Polypeptide components of the apamin receptor associated with a calcium activated potassium channel

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Photoaffinity labeling of rat brain membranes with [^{125}I]ANPAA-apamin incorporated radioactivity into polypeptides of 86 and 59 kDa and occasionally a more weakly labeled component of 45 kDa. These polypeptides were immunoprecipitated with anti-apamin antibodies and treated with glycosidases. Neither the 86 nor the 59 kDa polypeptide apppeared to b ^{N}-glycosylated. Partial proteolytic mapping of affinity labeled polypeptides with chymotrypsin or V8 protease generated an identical pattern. Thes results suggest that the 59 and 45 kDa components are not additional subunits of an oligomeric protein but result from cleavage of the 86 kDa polypeptide.

Apamin receptor polypeptide; Potassium channel; Calcium activation; Rat brain membrane

1. INTRODUCTION

Apamin, a 2 kDa peptide from bee venom, blocks the small conductance Ca^{2+}-activated K^+ channel that underlies the long-lasting afterhyperpolarization in certain electrically excitable cells [1,2]. [^{125}I]Apamin has been used to detect high affinity receptors, in neurons [3], glial cells [4], skeletal [5], cardiac and smooth muscle membranes and hepatocytes [6-8]. Although solubilization of the apamin receptor has been achieved and its size estimated from sucrose gradient centrifugation [9], the density of receptors expressed in all tissues studied is extremely low and has precluded purification. Our present knowledge of receptor structure therefore is mainly derived from the use of covalent labeling techniques. Affinity labeling with a variety of crosslinking reagents has identified at least three major receptor associated polypeptides of 86, 59 and 30 kDa [4,6,10-13]. Interestingly, whereas the 86 kDa polypeptide appears to be a constant receptor component, the 59 kDa polypeptide and a more variable, weakly labeled, 45 kDa band have only been detected in certain tissues or cell types leading to the hypothesis that two structurally distinct apamin receptor subtypes may exist [4,12,13].

In the present paper the relationship between these polypeptides is re-examined using ligand directed antibodies, endoglycosidase-F treatment, and partial proteolytic mapping.

2. MATERIALS AND METHODS

Rat brain synaptic membranes were prepared as previously described in the presence of protease inhibitors: 0.1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 mM iodoacetamide and 1 mM EDTA [12]. Apamin was iodinated [3] and a mono-[^{125}I]iodoapamin derivative separated by SP-Sephadex C-25 (Pharmacia) chromatography [14]. Binding experiments with [^{125}I]apamin were carried out in a buffer containing 25 mM Tris, 10 mM KCl, and 0.1% bovine serum albumin adjusted to pH 7.4 with HCl, at 4°C for 60 min. Bound ligand was separated by rapid filtration over polyethyleneimine pretreated Whatman GFC filters. Non-specific binding was determined in the presence of 0.1 μM unlabeled apamin. Photoreactive azidonitrophenylaminoacetylmono-[^{125}I]iodoapamin ([^{125}I]ANPAA-apamin) was prepared as previously described using 4-azido-2-nitrophenylaminoacetylsuccinimidyl ester (a gift of Dr K. Angelides) and immediately used to photolabel apamin receptors [11]. Membrane samples were solubilized in 3% SDS, 10 mM EDTA, 10% glycerol, 70 mM Tris adjusted to pH 9 with HCl (buffer A), heated to 100°C for 5 min, then analyzed by SDS-PAGE on a 5-15% gradient gel and autoradiography.

2.1. *Immunoprecipitation*

In certain cases denatured samples were diluted 4-fold with 25 mM Tris, 75 mM KCl, 25 mM EDTA, 1% Triton X-100, 50 mM KH_2PO_4, casein 0.1%, adjusted to pH 7.5 with NaOH (buffer B) and immunoprecipitated with anti-apamin antibodies. Rabbit anti-apamin [15] or control serum was added and incubation was carried out overnight at 4°C. Pansorbin (Calbiochem) was then added (40 μl/μl serum) and samples were rotated for 1 h at 4°C, followed by centrifugation at 10 000 × *g* for 1 min. The pellet was washed twice with buffer B and then twice with 25 mM Tris, 10 mM KCl, adjusted to pH 7.5 with HCl. Pellets were then resuspended in buffer A containing 1% β-mercapteothanol and heated to 100°C for 5 min. After centrifugation to remove Pansorbin the supernatant was loaded onto a 5-15% SDS-polyacrylamide gel.

2.2. *Deglycosylation*

Affinity-labeled proteins were located by autoradiography, excised, and homogenized in 10 mM Tris, 5 mM EDTA, 0.1% Nonidet NP-40, and 0.01% SDS adjusted to pH 8 with HCl and then extracted overnight. Polyacrylamide fragments were removed by centrifugation and the supernatants lyophilized and then dissolved in 90 μl 1% β-mercaptoethanol. 2 U of endoglycosidase-F (Boehringer) were added and samples were incubated for 70 h at 37°C. In certain cases samples were treated with 50 mU neuraminidase (Calbiochem) in a 50 mM Na-acetate buffer at pH 5.5 containing 1 mM $CaCl_2$ prior to endoglycosidase-F treatment. Enzymes were inactivated by boiling in buffer A and samples were analyzed by SDS-PAGE and autoradiography.

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2.3. *Partial proteolysis*

Affinity labeled proteins were extracted from gel slices as above in 125 mM Tris, 1% glycerol, 0.01% SDS, adjusted to pH 7.4 with HCl, and concentrated by lyophilization. Samples were treated with 10 μg chymotrypsin for 30 min (Sigma) or 2 μg *Staphylococcus aureus* V8 protease (Sigma) for 60 min at 37°C and analyzed as above.

3. RESULTS

[^{125}I]Apamin binds to high affinity receptors in rat brain synaptic membranes with a K_d of about 40 pM as calculated from the competition curve shown in Fig. 1A. Scatchard analysis of data from saturation experiments carried out with this membrane preparation (results not shown) confirmed previous observations [12,14] of a single class of receptors with a binding capacity in the range of 10–20 fmol/mg of protein. Photoaffinity labeling of synaptic membranes (Fig. 1B) revealed two major labeled polypeptides of 86 and 59 kDa. Labeling was specific as it was inhibited when receptor sites were protected by competing unlabeled apamin, with partial reduction of photolabeling with 0.1 nM apamin and almost complete inhibition at 1 nM.

The displacement of [^{125}I]apamin from its high affinity site (Fig. 1A) and the competitive attenuation of affinity labeling of both the 86 and 59 kDa polypeptides by unlabeled apamin clearly occurs in the same concentration range. This confirms that both polypeptides are associated with the high affinity apamin receptor.

Polyclonal anti-apamin antibodies were tested for their ability to immunoprecipitate [^{125}I]ANPAA-apamin labeled receptor polypeptides. Photolabeled membranes were solubilized and denatured in the presence of 3% SDS, samples were then diluted, incubated with anti-apamin or control serum and then immunoprecipitated (Fig. 2A). About 80% of the total radioactivity was recovered when SDS was diluted to a final concentration of 0.7% or less before the addition of antiserum. Immunoprecipitated material was eluted from the Pansorbin pellet by boiling in SDS-PAGE sample buffer and analyzed by PAGE and autoradiography (Fig. 2B). It contained both free [^{125}I]ANPAA-apamin that migrated with the dye front and the covalently labeled 86 and 59 kDa polypeptides. This procedure removed the majority of the synaptic

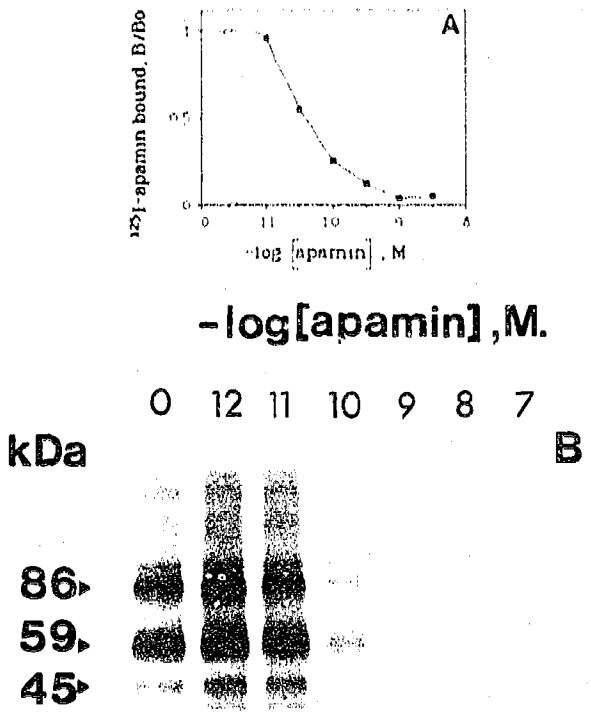

Fig. 1. Photoaffinity labeling of the apamin receptor. Brain membranes were incubated with (a) [^{125}I]apamin or (b) [^{125}I]ANPAA-apamin in the presence of increasing concentrations of unlabeled apamin. In (A) bound [^{125}I]apamin was measured by vacuum filtration and γ counting. B = bound; B_o = bound in the absence of unlabeled apamin. In (B) membranes were exposed to a UV lamp, recovered by centrifugation and analyzed by SDS-PAGE and autoradiography.

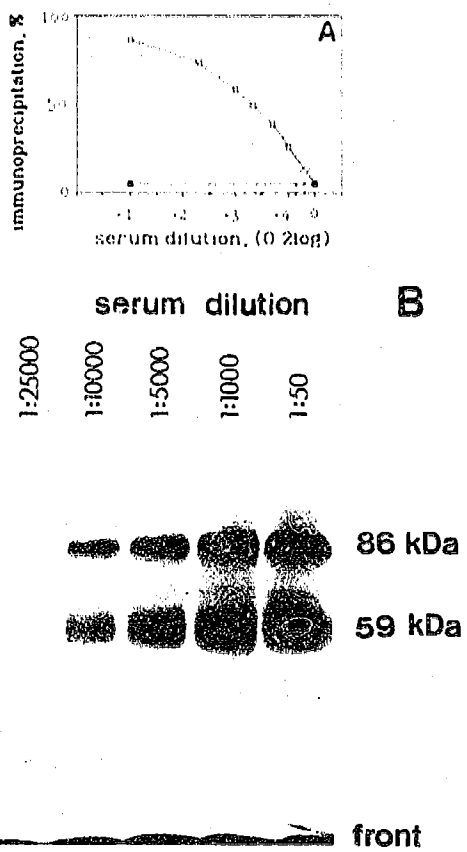

Fig. 2. Immunoprecipitation of affinity-labeled apamin receptors. Brain membranes were affinity labeled with [^{125}I]ANPAA-apamin, solubilized and incubated with anti-apamin antiserum (□) or control serum (■). Immune complexes were then recovered by incubation with Pansorbin followed by centrifugation and were analyzed by (A) γ counting and by (B) SDS-PAGE and autoradiography.

membrane proteins, as only the immunoglobulin chains were revealed by Coomassie blue staining, indicating that significant receptor purification was achieved. As less than 10 fmol of receptor were loaded per lane, the detection of receptor polypeptides by conventional protein staining techniques was not feasible.

In order to determine whether the 86 and 59 kDa bands contain N-linked carbohydrate, immunoprecipitation, SDS-PAGE, and band excision and elution were used to enrich the photolabeled apamin receptor sufficiently to allow glycosidase treatment without interference from the large amounts of glycoproteins known to be present in synaptic membranes. Treatment of polypeptides with endoglycosidase-F (endo-F) for up to 70 h had no effect on the mobility of either the 86 or 59 kDa polypeptide (Fig. 3). Pretreatment with neuraminidase to remove sialic acid residues that might interfere with the action of endo-F was equally without effect (results not shown). Control samples were incubated in identical conditions in the absence of endo-F. The fact that endo-F was active in the conditions used was verified using [^{125}I]orosomucoid which showed the expected decrease in molecular mass from 44 to 26 kDa, following deglycosylation (Fig. 3). An additional control experiment was carried out on the β_1 subunit of voltage-dependent Na channel affinity labeled with [^{125}I]-ANPAA α-scorpion toxin (toxin V from *Leiurus quinquestratus quinquestratus*) [16]. In control conditions this polypeptide migrated at 36 kDa. After 17 h endo-F treatment the major band, which probably corresponds to a partially deglycosylated form migrated at 31 kDa, whereas a minor band representing the fully deglycosylated core polypeptide was detected at 22 kDa in agreement with previous reports [17] (results not shown).

Partial proteolytic digestion of the affinity labeld 86 and 59 kDa polypeptides was performed after SDS-PAGE, excision and elution of the appropriate gel regions. Protease treatment was stopped by boiling in SDS-PAGE sample buffer, and samples were analyzed by electrophoresis, autoradiography and densitometric scanning. Chymotrypsin digestion gave a very similar pattern for both the 86 and the 59 kDa polypeptides with, in each case, the formation of three major proteolytic products of 36, 20, and a doublet peaking at about 15 kDa (Fig. 4A). In certain experiments a minor, specifically labeled component of 45 kDa was detected as previously reported [11,14]. After chymotrypsin digestion of the 45 kDa band proteolytic fragments of 36, 20, and a doublet at about 15 kDa were again identified (not shown).

Partial proteolysis of the 86 and 59 kDa bands with V8 protease (Fig. 4B) also gave an identical profile with, in both cases, two major degradation products of 46 and 37 kDa. Although Fig. 4 shows identical patterns for the 86 and 59 kDa bands, differences were apparent when proteolysis was stopped at earlier stages. A shorter incubation with V8 protease produced two peptides from the 86 kDa chain that migrated between 86 and 59 kDa (not shown). However neither protease generated a 59 kDa form from the 86 kDa chain.

4. DISCUSSION

Ca^{2+} activated K^+ channels are important regulators of the electrical activity of excitable cells but are as yet poorly characterized at the molecular level. Apamin, a peptide neurotoxin, specifically blocks one type of Ca^{2+}-activated K^+ channel and binds to a high affinity receptor site presumably located on the channel protein. Affinity labeling with [^{125}I] apamin derivatives has provided the only structural insight to date into this class of channel allowing the identification of at least

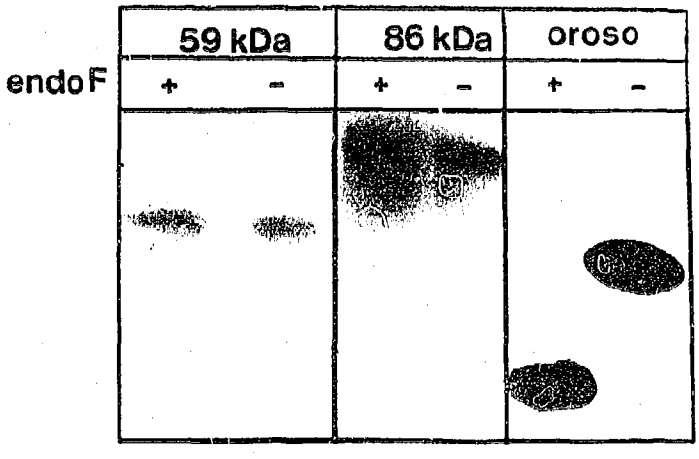

Fig. 3. Glycosidase treatment of affinity-labeled apamin receptors. Apamin receptors were partially purified by immunoprecipitation, and SDS-PAGE. After elution from gel slices polypeptides were treated with endoglycosidase F and analyzed by SDS-PAGE and autoradiography. OROSO indicates control experiments with [^{125}I]orosomucoid.

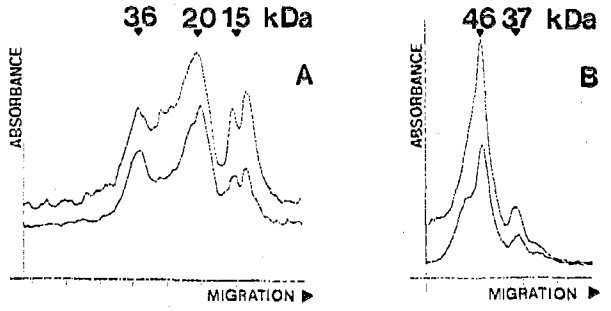

Fig. 4. Partial proteolysis of affinity labeled apamin receptors. Apamin receptors were partially purified by SDS-PAGE, the 86 kDa (upper trace) and the 59 kDa (lower trace) polypeptides were cut out and then treated with (A) chymotrypsin or (B) V8 protease and analyzed by SDS-PAGE, autoradiography and densitometric scanning.

three major polypeptides of 86, 59 and 30 kDa and a possible component at 45 kDa [4,6,10-12]. Apamin contains two amines amenable to modification: αNH_2-Cys_1 and ϵNH_2-Lys_4. Photoaffinity labeling with two different, chemically characterized, [^{125}I]-ANPAA apamin derivatives has shown that the 86 and 59 kDa membrane polypeptides are crosslinked by an ANPAA group coupled at the αCys_1 position whereas the 30 kDa polypeptide is crosslinked by ANPAA at ϵLys_4 [12], which suggested that the apamin binding site is located at the zone of interaction between different subunits of an oligomeric protein.

In experiments with a range of membrane preparations and cultured cells we have shown that, while the 86 kDa component was always detected, the 59 kDa polypeptide was present in brain membranes [11,12], primary cultured astrocytes [4], and liver membranes [6] but absent in primary cultured neurons [11,12], and intestinal smooth muscle and heart membranes [6]. This observation led us to the conclusion that structural subtypes of apamin receptors may occur that differ by the accessibility of a 59 kDa subunit to covalent labeling by an ANPAA-apamin derivative occupying the binding site that is presumably located on the 86 kDa polypeptide [4,6,12]. An affinity labeling strategy using several different reagents to crosslink the toxin to its receptor and two different receptor sources has much more recently led Auguste and colleagues to independently propose a similar hypothesis suggesting the existence of receptor subtypes [13]. The experiments described in the present paper were designed to determine whether the 86 and 59 kDa polypeptides are in fact both associated with the high affinity apamin binding site and, if so, to further investigate their structure.

A protocol in which photolabeling was inhibited by competition with increasing concentrations of unlabeled apamin demonstrated that label incorporation into the 86 and 59 kDa polypeptides was inhibited to an equivalent degree at concentrations compatible with occupancy of the apamin binding site. Furthermore both the 86 and 59 kDa components were specifically immunoprecipitated by anti-apamin antibodies, after denaturation at 100°C in the presence of SDS. These polypeptides are therefore associated with the high affinity binding site and must in fact be situated in close proximity as we have previously shown they can only be crosslinked to the αNH_2 of Cys_1 and not to the ϵNH_2 of Lys_4 of receptor-bound apamin [12].

Treatment with a range of enzymes strongly suggests in fact that these two polypeptides are closely related. Glycosidase treatment indicates that neither are *N*-glycosylated, whereas proteolytic mapping with two different proteases points to a striking homology. On the strength of these observations the hypothesis that the 59 kDa component is an additional subunit that is associated with the 86 kDa polypeptide in a hetero-oligomeric complex now appears unlikely. A more reasonable explanation is that the 59 kDa chain is a segment of the 86 kDa polypeptide that includes the apamin binding site.

Is the 59 kDa chain a short form of the receptor polypeptide that is only synthesized in certain cell types, or is it a proteolytic fragment? The latter explanation is more likely as in the six different tissue/cell types that we have tested to date we have detected either both the 86 and the 59 kDa chains or the 86 kDa polypeptide alone but never the 59 kDa component alone. This suggests that the 59 kDa chain is always derived post-translationally from the longer polypeptide. The same is probably true of the minor 45 kDa polypeptide occasionally detected by ourselves and other investigators [11,13].

Is proteolytic cleavage of the 86 kDa polypeptide a physiologically relevant process that occurs in the living cell or an artefactual event that takes place following cell disruption? We can only speculate as to the answer. It is difficult to be certain that proteolysis does not occur during sample preparation. However, it is worth pointing out that the 59 kDa fragment is detected when apamin receptors are labeled on cultured astrocytes that are rapidly processed for SDS-PAGE [4], but absent in smooth muscle or cardiac membranes [6], which require lengthy preparation and are, a priori, much more liable to proteolysis indicating that truncated receptor polypeptides may be produced in intact cells. It is interesting to note that two size forms of the α_1 subunit of the dihydropyridine-sensitive calcium channel have been detected in transverse tubule preparations and in cultured muscle cells [18] suggesting that post-translational proteolytic cleavage of ion channel proteins may occur as part of a normal regulatory process.

In summary the present paper simplifies our view of the structure of the apamin binding component of the Ca^{2+}-activated K^+ channel. We now propose that the apamin binding site is situated in a region where two channel polypeptides of 86 and 30 kDa interact and that previously detected 59 and 45 kDa polypeptides are fragments of the 86 kDa chain. Further investigation will be required to determine why truncated apamin receptor polypeptides are only produced in certain tissues.

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