Volume 248, number 1,2, 150–154 FEB 07142 May 1989

Polypeptide constitution of receptors for apamin, a neurotoxin which blocks a class of Ca^{2+}-activated K^+ channels

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Received 10 February 1989

Affinity labelling experiments with different azido-^{125}I-apamin derivatives were carried out to identify polypeptide components of the apamin-sensitive Ca^{2+}-activated K^+ channel in brain and pheochromocytoma cell membranes. Different polypeptides were labelled with different apamin derivatives. The major component has a molecular mass of about 30 kDa but other components at 45, 58 and 86 kDa were also identified. Results obtained with brain membranes on one hand and pheochromocytoma cells on the other were not exactly identical and suggest that there are sub-types of apamin receptors.

Apamin; Ca^{2+}-activated K^+ channel

1. INTRODUCTION

Apamin is a bee venom neurotoxin of 18 amino acids [1] that blocks with a high affinity a class of Ca^{2+}-sensitive K^+ channels [2,3]. This Ca^{2+}-activated K^+ channel is responsible for the after-hyperpolarization observed in a number of excitable cells [4–7] and is known to have a small conductance [4,8].

Although extensive information is available concerning the localization of apamin receptors (i.e. putative apamin-sensitive Ca^{2+}-activated K^+ channels) in the brain [9,10], the exact subunit structure of the toxin-binding-site component is not yet known. The total molecular mass of the receptor protein has been found to be 250 kDa by radiation inactivation techniques [11] and 350 kDa by sucrose gradient centrifugation [12]. A first series of affinity labelling experiments with DSS has identified a polypeptide at ~30 kDa [11,13], however other authors have later identified polypeptides of 44 kDa, 59 kDa and 86 kDa [14,15].

In order to resolve these apparent discrepancies and to definitely identify polypeptides involved in the formation of the apamin-sensitive Ca^{2+}-activated K^+ channel, we have in this work used (i) 2 types of membrane preparations, one from rat brain and one from PC12 cells which have been shown to overexpress the apamin receptor [16], and (ii) 6 different ways to graft the toxin to its protein receptor.

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Abbreviations: ANBNOS, *N*5-azido 2-nitrobenzoyloxysuccinimide; ANPAA, succinimidyl ester azidonitrophenyl aminoacetate; DSS, disuccinimidyl suberate; HSAB, *N*-hydroxysuccinimidyl 4-azidobenzoate; SANPAH, *N*-succinimidyl 6-(4′-azido 2′-nitrophenylamino) hexanoate

2. MATERIALS AND METHODS

Apamin was radioiodinated according to Hugues et al. [17] except that ^{125}I-monoiodoapamin was purified by high-performance liquid chromatography (HPLC) using a TSK SP-5PW column (0.75 × 7.5 cm). Monoiodoapamin was eluted

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with a linear 0 to 400 mM NaCl gradient (eluent B) in 50 mM phosphate buffer, pH 6, with a slope of 2% B per min and a flow rate of 1 ml/min. HPLC purified ^{125}I-monoiodoapamin was eluted at 230 mM NaCl. Under these conditions ^{125}I-monoiodoapamin was completely devoid of any contamination by native apamin.

Photoreactive ^{125}I-monoiodoapamin with a specific radioactivity of 2000 Ci/mmol was prepared as described by Seagar et al. [14] except that the incubation was performed in 100 mM carbonate buffer at pH 9.

PC12 cells were grown in H16 medium, supplemented with 10% horse serum and 5% fetal calf serum, at 37°C and 5% CO_2. After 7 days of culture, cells were scraped off and washed with a 10 mM Tris-Cl buffer at pH 7.5, containing a mixture of protease inhibitors (1 μM pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 5 mM EDTA). Cells were pelleted at 5000 × g, resuspended in the same buffer and then homogenized with a Potter homogenizer. Protein concentrations were measured by the Bradford method [18] (Biorad), using bovine serum albumin as a standard. Homogenized PC12 cells were frozen and stored in liquid nitrogen until use.

Rat brain membranes were prepared with the same mixture of protease inhibitors used in PC12 cell preparations. Briefly, rats were killed by decapitation, brains were collected and homogenized with a Potter homogenizer in ice-cold Tris-Cl (20 mM) at pH 7.5, containing 0.32 M sucrose. The homogenate was centrifuged at 1500 × g during 7 min. The supernatant was collected and centrifuged for 20 min at 20000 × g. The pellet was collected, resuspended in a 5 mM Tris-Cl buffer at pH 7.5 and lysed during 60 min at 4°C. Lysed membranes were centrifuged for 30 min at 70000 × g. The pellet was collected and frozen in liquid nitrogen until use.

In photolabelling experiments, brain membranes or PC12 cell membranes (1 mg/ml of protein) were incubated in a buffer containing 100 mM Tris-Cl at pH 8.2, 5 mM KCl, 0.05% bovine serum albumin (buffer A), in the presence of the appropriate ^{125}I-apamin derivative at a concentration of 0.3 nM (PC12) or 0.1–0.2 nM (brain membranes) in the presence or absence of 0.1 μM unlabelled apamin to evaluate the contribution of the non-specific labelling component. Incubation was carried out in the dark at 4°C for 30 min, membranes were then centrifuged at 12000 × g for 15 min. Pellets were resuspended in buffer A and irradiated for 3 min with a UV lamp (Philips 60 W (λ = 253 nm)) at a distance of 5 cm. Membranes were then centrifuged and washed twice with buffer A. Pellets were resuspended in the denaturation buffer containing 50 mM Tris-Cl at pH 6.8, 0.6% SDS, 13.5% glycerol with or without 2% β-mercaptoethanol and heated 5 min at 100°C before analysis by electrophoresis. When DSS was used as a cross-linker reagent, and after incubation with apamin, pellets were resuspended in 50 mM carbonate buffer, pH 9, and incubated with increasing concentrations of DSS (0.01–1 mM) for 30 min. All experiments described above were performed in the presence of the protease inhibitor mixture described above. Samples obtained from labelling experiments were analysed by SDS-gel electrophoresis with an isocratic concentration of polyacrylamide (10%) [19] and submitted to autoradiography using Kodak XAR films and an intensifying screen.

Chemicals: HSAB, ANBNOS, SANPAH and DSS were purchased from Sigma. ANPAA was a gift of Dr Angelides. ^{125}I-Na (IMS 30) was purchased from Amersham.

3. RESULTS

The different cross-linking systems used in this work are listed in fig.1. Labelling patterns obtained with brain membranes were different according to the cross-linkers used (fig.2). A protein of 30 kDa was labelled with all ^{125}I-apamin derivatives used and was in 3 out of the 4 conditions used in fig.2 the most intensely labelled band. The most intensely labelled band with the ANPAA derivative of apamin was a protein of 86 kDa. In addition two proteins of 58 and 45 kDa were also labelled by ANPAA and SANPAH derivatives of the toxin. Finally a 28 kDa protein was also labelled with the HSAB derivative. The same autoradiographic pattern was obtained under non-reducing conditions (not shown).

The major protein labelled with PC12 cell homogenates and all the apamin derivatives is a 30 kDa protein under reducing conditions (fig.3, lanes 1–10). An 86 kDa protein was also labelled with ANPAA. Proteins were slightly labelled at 45 and 58 kDa when using HSAB or SANPAH. As previously observed with rat brain membranes, a 28 kDa protein was detectable in cross-linking ex-

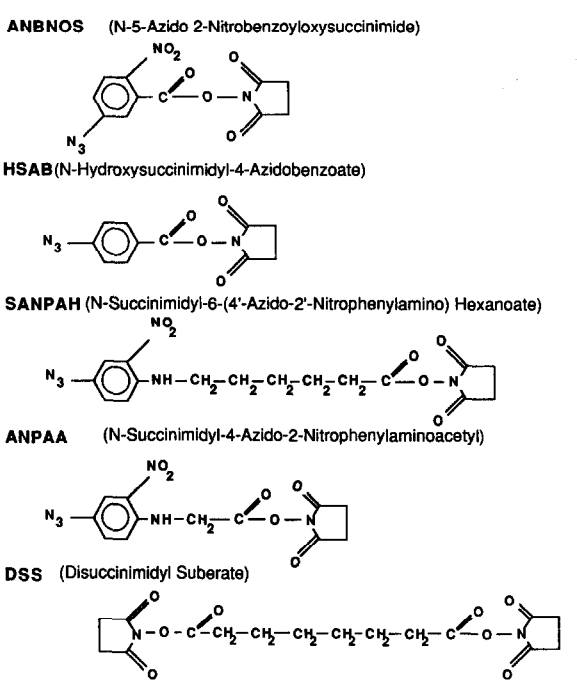

Fig.1. Cross-linking reagents used in this work.

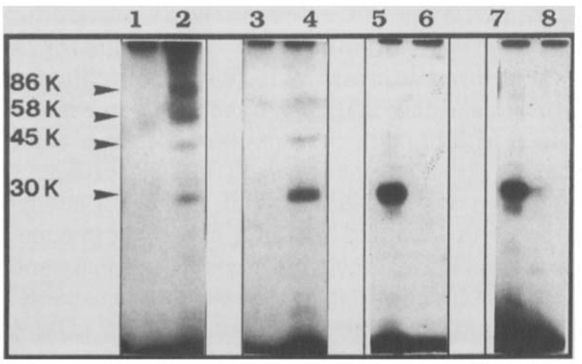

Fig.2. Affinity labelling of rat brain membranes with different apamin derivatives. Lanes: 1,2, ANPAA; 3,4, SANPAH; 5,6, ANBNOS; 7,8, HSAB. Lanes 1,3,6,8 correspond to experiments carried out in the presence of 0.1 μM unlabelled apamin (non-specific labelling). All results presented here correspond to non-reducing conditions.

periments with the HSAB derivative. Cross-linking experiments with DSS and PC12 cell membranes gave results similar to those previously described for microsomal brain membranes [11,13], i.e. a single labelled polypeptide at 30 kDa. Under non-reducing conditions (fig.3, lanes 11,12) gel patterns revealed that the major protein labelled had a molecular mass of 25 kDa. However several slightly labelled proteins were also seen, in particular a 18 kDa protein. The same pattern was obtained with all cross-linking reagents used.

All labelling patterns corresponded to specific labelling since complete protection was observed in the presence of an excess of native apamin. Results are summarized in table 1.

Table 1

Labelling of the apamin receptor obtained with different cross-linker reagents

	Rat brain membranes					PC12 cell membranes			
MR:	86	58	45	30	28	86	58	30	28
ANPAA	xx	x	x	x		x		xx	
SANPAH		x	x	xx			x	xx	
ANBNOS				xx				xx	
HSAB				xx	x			xx	x
DSS				xx				xx	

MR, molecular mass, in kDa, of the protein; xx, intense labelling; x, less intense labelling

4. DISCUSSION

This paper describes photoaffinity labelling by cross-linking ^{125}I-apamin to its receptor using several reagents which are different in their structure but not in their photoreactive function (except for DSS). Polypeptide labelling patterns are different with the different cross-linking reagents used. The shortest reagents used (ANBNOS, HSAB, fig.1) only differ by the position of the nitrene and by the presence of a nitro function (ANBNOS) on the phenyl group. It seems that these limited differences are sufficient to give a different labelling of the apamin receptor in both PC12 and brain membranes. ANBNOS only labelled a 30 kDa protein while HSAB labelled both 28 and 30 kDa proteins.

Labelling experiments with ANPAA (fig.1) gave

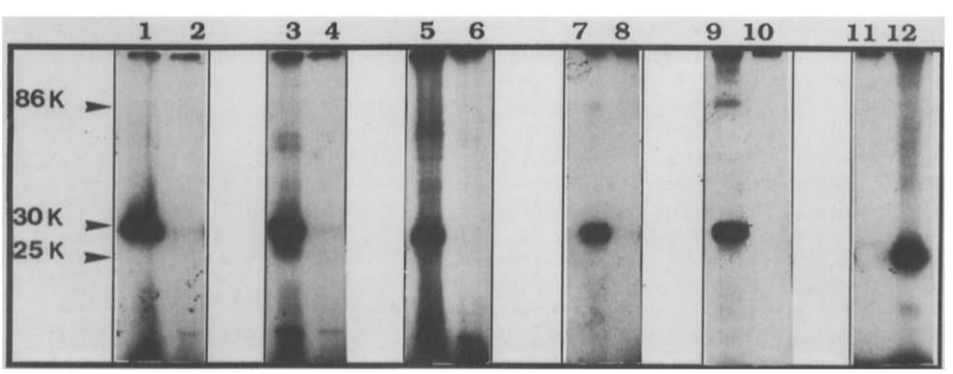

Fig.3. Affinity labelling of PC12 cell homogenates with different apamin derivatives. Lanes: 1,2, ANBNOS; 3,4,11,12, HSAB; 5,6, SANPAH; 7,8, DSS; 9,10, ANPAA. Lanes: 1–10, reducing conditions; 11,12, non-reducing conditions. Lanes 2,4,6,8,11, non-specific cross-linking in the presence of 0.1 μM apamin.

a labelling of 86, 58, 45 and 30 kDa polypeptides in brain membranes and of 86 and 30 kDa polypeptides in PC12 cells. This reagent only differs from ANBNOS and HSAB by the presence of a CH_2-NH between the activated ester function and the phenyl group bearing the photoreactive function. SANPAH (fig.1), which is similar to ANPAA with respect to its functional group, only allows the labelling of the 30 kDa protein and a less marked labelling of 45 and 58 kDa proteins. SANPAH differs from ANPAA by the number of $-CH_2-$ (5 instead of 1) between the two reactive groups. This difference could be the explanation for the absence of labelling of the 86 kDa band.

Differences in polypeptides identified by affinity labelling of a toxin receptor using different cross-linkers have already been observed with ω-conotoxin [20], a polypeptide toxin specific for one class of voltage-sensitive Ca^{2+} channels.

The situation is different in PC12 cell membranes. ANBNOS only labels a 30 kDa band while HSAB seems to label two minor bands at 58 and 45 kDa and a double band in the 30 kDa region. SANPAH gives a major labelling at 30 kDa and the same two minor bands at 58 and 45 kDa. As for brain membranes, DSS only cross-links a protein at 30 kDa. Surprisingly ANPAA, unlike in brain membranes, primarily labels a band at 30 kDa, the 86 kDa protein being only slightly labelled. Under non-reducing conditions, the 30 kDa protein identified in PC12 cell membranes behaves as a 25 kDa protein. Increases in apparent molecular mass after reduction have been found for several receptors including the β2 adrenergic receptor [21], the opioid receptor [22] and the neurotensin receptor [23]. This difference in molecular mass suggests the presence of intramolecular disulfide bonds. This difference found with PC12 membranes is not observed with brain membranes.

All these results taken together (table 1) strongly suggest that, as already indicated before [11,13], the ~30 kDa protein is an important element of the apamin receptor. Other subunits at 45, 58 and 86 kDa, however, are also clearly associated with the apamin-sensitive Ca^{2+}-activated K^+ channel. The difference observed (table 1) between results obtained with brain membranes on one hand and PC12 cell membranes on the other hand indicate that the apamin receptor is similar but not identical in the two types of membranes. This view is consistent with the observation that the K_d value for the apamin-receptor complex is 20 pM in brain membranes [17] while it is 350 pM in PC12 membranes [16].

Acknowledgements: This work was supported by the Centre National de la Recherche Scientifique and the Ministère de la Défense Nationale (grant DRET 85/138). We thank Dr Angelides for a generous gift of ANPAA. The skilful technical assistance of F. Aguila, M.-T. Bohn and C. Roulinat-Bettelheim is gratefully acknowledged.

REFERENCES

[1] Habermann, E. (1972) Science 177, 314–322.
[2] Jenkinson, D.H. (1981) Trends Pharmacol. Sci. 2, 318–320.
[3] Lazdunski, M., Romey, G., Schmid-Antomarchi, H., Renaud, J.-F., Mourre, C., Hugues, M. and Fosset, M. (1988) in: Handbook of Experimental Pharmacology, vol.83 'Calcium in Drug Action' (Baker, P.F. ed.) chap.7, pp.135–145, Springer, Berlin.
[4] Bernardi, H., Bidard, J.-N., Fosset, M., Hugues, M., Mourre, C., Rehm, H., Roey, G., Schmid-Antomarchi, H., Schweitz, H., De Weille, J.R. and Lazdunski, M. (1989) Drug Res. 39, 159–163.
[5] Romey, G. and Lazdunski, M. (1984) Biochem. Biophys. Res. Commun. 118, 669–674.
[6] Hugues, M., Romey, G., Duval, D., Vincent, J.-P. and Lazdunski, M. (1982) Proc. Natl. Acad. Sci. USA 79, 1308–1312.
[7] Hugues, M., Schmid, H., Romey, G., Duval, D., Frelin, C. and Lazdunski, M. (1982) EMBO J. 1, 1039–1042.
[8] Blatz, A.L. and Magleby, K.L. (1986) Nature 323, 718–720.
[9] Mourre, C., Schmid-Antomarchi, H., Hugues, M. and Lazdunski, M. (1984) Eur. J. Pharmacol. 100, 135–136.
[10] Mourre, C., Cervera, P. and Lazdunski, M. (1987) Brain Res. 417, 21–32.
[11] Schmid-Antomarchi, H., Hugues, M., Norman, R.I., Ellory, C., Borsotto, M. and Lazdunski, M. (1984) Eur. J. Biochem. 142, 1–6.
[12] Seagar, M.J., Marqueze, B. and Couraud, F. (1987) J. Neurosci. 7, 565–570.
[13] Hugues, M., Schmid, H. and Lazdunski, M. (1982) Biochem. Biophys. Res. Commun. 107, 1577–1582.
[14] Seagar, M.J., Labbe-Jullie, C., Granier, C., Van Rietshoten, J. and Couraud, F. (1985) J. Biol. Chem. 260, 3895–3898.
[15] Marqueze, B., Seagar, M.J. and Couraud, F. (1987) Eur. J. Biochem. 169, 295–298.
[16] Schmid-Antomarchi, H., Hugues, M. and Lazdunski, M. (1986) J. Biol. Chem. 261, 8633–8637.
[17] Hugues, M., Duval, D., Kitabgi, P., Lazdunski, M. and Vincent, J.-P. (1982) J. Biol. Chem. 257, 2762–2769.

[18] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[19] Laemmli, U.K. (1970) Nature 227, 680–685.
[20] Barhanin, J., Schmid, A. and Lazdunski, M. (1988) Biochem. Biophys. Res. Commun. 150, 1051–1062.
[21] Fraser, C.M. and Venter, J.C. (1980) Proc. Natl. Acad. Sci. USA 261, 9094–9097.
[22] Gionnini, T.L., Howard, A.D., Miller, J.M. and Simon, E.J. (1985) J. Biol. Chem. 260, 15117–15121.
[23] Mills, A., Demoliou-Mason, C.D. and Barnard, E.A. (1988) J. Biol. Chem. 263, 13–16.