Lee, C. A., & Saier, M. H., Jr. (1983) *J. Biol. Chem. 258*, 10761–10767.
Lee, C. A., Jacobson, G. R., & Saier, M. H., Jr. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7336–7340.
Leonard, J. E., & Saier, M. H., Jr. (1983) *J. Biol. Chem. 258*, 10757–10760.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Postma, P. W., & Lengeler, J. W. (1985) *Microbiol. Rev. 49*, 232–269.
Roossien, F. F., & Robillard, G. T. (1984a) *Biochemistry 23*, 211–215.
Roossien, F. F., & Robillard, G. T. (1984b) *Biochemistry 23*, 5682–5685.
Roossien, F. F., Blaauw, M., & Robillard, G. T. (1984) *Biochemistry 23*, 4934–4939.
Roossien, F. F., van Es-Spiekman, W., & Robillard, G. T. (1986) *FEBS Lett. 196*, 284–290.
Saier, M. H., Jr. (1977) *Bacteriol. Rev. 41*, 856–871.
Saier, M. H., Jr. (1985) *Mechanisms and Regulation of Carbohydrate Transport in Bacteria*, Academic, New York.
Waygood, E. B., Mattoo, R. L., & Peri, K. G. (1984) *J. Cell. Biochem. 25*, 139–159.

Molecular Structure of Rat Brain Apamin Receptor: Differential Photoaffinity Labeling of Putative K^+ Channel Subunits and Target Size Analysis†

Michael J. Seagar,*,‡ Catherine Labbé-Jullié,‡ Claude Granier,‡ Alexandra Goll,§ Hartmut Glossmann,§ Jurphaas Van Rietschoten,‡ and François Couraud‡

Laboratoire de Biochimie, Faculté de Médecine, Secteur Nord, Unité 172, Institut National de la Santé et de la Recherche Médicale, 13326 Marseille Cedex 15, France, and Institut fur Biochemische Pharmakologie, 6020 Innsbruck, Austria

Received November 15, 1985; Revised Manuscript Received February 10, 1986

ABSTRACT: Two photoreactive apamin derivatives were prepared with an aryl azide [[(azidonitrophenyl)amino]acetate (ANPAA)] group coupled at different positions on the neurotoxin molecule. These ligands were used to identify membrane components in the environment of the neuronal binding site that is associated with a Ca^{2+}-activated K^+ channel. ^{125}I-[α-ANPAA-Cys_1]apamin labeled a single M_r 86 000 chain in cultured neurons whereas two bands corresponding to M_r 86 000 and 59 000 were detected in synaptic membrane preparations, suggesting that the M_r 59 000 polypeptide may be a degradation product. ^{125}I-[ϵ-ANPAA-Lys_4]apamin however incorporated uniquely into two smaller components with M_r 33 000 and 22 000 in both cultured neurons and synaptic membranes. Randomly modified ^{125}I-ANPAA-apamin gave a cross-linking profile equivalent to the sum of those obtained with the two defined derivatives. The apamin binding site seems to be located at the frontier between three or more putative K^+ channel subunits which are only accessible from limited regions of the receptor-associated photoprobe. Irradiation of frozen rat brain membranes with high-energy electrons led to a reduction in ^{125}I-apamin receptor capacity, yielding a target size for the functional binding unit of M_r 84 000–115 000, which could be constituted by the M_r 86 000 subunit alone or by the M_r 86 000 subunit in conjunction with one of the two smaller subunits.

Considerable advances in the molecular characterization of ion channel proteins, responsible for action potential generation in excitable cells, have been achieved in recent years. The purification and functional reconstitution of the voltage-sensitive Na^+ channel have been accomplished with radiolabeled neurotoxins as biochemical probes (Catterall, 1984). Similarly, components of the voltage-dependent Ca^{2+} channel have been isolated (Curtis & Catterall, 1984), in association with receptors for labeled Ca^{2+} antagonists (Glossmann & Ferry, 1985). This type of approach has not been widely applicable to K^+ channels due to an almost total lack of specific toxins or drugs that can be used in ligand binding assays.

Apamin, a 2000-dalton peptide purified from bee venom, seems at the present time to be an exception. Nanomolar concentrations of apamin specifically block a K^+ permeability, present in a variety of cell types, that is activated by an increase in the intracellular free Ca^{2+} concentration. In neuroblastoma and skeletal muscle cells it inhibits a macroscopic slow K^+ current which underlies the long-lasting after hyperpolarization (Hugues et al., 1982b; Romey & Lazdunski, 1984; Cognard et al., 1984). However, the tetraethylammonium-sensitive Ca^{2+}-activated K^+ conductance, which has been extensively studied by single-channel recording techniques, is not blocked by apamin (Romey & Lazdunski, 1984). This apparent contradiction has been clarified by recent studies in sympathetic ganglia that indicate that two types of Ca^{2+}-activated K^+ current coexist in the same neuron, each having a distinct physiological role. The fast current which is blocked by tetraethylammonium contributes to spike repolarization whereas the apamin-sensitive slow current produces the prolonged hyperpolarization which may modulate repetitive firing characteristics (Pennefather et al., 1985). Recent work has involved the use of apamin and its derivatives in an attempt to gain an insight into the structure of this neuronal K^+ channel.

We have detected high-affinity binding sites for mono-[^{125}I]iodoapamin on primary cultured neurons and have correlated receptor occupancy to an inhibition of ion efflux

† This research was supported by grants from the Centre National de la Recherche Scientifique (U.A. 553) and the Institut National de la Santé et de la Recherche Médicale (U. 172).
* Address correspondence to this author.
‡ Institut National de la Santé et de la Recherche Médicale.
§ Institut fur Biochemische Pharmakologie.

0006-2960/86/0425-4051$01.50/0 © 1986 American Chemical Society

(Seagar et al., 1984). To try to identify receptor-associated polypeptides, we first randomly derivatized ^{125}I-apamin with a photolabile arylazide and demonstrated covalent incorporation into membrane proteins (Seagar et al., 1985). Interpretation of these observations was difficult as several specific radioactive bands were resolved, the labeling pattern in cultured neurons was different from that in synaptic membranes, and considerable discrepancy existed between our data and reports using a homobifunctional cross-linker (Schmid-Antomarchi et al., 1984). We have now partially resolved these inconsistencies by the synthesis of two different probes with arylazide groups at different positions on the apamin molecule. Photoaffinity labeling showed, surprisingly, that completely different polypeptides are labeled from each position on the receptor-bound ligand. Target size analysis was carried out to have an indication as to which of these putative K^+ channel subunits contributes functionally to the apamin binding site.

Materials and Methods

Preparation of ^{125}I-ANPAA-Apamin[1] Derivatives. Apamin was isolated from bee venom (Banks et al., 1981) and shown to be pure by HPLC, amino acid analysis, and toxicity testing. It was then radioiodinated at His_{18} (Seagar et al., 1984) and a mono[^{125}I]iodoapamin derivative with a specific radioactivity of about 2000 Ci/mmol was separated by SP- (sulfopropyl-) Sephadex C-25 (Pharmacia) chromatography (Hugues et al., 1982a). A 200-μL aliquot of 30–40 nM mono[^{125}I]iodoapamin was adjusted to pH 8.5 and to a final concentration of 1 mg/mL bovine serum albumin by addition of a stock solution in 0.1 M NaOH. All following operations were then carried out in a darkroom under a red photographic lamp. ANPAA-OSu[1] (a gift of Dr. K. Angelides, University of Florida, Gainesville) was then added in acetonitrile to give molar ratios from 1:1 to 10:1 ANPAA-OSu:primary amines (of the ^{125}I-apamin plus the serum albumin) in a final volume of 230 μL. At the indicated times the reaction was quenched by dilution into 7 mL of H_2O, to which was added 100 μL of 0.1 M acetic acid, and then loaded onto an 8 × 0.4 cm column of SP-Sephadex C-25 equilibrated in 10 mM NaP_i buffer at pH 7 containing 10 μM serum albumin. Elution was carried out in three stages, first with 5 mL of the equilibrating buffer, then with a linear gradient from 0 to 100 mM NaCl, and finally with a step to 300 mM NaCl in the same buffer. The peaks were detected by γ counting with 40% efficiency.

Preparation of Biotinylapamin Derivatives. Apamin (100 nmol) in 450 μL of 0.1 M sodium borate buffer at pH 8.5 was reacted with biotin-OSu[1] (IBF) at a molar ratio of 1:1 or 10:1 biotin-OSu:primary amines of apamin for 1 h at room temperature. The reaction medium was then adjusted to a conductivity of 0.7 mS and to pH 5.3 by addition of 500 μL of H_2O and 100 μL of 0.1 M acetic acid. The derivatives were purified by ion-exchange chromatography on an 8 × 0.4 cm CM-52 column (Whatman) in four steps: first, elution with 10 mL of ammonium acetate (1.1 mS), then a linear gradient from 1.1 to 5 mS, then equilibrium at 5 mS, and finally a second linear gradient from 5 to 25 mS. The reaction products were identified from their elution position, detected by absorbance at 214 nm, and by amino acid analysis of the acid hydrolysates after dansylation of the peaks.

Iodination of [ϵ-Biotinyl-Lys_4]apamin. [ϵ-Biotinyl-Lys_4]-apamin (1 nmol) was reacted with 0.5 mCi of Na^{125}I (Amersham) and 5 nmol of iodogen (Pierce Chemical Co.) in 0.1 M Tris-HCl buffer, pH 8.6, in a final volume of 50 μL at 37 °C for 15 min. After addition of 35 μL of 0.1 M HCl, separation was carried out on an 8 × 0.4 cm SP-Sephadex C-25 column equilibrated in a 10 mM NaP_i buffer, pH 7, containing 10 μM serum albumin in three steps: first, elution with 30 mL of equilibration buffer, then elution with 40 mL of 50 mM NaCl, and finally elution with 200 mM NaCl in the same buffer. Tubes containing ^{125}I-[ϵ-biotinyl-Lys_4]apamin were pooled to give a final concentration of about 10 nM. Batch incubation with Ultrogel-A4R–avidin (IBF) was used to verify the presence of ^{125}I-biotinylpeptide.

Preparation of ^{125}I-[α-ANPAA-Cys_1,ϵ-biotinyl-Lys_4]apamin. A 400-μL aliquot of ^{125}I-[ϵ-biotinyl-Lys_4]apamin was adjusted to pH 8.5 by addition of 0.1 M NaOH and reacted with ANPAA-OSu at a molar ratio of 2:1 ANPAA-OSu:total primary amines for 1 h at room temperature.

Binding Studies and Photolabeling Procedure. Cerebral hemispheres from 16-day Wistar rat embryos were dissociated and cultured in poly(L-lysine)-treated 60-mm dishes (Corning) as previously described (Seagar et al., 1984). Synaptic membranes were prepared as in Seagar et al. (1985). The binding buffer for neuronal cultures contained 25 mM Hepes, 10 mM glucose, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 0.1% serum albumin adjusted to pH 7.5 with Tris base. The binding buffer for all membrane preparations contained 25 mM Tris, 10 mM KCl, and 0.1% serum albumin adjusted to the required pH with HCl. Equilibrium binding studies with synaptic membranes (300–400 μg/mL) were carried out in 0.5 mL of buffer at 1 °C, and bound ligand was separated by rapid filtration over cellulose acetate (EH-Millipore) or poly(ethylenimine) pretreated glass fiber (GFB-Whatman) filters, followed by washing 4 times with 1.5 mL of ice-cold buffer, and quantified by γ counting with 60% efficiency.

Cells and membranes were labeled by using photoreactive ligands and then processed, in the presence of the protease inhibitors phenanthroline (1 mM), pepstatin A (1 μM) phenylmethanesulfonyl fluoride (100 μM), iodoacetamide (1 mM), and EDTA (1 mM), for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (5–15% gradient) and autoradiography as previously described (Seagar et al., 1985). Protein was assayed by the Bradford (1976) method or a modified Lowry (1951) method, using a bovine serum albumin standard.

Target Size Analysis. Crude synaptosomal membranes (Seagar et al., 1985) from Wistar rat cerebral cortex were prepared. After resuspension (10–15 mg of protein/mL) in degassed, serum albumin free binding buffer, supplemented with 100 μM phenylmethanesulfonyl fluoride and 1 mM EDTA, they were frozen in screwtop polypropylene vials (Nunc) and stored prior to and after irradiation in liquid nitrogen. Samples were irradiated with 10 MeV electrons from the linear accelerator (CSF Thompson, Paris, France) of Justus Liebig Universitat, Giessen, FRG, at temperatures between −130 and −100 °C maintained by a stream of liquid nitrogen. Dosimetry, determined from radiochromic dye detectors, was carried out as previously described (Ferry et al., 1983). Apamin receptors were titrated as above with 0.1 nM ^{125}I-apamin at pH 7.5, and nonspecific binding was determined in the presence of 0.1 μM native apamin. α_1-Adrenoreceptors,

[1] Abbreviations: ANPAA, [(4-azido-2-nitrophenyl)amino]acetyl; ANPAA-OSu, [(4-azido-2-nitrophenyl)amino]acetic acid succinimidyl ester; biotin-OSu, biotin succinimidyl ester; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; ^{125}I-HEAT, *dl*-2-[[[2-(3-[^{125}I]iodo-4-hydroxyphenyl)ethyl]amino]ethyl]tetralone; P_i, inorganic phosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SP, sulfopropyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

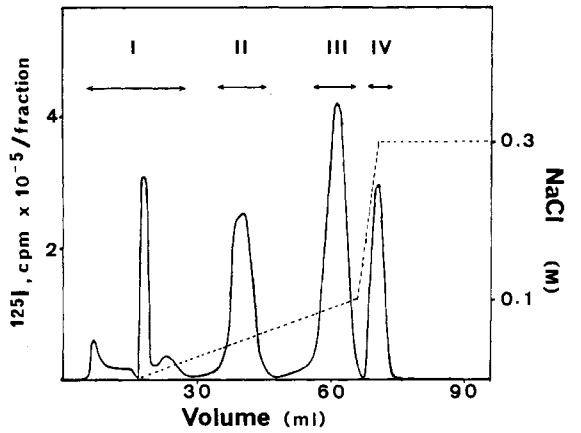

FIGURE 1: Separation of photoreactive ^{125}I-apamin derivatives by ion-exchange chromatography. Mono[^{125}I]iodoapamin was reacted with a 2-fold molar excess of ANPAA-OSu at pH 8.5 for 1 h. The reaction products were separated by chromatography on a SP-Sephadex C-25 column (0.4 × 8 cm) in three steps as indicated, in 10 mM NaP_i and 10 μM serum albumin buffer at pH 7. One-milliliter fractions were collected. The peaks were located by γ counting.

β-scorpion toxin receptors, and acetylcholinesterase were followed as internal molecular weight standards. α_1-Adrenoreceptors were assayed by incubating 40 μg of membrane protein, in 1 mL of 50 mM Tris, 150 mM NaCl, and 1 mM EDTA adjusted to pH 7.4 with HCl, with 0.2 nM ^{125}I-HEAT[1] (specific activity, 2000 Ci/mmol, Amersham Corp.) in the presence and absence of 1 μM HEAT (Amersham Corp.) for 60 min at 30 °C. Bound ligand was separated by rapid filtration over glass fiber filters (GFC, Whatman) (Lubbecke et al., 1983). β-Scorpion toxin receptors were titrated by using [^{125}I]iodotoxin VI from *Centruroides suffusus suffusus* as previously described (Jover et al., 1984). Receptor-associated ligand was measured by γ counting with 60% efficiency. Acetylcholinesterase was assayed according to Ellman et al. (1961). The radiation dose for which 37% of the original binding activity remained (D_{37}) was calculated by linear regression of a ln B/B_0 vs. D plot (where D = dose, B = bound ligand, and B_0 = bound ligand when $D = 0$). The molecular weight was calculated by using the formula of Kepner and Macey (1968):

$$\text{molecular weight} = [(6.4 \times 10^5)/[D_{37}\ (\text{rad})]]S$$

where S is a temperature correction factor of 2.8 (Kempner & Haigler, 1982). The indicated errors are derived from the standard deviation of the slope passing through the origin.

Results

Preparation of Photoaffinity Probes. Mono[^{125}I]iodoapamin was coupled with an amine-directed succinimidyl ester of the photosensitive azide ANPAA in the presence of bovine serum albumin as a carrier protein. The reaction mixture was then separated by ion-exchange chromatography, as shown in Figure 1, into four peaks, which were tentatively assigned as follows from their relative elution positions: I, run through; II, ^{125}I-$(ANPAA)_2$-apamin; III, ^{125}I-ANPAA-apamin; and IV, ^{125}I-apamin. The elution position of nonmodified ^{125}I-apamin coincided with that of peak IV. Both increasing the molar ratio of activated ANPAA:primary amine of ^{125}I-apamin plus serum albumin carrier in steps from 1:1 to 10:1 and increasing the reaction time (see Table I) led to a corresponding increase in peak II compatible with its being the dimodified derivative and peak III the monomodified derivative. Further characterization of picomole quantities of these radiolabeled derivatives was impractical.

Table I: Effects of ANPAA-OSu Concentrations and Reaction Time on Distribution of Radioactivity in Chromatogram Fractions[a]

molar ratio of ANPAA-OSu:reactive amine	reaction time (h)	% radioactivity			
		peak I	peak II	peak III	peak IV
0		25			75
1:1	1	28	10	30	32
2:1	1	15	26	41	18
10:1	3	27	63	7	3

[a] After modification at indicated reagent concentrations and reaction times, products were fractionated into four peaks by SP-Sephadex C-25 chromatography as in Figure 1.

Table II: Amino Acid Analysis of Apamin and Dansylated (DNS) Peptides

amino acid	apamin	DNS-apamin	DNS-dibiotinyl-apamin	DNS-monobiotinyl-apamin
Asp	1.00	1.05	1.13	1.02
Thr	1.11	1.01	1.13	1.05
Glu	2.80	3.18	3.06	3.08
Pro	1.11	0.90	0.86	0.99
Ala	2.98	2.92	3.00	3.01
Cys	3.60	2.35	3.21	2.40
Leu	1.01	0.99	1.03	1.02
His	1.00	0.98	0.71	1.06
Lys	1.00	0.24	0.98	0.99
Arg	2.05	1.89	1.91	1.89

Apamin was therefore modified with biotin in a similar way but on a preparative scale to permit identification of the reaction products. This reagent was chosen to avoid consuming large amounts of a limited supply of ANPAA-OSu, and with the advantage that biotinylated peptides can be identified by affinity adsorption to immobilized avidin. Derivatization of apamin with biotin, also activated as a succinimidyl ester, followed by ion-exchange chromatography gave an elution profile identical with that in Figure 1. Portions of each of these peaks were treated with dansyl chloride, which reacts with free primary amines; these were then hydrolyzed and subjected to amino acid analysis. This methodology leads to the loss of dansylated amino acids, whereas residues protected by biotin, coupled in a labile amide bond, are quantitatively recovered. Peaks II, III, and IV were in this way identified as the disubstituted (α-Cys_1,ϵ-Lys_4) derivative, the monosubstituted (ϵ-Lys_4) derivative, and unmodified apamin, respectively (see Table II). The activity of the dimodified derivative was checked by a competition binding assay. [α-Biotinyl-Cys_1, ϵ-biotinyl-Lys_4]apamin had an equilibrium dissociation constant (K_d) of 40–50 pM at pH 7.5 and 4 °C, which is only 2–3 times less potent than native apamin.

By analogy with the biotinylated peptides, we therefore consider peak III, obtained after fractionation of ANPAA-treated ^{125}I-apamin, in Figure 1, as ^{125}I-[ϵ-ANPAA-Lys_4]-apamin, which we will refer to as ^{125}I-ϵANPAA-apamin. In order to obtain a second chemically defined photoprobe with the reactive azide at the α-Cys_1 position, [ϵ-biotinyl-Lys_4]-apamin was used as the starting material. Biotinylapamin was iodinated as described under Materials and Methods, and the iodination mixture was separated by ion-exchange chromatography as shown in Figure 2. A single radioactive peak eluted after a step to 50 mM NaCl. The fact that this peak contained ^{125}I-biotinylpeptide was verified by batch incubation with Ultrogel-A4R–avidin. After intensive washing, >80% of the radioactivity remained bound to the gel matrix compared to <0.01% for mono[^{125}I]iodoapamin. The specific radioactivity was estimated by direct binding to a calibrated receptor

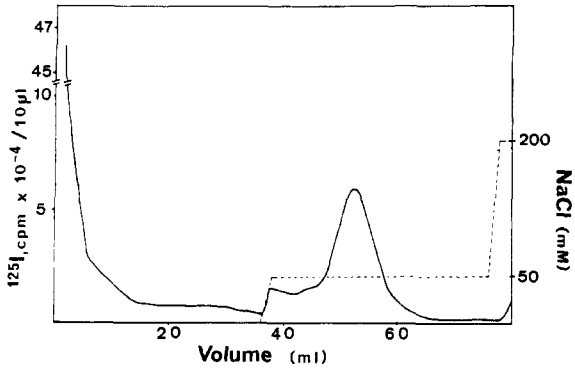

FIGURE 2: Preparation of ^{125}I-ε-biotinylapamin. One nanomole of monobiotinylapamin was reacted with 0.5 mCi of Na^{125}I and 5 nmol of iodogen in 10 mM Tris adjusted to pH 8.6 with HCl. Separation was achieved on a SP-Sephadex C-25 column in conditions similar to Figure 1. ^{125}I-ε-Biotinylapamin eluted after a step to 50 mM NaCl. One-milliliter fractions were collected.

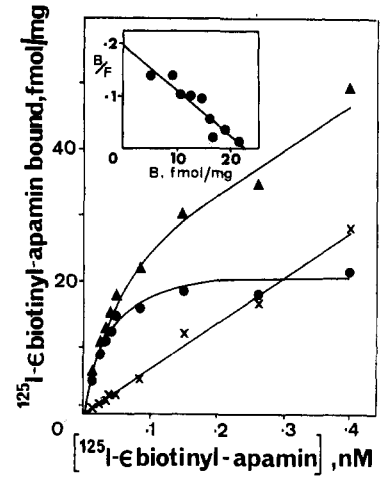

FIGURE 3: Equilibrium binding of ^{125}I-ε-biotinylapamin to rat brain membranes. Crude synaptic membranes (0.3 mg of protein/mL) were incubated with increasing concentrations of ^{125}I-ε-biotinylapamin in the presence (×) and absence (▲) of 0.1 µM apamin for 90 min at 1 °C in 25 mM Tris, 10 mM KCl, and 0.1% serum albumin adjusted to pH 9 with HCl. Membrane-associated radioactivity was determined after rapid filtration. The saturable, specific binding component (●), which is the difference between total (▲) and nonspecific (×) binding, is represented as a Scatchard plot (inset), where B = bound and F = free.

preparation as follows. First, the receptor capacity of a synaptic membrane preparation was titrated by using pure mono[^{125}I]iodoapamin (2000 Ci/mmol). A saturation curve was then established for ^{125}I-[ε-biotinyl-Lys$_4$]apamin by using the same membrane preparation. The radioactivity specifically bound at saturation was found to be only 25% of that obtained with the monoiodinated ^{125}I-labeled ligand. The receptor capacity for the two ligands was assumed to be the same, as derivatization would only modify affinity. ^{125}I-[ε-Biotinyl-Lys$_4$]apamin therefore has a specific radioactivity of 500 Ci/mmol, which implies that complete separation of labeled and unlabeled biotinylpeptides was not achieved. High-affinity binding was however conserved with K_d = 35 pM at 4 °C and pH 9, as calculated from the data shown in Figure 3. This ^{125}I-derivative, which has the ε-NH$_2$ protected by biotin, was then treated with ANPAA-OSu to obtain a single possible radiolabeled photoreactive apamin species, ^{125}I-[α-ANPAA-Cys$_1$,ε-biotinyl-Lys$_4$]apamin, which will be referred to as ^{125}I-α-ANPAA-apamin. No separation of the reaction products was attempted as the concentration and specific radioactivity of the initial ligand was in this case too low to allow any further dilution.

Photolabeling of Primary Cultured Neurons and Synaptic Membranes. The defined photosensitive apamin derivatives were used to label receptor-associated polypeptides in two different biological systems. Primary cultured neurons from the embryonic rat brain were tested in the hope that proteolytic degradation of receptor proteins would be minimized in a whole cell system. Rat brain synaptic membranes, which are a convenient and, as yet, the richest source (20–30 fmol of receptor/mg of protein) of apamin binding sites reported, were labeled in parallel to explore receptor structure in what could be the starting material for purification.

The labeling pattern obtained with both characterized photoreactive derivatives and a nonseparated reaction mixture is shown in Figure 4. Only receptor-associated polypeptides were labeled, as complete protection from covalent incorporation occurred when 0.1 µM native apamin was included. Although 0.1 µM apamin was routinely used to define specific labeling, experiments over a range of concentrations showed that partial protection started between 10 and 100 pM and was complete with 1 nM apamin. No covalent incorporation of radioactivity into macromolecular constituents was observed when ^{125}I-apamin or ^{125}I-[ε-biotinyl-Lys$_4$]apamin was photolyzed after incubation with membranes (not shown). Protease inhibitors were present in all experiments from tissue homogenization onward. The two photoprobes labeled completely different polypeptides. ^{125}I-εANPAA-apamin incorporated into components of M_r 33 000 and 22 000 in both cultured neurons and synaptic membranes (lanes 1 and 3) although bands in synaptic membranes were rather heterogeneous. ^{125}I-αANPAA-apamin cross-linked a single polypeptide at M_r 86 000 in cultured neurons (lane 5) whereas in synaptic membranes two major chains at M_r 86 000 and 59 000 were labeled (lane 7).

Clearly, the labeling pattern obtained in neuronal cultures by using a nonfractionated reaction mixture (lane 9) is the sum of that obtained with the two derivatives (lanes 1 and 5). Although less obvious, this is in fact also the case with synaptic membranes. Labeling of the components at M_r 86 000 and 59 000 is relatively much more intense than in cell cultures, necessitating an overexposure of the autoradiograms to reveal the lower molecular weight chains. The autoradiogram shown in lane 3 (^{125}I-εANPAA-apamin) required an exposure 3–4 times longer than that in lane 7 (^{125}I-αANPAA-apamin). For this reason cross-linking of the lower molecular weight chains in synaptic membranes was overlooked in our first series of experiments using the undefined reaction mixture (Seagar et al., 1985). The omission of β-mercaptoethanol during denaturation did not modify the migration of any of the detected bands.

Target Size Analysis. The exposure of frozen brain membranes to 10-MeV electrons from a linear accelerator resulted in decreased ^{125}I-apamin binding.

As shown in Figure 5, binding decayed monoexponentially with increasing doses of ionizing radiation, and this was due to a reduction in receptor capacity (see inset to Figure 5). Linear regression analysis of the decay curve of results pooled from three independent irradiations gave D_{37} = 21.3 ± 0.3, which yields a target size of M_r 84 000 ± 1000.

The internal standards however gave somewhat lower values than in published reports using frozen samples. The α_1-adrenoreceptor with M_r 63 000 ± 2000 was reasonably close to target size data using [^{3}H]prazosin (M_r 71 500 ± 17 000 (Lubbecke et al., 1983) but lower than that using ^{125}I-HEAT [M_r 85 000 ± 1600 (Lubbecke et al., 1983)] as the labeling ligand. The β-scorpion toxin receptor, which is thought to be

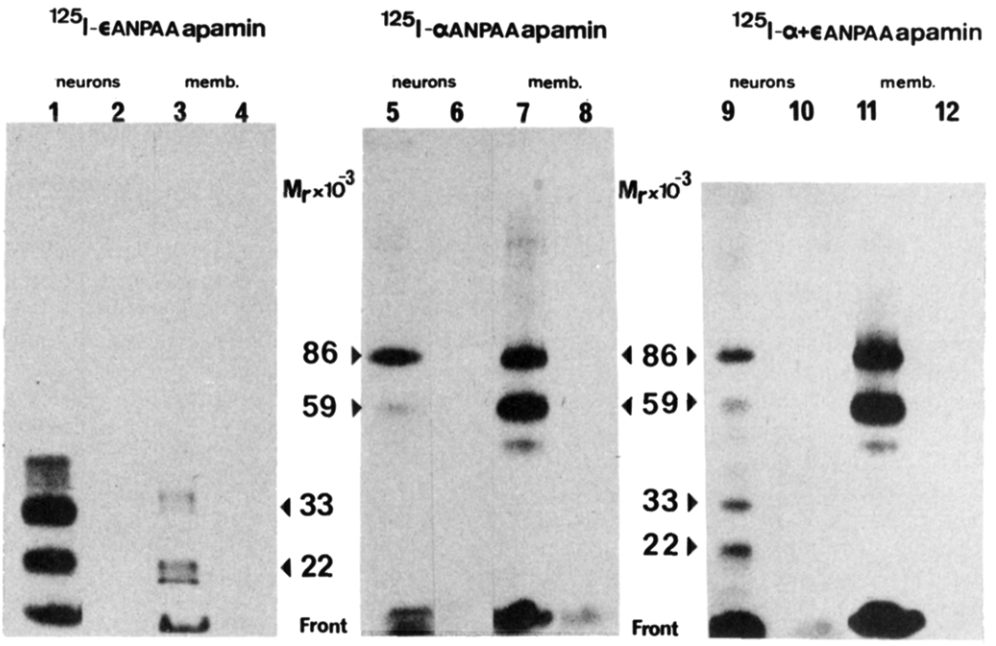

FIGURE 4: Differential photoaffinity labeling of receptor-associated polypeptides. The photoreactive derivatives (50–100 pM) were allowed to equilibrate in the dark with receptors in primary cultured neurons or crude synaptic membranes at 1 °C in the presence (even-numbered lanes) or absence (odd-numbered lanes) of 0.1 μM apamin. After photolysis membrane preparations were processed for SDS–PAGE, and labeled components were detected by autoradiography. Protease inhibitors were present from tissue homogenization onward. The relative molecular masses shown indicate the average values obtained from several experiments after subtraction of 2000, the approximate contribution of the apamin derivatives. The abbreviations used for the photoaffinity compounds are as follows: ^{125}I-ϵANPAA-apamin = ^{125}I-[ϵ-ANPAA-Lys$_4$]apamin (i.e., peak III in Figure 1); ^{125}I-αANPAA-apamin = ^{125}I-[α-ANPAA-Cys$_1$,ϵ-biotinyl-Lys$_4$]apamin (i.e., ANPAA-OSu-treated ^{125}I-[ϵ-biotinyl-Lys$_4$]apamin); ^{125}I-α+ϵANPAA-apamin = ^{125}I-[α-ANPAA-Cys$_1$,ϵ-ANPAA-Lys$_4$]apamin + ^{125}I-[ϵ-ANPAA-Lys$_4$]apamin + ^{125}I-apamin (i.e., nonfractionated reaction mixture, peaks I, II, III, and IV in Figure 1).

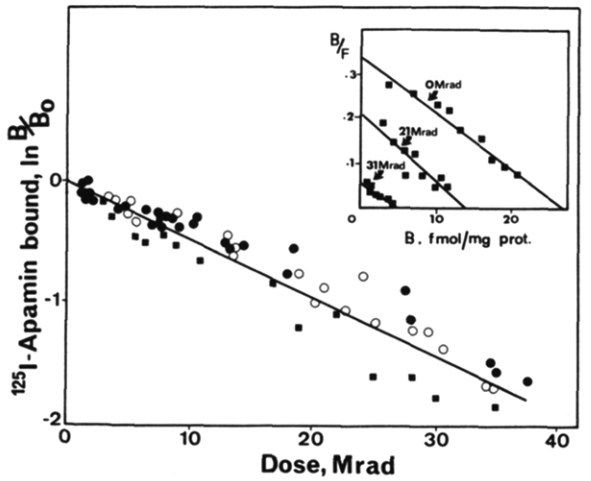

FIGURE 5: Radiation inactivation of rat brain apamin receptor. Crude synaptic membranes were frozen in liquid nitrogen and then exposed to 10-MeV electrons from a linear accelerator at about −120 °C. ^{125}I-Apamin binding was assayed, and nonspecific binding, determined in the presence of 0.1 μM apamin, was subtracted. Specific binding, represented as ln B/B_0, was plotted vs. the radiation dose (B = bound, b_0 = bound when dose = 0 Mrad). The symbols ○, ●, and ■ represent three totally independent irradiation experiments performed on different dates. Data points are the mean of duplicate determinations. D_{37} was obtained by linear regression of the pooled results and used to calculate the molecular size of the apamin binding unit (see Materials and Methods), yielding M_r = 84 000 ± 1000 (±standard deviation, calculated from the standard deviation of the slope passing through the origin). Inset: Scatchard plot of equilibrium binding data obtained at different radiation doses (B = bound, F = free).

functionally associated with the β_1 subunit of the voltage-sensitive Na^+ channel (Angelides et al., 1985), also gave a slightly lower value, M_r 35 000 ± 1000, than that previously reported from irradiation of frozen rat brain synaptosomes [M_r 43 000 (Angelides et al., 1985)]. It was nevertheless in good agreement with photoaffinity labeling data [M_r 35 000 (Darbon et al., 1983)] and the molecular weight of the β_1 subunit of the purified saxitoxin receptor [M_r 39 000 (Catterall, 1984)]. Acetylcholinesterase yielded a unit size of M_r 52 000 ± 2000. Although previous results with the membrane-bound monomer have indicated target sizes ranging from M_r 75 000 to M_r 62 000 (Levinson & Ellory, 1974; Angelides et al., 1985; Leigh et al., 1984), our data differ notably from the generally accepted unit size of M_r 70 000. If the apamin receptor target size of M_r 84 000 is normalized by using this last, most diverging, standard, M_r 115 000 is obtained, which should indicate the upper limit of calibration error. As all standards indicate an underestimation, it seems reasonable to assume that the true target size lies between M_r 84 000 and M_r 115 000.

Discussion

The α-amino of Cys_1 and the ϵ-amino of Lys_4 of apamin can be modified without any notable loss in toxicity (Vincent et al., 1975; Cheng-Raude et al., 1976) or receptor binding activity (this paper). Coupling a photoreactive group at these positions was therefore a reasonable way to obtain an active photoaffinity ligand that could be used to probe the structure of the apamin-sensitive, Ca^{2+}-activated K^+ channel.

The simplest experimental protocol, which has been described in a previous report (Seagar et al., 1985), was to treat ^{125}I-apamin with an aryl azide activated as a succinimidyl ester, followed by quenching the reaction mixture into the binding buffer and subsequent photolabeling. In this way, we identified specifically labeled polypeptides at M_r 86 000, 30 000, and 22 000 in cultured neurons and M_r 86 000 and 59 000 in rat brain synaptic membranes but could not explain the puzzling difference between these two systems. A possible source of artifact with this type of approach was the use of a chemically undefined photoprobe and in particular the probable presence

of some ^{125}I-(ANPAA)$_2$-apamin. Experiments using disuccinimido suberate to cross-link ^{125}I-apamin to its receptor identified a labeled band at about M_r 33 000 (Schmid-Antomarchi et al., 1984), which could be a subunit of a larger oligomeric protein. If a single type of subunit is present, and if each carries an apamin binding site, bifunctional photoaffinity ligands could conceivably cross-link two or three subunits, giving rise to artifactual covalent dimers or trimers with molecular weights close to the M_r 59 000 and 86 000 chains that we detected.

^{125}I-Monoazidoapamin derivatives were therefore synthesized to try to eliminate this possibility with the hope that this might also clarify the observed differences between receptors on intact neurons and synaptic membranes. Ion-exchange chromatography of the reaction mixture, after treatment of ^{125}I-apamin with ANPAA-OSu, resulted in the elution of two ^{125}I-ANPAA derivatives at different salt concentrations, indicating a difference in charge. Direct characterization of these radiolabeled peptides was impossible as only picomole amounts were prepared in the presence of a high concentration of bovine serum albumin (BSA). However, experiments using a range of ANPAA-OSu:peptide molar ratios suggested that the derivatives were di- and monomodified apamin. This was then confirmed by an analogous protocol with biotin-OSu and peptide quantities that allowed clear identification of the products. ^{125}I-ANPAA-apamin, separated from the reaction mixture, labeled two polypeptides with M_r 33 000 and 22 000 in both intact neurons and synaptic membranes whereas ^{125}I-(ANPAA)$_2$-apamin when tested on synaptic membranes gave a labeling pattern (not shown) identical with that obtained with the reaction mixture (see Figure 4, lane 11) with major bands at M_r 86 000 and 59 000. At first sight this result seemed to support the hypothesis that the bifunctional ligand can cross-link lower molecular weight subunits. However, another possible interpretation was that the mono-ANPAA derivative we obtained was not a mixture of ligands monosubstituted at either the α- or ϵ-amino positions but [as in Dempsey (1982)] a single chemical species monosubstituted on the most reactive of the two amino groups. In this case the high molecular weight receptor components could perhaps only be reached from the least reactive of the amino groups, which was only modified in the di-ANPAA derivative. This hypothesis was subsequently verified by biotinylation experiments in quantities that allowed product analysis and showed that the monoderivative was entirely [ϵ-biotinyl-Lys$_4$]apamin and by analogy that ^{125}I-ANPAA-apamin was modified on the same residue. Iodination and coupling of ANPAA to the unprotected α-amino group of [ϵ-biotinyl-Lys$_4$]apamin then allowed photolabeling of the higher molecular weight receptor components in both cultured neurons and synaptic membranes. This experimental protocol led to the unanticipated observation that aryl azide groups coupled at different positions on a relatively small peptide ligand can, after photolysis, react with different membrane components. An idea of the distances involved has been obtained by construction of a Nicholson molecular model of apamin based on published NMR data (Bystrov et al., 1980; Wemmer & Kallenbach, 1983). This indicated that the α- and ϵ-amino groups are separated by 13 Å maximum, with ANPAA, after photogeneration of a nitrene, contributing an arm of about 9 Å maximum at either of these positions. These results suggest therefore that the apamin binding site is located at the frontier between several polypeptide chains. Similar observations have been reported with two photoreactive α-scorpion toxin derivatives that both bind to pharmacological site 3 on the voltage-sensitive Na^+ channel in rat brain but differentially label the α and β_1 subunits (Sharkey et al., 1984).

These experiments also suggest an explanation for the discrepancy between our published photoaffinity data (Seagar et al., 1985) and reports using a homobifunctional cross-linker (Schmid-Antomarchi et al., 1984). We have shown that the ϵ-amino of Lys$_4$ is the most reactive of the two amines at pH 8.5, which was unexpected as the pK's for the α- and ϵ-amino groups are 6.7 and 10.3, respectively (Vincent et al., 1975). This is probably due to its greater accessibility, for which reason it is likely to be the favored cross-linking position when disuccinimido suberate is added to membranes containing receptor-bound ^{125}I-apamin. As the higher molecular weight receptor subunits cannot be attained from this residue, the observation that a M_r 33 000 chain was labeled (Schmid-Antomarchi et al., 1984) is compatible with our results. A second lower molecular weight peptide with M_r 16 000 has also been detected by using disuccinimido suberate but was thought to be a degradation product (Schmid-Antomarchi et al., 1984). This could well correspond to the M_r 22 000 chain that we also label from the ϵ-amino position. However, as it is clearly present on intact neurons, subsequently processed in the presence of protease inhibitors, we do not consider it to be a proteolytic fragment.

Two important differences between cultured neurons and synaptic membranes persist. First, labeling of the M_r 33 000 and 22 000 components is much weaker in synaptic membranes than in cells. Two interpretations can be envisaged. Either a smaller proportion of synaptic receptor proteins contain these two polypeptides or synaptic membrane receptors assume a conformation in which the M_r 33 000 and 22 000 polypeptides are less accessible to the receptor-bound probe and the probability for photoincorporation is thus lower than in cultured neurons. For this reason these polypeptides were not detected in membranes in our first experiments with a noncharacterized photoaffinity ligand. Second, a M_r 59 000 polypeptide is a major component in synaptic membranes but not in cultured neurons. The most obvious interpretation is that this is a fragment of the M_r 86 000 subunit, created by proteolysis during membrane preparation, in spite of the presence of five protease inhibitors. However, the possibility that it is another associated subunit cannot be ruled out. In this case the arguments used above are also applicable. The M_r 59 000 polypeptide is either absent in cultured neurons or present but too far from the neurotoxin binding site to be labeled. To summarize, the differences in the photolabeling pattern observed could indicate the existence of two structurally different receptor populations. Our data do not allow us to conclude as to whether receptors differ only at the conformational level or whether a change in the number or type of polypeptide chains associated with the apamin receptor is involved. If two different receptor types exist, this probably has some functional significance; however, no physiological correlation can as yet be proposed.

The molecular size of the functional unit necessary for ^{125}I-apamin binding, as estimated by radiation inactivation, was from M_r 84 000 to M_r 115 000. It may be useful to speculate as to which of the photolabeled polypeptides are most likely to contribute to this entity. Photoaffinity labeling provides a clue in that the M_r 86 000 chain is invariably a major cross-linked component in both cultured neurons and synaptic membranes, whereas the intensity of the other three polypeptides fluctuates, which suggests that the M_r 86 000 polypeptide contributes to the binding site. The simplest interpretation would be that this subunit alone is required for

neurotoxin binding, which is strongly suggested by the uncorrected target size of M_r 84000. However, internal controls indicated the possibility of an underestimation of the target size, perhaps due to difficulty in measuring the absolute radiation dose received during sample exposure. The target size obtained after normalization with respect to the most divergent standard (M_r 115000) would be compatible with the sum of the M_r 86000 and either the M_r 33000 or the M_r 22000 polypeptides. These subunits, which do not appear to be covalently associated, could nevertheless be conformationally interdependent and behave as a single radiation-sensitive unit containing the binding site. The present data do not allow us to choose between these two models; however, the possibility that the binding site is either formed solely by one of the smaller subunits (target size $M_r \leq 59000$) or requires a tightly interacting complex containing at least one of each of the detected polypeptides (target size $M_r \geq 200000$) can be eliminated.

Lastly, an alternative hypothesis should be pointed out. ^{125}I-Apamin binding to rat brain membranes and cultured neurons requires the presence of K^+ ions (Habermann & Fisher, 1979; Seagar et al., 1984), which implies that a K^+-selective ionic binding site, present on the apamin receptor, must be occupied for binding to occur. If the K^+ site and the apamin binding site are located on separate structures with different radiation sensitivities, it is conceivable that the measured target size could be uniquely that of the K^+ binding regulatory unit.

Our results diverge considerably from a previous radiation inactivation study on the apamin receptor that indicated a target size of 250000 (Schmid-Antomarchi et al., 1984). A possible source of discrepancy is that this higher target size was determined by irradiating lyophilized membranes at 10 °C and then rehydrating them, whereas we irradiated frozen membranes at −120 °C. This difference in sample preparation has been shown, in work on neurotoxin receptors on the rat brain Na^+ channel, to have a dramatic effect on the resulting molecular mass, and this suggests that lyophilization can perturb the channel environment, yielding a larger target size (Angelides et al., 1985).

Photoaffinity labeling with defined derivatives indicates that apamin binds at a zone of interaction between three or four polypeptide chains which may be K^+ channel subunits. The largest of these with M_r 86000 probably carries the binding site. Although these techniques may yet yield further information about receptor structure, the unequivocal demonstration that these are components of an oligomeric protein that forms a transmembrane K^+-selective ionophore will require detergent extraction, purification, and functional reconstitution. However, given the very low density of receptors in brain membranes, this will be a difficult task.

ACKNOWLEDGMENTS

We thank Drs. Sattler and Doell (Strahlenzentrum der Justus-Liebig-Universitat, Giessen, West Germany) for providing linear accelerator facilities and carrying out the dosimetry, Brigitte Ceard, Paule Deprez, Paule Frachon, Barbara Habermann, and Christian Leveque for technical assistance, Kim Angelides for his gift of reagents, Hervé Darbon, David Ferry, and Emmanuel Jover for helpful discussions, and Hervé Rochat for his continued interest and support.

Registry No. ANPAA-OSu, 60177-06-2; K, 7440-09-7; biotin-OSu, 35013-72-0; [ε-biotinyl-Lys_4]apamin, 102574-98-1; ^{125}I-[ε-biotinyl-Lys_4]apamin, 102586-46-9; ^{125}I-[α-ANPAA-Cys_1,ε-biotinyl-Lys_4]-apamin, 102586-47-0; ^{125}I-[ε-ANPAA-Lys_4]apamin, 102586-48-1; apamin, 24345-16-2.

REFERENCES

Angelides, K. J., Nutter, T. J., Elmer, L. W., & Kempner, E. S. (1985) *J. Biol. Chem. 260*, 3431–3439.
Banks, B. E. C., Dempsey, C. E., Pearce, F. L., Vernon, C. A., & Wholley, T. E. (1981) *Anal. Biochem. 116*, 48–52.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Bystrov, V. F., Okhanov, V. V., Miroshnikov, A. I., & Ovchinnikov, Yu. A. (1980) *FEBS Lett. 119*, 113–117.
Catterall, W. A. (1984) *Science (Washington, D.C.) 223*, 653–661.
Cheng-Raude, D., Treloar, M., & Habermann, E. (1976) *Toxicon 13*, 465–473.
Cognard, C., Traorê, F. Potreau, D., & Raymond, G. (1984) *Pfluegers Arch. 402*, 222–224.
Curtis, B. M., & Catterall, W. A. (1984) *Biochemistry 23*, 2113–2118.
Darbon, H., Jover, E., Couraud, F., & Rochat, H. (1983) *Biochem. Biophys. Res. Commun. 115*, 415–422.
Dempsey, C. E. (1982) *J. Chem. Soc., Perkin Trans. 1*, 2625–2629.
Ellman, G., Courtney, D., Andres, O., & Featherstone, L. (1961) *Biochem. Pharmacol. 7*, 88–95.
Ferry, D. R., Goll, A., & Glossmann, H. (1983) *Naunyn-Schmiedeberg's Arch. Pharmacol. 323*, 292–297.
Glossmann, H., & Ferry, D. R. (1985) *Methods Enzymol. 109*, 513–550.
Habermann, E., & Fisher, K. (1979) *Eur. J. Biochem. 94*, 355–364.
Hugues, M., Duval, D., Kitabgi, P., Lazdunski, M., & Vincent, J. P. (1982a) *J. Biol. Chem. 257*, 2762–2769.
Hugues, M., Schmid, H., Romey, G., Duval, D., Frelin, C., & Lazdunski, M. (1982b) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1308–1312.
Jover, E., Bablito, J., & Couraud, F. (1984) *Biochemistry 23*, 1147–1152.
Kempner, E. S., & Haigler, H. T. (1982) *J. Biol. Chem. 257*, 13297–13299.
Kepner, G. R., & Macey, R. I. (1968) *Biochim. Biophys. Acta 163*, 188–203.
Leigh, P. J., Cramp, W. A., & Mac Dermot, J. (1984) *J. Biol. Chem. 259*, 12431–12436.
Levinson, S. R., & Ellory, J. C. (1974) *Biochem. J. 137*, 123–125.
Lowry, O. M., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Lubbecke, F., Ferry, D. R., Glossmann, H., Sattler, E. L., & Doell, G. (1983) *Naunyn-Schmiedeberg's Arch. Pharmacol. 323*, 96–100.
Pennefather, P., Lancaster, B., Adams, P. R., & Nicoll, R. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3040–3044.
Romey, G., & Lazdunski, M. (1984) *Biochem. Biophys. Res. Commun. 118*, 669–674.
Schmid-Antomarchi, H., Hughes, M., Norman, R., Ellory, C., Borsotto, M., & Lazdunski, M. (1984) *Eur. J. Biochem. 142*, 1–6.
Seagar, M. J., Granier, C., & Couraud, F. (1984) *J. Biol. Chem. 259*, 1491–1495.
Seagar, M. J., Labbê-Jullié, C., Granier, C., Van Rietschoten, J., & Couraud, F. (1985) *J. Biol. Chem. 260*, 3895–3898.
Sharkey, R. G., Beneski, D. A., & Catterall, W. A. (1984) *Biochemistry 23*, 6078–6086.
Vincent, J. P., Schweitz, H., & Lazdunski, M. (1975) *Biochemistry 14*, 2521–2525.
Wemmer, D., & Kallenbach, N. R. (1983) *Biochemistry 22*, 1901–1906.