our results indicate that TTX, the most used and studied sodium channels blocker, should be considered as an allosteric modifier of sodium channels, and new allosteric interactions are elucidated between receptor sites 1, 5, and 3, using scorpion α-toxin as probe. The increase in scorpion α-toxin binding by TTX (Refs. 15 and 16; Fig. 3) has been attributed previously to its block of sodium channel conductance and prevention of possible depolarization (known to reduce the scorpion α-toxins binding, 21, 22). In contrast to previous interpretations (15, 16), we suggest that this enhancement may be attributed to a direct allosteric effect of TTX on receptor site 3, resulting in shifting more receptors to the high affinity state for scorpion α-toxin, since all experiments have been performed in sodium-free media (Refs. 15 and 16; Fig. 3). The reversal of brevetoxin inhibition by TTX further supports its allosteric effect on receptor site 3.

These results indicate also the presence of allosteric interactions between TTX and brevetoxin receptor sites that are mainly detected at the region of scorpion α-toxin receptor site. Previously, enhancement of 13H]saxitoxin binding by brevetoxin was reported in neuroblastoma cells (6) but not in rat brain synaptosomes (9). Tritiated brevetoxin binding was slightly enhanced (5–10%) in the presence of higher concentrations of saxitoxin or TTX (8). Together, previous and present results suggest the presence of allosteric interactions between TTX and brevetoxin receptor sites. However, such interaction may result in very limited conformational changes, as detected by the limited changes in binding at receptor sites 1 and 5.

Our present results may indicate that the binding of TTX and brevetoxin to their distinct receptor sites may induce additional, previously undetectable conformational changes on the sodium channel protein. Such conformational changes could not be detected by the study of TTX and PbTx-1 alone, suggesting that they may not be directly related to the function of each toxin. Rather, other conformational changes resulting from the binding of these toxins, may be detected using scorpion α-toxin as sensitive probe for subtle conformational alterations in a different region comprising receptor site 3 on sodium channels. The significant reversal of brevetoxin-induced binding inhibition of AaH II by TTX is comparable with that previously observed by veratridine (14). Both sodium channel effectors (TTX and veratridine) could enhance the scorpion α-toxin binding more efficiently in the presence of the negative modulator, PbTx-1 (Fig. 3; Ref. 14). Brevetoxins have been shown to allosterically enhance the binding of alkaloid toxins (9, 11). Our results suggest that allosteric interactions between TTX and PbTx-1 receptor sites are also present, but expressed more significantly in an indirect conformational changes sensed by scorpion α-toxin binding. These may suggest an intimate dynamic relationship between at least some recognition sites comprising receptor site 3 and those of receptor sites 1 and 5.

This study may pave the way to a new insight into the dynamic conformational changes induced on the channel protein upon neurotoxin binding and action, revealing dynamic relationship between the pore region (receptor site 1) (23, 24),

enlargement may not be explained by an additive effect. In the presence of brevetoxin, TTX increases AaH II binding by about 2-fold, as compared to about 1.3-fold increase by TTX alone (Fig. 3A). The enhancement of AaH II binding by TTX (with or without brevetoxin) is evident only at the resting membrane potential; at depolarized conditions (by high external potassium concentration; Ref. 16), TTX is not able to negate the decrease in scorpion α-toxin binding (data not shown).

Scatchard analysis of AaH II binding reveals that the reversal of brevetoxin’s negative effect on scorpion α-toxin binding by TTX is due to a 2-fold increase in AaH II binding affinity (Fig. 3B). Brevetoxin at half saturating concentration (30 nM) increases the Kd of AaH II about 2-fold, in accordance with our previous results (14), and the presence of TTX decreases the Kd back to its original value. Thus, in the presence of both TTX and PbTx-1, the binding affinity of AaH II is about its value with no effectors (Fig. 3B; Ref. 14). This suggests that TTX in the presence of brevetoxin induces an allosteric effect on receptor site 3 that reverses or prevents the decrease in affinity induced by brevetoxin alone, resulting in scorpion α-toxin binding around control level.

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the transmembrane regions of segments S5 and S6 (receptor site 5) (5), and extracellular amino acid loops (receptor site 3) (20, 25) of the different domains of sodium channels. Considering the molecular mapping of these receptor sites (5, 20, 23–25), our study may contribute to the elucidation of the structural basis underlying the dynamic conformational changes responsible for the function of sodium channels.

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