Isoform-specific interaction of the α1A subunits of brain Ca\(^{2+}\) channels with the presynaptic proteins syntaxin and SNAP-25

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Abstract

Presynaptic Ca\(^{2+}\) channels are crucial elements in neuronal excitation-secretion coupling. In addition to mediating Ca\(^{2+}\) entry to initiate transmitter release, they are thought to interact directly with proteins of the synaptic vesicle docking/fusion machinery. Here we report isoform-specific, stoichiometric interaction of the BI and rbA isoforms of the α1A subunit of P/Q-type Ca\(^{2+}\) channels with the presynaptic membrane proteins syntaxin and SNAP-25 in vitro and in rat brain membranes. The BI isoform binds to both proteins, while only interaction with SNAP-25 can be detected in vitro for the rbA isoform. The synaptic protein interaction ("synprint") site involves two adjacent segments of the intracellular loop connecting domains II and III between amino acid residues 722 and 1036 of the BI sequence. This interaction is competitively blocked by the corresponding region of the acid residues 722 and 1036 of the BI sequence. This interaction ("synprint") site involves two adjacent segments of the intracellular loop connecting domains II and III between amino acid residues 722 and 1036 of the BI sequence.

Numerous studies (1–4) show that neurotransmitter release in the mammalian central nervous system is initiated by Ca\(^{2+}\) entry through both ω-conotoxin GVIA-sensitive N-type Ca\(^{2+}\) channels, which contain the α1B subunit (5, 6), and ω-agatoxin IVA- and ω-conotoxin MVIIIC-sensitive P-type and Q-type Ca\(^{2+}\) channels, which are thought to contain α1A subunits (7–11). cDNAs encoding α1A subunits have been cloned from rabbit (BI; ref. 7) and rat (rbA; ref. 8). They share an overall amino acid sequence identity of 92%, and the corresponding RNAs direct the synthesis in Xenopus oocytes of Q-type Ca\(^{2+}\) channels having intermediate affinity for ω-agatoxin IVA (9, 10). α1A subunits isolated from brain by immunoprecipitation bind ω-conotoxin MVIIIC with a K\(_d\) of 100 pM and are localized in the presynaptic terminals of many central neurons (11, 12). α1I subunits that are able to form P-type Ca\(^{2+}\) channels having high affinity for ω-agatoxin IVA have not yet been identified, but similarities in physiological properties and localization of α1A and P-type Ca\(^{2+}\) channels suggest that a specific isoform of α1A may also form P-type channels (10, 11).

Vesicle docking and fusion are mediated by a complex of proteins including the synaptic vesicle protein VAMP/synaptobrevin and the presynaptic plasma membrane proteins syntaxin and SNAP-25 (synaptosome-associated protein of 25 kDa) (13, 14). Antibodies against syntaxin, a 35-kDa integral membrane protein of the presynaptic plasma membrane, immunoprecipitate N-type channels from rat brain (15, 16), and a stoichiometric complex of syntaxin and N-type Ca\(^{2+}\) channels has been isolated (17). An 87-amino acid sequence from the cytoplasmic loop between homologous repeats II and III (L\(_{II}III\) of α1B) is sufficient to interact with syntaxin 1A (18). This α1B peptide can block the binding of native N-type Ca\(^{2+}\) channels to syntaxin, indicating that this binding site is required for stable interaction of syntaxin with the N-type Ca\(^{2+}\) channel. Peptides from the same region of α1B also bind SNAP-25 (19). We suggest the term "synprint" for this synaptic protein interaction site on the Ca\(^{2+}\) channel.

In contrast to N-type Ca\(^{2+}\) channels, Sheng et al. (18) found that the corresponding segment of L\(_{II}III\) from the rbA isoform of α1A (8) did not bind to syntaxin. This result raised the possibility that P/Q-type Ca\(^{2+}\) channels might not have a synprint that is able to bind to presynaptic plasma membrane proteins involved in the transmitter release process. However, examination of the amino acid sequence differences between the BI and rbA isoforms of α1A shows considerably lower identity (78.4%) in L\(_{II}III\) than in the remainder of the protein, suggesting that this segment may be subject to alternative splicing. In support of this, we have found that α1A isoforms with amino acid sequences characteristic of L\(_{II}III\) from both rbA and BI are present in both rat and rabbit brain using site-directed antibodies (20). Here we show that two adjacent, interacting regions within L\(_{II}III\) of the BI isoform of α1A bind to both syntaxin and SNAP-25 and that the corresponding segment of the rbA isoform binds to SNAP-25 but not to syntaxin. Our results provide a molecular basis for close association of P/Q-type Ca\(^{2+}\) channels with synaptic vesicles during transmitter release and suggest the possibility that differential interactions of Ca\(^{2+}\) channel isoforms with syntaxin and SNAP-25 may modulate the efficiency of synaptic transmission.

**Experimental Procedures**

cDNA Cloning. A human BI (hBI) clone was isolated by screening a cDNA library generated from the human small cell lung carcinoma line, SCC-9 (21). Briefly, ~500,000 cDNAs were screened with a randomly primed \(^{32}\)P-radiolabeled 1.9-kb EcoRI fragment from the rat brain class A cDNA, rbA-73 (22). Hybridization was carried out overnight at 62°C in 5× SSPE (× SSPE = 0.18 M NaCl/1 mM EDTA/10 mM sodium phosphate, pH 7.4), 0.3% SDS, and 0.2 mg of denatured salmon sperm DNA per ml. Filters were washed four times for 20 min in 0.5× SSPE at 60°C. A human brain BI clone was isolated by screening a human hippocampus cDNA library (Stratagene) with a \(^{32}\)P-radiolabeled 1.3-kb EcoRV–SsrI fragment of the small cell lung carcinoma cDNA. DNA sequencing was performed on double-stranded plasmid DNA with modified T7 polymerase (Sequenase 2.0; United States Biochemical).

**Abbreviations:** BI, the BI isoform of α1A subunits; rbA, the rbA isoform of α1A subunits; hBI, human BI; GST, glutathione S-transferase.

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Preparation of Fusion Proteins. A glutathione S-transferase (GST) fusion protein including full-length SNAP-25 was prepared by ligating an EcoRI–XhoI fragment of SNAP-25 (ref. 23; amplified by PCR with synthetic oligonucleotides including the appropriate restriction sites) into the pGEX4T vector (Pharmacia LKB). Histidine-tagged (His)-fusion proteins of α1A were generated by using cDNAs encoding the rat brain rbA-126 isoform (8) and a human homolog of the BI-I isoform (see above) from a small cell lung carcinoma cell line (21) as a template and synthetic oligonucleotides with overhanging restriction sites as primers in a PCR. The amplified products were directionally cloned into the pTrrHis expression vector (Invitrogen) to yield cDNAs expressing the His-fusion proteins rbA 724-869, rbA 844-981, rbA 724-981, hBI 722-895, hBI 843-1036, and hBI 722-1036. Constructions of full-length GST–syntaxin 1A, GST–syntaxin 1A N-terminus, GST–syntaxin 1A C-terminus, and His-fusion proteins of the N-type Ca<sup>2+</sup> channel were performed as described (18). All constructs were verified by DNA sequence analysis. The protease-deficient Escherichia coli strain BL26 (Novagen) was used for expression of all fusion proteins following standard procedures (18, 24). The amount of each fusion protein in the supernatant was estimated with a standard curve relating the intensity of the immunoblotting signal to the amount of a standard fusion protein (T7 gene 10; Novagen) applied.

**In Vitro Binding Assays and Immunoblot Analyses.** GST fusion proteins (150 pmol) were bound to glutathione Sepharose beads (30 μl; Pharmacia LKB) in phosphate-buffered saline (PBS; 140 mM NaCl/2.7 mM KCl/10.1 mM Na2HPO4/1.8 mM KH2PO4, pH 7.3) containing 0.1% Triton X-100, pepstatin, aprotinin, and leupeptin (each at 4 μM) and 0.4 μM phenylmethylsulfonyl fluoride. The mixture was incubated at 4°C for 1 hr with constant agitation. The beads were then washed with PBS and incubated with identical concentrations of expressed His-fusion proteins of different Ca<sup>2+</sup> channels for 3 hr at 4°C. Beads were then washed three times in ice-cold PBS/0.1% Triton X-100 and two times in 50 mM Tris-HCl (pH 8) and 0.1% Triton X-100. Bound proteins were eluted from the beads by competition with 15 mM reduced glutathione/50 mM Tris-HCl (pH 8) (20 μl) for 20 min with gentle mixing. Eluates were separated from the beads by centrifugation at 10,000 × g for 1 min, mixed with 10 μl 3× Tricine sample buffer, and boiled for 2 min. Proteins were separated on 10–20% Tricine gradient gels (Novex) and transferred overnight to nitrocellulose (0.2 μm; Schleicher & Schuell). Immunoblot analyses were performed as previously described with Anti-T7.Tag monoclonal antibody (1:10,000; Novagen). The immunoreactive bands were visualized by enhanced chemiluminescence (ECL system; Amersham). Bound antibodies were quantitated by PhosphorImager (Molecular Dynamics). In all experiments, the concentration of fusion protein bound to glutathione-Sepharose was quantitated by protein staining with Ponceau S and found to be equivalent for all samples.

**RESULTS AND DISCUSSION**

**Identification of a Second Syntaxin-Binding Segment in the α<sub>1B</sub> Subunit of N-type Ca<sup>2+</sup> Channels.** Deletion analysis revealed a minimum requirement of 87 amino acids in the intracellular loop LII-III (rbA 773-859) to maintain binding activity (18). However, the affinity of Ca<sup>2+</sup>-channel fusion proteins for syntaxin is progressively reduced as their size is decreased toward the minimum sequence of 87 amino acid residues (unpublished results). Therefore, we measured the binding of equivalent concentrations of larger fusion proteins from LII-III to identify additional regions that influence the affinity of syntaxin binding. As shown in Fig. L1, rbB 718-963 binds with substantially higher affinity to syntaxin than rbB 718-859. The contribution of rbB 832-963 is responsible for this difference in affinity as the sum of signal intensities of rbB 718-859 and rbB 832-963 is approximately equal to the intensity of rbB 718-963. No binding to GST alone was detected for these three fusion proteins (Fig. L1A, right lanes), demonstrating the specificity of the interactions. We therefore conclude that the syntaxin-binding site of N-type Ca<sup>2+</sup> channels is composed of two adjacent regions of the intracellular loop LII-III.

**The BI Isoform of Human α<sub>1A</sub>.** Screening of cDNA libraries generated from human hippocampus and a small cell carcinoma cell line (21) resulted in the isolation of clones encoding a human homologue of the BI isoform of α<sub>1A</sub>. The predicted amino acid sequences of the human hippocampal and small cell carcinoma BI isoforms were identical in the LII-III region and share 92% and 82% identity with the rabbit BI and rat rbA isoforms of α<sub>1A</sub>. The predicted BI-1 isoform (8) and a human homolog of the BI-1 isoform (see above) were used to generate BI-1A fusion proteins of different Ca<sup>2+</sup> channel configurations to determine the specificity of the interactions. We therefore conclude that the syntaxin-binding site of N-type Ca<sup>2+</sup> channels is composed of two adjacent regions of the intracellular loop LII-III.

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**The BI Isoform of Human α<sub>1A</sub>**. Screening of cDNA libraries generated from human hippocampus and a small cell carcinoma cell line (21) resulted in the isolation of clones encoding a human homologue of the BI isoform of α<sub>1A</sub>. The predicted amino acid sequences of the human hippocampal and small cell carcinoma BI isoforms were identical in the LII-III region and share 92% and 82% identity with the rabbit BI and rat rbA isoforms of α<sub>1A</sub>.
isoforms, respectively (Fig. 2). Alignment of the three sequences shows that relative to rbA, the human α1A isoform contains several insertions of nearly identical sequence at the same positions as those of rabbit BI, indicating that the human α1A cDNAs correspond to the BI isoform. Using anti-peptide antibodies that specifically recognize LII-III of the BI and rbA isoforms of α1A, we have identified α1A subunits with sequences in LII-III characteristic of both BI and rbA in both rat and rabbit brain (20). Together, these results support the hypothesis that BI and rbA are different isoforms of α1A which are both present in rat, rabbit, and human.

Only the BI Isoform of α1A Binds to Syntaxin. Based on the binding behavior of the α1B fusion proteins described above, we expressed His-fusion proteins containing the corresponding regions of LII-III of the hBI and the rbA isoforms. Recombinant GST–syntaxin or GST alone was bound to glutathione-Sepharose beads and incubated with equal concentrations of the six α1A His-fusion proteins. Immunoblot detection of the specifically eluted fusion proteins revealed that the largest fusion protein of the hBI isoform (hBI 722-1036) is able to bind with high affinity to GST–syntaxin (Fig. 1B). The two smaller hBI fusion proteins (hBI 722-895 and hBI 843-1036, respectively) show no detectable binding (Fig. 1B), and no binding to GST alone was detected for any of the fusion proteins (data not shown). Titration studies demonstrated that, under our binding conditions, half-maximal binding occurs at ~1–2 μM hBI 722-1036 and that ~0.7 mol of hBI 722-1036 are bound per mol of syntaxin at a saturating concentration of hBI 722-1036 (Fig. 1C). These results with the BI isoform of α1A contrast with the binding studies with the N-type channel where the binding contribution of the two smaller fusion proteins rbB 718-859 and rbB 832-963 are additive (Fig. 1A). The affinity of the fusion protein containing both these segments (rbB 718-963) is approximately 10-fold greater (0.2 μM; ref. 18).

Surprisingly, all three rbA fusion proteins (rbA 724-869, rbA 844-981, and rbA 724-981) show no detectable binding to GST–syntaxin (Fig. 1B), and no binding to GST alone was detected for any of the fusion proteins (data not shown). Titration studies demonstrated that, under our binding conditions, half-maximal binding occurs at ~1–2 μM hBI 722-1036 and that ~0.7 mol of hBI 722-1036 are bound per mol of syntaxin at a saturating concentration of hBI 722-1036 (Fig. 1C). These results with the BI isoform of α1A contrast with the binding studies with the N-type channel where the binding contribution of the two smaller fusion proteins rbB 718-859 and rbB 832-963 are additive (Fig. 1A), and the affinity of the fusion protein containing both these segments (rbB 718-963) is approximately 10-fold greater (0.2 μM; ref. 18).

Because fusion proteins from both α1B and the BI isoform of α1A bound to the C-terminal one-third of syntaxin 1A, we investigated whether both occupy the same or overlapping binding sites in competition binding assays. GST–syntaxin 1A was bound to glutathione-Sepharose beads and incubated with a constant concentration (5 μM) of hBI 722-1036 from α1A and increasing concentrations of rbB 718-963 from α1B. As shown in Fig. 1C, the signal intensity of hBI 722-1036 of the P/Q-type channel (upper band) diminishes progressively while the signal intensity of rbB 718-963 of the N-type channel (lower band) increases. These results demonstrate that synprint sites from the other rbA fusion proteins, no binding to syntaxin was observed (data not shown), suggesting that the rbA isoform of α1A does not interact with syntaxin.

Synprint Sites from α1A and α1B Compete for Binding to the C-Terminal One-Third of Syntaxin. We have previously shown that rbB 718-859 from α1B binds to a GST–syntaxin deletion construct containing only the C-terminal one-third (amino acids 181-288) of syntaxin 1A (18). However, it remained possible that the second syntaxin-binding segment of α1B (rbB 832-963) binds to another site on syntaxin. Binding studies with rbB 718-859, rbB 832-963, rbB 718-963, and the C-terminal segment of syntaxin show that all three fusion proteins bind to the C-terminal one-third (amino acids 181-288) of syntaxin 1A (Fig. 1D). No binding to GST–syntaxin–NT (amino acids 2-190) or GST alone could be detected, suggesting that both of the syntaxin-binding regions of N-type Ca\(^{2+}\) channels interact with a C-terminal site on syntaxin near the intracellular surface of the presynaptic plasma membrane.

We also performed binding studies with the corresponding fusion proteins of the two isoforms of α1A. Similar to full-length syntaxin 1A, only the largest fusion protein of the BI isoform showed any detectable binding to syntaxin fragments (Fig. 1B). It binds specifically to the C-terminal one-third of syntaxin 1A (amino acids 181-288), whereas no binding to GST–syntaxin–NT or GST alone occurred. In contrast, all three fusion proteins of rbA failed to show any detectable binding (data not shown), in agreement with the data in Fig. 1B.


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**Fig. 2.** Amino acid sequence of LII-III of the BI isoform of α1A cDNAs encoding a human isoform of the α1A subunit were isolated from a small cell carcinoma cell line and from human hippocampus and sequenced as described. The sequences of the rabbit isoform of α1A (7), the rat rbA isoform of α1A (8), and the human hBI isoform of α1A are compared, and the positions of the N and C termini of the fusion proteins used in these experiments are noted by arrows.
LII-III of P/Q-type and N-type channels do indeed compete for the same binding region on syntaxin 1A.

Synprint Sites from α1A and α1B Compete for Binding to SNAP-25.

The α1B subunits of N-type Ca\textsuperscript{2+} channels interact with SNAP-25 as well as syntaxin (19). To determine whether α1A subunits interact with SNAP-25 also, a GST fusion protein of SNAP-25 was bound to glutathione-Sepharose beads and incubated with equal concentrations of the three α1B fusion proteins and the six rbA and hBI α1A fusion proteins. All three fusion proteins of the N-type channel (rbB 718-859, rbB 832-963, and rbB 718-963) bound to GST–SNAP-25 (Fig. 4A). The signal intensities of rbB 718-859 and rbB 832-963 were comparable to the intensity of the rbB 718-963 signal.

Fusion proteins derived from α1A also bind to SNAP-25. The hBI 722-1036 fusion protein of the P/Q-type Ca\textsuperscript{2+} channel shows the most intense immunoblot signal (Fig. 4A). Titration studies (Fig. 4B) demonstrate half-maximal saturation at \(~1–2\) µM and binding of 0.8 mol of hBI 722-1036 per mol of SNAP-25 at saturation, comparable to that for syntaxin. The long exposure shown in Fig. 4A reveals weak binding of hBI 843-1036 to SNAP-25, whereas no binding of hBI 722-895 is observed. These data support the hypothesis that both regions of the synprint site on the BI isoform of α1A are required for high affinity binding of presynaptic proteins.

In contrast to the BI isoform, the rbA 724-981 fusion protein shows only weak binding to GST–SNAP-25. Nevertheless, this binding is specific because no binding to GST alone is observed (data not shown). In addition, a fusion protein containing the entire LII-III loop of the rbA binds with comparable intensity (data not shown). Although the binding is much weaker than...
the corresponding region of the BI isoform, these data suggest that the rbA isoform of the P/Q-type channel can also interact with proteins of the docking and fusion machinery. Evidently, the sequence differences in LII-III between BI and rbA isoforms result in different binding affinities of their synprint sites for SNAP-25.

To test if the binding regions on α₁B and the BI isoform of α₁A compete for binding to SNAP-25, we performed a competition assay with GST–SNAP-25 bound to glutathione-Sepharose beads. As shown in Fig. 4C, increasing concentrations of rbB 718-963 of α₁B displace hBI 722-1036 of α₁A from its binding site on SNAP-25. Evidently, the synprint sites of α₁A and α₁B interact with identical or overlapping regions on SNAP-25 as well as on syntaxin.

Association of α₁A, Syntaxin, and SNAP-25 in the Brain. To determine whether syntaxin and SNAP-25 are associated with α₁A in vivo, we solubilized P/Q-type Ca²⁺ channels from rat brain, partially purified them by chromatography on wheat germ agglutinin-Sepharose, and analyzed them by immunoprecipitation with specific antibodies as described (11, 20). An antibody against a fusion protein with the amino acid sequence of LII-III of the rbA isoform of α₁A immunoprecipitated class A Ca²⁺ channels (20) and coimmunoprecipitated SNAP-25 and syntaxin (Fig. 5). This immunoprecipitation was blocked by pre-incubation of the antibody with the corresponding fusion protein and was not observed with nonimmune IgG, indicating that the association of α₁A with syntaxin and SNAP-25 is specific. These results provide further support for the conclusion that syntaxin and SNAP-25 specifically associate with class A Ca²⁺ channels through interaction with a site in LII-III of α₁A.

Coiled–Coiled Domains in Synprints. It has been suggested that the interactions among syntaxin, SNAP-25, and VAMP/synaptobrevin are mediated by α-helical structures that form coiled coils (25–27). Syntaxin possesses three segments with a high predicted probability of forming coiled coils (amino acids 30-64, 68-112, and 189-231), and the latter of these is believed to be responsible for binding of SNAP-25 (26) and VAMP/synaptobrevin (25). Because the binding site for N-type channels and the BI isoform of P/Q-type channels resides in the same segment of syntaxin, we analyzed the binding regions of the channels by using an α-helical coiled–coiled prediction program (28). Scores above 1.1 generally correspond to regions that form extended amphipathic α-helices in globular proteins, and scores above 1.3 have a high probability of forming coiled–coiled structures. For the N-type channel, the program predicts two putative coiled–coiled regions, one with four heptad repeats (amino acids 719-746, average score 1.78) and one with three heptad repeats (amino acids 769-791, average score 1.41). The 87-amino acid region (amino acids 773-859) which we previously reported as a minimum requirement for binding syntaxin (18) comprises the second predicted coiled-coil region. Our present data indicate that another region of LII-III (amino acids 832-963) also contributes to the syntaxin-binding of the N-type channel (see Fig. 1B). Within that region, there are no predicted α-helices. Therefore, it seems likely that binding of the N-type channel to syntaxin may be mediated partially, but not exclusively, by α-helices which form coiled–coiled structures.

Analysis of the two isoforms of α₁A, rbA and BI, reveals two putative coiled–coiled regions at virtually identical positions (amino acids 728-748 and 779-799 for rbA; amino acids 726-746 and 777-797 for BI). Only the BI isoform displays detectable binding to syntaxin 1A. Furthermore, the BI fusion protein LII-III 722-895, which contains both putative coiled–coiled regions, does not bind syntaxin. Taken together, these binding data argue against an exclusive role of these putative coiled–coiled regions in binding of the BI isoform of α₁A to syntaxin, but these structures may mediate the binding interaction together with adjacent sequences in LII-III 843-1036.

Isoforms of Class A Ca²⁺ Channels Interact Differentially with Presynaptic Membrane Proteins. Six different α₁ subunit genes have been cloned and shown to encode proteins with distinct biophysical and pharmacological properties when expressed in heterologous expression systems (29). An even greater variety is generated by alternative splicing. For the BI isoform of α₁A, two different full-length cDNAs have been described (BI-1 and BI-2) that differ in the C-terminal domain (7). Multiple alternatively spliced forms of the rbA isoform of α₁A also exist and encode proteins with altered voltage dependence of activation and kinetics of inactivation (30). The BI and rbA isoforms of α₁A subunits share >98% amino acid identity in regions flanking LII-III (amino acids 1-715, 98.5% identity; amino acids 1249-1857, 98.2% identity) but show distinct regions of divergence in LII-III in which the amino acid sequence identity is only 78.4%. The distinctly different levels of amino acid sequence identity in these regions suggest alternative splicing. In support of this hypothesis, both human and rat genomes contain a single α₁A gene (C.D.H. and T.P.S., unpublished observations). Furthermore, our studies with site-directed anti-peptide antibodies show directly that both the BI and rbA isoforms of α₁A are expressed in both rat and rabbit brain (20).

Our binding data with fusion proteins containing the intra-cellular loop LII-III of these two α₁A variants demonstrate that they bind with different affinities to the presynaptic proteins syntaxin and SNAP-25. The BI isoform has higher affinity for binding to both syntaxin and SNAP-25 than the rbA isoform. Using our in vitro assay, binding of rbA to SNAP-25 is clearly detected, while binding to syntaxin is not. Because these differences in affinity were observed for fusion proteins containing both fragments of LII-III and full-length LII-III, it is likely that they reflect intrinsic differences in affinity of the two isoforms for syntaxin and SNAP-25 and are not due to differences in our selection of segments for construction of the fusion proteins. If these binding interactions are required for efficient coupling of Ca²⁺ influx with synaptic vesicle fusion as previously suggested (18, 19), these data imply that a neuron could modulate the efficiency of synaptic transmission by regulating the expression of different isoforms of a single class A Ca²⁺ channel gene. Further studies examining the cellular and subcellular distributions of the BI and rbA isoforms of α₁A are required to address this possibility.

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