Volume 245, number 1,2, 235–237 FEB 06926 March 1989

Isolation and reconstitution of the high-affinity choline carrier

M. Knipper, I. Boekhoff and H. Breer

University Stuttgart-Hohenheim, Institute for Zoophysiology, Garbenstr. 30, 7000 Stuttgart 70, FRG

Received 26 January 1989

Monoclonal antibodies, which block the high-affinity uptake of choline in synaptosomal ghosts, have been used to purify a membrane polypeptide (80 kDa) from insect synaptosomal membranes. This isolated protein was found to catalyse the sodium-dependent, hemicholinium-sensitive accumulation of choline after reconstitution into liposomes, thus, apparently represents the high-affinity choline transporter.

Choline transporter; Purification; Reconstitution; Synaptosomal membrane

1. INTRODUCTION

The high-affinity carrier localized in the presynaptic membranes of cholinergic nerve terminals catalyzes the translocation of choline, which is supposed to be the rate-limiting step in the synthesis of acetylcholine [1–3]. During the last few years, this process has been studied in depth using membrane vesicles derived from synaptosomal membranes [4–7]. It has been found that the transporter catalyzes electrogenic cotransport of sodium, chloride and choline and the stoichiometry of the system was estimated to be $2Na^+ : Cl^- :$ choline.

However, the molecular basis of choline transport is not known. The purification of the transporter in an active form is an essential step towards this goal. In photoaffinity labelling approaches using tritiated hemicholinium-3, a 80 kDa polypeptide has been identified as a putative candidate for a carrier constituent [8] and monoclonal antibodies which block choline accumulation recognized the same polypeptide in Western blots [9]. These antibodies have been used in this study to purify the transporter polypeptides which were subsequently reconstituted in liposomes and assayed for transport activity.

Correspondence address: M. Knipper, University Stuttgart-Hohenheim, Institute for Zoophysiology, Garbenstr. 30, 7000 Stuttgart 70, FRG

2. MATERIALS AND METHODS

2.1. *Materials*

[*N-Me*-^{3}H]Choline chloride (spect. act. 80 Ci/mmol) was obtained from New England Nuclear. Hemicholinium-3, choline bromide, soybean phospholipids (asolectin), sodium deoxycholate, octylglycoside, Chaps were received from Sigma. Standard proteins for SDS gel electrophoresis were from Pharmacia. All other reagents were obtained in the purest form commercially available.

2.2. *Purification of the transporter protein*

Properties of the monoclonal antibodies used in this study (mab VIB6F5) were described previously [9].

Synaptosomal membranes were prepared from locust nervous tissue as described previously [4]. Proteins were solubilized by incubation with Tris-HCl (pH 8.0) buffered deoxycholate (0.9%) containing 400 mM NaCl. The detergent of the extract was exchanged for 0.05% Chaps and the polypeptides were separated on a Mono-Q HR 5/5 column (Pharmacia) using a 0–2 M KCl gradient. The fractions were assayed for immunoreactivity and positive fractions were applied to an immunoaffinity column produced by coupling VIB6F5 antibodies onto Tresyl-activated Sepharose 4B (Pharmacia). Polypeptides retained on the column were eluted with 10 mM phosphate-buffered 150 mM NaCl (pH 9.5) containing 0.05% Chaps, 5 mM EDTA, 5 mM EGTA. The eluate was immediately neutralized by adding appropriate volumes of 60 mM Na-acetate, pH 4.0, and was concentrated using Centricon-30 (Amicon). Samples were prepared and analyzed on discontinuous SDS-polyacrylamide gels (4% stacking gel, 12.5%

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

separating gel) as described [10]. The gels were stained with Coomassie blue.

2.3. *Reconstitution*

Affinity purified polypeptides were mixed with partially purified asolectin (10 mg/ml) resuspended in Tris-HCl (pH 7.4)-buffered octylglycoside (4.6 mM) containing 100 mM KCl. After 10 min on ice the mixture was dialyzed against 100 mM KCl, 25 mM sucrose, 0.5 mM DTT, 0.02% deoxycholate buffered with 5 mM Tris-HCl, pH 7.4, for 48 h.

2.4. *Choline transport assay*

The influx of choline was measured using an inwardly directed sodium gradient and an outwardly directed potassium gradient as described in [5]. For each time point 200 μl of proteoliposomes (1–10 μg protein) were added to 200 μl of influx solution containing 0.15 M NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 μM [*N-Me*-^{3}H]choline buffered with 10 mM Tris-HCl, pH 7.4. Reactions were stopped by filtration (Whatman GF/C filter) and samples were counted as described [4].

3. RESULTS

The reconstituted liposomes containing affinity purified polypeptides (see lane B in fig.2) showed a time-dependent, rapid uptake of choline (fig.1). The initial rate of uptake was estimated as about 950 pmol/mg of protein for the first 15 s. The extent of choline accumulated after 1 min was more than 2500 pmol/mg of protein. When Na^+ in the transport medium was substituted by K^+, only a very small amount of choline accumulated (fig.1). Thus, the observed accumulation was obviously due to the inwardly directed Na^+ gradient [5]. In the presence of hemicholinium-3, the specific blocker of choline uptake in synaptosomes, choline was not transported; a similar degree of choline entrapment was observed in liposomes without protein indicating that the rapid accumulation of choline in proteoliposomes is due to a protein component isolated from synaptosomal membranes incorporated into liposomes.

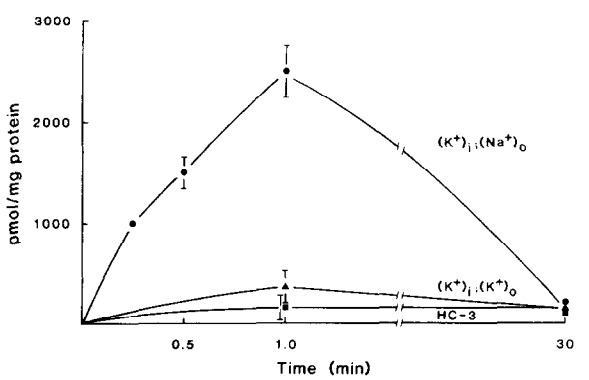

Fig.1. Transport of choline by proteoliposomes produced by reconstituting affinity purified polypeptides (fig.2, lane B) in liposomes. Choline accumulates rapidly during the first 60 s. The concentrative uptake depended essentially on extravesicular sodium and was completely blocked by hemicholinium-3.

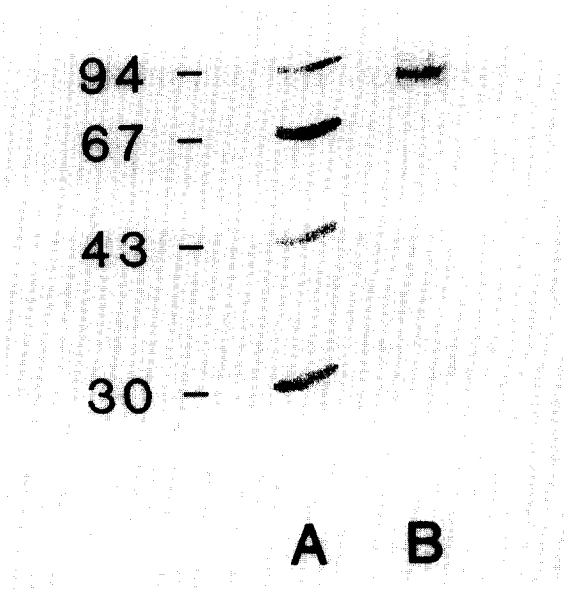

Fig.2. Polyacrylamide gel electrophoresis of immunoaffinity purified polypeptides from synaptosomal membranes. Membrane proteins were solubilized by 0.9% sodium deoxycholate, separated by ion-exchange chromatography and further purified by immunoaffinity chromatography. Lane A shows the standard proteins: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa). Lane B, polypeptides eluted from the affinity column; there is only one major polypeptide band (80 kDa).

Analysis of the fractions containing transport activity for its protein composition using SDS-polyacrylamide gel electrophoresis and visualisation by different staining procedures revealed the presence of one polypeptide band corresponding to a molecular mass of about 80 kDa (fig.2) suggesting that the 80 kDa polypeptide represents the high-affinity choline transporter.

4. DISCUSSION

Proteoliposomes containing immunoaffinity

purified polypeptides from insect synaptosomal membranes (lane B in fig.2) display a concentrative accumulation of choline. This accumulation is only observed in the presence of extravesicular Na^+ and an inwardly directed Na^+ gradient, and is completely blocked by hemicholinium-3. These results are consistent with the idea that choline is transported into the reconstituted liposomes via a specific transport system. Proteoliposomes with transport activity for choline contain only one population of polypeptides (80 kDa). The size of the polypeptides corresponds to proteins affinity labelled with tritiated hemicholinium-3 [8] and recognized by specific monoclonal antibodies which block choline uptake into synaptosomes [9]. Interestingly the apparent molecular mass of the purified polypeptides showing transport activity is very similar to the recently identified transport proteins for glucose [11] and GABA [12], thus, it is likely that the 80 kDa polypeptide is the only component of the high-affinity choline carrier.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft. We are grateful to C. Kahle for expert technical assistance.

REFERENCES

[1] Kuhar, M.J. and Murrin, L.C. (1978) J. Neurochem. 30, 15–21.
[2] Jope, R.S. (1979) Brain Res. Rev. 1, 313–344.
[3] Tucek, S. (1985) J. Neurochem. 44, 11–24.
[4] Breer, H. and Lueken, W. (1983) Neurochem. Int. 5, 713–720.
[5] Breer, H. and Knipper, M. (1985) Cell. Mol. Neurobiol. 5, 285–296.
[6] Ducis, I. and Whittaker, V.P. (1985) Biochim. Biophys. Acta 815, 109–127.
[7] Vyas, S. and O'Regan, S. (1985) J. Membr. Biol. 85, 111–119.
[8] Knipper, M., Krieger, J. and Breer, H. (1989) Neurochem. Int., in press.
[9] Knipper, M., Strotmann, J., Mädler, U., Kahle, C. and Breer, H. (1989) Neurochem. Int., in press.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Schmidt, U.M., Eddy, B., Fraser, C.M., Venter, J.C. and Semenza, G. (1983) FEBS Lett. 161, 279–283.
[12] Radian, R., Bendahan, A. and Kanner, B.I. (1986) J. Biol. Chem. 261, 15437–15441.