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Neuronal and glial γ-aminobutyric acid$^+$ transporters are distinct proteins

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In the central nervous system, two subtypes of sodium- and chloride-coupled GABA transporter exist. One is sensitive to ACHC, the other to β-alanine. They are thought to be of neuronal and glial origin, respectively. GABA transport in membrane vesicles derived from astroglial cells was found to be sodium- and chloride-dependent, electrogenic and much more sensitive to β-alanine than to ACHC. Immunoblotting with antibodies directed against a variety of sequences of the ACHC-sensitive transporter indicated that none of these epitopes was shared by the glial transporter.

Astroglial cell; Membrane vesicle; GABA transport; Immunofluorescence; Sequence directed antibody; Immunoblotting

1. INTRODUCTION

High-affinity, sodium-dependent neurotransmitter uptake is thought to be the mechanism by which the overall process of synaptic transmission is terminated [1–3]. GABA is the major inhibitory transmitter in the central nervous system. Its high-affinity uptake system co-transports GABA with sodium and chloride [4,5]. In rat brain there are at least two different transporters. One – $GABA_A$ – is inhibited by ACHC, while the other – $GABA_B$ – is sensitive to β-alanine [6]. They are thought to be of neuronal [7,8] and glial origin [9], respectively.

One of the GABA transporters has been purified [10] and was subsequently cloned [11]. This transporter is sensitive exclusively to ACHC [6,12] and thus is probably of neuronal origin. This is reinforced by immunocytochemical localization studies employing an antibody raised against the pure transporter [13].

At the present time it is not clear if $GABA_A$ and $GABA_B$ transporters are two forms of the same protein – for instance phosphorylated and dephosphorylated – or two distinct proteins. Therefore we have studied GABA transport in membranes derived from astroglial cells and have employed sequence-directed antibodies against the $GABA_A$ transporter to answer the above question.

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Abbreviations: GABA, γ-aminobutyric acid; ACHC, *cis*-3-aminocyclohexane carboxylic acid; GFAP, glial fibrillary acid protein.

2. MATERIALS AND METHODS

2.1. *Materials*

[^{3}H]GABA (47.6 Ci/mmol) was obtained from the Nuclear Research Center, Negev. [^{125}I]Protein A (30 mCi/mg) was from Amersham. Reagents for tissue culture, including Dulbecco minimal essential medium, MEM HEPES, and fetal calf serum were from Gibco/BRL. ACHC was synthesized for us by Erwin Gross, from the Pilot Plant at the School for Applied Science of the Hebrew University, according to the method of Johnston et al. [14]. Valinomycin and fluorochrome-labelled secondary antibodies were from Sigma; nigericin from Calbiochem. Monoclonal antibodies against GFAP were purchased from Boehringer. Antisera against synthetic peptides corresponding to the aminoterminus (P_{NH_2}; residues 6–19); residue 410–422 ($P_{410-422}$) or residues 480–493 ($P_{480-493}$) of the neuronal GABA transporter molecule were generated in rabbits as previously described [15].

Antiserum against the carboxy terminal as well as its corresponding peptide (P_{COOH}; residues 571–586) were a generous gift from Dr. Reinhard Jahn (Yale University Medical School, New Haven, CT). The antibody against the intact transporter was prepared as described [10]. All antibodies were purified by binding to Affi-gel 15 (Bio-Rad) carrying the partially purified transporter as described [13].

2.2. *Cell culture*

Primary cultures of mouse cortical astrocytes were obtained from two-day-old BALB/c mice essentially as described [16]. Cells were grown on poly-D-lysine (50 mg/l) -coated 250 ml culture flasks at 37°C in a moist atmosphere containing 5% CO_2. Cultures were fed twice a week with Dulbecco minimal essential medium (high glucose formulation) supplemented with 10% fetal calf serum, 2 mM glutamine, 2.4 g/l sodium bicarbonate and 100 mg/l gentamycin. For immunofluorescent staining, glial precursor cells were shaken off mechanically from the surface of the monolayer and subcultured in 35-mm petri dishes in the presence of 10% serum to promote differentiation into type 2 astrocytes.

2.3. *Preparation of astroglial membrane vesicles*

After three weeks in culture cells were washed with 0.32 M mannitol and 1 mM Na-EDTA, pH 7.4, and harvested with a rubber policeman. Subsequently they were homogenized on ice in 0.1 M sodium phosphate buffer containing 1% glycerol, 1 mM $MgSO_4$, 0.5 mM EDTA and 5 mM TRIS-sulfate, pH 7.4 (homogenization buffer) and centrifuged for 10 min at 3,000 × *g* to remove nuclei and undisrupted cells. The supernatant was centrifuged for 20 min at 27,000 × *g* at 4°C. The

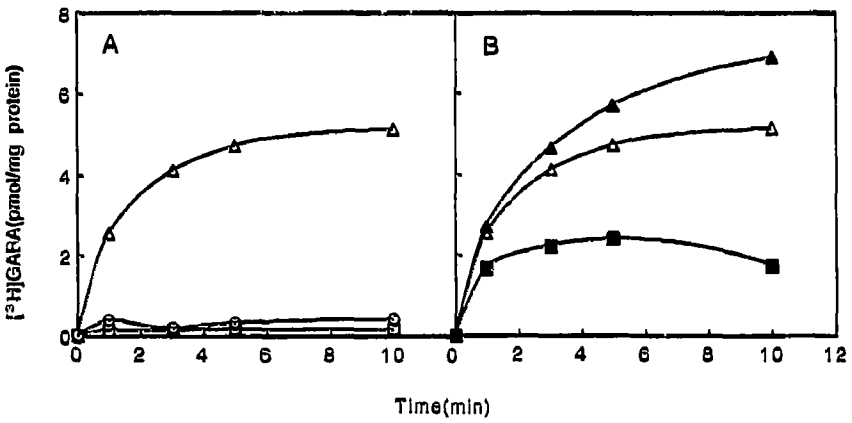

Fig. 1. Ion-dependence and effects of ionophores on GABA transport. Transport of GABA was measured for the indicated times, using astroglial membrane vesicles at 100 μg of protein per time point. In each mixture (approx. 190 μl) 1 μCi of [³H]GABA was present (i.e. 0.1 μM GABA). (A) The composition of the external medium was 0.15 M NaCl (△), 0.15 choline chloride (□) or 0.15 M sodium isethionate (○). (B) The external media contained 0.15 M NaCl in the presence of one of the following ionophores: none (△), 5 μM nigericin (■) or 2.5 μM valinomycin (▲).

resulting pellet was suspended and diluted in 5 mM TRIS-HCl and 1 mM Na-EDTA, pH 7.4. After the osmotic shock, the membranes were collected by centrifuging at 27,000 × *g* for 20 min. The pellet was resuspended in homogenization buffer, divided into aliquots and frozen in liquid nitrogen.

2.4. *Preparation of synaptosomal membrane vesicles*

Crude synaptosomal membrane vesicles were prepared from rat or mouse brain as described [4].

2.5. *Transport*

Membrane vesicles were thawed rapidly at 37°C, loaded with 0.1 M KPi, pH 6.9 and assayed for GABA transport as described [4]. Time points were done in triplicate. Standard deviations were 2–4%. Therefore, only mean values are indicated.

2.6. *Immunofluorescence*

Cultures were fixed in ethanol/acetic acid (5:1, v/v) for 10 min at −20°C. After several washes with MEM-HEPES and MEM-HEPES containing 1% bovine serum albumin, cells were sequentially incubated for 30 min at room temperature with antibodies against a peptide fragment of the neuronal GABA transporter molecule (diluted 1:100 in MEM-BSA) and with a monoclonal anti-GFAP antibody (diluted 1:20). After four washes in MEM the secondary antibody mixture (FITC-conjugated goat anti-mouse IgG and TRITC-conjugated goat anti-rabbit IgG, both diluted 1:50 in MEM-BSA) was added for 30 min. After rinsing in MEM-HEPES, preparations were mounted in gelvatol and examined in an Olympus IMT2 inverted microscope.

2.7. *Other methods*

SDS-PAGE [17] was done with 4% stacking gel and 10% separating gel, exactly as described [10]. Analysis of the specificity of the antibodies was done essentially as described [10,18]. Protein determination was done according to the Bradford method [19].

3. RESULTS AND DISCUSSION

When membrane vesicles derived from astroglial cells were loaded with potassium phosphate and diluted into a NaCl-containing medium, accumulation of [³H]GABA was observed (Fig. 1A). In the absence of either external sodium or chloride, uptake was abolished (Fig. 1A). The potassium-specific ionophore, valinomycin, is expected, under these conditions, to enhance the membrane potential (interior negative). Addition of this ionophore stimulates uptake by about 25% (Fig. 1B), indicating that the process is electrogenic. The exchange ionophore, nigericin, is expected to exchange

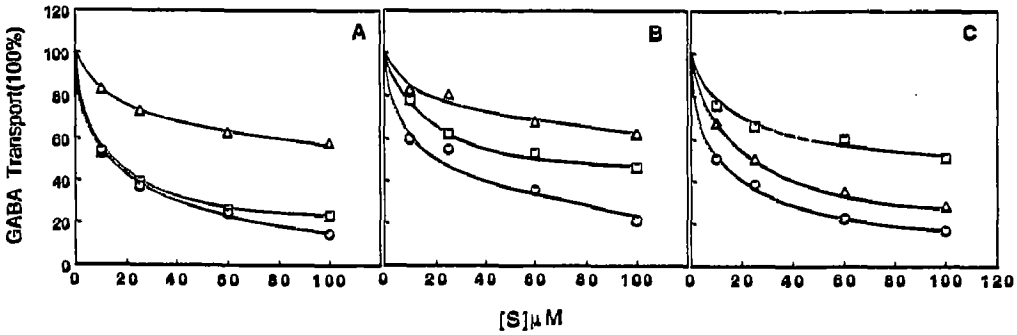

Fig. 2. Inhibition of [³H]GABA transport by substrate analogues. (A) GABA transport rates were measured for 4 min using astroglial membrane vesicles at 100 μg of protein per time point. (B and C) GABA transport rates were measured for 2 min using crude synaptosomal membrane vesicles from mouse or rat brain, respectively, at 200 μg of protein per time point. In each reaction 1 μCi of [³H]GABA was present. Substrate analogues were present in the influx medium (0.15 M NaCl) only at the indicated concentration, except in the case where β-alanine and ACHC were added together. There it reflects the total concentration, consisting of an identical contribution from each. △, ACHC; □, β-alanine; ○, ACHC and β-alanine together. 100% transport was 0.88, 75.6 or 19.6 pmol/min·mg protein in A, B or C, respectively.

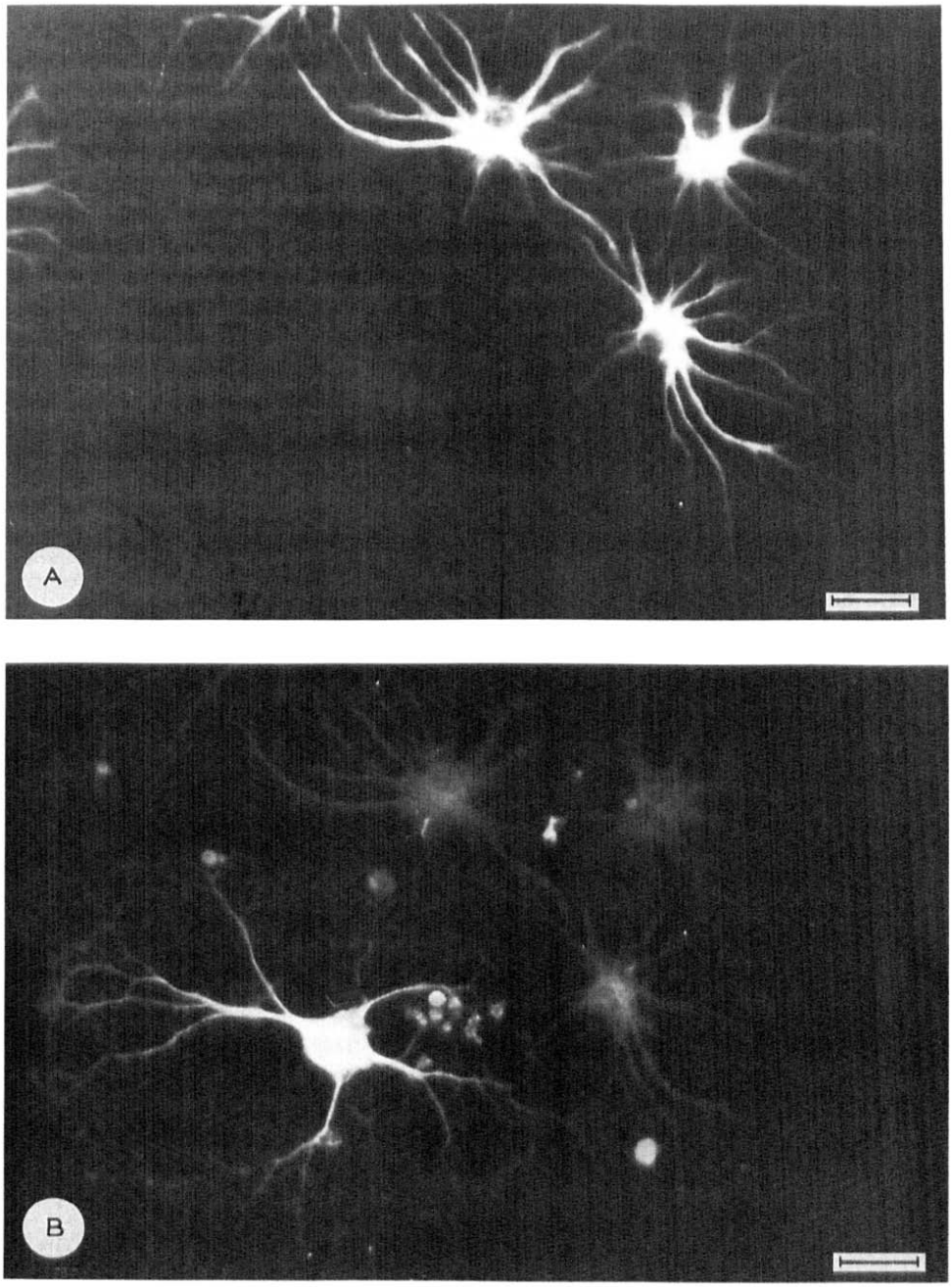

Fig. 3. Double-labeling immunostaining of cultured astrocytes from mouse brain using monoclonal anti-GFAP antibodies (A) in conjunction with anti-$P_{410-422}$ antibodies (B). Scale bars indicate 26.7 μm.

external sodium and internal potassium, thereby collapsing the sodium gradient and probably also the membrane potential. It can be observed that nigericin causes a time-dependent inhibition of [^{3}H]GABA uptake (Fig. 1B). This GABA transport in glial membrane vesicles is in many aspects reminiscent of that in membranes from rat brain [4]. The sodium- and chloride-dependence of the process in astroglial membranes can actually be predicted from previous observations that in membrane vesicles from rat brain, where both subtypes are expressed [6], no GABA uptake at all was observed in the absence of either sodium or chloride [4,6]. Also, membrane vesicles from rat brain exhibited a single high-affinity K_m for GABA (2–3 μM), similar to that of the isolated $GABA_A$ transporter [4,10]. Thus, a similar value is expected for the $GABA_B$ transporter. Indeed, a K_m of about 2 μM was observed in the glial membrane vesicles. The value of V_{max} was 20–30 pmol/min·mg protein.

The data presented in Fig. 2 indicate that the glial GABA transporter exhibits the pharmacological profile expected for the $GABA_B$ transporter. Thus, β-alanine was a much more potent inhibitor than ACHC in the glial membrane vesicles (Fig. 2A). On the other hand, both compounds inhibited GABA transport in membrane vesicles from either mouse or rat brain and their

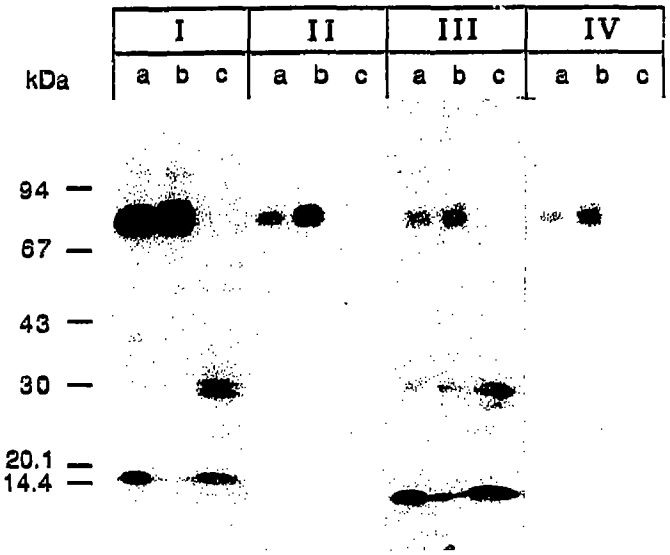

Fig. 4. Immunoreactivity of membrane vesicles towards different antibodies. Membrane vesicles from rat brain (lanes a), mouse brain (lanes b) or cultured astroglial cells (lanes c) were subjected to SDS-PAGE and immunoblotted with the following antibodies: I, antibody against the whole $GABA_A$ transporter; II, anti-P_{NH_2} antibody; III, anti-$P_{410-422}$ antibody; and IV, anti-P_{COOH} antibody. 40 μg of membrane protein were loaded per each lane of a and b, and 450 μg for that of c lanes.

inhibition was additive (Fig. 2B and C). No such additivity was observed in glial membrane vesicles (Fig. 2A). Thus, the glial membranes feature only one transporter, namely the $GABA_B$ subtype.

3.1. *Double-labeling immunostaining of cultured astrocytes*

Further evidence for molecular differences between the neuronal and the glial GABA transporter was gained from double-labeling immunostaining of cultured astrocytes as shown in Fig. 3. The antibody against GFAP, a specific molecular marker for astrocytes, stains nearly all of the stellate-shaped cells in the culture. However, the anti-$P_{410-422}$ antibody of the $GABA_A$ transporter does not stain any of these cells. The same was true for anti-P_{NH_2} or anti-$P_{480-493}$antibodies (data not shown). The only cells which light up are GFAP-negative cells with an oligodendrocyte morphology occasionally occuring in the culture. It should be noted, however, in this context, that oligodendrocytes previously were suggested to have a neuronal-like GABA uptake system [20].

Fig. 4 shows immunoblotting data with several sequence-directed antibodies against various parts of the $GABA_A$ transporter as well as with one raised against the whole transporter. When membrane vesicles from both rat and mouse brain were probed with these antibodies a band of around 80 kDa lit up (Fig. 4, lanes a and b). This agrees with the well-known mobility of the pure $GABA_A$ transporter [10,15]. On the other hand, the glial membrane did not react with any of the antibodies (Fig. 4, lanes c), notwithstanding the fact that the amount of GABA transport activity loaded on the gel was the same for all types of membranes. Thus, out of a variety of epitopes present in the $GABA_A$ transporter, none is shared by its $GABA_B$ counterpart. It is therefore concluded that the two are distinct proteins. However, this does not exclude (possible) homologous sequences, e.g. in the transmembrane domains. As neurotransmitter transporters appear to belong to a novel superfamily [21–25], it should be possible to clone the $GABA_B$ transporter with a PCR-type of approach. Comparison of cDNA clones encoding for the two GABA transporters with those encoding for other members of the family may indicate features such as the structural basis of the GABA binding site.

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REFERENCES

[1] Iversen, L.L. (1971) Br. J. Pharmacol. 41, 571–591.
[2] Iversen, L.L. (1973) Br. Med. Bull. 29, 130–135.
[3] Bennet Jr., J.P., Mulder, A.H. and Snyder, S.M. (1974) Life Sci. 15, 1045–1056.
[4] Kanner, B.I. (1978) Biochemistry 17, 1207–1211.
[5] Keynan, S. and Kanner, B.I. (1988) Biochemistry 27, 12–27.
[6] Kanner, B.I. and Bendahan, A. (1990) Proc. Natl. Acad. Sci. USA 87, 2550–2554.
[7] Bowery, N.G., Jones, G.P. and Neal, M.J. (1976) Nature 264, 281–284.
[8] Neal, M.J. and Bowery, N.G. (1977) Brain Res. 138, 169–174.
[9] Schon, F. and Kelly, J.S. (1975) Brain Res. 86, 243–257.
[10] Radian, R., Bendahan, A. and Kanner, B.I. (1986) J. Biol. Chem. 261, 15437–15441.
[11] Guastella, J., Nelson, N., Nelson, H., Czyzyk, L., Keynan, S., Miedel, M.C., Davidson, N., Lester, H.A. and Kanner, B.I. (1990) Science 249, 1303–1306.
[12] Keynan, S., Suh, Y.-J., Kanner, B.I. and Rudnick, G. (1992) Biochemistry (in press).
[13] Radian, R., Ottersen, O.P., Storm-Mathisen, J., Castel, M. and Kanner, B.I. (1990) J. Neurosci. 10, 1319–1330.
[14] Johnston, T.P., McCaleb, G.S., Clayton, S.D., Frye, J.L. and Krauth, C.A. (1977) J. Med. Chem. 20, 279–290.
[15] Mabjeesh, N.J. and Kanner, B.I. (1992) J. Biol. Chem. (in press).
[16] McCarthy, K.D. and de Vellis, J. (1980) J. Cell. Biol. 85, 890–902.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[19] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[20] Reynolds, R. and Herschkowitz, N. (1984) Brain Res. 322, 17–31.
[21] Pacholczyk, T., Blakely, R.D. and Amara, S.G. (1991) Nature 350, 350–354.
[22] Shimada, S., Kitayama, S., Lin, C.-L., Patel, A., Nanthakumar, E., Gregor, P., Kuhar, M. and Uhl, G. (1991) Science 254, 576–578.
[23] Kilty, J.E., Lorang, D. and Amara, S.G. (1991) Science 254, 578–579.
[24] Hoffman, B.J., Mezey, E. and Brownstein, M.J. (1991) Science 254, 579–580.
[25] Blakely, R.D., Berson, H.E., Fremeau Jr., R.T., Caron, M.G., Peek, M.M., Prince, H.K. and Bradley, C.C. (1991) Nature 354, 66–70.