Allosteric interactions among pyrethroid, brevetoxin, and scorpion toxin receptors on insect sodium channels raise an alternative approach for insect control

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Abstract Intensive pyrethroid use in insect control has led to resistance buildup among various pests. One alternative to battle this problem envisions the combined use of synergistically acting insecticidal compounds. Pyrethroids, scorpion α- and β-toxins, and brevetoxins bind to distinct receptor sites on voltage-gated sodium channels (NaChs) and modify their function. The binding affinity of scorpion α-toxins to locust, but not rat-brain NaChs, is allosterically increased by pyrethroids and by brevetoxin-1. Brevetoxin-1 also increases the binding of an excitatory β-toxin to insect NaChs. These results reveal differences between insect and mammalian NaChs and may be exploited in new strategies of insect control.

Key words: Pyrethroid; Scorpion toxin binding; Allosteric effect; Insect sodium channel; Insect control; Selective insecticide

1. Introduction

Voltage-gated sodium channels (NaChs) are critical for generation of action potentials in excitable cells, and are targeted by a large variety of chemically distinct compounds that bind to several receptor sites on the pore-forming α-subunit [1,2]. Most lipid-soluble NaCh activators, including pyrethroid insecticides, toxic alkaloids (e.g. veratridine and batrachotoxin) and marine cyclic polyether toxins (e.g. brevetoxins), affect NaChs of both insects and mammals. However, several scorpion toxins show selectivity for insect and mammalian NaCh subtypes [3–5], which may be harnessed for design of selective drugs and insecticides.

Pyrethroids are widely used to control insect pests in agriculture and for protection of public health (>25% of the world insecticide market). They cause a fast knockdown (*kdr*) effect on insects by modifying the gating kinetics of neuronal NaChs [6]. However, their intensive use in the last two decades led to resistance buildup in many insect species [7], which in many instances was associated with point mutations in the insect NaCh gene (*kdr* mutations; [8–10]). Pyrethroids bind to transmembrane hydrophobic channel regions where many *kdr* mutations were assigned [7–10], and modify mainly the channel activation [11]. Moreover, similar hydrophobic regions of the channel are also targeted by alkaloid toxins, such as veratridine and batrachotoxin, which bind to receptor site-2 [12,13], and by brevetoxins, which bind receptor site-5 [1,2,14,15]. Although the various toxin receptor sites are topologically distinct, they are involved in various allosteric interactions in insect and mammalian NaChs [1,2,16–20]. Variation in these interactions between insect and mammalian NaChs illuminate subtle, yet functionally relevant differences that may be exploited for the design of anti-insect selective compounds [16,21].

Scorpion toxins that modulate NaCh gating are divided into α and β classes according to their mode of action and binding properties [3,22]. α-Toxins (e.g. LqhαIT, Lqh3 and Lqh2 (from *Leiurus quinquestriatus hebraeus*)) slow down Na^+ current inactivation upon binding to receptor site-3, which involves extracellular loops in domains 1 and 4 of the NaCh [1,2]. They are divided to several groups according to their affinity to insect (e.g. LqhαIT) and various mammalian NaChs (e.g. Lqh2 and Lqh3) [3–5,20]. β-Toxins shift the voltage dependence of activation to more negative membrane potentials upon binding to receptor site-4, assigned mainly to external loops in domain 2 [23,24]. Two groups of the β-toxin class, the excitatory (e.g. AahIT (from *Androctonus australis hector*) and Bj-xtrIT (from *Buthotus judaicus*)) and the depressant (e.g. LqhIT2) toxins, are selective for insect NaChs [3,25–27], and affect insects synergistically [28,29].

The present work demonstrates allosteric modulation of the binding of α- and β-scorpion toxins by pyrethroids and brevetoxin on insect NaChs, and suggests that the combined application of pyrethroids with scorpion toxins may reduce resistance buildup [16,21].

2. Materials and methods

2.1. Toxins

Bj-xtrIT and LqhαIT were produced in a recombinant form [30,31]. AahIT was kindly provided by M. Elazar and E. Zlotkin, The Hebrew University of Jerusalem. Pyrethroids were a generous gift of K. Naumann, Bayer AG, Germany. Veratridine was from Sigma (Steinhem,

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Abbreviations: NaCh, voltage-gated sodium channel; AahIT, excitatory toxin from *Androctonus australis hector*; Bj-xtrIT, excitatory toxin from *Buthotus judaicus*; PbTx-1, brevetoxin from the marin dinoflagellate *Ptychodiscus brevis*; LqhαIT, Lqh2 and Lqh3, scorpion α-toxins from *Leiurus quinquestriatus hebraeus*

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Germany). Brevetoxin PbTx-1 (from the marin dinoflagellate *Ptychodiscus brevis*) and Lqh2 were from Latoxan (Valance, France). Iodogen was from Pierce Chemicals (Rockford, IL, USA). Carrier-free $Na^{125}I$ was from Amersham (Buckinghamshire, UK). All other chemicals were of analytical grade.

2.2. Neuronal membrane preparation

Locust synaptosomes, prepared from dissected brains and ventral nerve cords of adult locusts (*Locusta migratoria*), and rat brain synaptosomes were prepared by established methods [20,32].

2.3. Radioiodination

AahIT, BjxtrIT, Lqh2 and LqhαIT were radioiodinated by Iodogen using 5 μg toxin and 0.5 mCi carrier-free $Na^{125}I$, as was described [20,30,32,33]. The concentration of the monoiodotoxins was determined according to the specific activity of ^{125}I corresponding to 2500–3000 dpm/fmol of monoiodotoxin [33].

2.4. Binding assays

Equilibrium competition and cold saturation assays were performed using increasing concentrations of the unlabeled toxin in the presence of a constant low concentration of the radioactive toxin. Hot saturation was performed using increasing concentrations of the labeled toxin. Saturation experiments were analyzed by the iterative computer program LIGAND (Elsevier Biosoft, Cambridge, UK). Competition binding experiments were analyzed by KaleidaGraph (Synergy Software, USA) using a non-linear Hill equation [18,33].

2.5. Kinetic of dissociation

Dissociation was initiated with excess cold toxin and the kinetic data was subjected to analysis according to Weiland and Molinoff [34]. The dissociation rate constant (k_{off}) was determined directly from a first order plot of ligand dissociation versus time or from the non-linear analysis of the data by KaleidaGraph. Non-specific toxin binding was determined in the presence of excess cold toxin

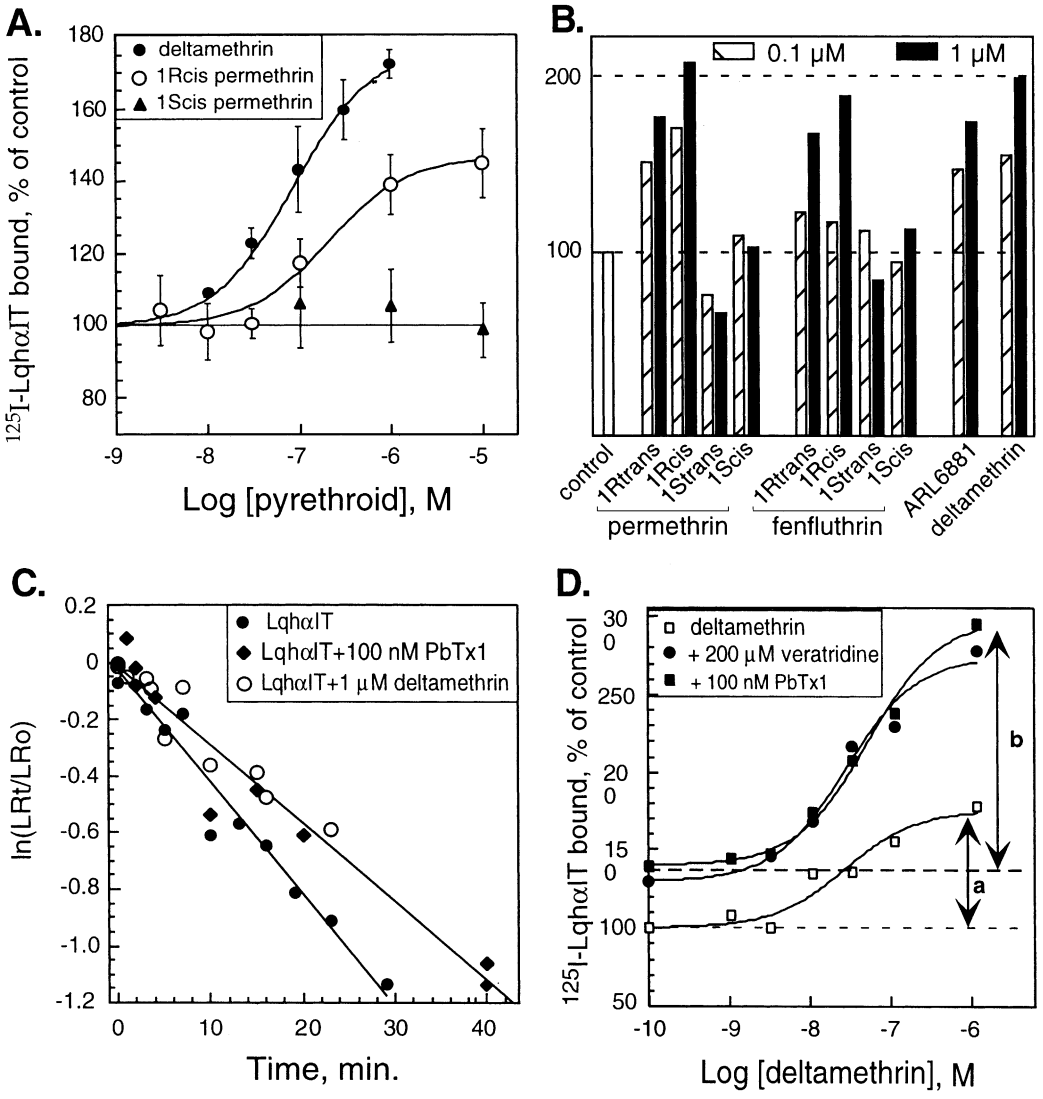

Fig. 1. Effect of pyrethroids on LqhαIT binding to locust neuronal membranes. A: Enhancement of [^{125}I]LqhαIT binding by increasing concentrations of deltamethrin, 1Rcis permethrin, and 1Scis permethrin. Membranes were incubated with 120 pM [^{125}I]LqhαIT for 1 h. Non-specific binding, measured in the presence of 1 μM LqhαIT, was subtracted. Values represent percentage of the maximal specific binding without pyrethroids (100%) and are the mean of three independent experiments ± S.D. (error bars). B: Effects of different pyrethroids on [^{125}I]LqhαIT binding. Membranes were incubated with [^{125}I]LqhαIT as in A, in the presence of 0.1 or 1 μM pyrethroid. Different conformers of permethrin and fenfluthrein were tested. The mean of two independent experiments is presented. C: Kinetics of [^{125}I]LqhαIT dissociation. Membranes were incubated with 0.1 nM [^{125}I]LqhαIT for 1 h and dissociation was initiated with 1 μM LqhαIT in the absence and presence of 100 nM brevetoxin PbTx-1 or 1 μM deltamethrin. A representative experiment is shown. D: Synergic effect of deltamethrin on LqhαIT binding in the presence of veratridine and PbTx-1. Arrows show the maximal increase in [^{125}I]LqhαIT binding in the presence of saturating concentrations of deltamethrin alone (a), and together with 200 μM veratridine or 100 nM PbTx-1 (b). Maximal specific binding of [^{125}I]LqhαIT alone (control 100%), and with veratridine or brevetoxin (without pyrethroid) are indicated by the lower and upper dashed lines, respectively.

and was typically 10%, 20%, 15%, and 10% of total binding for [^{125}I]LqhαIT, [^{125}I]AahIT and [^{125}I]Bj-xtrIT, and [^{125}I]Lqh2, respectively (see [33] for details).

3. Results and discussion

3.1. Effects of pyrethroids on the binding of LqhαIT to locust neuronal membranes

The pyrethroids deltamethrin and 1Rcis permethrin increased the binding of [^{125}I]LqhαIT to locust synaptosomes 175 ± 28% and 144 ± 38% ($n = 3$), respectively, above control level with effective concentration 50% (EC_{50}) of 58 ± 25 nM and 131 ± 54 nM ($n = 3$, Fig. 1A). The pyrethroid effect was stereospecific, and was obtained only with the 1Rcis and 1Rtrans isomers of permethrin and fenfluthrein (Fig. 1B). Since a similar effect on [^{125}I]LqhαIT binding was obtained with brevetoxin PbTx-1 [19,20], the kinetics of [^{125}I]LqhαIT dissociation from receptor site-3 in the presence of 1 μM deltamethrin was compared to that obtained in the presence of 100 nM PbTx-1 (Fig. 1C). The dissociation rate constant of bound LqhαIT ($k_{off} = 0.62 \pm 0.07 \times 10^{-3}$ s^{-1}, $n = 3$) was ~2-fold higher than that measured in the presence of deltamethrin ($k_{off} = 0.37 \pm 0.12 \times 10^{-3}$ s^{-1}, $n = 3$) or PbTx-1 ($k_{off} = 0.39 \pm 0.11 \times 10^{-3}$ s^{-1}, $n = 3$). These results suggest that both NaCh activators increase allosterically the stability of the α-toxin–receptor complex, which is in accordance with previous suggestions about positive allosteric interactions between receptor sites 3 and 5 [19]. Here we show that such interactions are possible also between receptor sites 3 and 7 (pyrethroid site; [15,16] of locust NaChs (Fig. 1C), which is in accordance with the synergic (i.e. more than additive) effects between pyrethroids and site-3 toxins observed in vivo and in insect NaCh preparations [35–37].

3.2. Allosteric interactions with other receptor sites

Binding of [^{125}I]LqhαIT to locust NaChs was measured in the presence of saturating concentrations of either veratridine (200 μM; [38] or PbTx-1 (100 nM; [19]) and increasing concentrations of deltamethrin. Veratridine and PbTx-1 increased [^{125}I]LqhαIT binding ~1.4-fold and deltamethrin 1.7-fold (Fig. 1D). Notably, deltamethrin had a synergic effect (three-fold increase) on [^{125}I]LqhαIT binding with any of

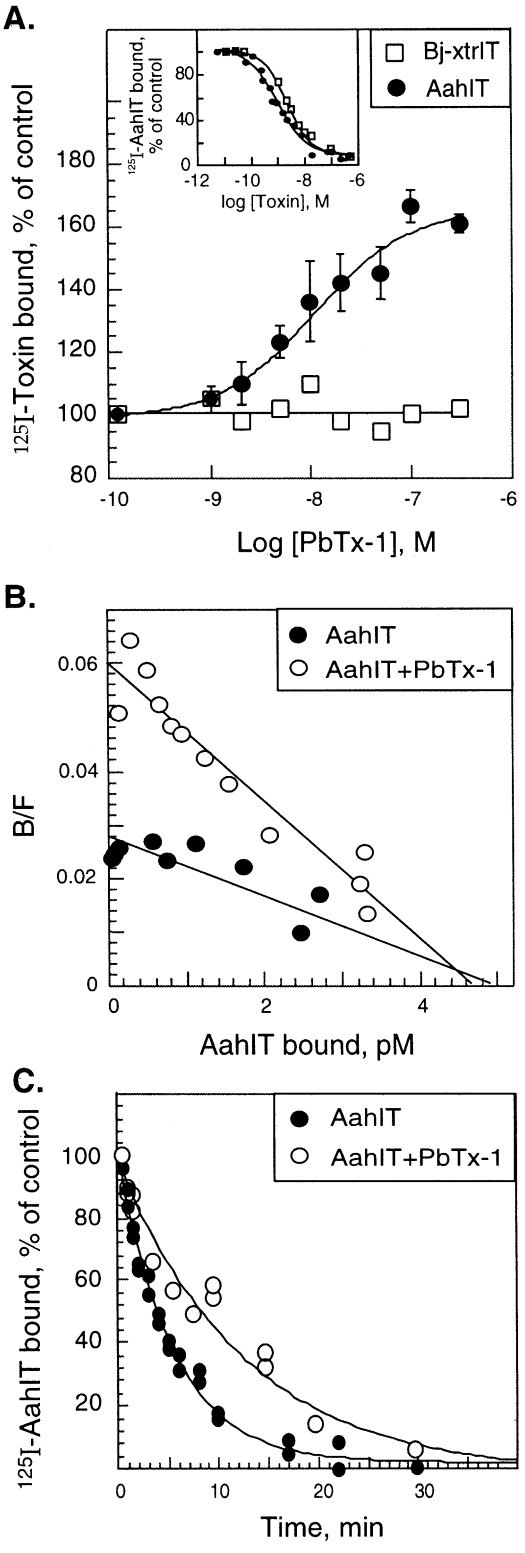

Fig. 2. Effects of brevetoxin-1 on the binding of excitatory toxins. A: PbTx-1 enhances the binding of AahIT but not Bj-xtrIT. 260 pM [^{125}I]AahIT or 85 pM [^{125}I]Bj-xtrIT were incubated with locust neuronal membranes in the presence of increasing concentrations of PbTx-1. The EC_{50} value for PbTx-1 effect on AahIT binding was 26 ± 9 nM, ($n = 3$) and the average increase obtained was 145 ± 25%. Experiments with the two excitatory toxins were performed in parallel using the same batch of PbTx-1 and membranes. Data points represent mean ± S.D. of three independent experiments. Inset (data from [30]): Competition of Bj-xtrIT for AahIT binding site. Cockroach neuronal membranes were incubated with 0.18 nM [^{125}I]AahIT and increasing concentrations of unlabeled AahIT or Bj-xtrIT. The K_i values obtained were: 0.67 ± 0.22 nM and 1.0 ± 0.2 nM for AahIT and Bj-xtrIT, respectively. B: Scatchard analysis of hot saturation curves of [^{125}I]AahIT binding in the absence or presence of 100 nM PbTx-1. Locust neuronal membranes were incubated with increasing concentrations of [^{125}I]AahIT in the absence (total binding) and presence of 200 nM Bj-xtrIT (non-specific binding). The data was analyzed by LIGAND (see Section 2). The equilibrium dissociation constants (K_D) obtained from two independent experiments were: for [^{125}I]AahIT alone, 0.85 and 1.85 nM (mean value 1.35 nM), and in the presence of 100 nM PbTx-1, 0.6 and 0.7 nM (mean value 0.65 nM). B_{max} mean values were 0.23 and 0.16 pmol/mg protein in the absence and presence of PbTx-1, respectively. C: Dissociation kinetics of bound [^{125}I]AahIT with and without PbTx-1. Locust neuronal membranes were incubated 1 h in the presence of 0.1 nM [^{125}I]AahIT. Dissociation was induced by addition of 200 nM Bj-xtrIT. Data is presented as percentage of maximal specific binding at equilibrium (100%). Each time-point presents the values of two independent experiments. The calculated dissociation rate constants from the two experiments were: $k_{off} = 3.15$ and 5.75×10^{-3} s^{-1} in the absence of PbTx-1, and $k_{off} = 1.67$ and 3.27×10^{-3} s^{-1} in the presence of 100 nM PbTx-1.

the other activators (see arrows in Fig. 1D). The positive allosteric interaction between receptor sites 2 (veratridine) and 5 (brevetoxin) with site-7 (deltamethrin) in insect NaChs is consistent with the enhancement of batrachotoxin binding by pyrethroids [39] and the lack of allosteric interaction between veratridine and brevetoxin sites in locust NaChs [20]. Interestingly, despite the 1.4-fold enhanced binding of a radiolabeled pyrethroid to rat brain NaChs by a scorpion anti-mammalian α-toxin, veratridine and brevetoxin were effectless [15]. None of the pyrethroids (2–10 μM) had an effect on [^{125}I]Lqh2 binding to rat brain synaptosomes (not shown). These results indicated that the allosteric interaction between the pyrethroid binding site and receptor site-3 was not reciprocal in rat brain NaChs, and differed markedly from the allosteric interactions observed in insect NaChs (Fig. 1).

3.3. Effects of pyrethroids and brevetoxin on the binding of excitatory toxin

In contrast to the positive allosteric interaction between insect NaCh receptors for pyrethroid and LqhαIT, the binding of the excitatory toxin, AahIT, to locust synaptosomes was unaffected by as high as 2 μM pyrethroids (not shown) or veratridine [16]. However, a dose-dependent increase in specific binding of [^{125}I]AahIT was observed in the presence of PbTx-1 (Fig. 2A). Scatchard analysis of [^{125}I]AahIT saturation binding curves in the presence of 100 nM PbTx-1 indicated a two-fold increase in affinity with no change in receptor site concentration (Fig. 2B). Dissociation kinetics of bound [^{125}I]AahIT from its receptor site in the presence of 100 nM PbTx-1 revealed a decrease in the dissociation rate constant ($k_{off} = 4.45 \pm 1.3 \times 10^{-3}$ s^{-1} and $2.47 \pm 0.8 \times 10^{-3}$ s^{-1} in the absence and presence of PbTx-1, respectively; Fig. 2C), implying that brevetoxin binding to receptor site-5 stabilized the complex AahIT–receptor site-4 in locust NaChs. This result resembles the enhancement in binding of the anti-mammalian β-toxin, Css2 (from *Centruroides suffusus suffusus*) by brevetoxin but not by veratridine in rat brain NaChs [40].

Another structurally unique excitatory toxin, Bj-xtrIT [30], competes on the high affinity sites of both excitatory (Fig. 2A, inset) and depressant toxins in insect NaChs, similarly to AahIT [16,30]. However, in contrast to AahIT, the binding of Bj-xtrIT was neither affected by brevetoxin (Fig. 2A) nor by veratridine or pyrethroids (not shown). These results suggest that the excitatory toxins may bind differently to receptor site-4 on locust NaChs.

3.4. Pyrethroids and scorpion toxins operate synergically

The effect of pyrethroids on insects was enhanced following their infection with a recombinant virus expressing AahIT [41]. Moreover, AahIT was found more effective on pyrethroid-resistant insect strains [41,42]. These results suggest that both effectors operate synergically, despite the lack of pyrethroid effect on AahIT binding (Fig. 2). The discrepancy between the in vivo toxicity and the in vitro binding assays could be related to the increased affinity of pyrethroids for NaChs in their open state following repetitive depolarization pulses induced by excitatory toxins [16,35], whereas most NaChs in insect synaptosomes usually open once and occupy mainly a slow inactivated state [32]. The binding of excitatory toxins is not affected by different NaCh states and membrane potential (Fig. 2; [16]), but the binding of pyrethroids to site-7 is affected. The apparent affinity of pyrethroids was approximately 50–100 nM (estimated from the EC_{50} values in Fig. 1), which corresponded to the concentration of deltamethrin required to modify *Drosophila* NaChs expressed in *Xenopus* oocytes after a single depolarization. However, following repetitive depolarization pulses, pyrethroid effect was observed at 1 nM [35].

Cohen and co-workers suggested that pyrethroids occupy two binding sites on insect NaChs, of which the second site could overlap to some extent with that of batrachotoxin and/or brevetoxin [35]. Therefore, it is possible that under our binding conditions, pyrethroids occupy mainly the second binding site, which does not interact with receptor site-4. Pyrethroid binding to this site affects, however, receptor site-3 on insect NaChs, which results in allosteric enhancement of LqhαIT binding (Fig. 1). Alternatively, it is possible that in insect NaChs binding of site-3, but not site-4, toxins enhances pyrethroid binding to site-7, which in turn, increases the binding of LqhαIT (Fig. 1). Since under physiological conditions binding of site-3 and site-4 toxins increase the probability for open channel states [1,2,17], it may enhance high binding affinity of pyrethroids to receptor site-7 [35], which leads to the observed synergic effects [35–37,41].

Our results offer an alternative approach to overcome pyrethroid resistance in the field. Since scorpion toxins bind to sites on insect NaChs that are distinct from those of pyrethroids, and since channel mutations that confer resistance do not decrease scorpion toxin binding [16,37,41,42], the combination of pyrethroids with scorpion toxins may minimize resistance buildup. The prominent synergic effect between site-3 toxins and pyrethroids on insect, but not mammalian NaChs (Fig. 1; [15,35–37]), provides a unique opportunity for insect control. Realization of such an approach requires utilization of anti-insect selective site-3 toxins. An extraordinary toxin candidate could be LqhαIT, which is very toxic to insects [16,20], and is amenable for molecular manipulations [31,43]. Should this approach succeed, we may envision design of small molecules that mimic the active site of an anti-insect selective LqhαIT, to be combined with pyrethroids in field application.

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