A Novel α-Conotoxin, PeIA, Cloned from Conus pergrandidis, Discriminates between Rat α9α10 and α7 Nicotinic Cholinergic Receptors*

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The α9 and α10 nicotinic cholinergic subunits assemble to form the receptor believed to mediate synaptic transmission between efferent olivocochlear fibers and hair cells of the cochlea, one of the few examples of postsynaptic function for a non-muscle nicotinic acetylcholine receptor (nAChR). However, it has been suggested that the expression profile of α9 and α10 overlaps with that of α7 in the cochlea and in sites such as dorsal root ganglion neurons, peripheral blood lymphocytes, developing thymocytes, and skin. We now report the cloning, total synthesis, and characterization of a novel toxin α-conotoxin PeIA that discriminates between α9α10 and α7 nAChRs. This is the first toxin to be identified from Conus pergrandidis, a species found in deep waters of the Western Pacific. α-Conotoxin PeIA displayed a 280-fold higher selectivity for α-bungarotoxin-sensitive α9α10 nAChRs compared with α-bungarotoxin-sensitive α7 receptors. The IC50 of the toxin was 6.9 ± 0.5 nM and 4.4 ± 0.5 nM for recombinant α9α10 and wild-type hair cell nAChRs, respectively. α-Conotoxin PeIA bears high resemblance to α-conotoxins MII and GIC isolated from Conus magus and Conus geographus, respectively. However, neither α-conotoxin MII nor α-conotoxin GIC at concentrations of 10 μM blocked acetylcholine responses elicited in Xenopus oocytes injected with the α9 and α10 subunits. Among neuronal non-α-bungarotoxin-sensitive receptors, α-conotoxin PeIA was also active at α3β2 receptors and chimeric α6/α3β2/β3 receptors. α-Conotoxin PeIA represents a novel probe to differentiate responses mediated either through α9α10 or α7 nAChRs in those tissues where both receptors are expressed.

Nicotinic acetylcholine receptors (nAChRs) are distributed widely in both the central and peripheral nervous system. In vertebrates, nine α subunits (α2–α10) and three β subunits (β2–β4) have been cloned. The rules of association for functional nAChRs are broadening and now permit receptors assembled from single α subunits (α7, α8, and α9) (1–3); receptors which contain multiple α subunits both with (α2α5β2, α3α5β2, α3α5β4), α4α5β2 (4–6) and without supplemental β subunits (α7α8, α9α10) (2, 7); receptors with single α and multiple β subunits (α5β2β4, α3β3β4) (8, 9); receptors with multiple α and β subunits (α3β2β4a5, α4a6β2β3) (10–12); as well as heteromeric nAChRs formed via pairwise combinations of α2, α3, α4, or α6 with either the β2 or β4 subunits (13–17). Thus, the number of potential molecular forms of nicotinic receptors is very large. Elucidation of the precise structure and function of various neuronal nAChRs in vivo is particularly challenging, in part because of the scarcity of ligands selective for specific receptor subtypes.

The venoms of predatory cone snails (Conus) represent a rich combinatorial-like library of evolutionarily selected, neuropharmacologically active peptides (18). There are more than 500 species of these snails. Each Conus venom appears to contain a unique set of 50–200 small disulfide-bonded peptides that target receptors and ion channels in a highly subtype-selective manner. For peptides where function has been determined, three classes of targets have been elucidated: voltage-gated ion channels, G protein-coupled receptors, and ligand-gated ion channels (19). Perhaps the most conserved feature of cone snail venoms is the α-conotoxins; these are a series of structurally and functionally related peptides that target nAChRs. Every venom examined thus far has its own distinct complement of nicotinic receptor antagonists, suggesting that, within the genus, there are literally thousands of novel peptides that act on nAChRs. A major advance in recent years in the neuropharmacology of nAChRs has been the ability to characterize particular neuronal subtypes more readily by using specific conotoxins.

We now report the cloning of a gene encoding a novel peptide of the α-conotoxin family from Conus pergrandidis, α-conotoxin PeIA (α-CTx PeIA). As far as we are aware, this is the first toxin to be characterized from this species found in deep waters (50–550 meters) of the Western Pacific. We show that the peptide has unusual targeting specificity; it has high affinity for recombinant α9α10-containing nAChR receptors and can readily discriminate this α-bungarotoxin-sensitive receptor from the neuronal α7 α-bungarotoxin-sensitive receptor. In addition, α-CTx PeIA blocks native cochlear hair cell nAChRs with a high potency, demonstrating an in vivo target for the peptide. Thus, α-CTx PeIA can be used to discern selectively between α9α10- and α7-mediated functions at those sites where both types of receptors are expressed.
**EXPERIMENTAL PROCEDURES**

**Identification and Sequencing of Genomic Clones Encoding α-CTx PeIA—**Genomic DNA was prepared from 50 mg of *C. pergrandis* hepatopancreas using a Gentra PUREGENE DNA Isolation Kit (Gentra Systems, Minneapolis, MN) according to the manufacturer’s standard protocol. It was used as a template for PCR with oligonucleotides corresponding to the conserved intron and 3′-untranslated region sequences of α-conotoxin prepropeptides. The resulting PCR products were purified using a High Pure PCR Product Purification Kit (Roche Applied Science). The eluted DNA fragments were annealed to pAMP1 vector, and the resulting products were transformed into competent DH5α cells using the CloneAmp pAMP System for rapid cloning of amplification products (Invitrogen). The nucleic acid sequences of the resulting α-CTx-encoding clones were determined according to the standard protocol for automated sequencing.

**Chemical Synthesis—**α-CTx PeIA (0.45 mmol/μg) was synthesized on an amid resin using Fmoc-(N-(9-fluorenyl)methoxycarbonyl) chemistry and standard side protection, except on cysteine residues. Cys residues were protected in pairs with either S-trityl on Cys1 and Cys2, or S-acetamidomethyl on Cys3 and Cys16. The peptide was removed from the resin and precipitated. A two-step oxidation protocol was used to fold the peptides selectively as described previously (20). Briefly, the disulfide bridge between Cys3 and Cys16 was closed by dripping the peptide into an equal volume of 20 mM potassium ferricyanide, 0.1 M Tris, pH 7.5. The solution was allowed to react for 30 min, and the monomeric peptide was purified by reverse-phase HPLC. Simultaneous removal of the S-acetamidomethyl groups and closure of the disulfide bridge between Cys2 and Cys8 were carried out by oxidation with sodium metaperiodate. The cyclic peptide and HPLC eluent were dripped into an equal volume of 10 mM H2O:trifluoroacetic acid:acetonitrile (78:2:20 by volume) and allowed to react for 10 min. The reaction was terminated by the addition of acetic acid, diluted 20-fold with 0.1% trifluoroacetic acid, and the bicyclic peptide was purified by HPLC on a reverse-phase C18 Vydac column using a linear gradient of 0.1% trifluoroacetic acid, 0.092% trifluoroacetic acid, 60% acetonitrile, remainder H2O. Mass Spectrometry—Matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry was utilized. 

**Expression of Recombinant Receptors in Xenopus laevis Oocytes—**Capped cRNAs were in vitro transcribed from linearized rat plasmid DNA templates using the mMessage mMachine Transcription Kit (Ambion Corporation, Austin, TX). The maintenance of DNA templates using the mMessage mMachine Transcription Kit (Amersham), and the production of the predicted mature toxin was performed. In *C. pergrandis* the amino acid sequence at the amino-terminus is identical in mature α-CTx-PeIA and α-CTx-PeIB. The toxin sequence is similarly conserved (18). We utilized conserved intronic and 3′-untranslated region sequences of the α-CTx gene structure to design oligonucleotide primers for PCR amplification of the α-CTx-coding region. The resulting sequence from *C. pergrandis* is shown in Fig. 1. The toxin was named α-CTx PeIA (“Pe” designating the species, *pergrandis*; Roman numeral “T” to designate the canonical α-CTx disulfide bond pattern; and “A” to indicate that it is the first α-CTx reported from this species).

**RESULTS**

**Cloning of α-CTx PeIA—**Although the mature α-CTx sequences are highly variable, the organization of their encoding genes is constant across species. The α-CTxs are proteolytically cleaved from a larger precursor protein. This prepropeptide is ~40 amino acids long, with the mature α-CTx moiety of ~13–18 amino acids located at the carboxyl terminus of the precursor. A basic amino acid, immediately preceding the mature toxin in the precursor sequence, acts as a processing site. In contrast to the highly variable sequence of the mature toxin, the precursor proteins of α-CTxs are highly conserved. The signal sequence region is practically invariant among the different α-CTx precursors, even in distantly related Conus species. Also, sequence segments in the 3′-untranslated region of the α-CTx mRNA and in the intron immediately preceding the toxin sequence are similarly conserved (18). We utilized conserved intronic and 3′-untranslated region sequences of the α-CTx gene structure to design oligonucleotide primers for PCR amplification of the α-CTx-coding region. The resulting sequence from *C. pergrandis* is shown in Fig. 1. The toxin was named α-CTx PeIA (“Pe” designating the species, *pergrandis*; Roman numeral “T” to designate the canonical α-CTx disulfide bond pattern; and “A” to indicate that it is the first α-CTx reported from this species).

**Chemical Synthesis of α-CTx PeIA—**Solid phase chemical synthesis of the predicted mature toxin was performed. In
synthesizing the peptide, it was assumed that the disulfide bridging pattern of α-CTX PeIA was the same as all previously characterized α-CTXts, that is, Cys\(^5\) to Cys\(^8\) and Cys\(^{16}\) to Cys\(^{19}\) (18). The glycine at the carboxyl terminus was assumed to be post-translationally modified to a carboxyl-terminal amide. Cys groups were orthogonally protected in pairs to direct disulfide bond formation. Acid-labile S-trityl groups were removed simultaneously with peptide cleavage from the resin, and closure of the disulfide bridge between these Cys residues was accomplished with FeCN. The single-bridge peptide was purified by HPLC, and the acid-stable acetimidomethyl groups were removed; the disulfide bridges formed by iodine oxidation. Acid-labile bridging pattern of α-CTX PeIA was the same as all previously processed to a carboxyl-terminal amide.

Effect of α-CTX PeIA on ACh-evoked Currents through nAChRs—Conotoxins have been used widely as a pharmacological tool to characterize neuronal nAChRs (26). We therefore decided to analyze the effects of α-CTX PeIA on different nAChRs. Fig. 2A shows representative responses of X. laevis oocytes expressing either the α-bungarotoxin-sensitive α9α10 or α7 nAChRs to ACh and blockade of responses in the presence of α-CTX PeIA. Complete block of ACh-evoked currents was obtained with 0.5 μM α-CTX PeIA in the case of α9α10 compared with 100 μM in the case of α7 nAChRs. As derived from the inhibition curves in Fig. 2B, currents elicited by ACh in α9α10 nAChRs were potently blocked by α-CTX PeIA with a mean IC\(_{50}\) and S.E. of 6.9 ± 0.5 nM (n = 6). In the case of α7, the IC\(_{50}\) value, 1.8 ± 0.1 μM (n = 5), was 260-fold higher than that obtained for α9α10, thus indicating a high degree of selectivity of the toxin for α9α10. The blockage produced by α-CTX PeIA on both types of receptors was reversible after washing with saline solution. Fig. 3 shows the washout kinetics in the case of α9α10. The effect of α-CTX PeIA was reversed relatively rapidly: >50% recovery in 3 min, and total recovery after 12-15 min.

Cochlear outer and developing inner hair cells are the main targets of descending cholinergic olivocochlear effenter fibers (27). The efferent fibers-hair cell synapse is most likely mediated by α9α10 nAChRs (7, 28, 29), providing one of the few postsynaptic functions for non-muscle nAChRs. However, α7 transcripts as well as α7 immunostaining have been reported in the mammalian organ of Corti (30, 31). Recordings from inner hair cells are an excellent tool to evaluate the effects of the toxin in native hair cell nAChRs. As shown in Fig. 4A, α-CTX PeIA potently blocked ACh-evoked responses in inner hair cells. The IC\(_{50}\) value obtained, 4.4 ± 0.5 nM, was similar to that found in recombinant α9α10 receptors, thus confirming the α9α10 identity of this nAChR.

The physiologic conditions of neurotransmission in vivo, for instance a synaptic regime, differ notably from the conditions of the oocyte recording or of a bath application of ACh to the organ of Corti. Transmitter released in a synaptic cleft, in close proximity to postsynaptic receptors, reaches millimolar ranges, sufficient to activate in the millisecond range receptors with a low affinity active state and a fast desensitization rate (32). We therefore studied the effect of α-CTX PeIA on synaptic currents evoked by the release of presynaptic ACh in the presence of 25 mM KCl. As observed in Fig. 5, 30 nM α-CTX PeIA also blocked responses to synaptically released ACh, thus indicating that the toxin is a valuable tool to examine in vivo responses mediated through α9α10 receptors. Fig. 5A shows K\(^+\)-evoked synaptic currents either in the absence or presence of α-CTX PeIA. The blocking effect of the toxin was rapidly reversible upon washing the preparation. The effect of α-CTX PeIA was a reduction of the amplitude (Fig. 5B) of the synaptic currents (from 57.0 ± 0.7 pA with 997 events and 3 cells, to 37.5 ± 0.7 pA with 766 events and 3 cells, p < 0.0001). This result confirms a postsynaptic effect of the toxin on the nAChRs present in cochlear hair cells. A reduction in the frequency of events (from 2.2 ± 0.5 to 1.2 ± 0.2 Hz, data not shown) was observed. This could be either because the blocking action of the toxin on the synaptic events of small amplitudes could not be resolved within the noise of the recordings or because of an additional presynaptic effect of the toxin. The latter was not investigated further.

To assess the effect of α-CTX PeIA on non-α-bungarotoxin-sensitive neuronal nAChRs, concentration-response analysis was conducted on rat α2β4, α2β2, α4β2, α4β4, α3β2, and α3β4 (Fig. 6 and Table 1). The toxin had little or no activity when bath-applied on α2β4, α2β2, and α4β4 nAChRs at concentrations as high as 10 μM. The percent response ± S.E. to ACh was, respectively, 98.2 ± 2.1%, 95.3 ± 1.9%, and 91.4 ± 2.3% (n = 3). Only a 39.2 ± 5.5% (n = 4) block of α2β2 was observed in the presence of 10 μM α-CTX PeIA. On the other hand, α-CTX PeIA appeared to be effective on α3-containing receptors, displaying a higher selectivity for α3β2 (IC\(_{50}\) = 23 ± 1 nM, n = 5) than for α3β4 (IC\(_{50}\) = 0.48 ± 0.03 μM, n = 4) nAChRs. α-CTX PeIA was also tested on rat α6/α3β2β3 nAChRs, where α6/α3 is a chimeric α subunit containing the amino-terminal 237 amino acids of α6 and the remainder of α3 (33). The toxin at 100 nM blocked 89.8 ± 1.8% of the response (n = 4).

Nicotinic AChRs are members of the “Cys-loop” family of neurotransmitter-gated ion channels, which also includes γ-aminobutyric acid types A and C, glycine, 5-erotonin type 3, and some invertebrate anionic glutamate receptors (34). To assess the selectivity of α-CTX PeIA for nAChRs, we tested the effect of the toxin on some other members of the family, such as the γ-aminobutyric acid type A and 5-serotonin receptors. Concentrations of α-CTX PeIA as high as 10 μM did not modify responses to 30 μM γ-aminobutyric acid or 10 μM serotonin in oocytes injected with the respective recombinant receptors (n = 4), thus confirming the selectivity of this α-CTX from C. pergrandidis on nAChRs.

The mature toxin sequence of α-CTX PeIA bears high resemblance to α-CTXs MII (α-CTX MII) and GIC (α-CTX GIC) isolated from Conus magus and Conus geographus, respectively (Table II). Although α-CTX MII potently targets α3- and α6-containing neuronal nAChRs, α-CTX GIC has a higher selectivity for α6-containing receptors (35). We therefore examined
the effect of these toxins in α9α10-expressing oocytes. At a 10 μM concentration, neither α-CTx MII nor α-CTx GIC blocked α9α10 nAChRs (n = 3).

**DISCUSSION**

By utilizing conserved sequences in CTx genes, we have cloned a gene encoding a novel peptide of the α-CTx family from *C. pergrandis*. This species was only very rarely collected and once regarded among the 10 most valuable Conus species for shell collectors (36). In recent years, however, commercial collectors in the Central Philippines have collected a moderate number of specimens. α-CTx PeIA is the first toxin to be characterized from this species and adds to the arsenal of peptides active at nAChRs that have been isolated from *Conus*. Thus, *C. pergrandis* represents a new source of useful pharmacological probes to characterize nAChRs. We note, however, that the peptide has not yet been isolated from the venom of *C. pergrandis*, and it is possible that the native venom-derived peptide has post-translational modifications not evident from inspection of the genetic sequence. In this report, we describe the properties of a synthetic version of the putative CTx. The peptide is 16 residues in length, with two disulfide bonds.
α-CTX PeIA Discriminates between a9α10 and a7 nACHRs

![Graph showing inhibition curves for PeIA on a3β2, a3β4, and a4β2 nACHRs.](image)

Fig. 6. Effect of α-CTX PeIA on a3β2, a3β4, and a4β2 nACHRs. Inhibition curves were obtained by the coapplication of 10 μM (a3β2), 100 μM (a3β4) or 10 μM ACh (a4β2) and increasing concentrations of α-CTX PeIA. Oocytes were incubated with each concentration of the toxin for 10 min prior to the addition of ACh. Peak current values are plotted, expressed as the percentage of the peak control current evoked by ACh. The mean ± S.E. of four to five experiments/group are shown.

**Table I**

| IC₅₀ (nM) | CT⁺ | Hill slope | CT⁺ | nᵃᵇ |
|-----------|-----|------------|-----|-----|
| a9α10     | 6.9 | 5.2–9.1    | 0.88| 0.74–1.02| 6 |
| α7        | 1,800| 1,396–2,206| 1.10| 0.86–1.33| 5 |
| a3β2      | 23  | 19.2–27.5  | 1.25| 0.97–1.50| 5 |
| a3β4      | 480 | 272–640    | 1.23| 0.98–1.58| 4 |
| a4β2      | 11,600| 8,811–15,170| 3.02| 1.76–7.81| 3 |

ᵃ CI, 95% confidence interval.
ᵇ n, no. of experiments.

In the case of a4β2, the IC₅₀ was derived from the regression.

**Table II**

Sequence comparison of α-CTXs

Bold letters indicate nonconserved amino acid substitutions among the three peptides.

| α-Conotoxin | Sequence          | IC₅₀ (a9α10) |
|-------------|------------------|-------------|
| PeIA        | GCCSFHPCSVHPELC  | 6.7         |
| MII         | GCCSFVPCHELHNLN  | >10,000     |
| GIC         | GCCSFHPCAGNHWC   | >10,000     |

α-CTX PeIA belongs to the A superfamily, as do the majority of Conus peptides that are known to affect the function of nACHRs (37). The spacing between Cys residues, four amino acids in the first loop and seven in the second loop, is typical of several previously isolated α-CTXs of the α4 family, four amino acids in the first loop and seven in the second loop, is typical of α-CTXs. These toxins differ pharmacologically from a7 in that nicotine and ICS 205–930 block the former, whereas they activate the latter (7, 38). In addition, strychnine, a well-characterized glycine antagonist, potently blocks α9α10 and α7 nACHRs, with a lower IC₅₀ for α9α10 (7, 39, 40). α-CTX PeIA is the first reported CTx to block α9α10 nACHRs with high affinity. Similar to some previously characterized α4/7 family CTxs, including α-CTX MII and α-CTX GIC, α-CTX PeIA also potently blocks the α3β2 nACHR. In contrast, however, α-CTX MII and α-CTX GIC show no activity at α9α10 nACHRs at concentrations of up to 10 μM.

Current data support the notion that a receptor assembled from both α9 and α10 nACHR subunits mediates synaptic transmission between effector olivocochlear fibers and cochlear hair cells (3, 7, 28, 29, 41). However, α7 transcripts as well as α7 immunostaining have been reported in the mammalian organ of Corti (30, 31). The fact that native hair cell nACHRs were potently blocked by α-CTX PeIA precludes the participation of α7 nACHRs in mediating ACh-evoked responses in inner hair cells. The situation for other tissues remains to be determined. Dorsal root ganglion neurons express multiple nACHR subtypes, including α7-like, α3β4-like, and α4β2-like (42). They also coexpress both α9 and α10 subunits (43, 44). In addition, peripheral blood lymphocytes and developing thymocytes have been shown to express cholinergic receptors, including the nACHR subunits α2–5, α7, α9, α10, and β4, which could participate at different steps in the regulation of the immune response (45–47). Finally, α7, α9, and α10 are expressed in skin keratinocytes and might be involved at different steps in the regulation of skin homeostasis (48–51). The observation that α-CTX PeIA has a 260-fold selectivity for α9α10 compared with α7 indicates that it is a useful probe to differentiate responses mediated either through α9α10 or α7 nACHRs in the above mentioned tissues where both receptors are expressed.

The carnivorous marine snails of the genus Conus are a rich source of peptides targeted to nACHRs. Major components of the complex venomous arsenal that the fish-eating Conus employ are toxins that act at the muscle nicotinic receptor type (18). Why might these snails have evolved a toxin with high affinity for α9α10 receptors? C. pergranatis is found in deep waters (50–530 meters) of the Western Pacific. The feeding habits of C. pergranatis are unknown, but Conus species in general prey upon fish, mollusks, and/or worms. Little is known about the nACHR subtypes found in these prey. However, the Fugu rubripes (pufferfish) genome contains three candidate α7, two α8, and four α9 subunit genes. Moreover, it has been described that this fish genome contains the largest family of vertebrate nACHR subunits reported to date (52). In addition three α9 subunit genes have been described in the rainbow trout (Oncorhynchus mykiss) (53). Although none of the Fugu nACHR sequences show close identity to the mammalian or avian α10 subunits, three of the α9-like subunits possess glycosylation sites also found in the higher vertebrate α10 subunit (52), suggesting that these α9-like subunits might coassemble to form a functional nACHR, much like the higher vertebrate homomeric α9 and/or heteromeric α9α10 nACHRs. Higher vertebrate α9 and α9α10 nACHRs mediate different cholinergic inhibition at cochlear and vestibular hair cells (3, 7, 28, 29, 41). The cholinergic pharmacology of efferent block in the fish lateral line organ is similar to that in hair cells of the cochlea, indicating that the same nicotinic cholinergic receptor is likely involved (54, 55). The proper orientation of mechanosensory hair cells along the lateral line organ of a fish is essential for the ability of the animal to sense directional water movements. This sensory system appears to be important in many behavioral tasks such as prey capture, orientation with respect to external environmental cues, navigation in low light conditions, and mediation of interactions with nearby animals (56–59). Thus, block of efferent modulation of the lateral line activity by Conus toxins could facilitate prey capture by these predatory snails.

α-CTX PeIA shows considerable sequence similarity to the α4/7 α-CTXs MII and GIC (26). The comparison of the structure of α-CTX PeIA, MII, and GIC indicates that the four Cys residues are placed identically, having the same disulfide connectivity. Moreover, a conserved proline and the identical placement of a histidine and an asparagine that are known to either initiate or immediately precede an α-helix in GIC and MII indicate that the peptide backbone topology of α-CTX PeIA is likely similar to that of the other two α-CTXs. Indeed, compared as a group, there are only four nonconservative amino acid
substitutions among the three peptides (see bold residues in Table II). The α4/7 toxins have a common structural scaffold. Their polypeptide backbones appear to be virtually identical: the canonical α helical structure and two β turns are prominent structural features of the family. Given the near identity of the peptide backbones the ability of the different α4/7 Conus peptides to discriminate among different neuronal nAChR subtypes must clearly be mediated through their divergent side chain groups (18). Although all three toxins, MI, GIC, and PeIA, have high affinity for the α3β2 nAChR (35, 60), only α-CTx PeIA has high affinity for α9α10. Thus, the amino acids shown in bold in Table II are likely structural determinants of the high selectivity of α-CTx PeIA for α9α10 nAChRs.

Conclusion—A remarkable accomplishment of the Conus genus has been the evolution of nAChR antagonist of diverse subtype specificities. The diversity of their venom products is likely a consequence of the complex marine environment in which slow moving and otherwise unarmed predators must certainly be mediated through their divergent side chain groups (18). Although all three toxins, MI, GIC, and PeIA, have high affinity for the α3β2 nAChR (35, 60), only α-CTx PeIA has high affinity for α9α10. Thus, the amino acids shown in bold in Table II are likely structural determinants of the high selectivity of α-CTx PeIA for α9α10 nAChRs.

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