SOLUBILISATION AND RECONSTITUTION OF THE γ-AMINOBUTYRIC ACID TRANSPORTER FROM RAT BRAIN

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1. Introduction

High affinity, sodium-dependent, active transport systems for various neurotransmitters have been detected in brain preparations such as synaptosomes [1–5]. These systems have been implicated in termination of transmitter action on post-synaptic receptors [1] as well as in maintaining constant levels of transmitters in the neurons [6]. Recently, the mode of active transport of one of these neurotransmitters, γ-aminobutyric acid (GABA), has been investigated in membrane vesicles derived from synaptosomes [7]. The idea that ion gradients are the immediate driving force for this active accumulation [5], has been supported more directly using these membrane vesicles [7]. The process is absolutely dependent on both external sodium and chloride ions, and both gradients (out > in) can drive GABA accumulation. The dependency on the chloride ion gradient cannot be explained by the electrogenicity of the process alone. Thus, either chloride ions may be translocated inward together with sodium ions and GABA, or these anions are necessary to bring the carrier to the conformation required for GABA translocation. Direct flux experiments are required to decide between these possibilities. These experiments probably will be successful only in reconstituted proteoliposomes containing highly purified GABA transporter preparations, which are expected to have a very low chloride permeability. In addition, the use of such a reconstituted system will be important for the study of other aspects of the mechanism of GABA translocation.

This report describes the solubilisation of the GABA transporter and its functional incorporation into liposomes. The active, electrogenic, GABA transport catalysed by these proteoliposomes displays the features of the native system such as the sodium and chloride ion dependency, energisation and inhibitor sensitivity.

2. Materials and methods

Octylglucoside was synthesised as in [8]. Biobeads SM-2 were purchased from Bio-Rad Laboratories and washed as in [9]. Soybean phospholipids (asolactin, Assoc. Conc.) were partially purified [10]. [^{3}H]GABA was from New England Nuclear, valinomycin and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) were from Sigma. Nigericin was a generous gift of Dr R. J. Hosley from Eli Lilly.

Synaptosomal fractions were isolated from rat brain and purified using Ficoll gradient centrifugation [7]. Membrane vesicles were obtained after osmotic shock of either the 2–8% interfaces and/or the 8–12% interfaces [7]. Aliquots were stored in liquid air.

For solubilisation of the GABA carrier, these stored membrane vesicles were used and all subsequent steps were performed at 0–4°C. Membrane vesicles (20–50 mg protein) were incubated at 2 mg/ml with 0.32 M sucrose, buffer A (10 mM Tris–sulfate, pH 7.4, 0.5 mM EDTA, 1 mM $MgSO_4$), 1 mM dithiothreitol and 0.5% Triton X-100 (last addition). After 2 min of stirring the mixture was centrifuged in a Beckman 50-Ti rotor for 1 h at 46 000 rev./min. To the supernatant Biobeads SM-2 [9], 0.3 g/ml, were added and the mixture was stirred for 2 h. The mixture was then centrifuged for 5 min at 10 000 rev./min

in a Sorvall SS-34 rotor, to remove the Biobeads. The supernatant was brought to 50% saturation by addition of an equal volume of ammonium sulfate. After 10 min stirring the mixture was centrifuged in the SS-34 rotor at 15 000 rev./min for 20 min. The floating pellet was collected and resuspended at 5–10 mg protein/ml in a solution containing 50 mM sucrose and 0.5 mM dithiothreitol in buffer A. Aliquots were stored in liquid air. Under these conditions the protein could be stored for at least 2 months, without loss in transport activity, assayed after reconstitution.

Reconstitution was performed using a variant of the cholate dialysis procedure [10]. Soybean phospholipids were dried under a stream of nitrogen and suspended, using a bath type sonicator, at 30 μmol/ml in dialysis buffer (see below) containing 33 mM octylglucoside. This phospholipid suspension, 18–20 μmol/ml, was incubated with the solubilised protein, 1 mg/ml in a solution containing 50 mM potassium phosphate, pH 6.8, 1 mM dithiothreitol, 0.32 M sucrose, buffer A and 0.8% cholic acid neutralised with NaOH (last addition). This mixture was dialysed for 18–20 h against 120–200 vol. dialysis buffer, which contained 120 mM potassium phosphate, pH 6.8, 0.32 M sucrose, buffer A, 1 mM dithiothreitol and 1% (v/v) glycerol. The proteoliposomes obtained after dialysis were used for the transport experiments.

Transport of GABA was measured by adding 15–40 μl of proteoliposomes to 0.36 ml of an incubation mixture containing (unless indicated otherwise in the figure legends) 0.15 M NaCl, 2 mM $MgSO_4$, 0.07–0.28 μM [^{3}H]GABA at 3.5–35 Ci/mmol, and the additions indicated in the figure legends. After incubation for various times at room temperature (20–23°C), the reactions were terminated by addition of 2 ml ice cold incubation mixture (not containing [^{3}H]GABA) and filtration through membrane filters (Millipore, 0.22 μm pore size). After washing of the filters with another 2 ml of the above solution, the retained radioactivity was determined using liquid scintillation spectrometry. Zero times were obtained by adding the stopping solution prior to the proteoliposomes. Experimental values were up to 10–30-fold the zero time values. NaCl was used at 0.15 M instead of 0.1 M used for the native vesicles [7] since with the former salt concentration 50% higher values in that system were obtained (B. K., unpublished observations). Protein was assayed as in [11].

3. Results and discussion

When the proteoliposomes, formed in a potassium phosphate solution, are diluted into 0.15 M NaCl in the presence of valinomycin, a time-dependent GABA uptake is observed (fig.1A). Under these conditions both a sodium and a chloride ion gradient (out > in) and also a membrane potential (interior negative) are generated. This uptake, both with regard to initial rate and extent, is quantitatively comparable (30–100%) to that under similar conditions in the native system [7]. Like in the native system the transport is absolutely dependent on external sodium and chloride ions. No transport at all is detected when the proteoliposomes are diluted into KCl or sodium phosphate (fig.1A), or into sodium sulfate (data not shown). Since in the experiments in which the proteoliposomes are diluted into sodium phosphate also valinomycin is present, it is highly unlikely (as well as in the native system) that the chloride dependency

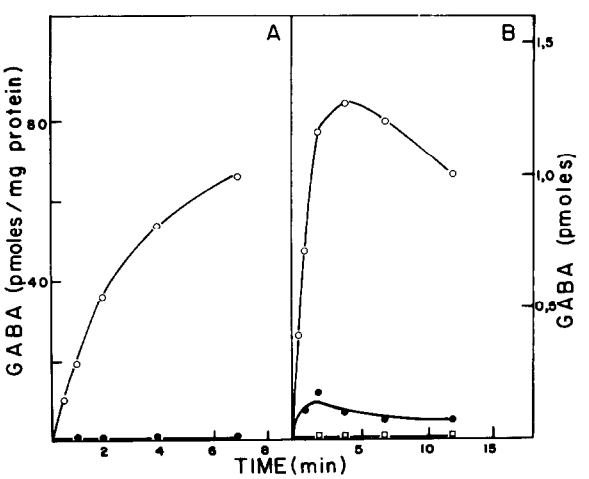

Fig.1.A. Ion dependence of GABA transport in reconstituted vesicles. Proteoliposomes (56 μg protein/reaction) were diluted into 150 mM NaCl (○–○); 150 mM KCl or 100 mM sodium phosphate, pH 6.8 + 50 mM sucrose (●–●). The media contained also 2.5 μM valinomycin and 0.07 μM [^{3}H]GABA, 7500 cpm/pmol. B. Dependence of reconstituted GABA transport activity on phospholipids and solubilised protein. Reconstituted vesicles containing soybean phospholipids (□–□); solubilised protein, 22 μg (●–●); or both (○–○) were diluted into 150 mM NaCl containing 2.5 μM valinomycin and 0.28 μM [^{3}H]GABA, 1650 cpm/pmol. Transport was measured as in section 2.

is merely due to the requirement for a membrane potential (interior negative). The requirement for external chloride ions in the native system is not absolute; other small monovalent anions such as bromide, nitrate and thiocyanate have been found to be effective to a small extent ([7], B. K. and I. Sharon, unpublished results). In the reconstituted system these anions are also partly effective, to a similar extent as in the native system. Thus, both with regard to initial rate and extent, the transport values expressed as percent of those in the presence of chloride are approx.: bromide 50%, nitrate 30% and thiocyanate 20%.

The optimal functional reconstitution of the GABA system requires both exogeneously-added phospholipids as well as the solubilised protein fraction (fig.1B). No transport at all was detected with only phospholipid present, and highly reduced transport

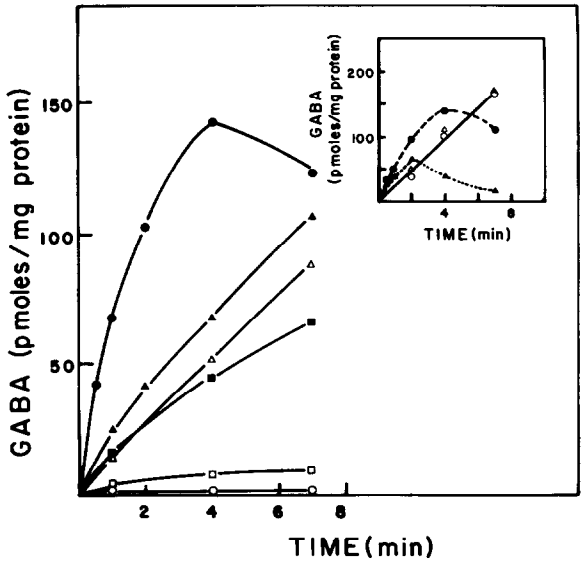

Fig.2. Effect of ionophores and inhibitors on GABA transport in reconstituted proteoliposomes. Transport assays were performed as in section 2 using 25 μg protein/assay. The medium contained 0.28 μM GABA, 1010 cpm/pmol and the following additions: valinomycin, 2.5 μM (●–●); none (▲–▲); CCCP, 5 μM (△–△); nigericin, 5 μM (○–○); triphenyltin chloride, 5 μM (□–□); or triphenyltin chloride, 5 μM and dithiothreitol, 1 mM (■–■). In the experiment depicted in the insert 15 μg protein was used and the medium contained 0.28 μM GABA, 1570 cpm/pmol as well as the following additions: valinomycin, 2.5 μM (●–●); none (○–○); CCCP, 5 μM (△–△); valinomycin, 2.5 μM and CCCP, 5 μM (▲–▲).

was obtained with the protein fraction alone (fig.1B). The presence of cholate at the onset of dialysis appears to be required for functional incorporation of the GABA transporter. When cholate is omitted from the incubation mixture no transport is detected when both phospholipids and the protein fraction are added or with the protein present alone (data not shown).

When the cation gradients artificially imposed across the proteoliposomes are abolished using the ionophore nigericin, GABA transport is strongly inhibited (fig.2). This is similar to observations on the native system [7]. Moreover, addition of the ionophore valinomycin stimulates GABA transport (fig.2) even stronger than in the native system [7]. Thus, the electrogenicity of GABA transport is preserved in the reconstituted system. The larger stimulation in the reconstituted system indicates that the passive permeability for potassium ions is lower in this system. It is not known yet if this is due to the excess of phospholipids added and/or to removal during the fractionation procedure of proteins causing potassium leakage. It is of interest to note that in the presence of valinomycin after 4–7 min a decay of GABA transport is observed, unlike in the control (fig.2). At longer times the phenomenon is even more pronounced (data not shown). An explanation may be that the decay of the sodium ion gradient occurs much faster when a potassium ion moves in the opposite direction, to preserve electroneutrality. This may also apply to the effects of the proton conductor CCCP on GABA transport (fig.2, insert). The proton conductor by itself has a small effect (fig.2). Triphenyltin chloride, the strongest known inhibitor of the native transport system [7] is also a very potent inhibitor in the reconstituted system (fig.2). Interestingly this inhibition is almost completely reversed by 1 mM dithiothreitol (fig.2). Dithiothreitol by itself does not affect the transport process (data not shown). Similar reversal of inhibition has been observed in the native system (B. K. and B. Raveh, unpublished observations).

When the detergent cholate is added to vesicles which have been previously allowed to accumulate GABA, an immediate loss of the solute is observed (fig.3), apparently because of destruction of the proteoliposome membrane. Efflux can also be observed under conditions when the membrane is not

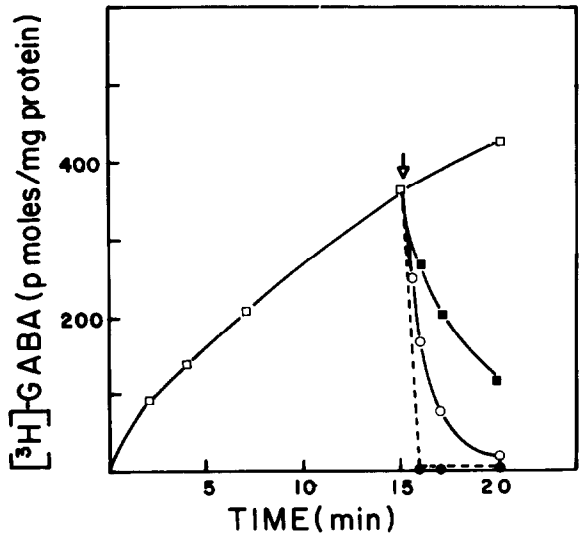

Fig.3. Efflux of [^{3}H]GABA from reconstituted proteoliposomes. Reconstituted vesicles, 15 μg protein, were allowed to accumulate [^{3}H]GABA, 0.28 μM, 1500 cpm/pmol, for 15 min. Then efflux was induced by 1 mM unlabelled GABA (▪–▪); 5 μM nigericin (○–○); or 1% cholate (●–●). Transport assays were performed as in section 2.

disrupted. Thus, addition of either a large excess of GABA or of nigericin results in a rapid efflux of the previously accumulated solute (fig.3).

These observations indicate that the solubilised GABA transporter has been functionally reconstituted and that the main features of active GABA transport in native membrane vesicles have been preserved in the reconstituted system. These features include sodium and chloride ion dependency, direct driving by artificially-imposed ion gradients, electrogenicity as well as inhibitor sensitivity. In addition, transport in the reconstituted system is also quantitatively similar to that observed in the native system. Since, at the same dilution factor, the initial rate of GABA transport is proportional to the protein concentration of the vesicles (data not shown) the reconstituted system provides an assay for the purification of the GABA transporter. This purification is now in progress.

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