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Two pharmacologically distinct sodium- and chloride-coupled high-affinity γ-aminobutyric acid transporters are present in plasma membrane vesicles and reconstituted preparations from rat brain

(*cis*-3-aminocyclohexanecarboxylic acid/β-alanine/additive and competitive inhibition/transporter fractionation)

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ABSTRACT **Electrogenic sodium- and chloride-dependent γ-aminobutyric acid (GABA) transport in crude synaptosomal membrane vesicles is partly inhibited by saturating levels of either of the substrate analogues *cis*-3-aminocyclohexanecarboxylic acid (ACHC) or β-alanine. However, both of them together potently and fully inhibit the process. Transport of β-alanine, which exhibits an apparent K_m of about 44 μM, is also electrogenic and sodium and chloride dependent and competitively inhibited by GABA with a K_i of about 3 μM. This value is very similar to the K_m of 2–4 μM found for GABA transport. On the other hand, ACHC does not inhibit β-alanine transport at all. Upon solubilization of the membrane proteins with cholate and fractionation with ammonium sulfate, a fraction is obtained which upon reconstitution into proteoliposomes exhibits 4- to 10-fold-increased GABA transport. This activity is fully inhibited by low concentrations of ACHC and is not sensitive at all to β-alanine. GABA transport in this preparation exhibits an apparent K_m of about 2.5 μM and it is competitively inhibited by ACHC ($K_i \approx 7$ μM). These data indicate the presence of two GABA transporter subtypes in the membrane vesicles: the A type, sensitive to ACHC, and the B type, sensitive to β-alanine.**

High-affinity neurotransmitter transport is held to be the mechanism for termination of the overall process of synaptic transmission by removing the transmitters from the synaptic cleft (1, 2). These transporters are sodium ion/neurotransmitter cotransporters and are able to accumulate the neurotransmitter against considerable concentration gradients by using the electrochemical gradient of sodium ions. Furthermore, many of these transporters are also absolutely dependent on other ions, such as chloride or potassium, and are apparently coupled to them as well (reviewed in refs. 3–5). One of the important and abundant transporters in rat brain is the one for γ-aminobutyric acid (GABA). It is absolutely dependent on sodium and chloride ions (6) and has been demonstrated to be an electrogenic sodium and chloride/GABA cotransporter (7, 8).

Pharmacological differences in GABA uptake have been observed between slices from tissue where the process is predominantly neuronal and those where it is largely glial. The GABA analogue *cis*-3-aminocyclohexanecarboxylic acid (ACHC) seems to be selective for neuronal GABA uptake (9, 10), whereas β-alanine is a better inhibitor for its glial counterpart (11). In rat brain, GABA transport is thought to be primarily neuronal (12). The observations that GABA transport shares synaptosomes and membrane vesicles with voltage-sensitive sodium channels (13, 14) seemed to support this conclusion. However, more recently glial cells have been found to contain voltage-sensitive sodium channels as well (15). Therefore, while it can be concluded that the process in rat brain is localized in membrane vesicles from the plasma membrane, it is possible that uptake of GABA is both neuronal and glial. In this study we have examined the inhibitor sensitivity of rat brain GABA transport in synaptosomal membrane vesicles as well as in partly purified reconstituted systems. We identify and characterize two distinct subtypes, one sensitive to ACHC, the other to β-alanine, possibly of neuronal and glial origin, respectively.

MATERIALS AND METHODS

Materials. [2,3-^{3}H]GABA (specific radioactivity 50 Ci/mmol; 1 Ci = 37 GBq) and β-[2,3-^{3}H]alanine (specific radioactivity 87.2 Ci/mmol) were purchased from the Nuclear Research Center, Negev, Israel, and New England Nuclear, respectively. 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.* (16). Sephadex G-50-80 was from Pharmacia, and nigericin was from Calbiochem. Valinomycin, cholic acid, and asolectin (soybean phospholipids, catalog no. P 5638) were from Sigma. The cholic acid was recrystallized and asolectin was purified by acetone extraction, both as described (17). Crude lipids were extracted from bovine brain (18). Membrane filters were from Schleicher and Schuell or from Millipore. All other reagents were the purest commercially available.

Methods. *Preparations.* Crude and Ficoll-gradient-purified synaptosomes from 3-week-old rats were prepared, and from them membrane vesicles were prepared upon osmotic shock (6). The membrane vesicles were quick-frozen in small aliquots in liquid air and stored at −70°C. The sodium- and chloride-coupled high-affinity GABA transport activity was solubilized from rat brain membranes, fractionated with ammonium sulfate, and reconstituted as described (19).

Transport. GABA transport was measured in KP_i-loaded membrane vesicles (6) and proteoliposomes (19) as described, using 10 μl per time point. Transport of β-alanine was measured exactly like that of GABA (6) except that β-[2,3-^{3}H]alanine was used. In most experiments the substrates were used carrier free (0.5–1.0 μCi per reaction), resulting in final concentration of 0.05–0.1 μM (GABA) or 0.02–0.03 μM (β-alanine). Since these concentrations are far below the respective K_m values, the rates of transport are much lower than V_{max}. Inhibitors were used without preincubation and were added directly to the 0.15 M NaCl influx solutions. The final reaction volume was 200 μl (membrane

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Abbreviations: GABA, γ-aminobutyric acid; ACHC, *cis*-3-aminocyclohexanecarboxylic acid.

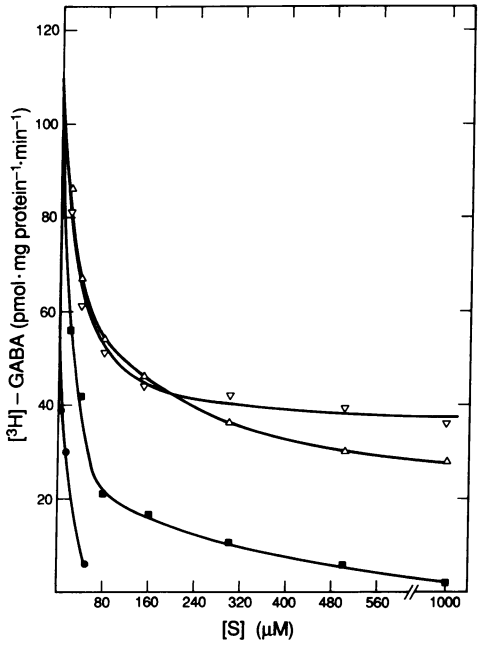

FIG. 1. Inhibition of [^{3}H]GABA transport by substrate analogues. GABA transport rates were measured for 1 min using crude synaptosomal membrane vesicles at 76 μg of protein per time point. In each reaction 1 μCi of [^{3}H]GABA was present. Substrate analogues were present in the influx medium only. Their final concentrations are indicated on the abscissa, except in the case where β-alanine and ACHC were added together. There it reflects the total concentration, consisting of identical contributions from each. ●, Unlabeled GABA; △, ACHC; ▽, β-alanine; and ■, ACHC and β-alanine together.

vesicles) or 370 μl (proteoliposomes). Experiments shown are typical. The values indicated in the figures represent means obtained for duplicates or triplicates. The error was usually smaller than 7%. When similar experiments were compared there was a considerable variation in absolute values between different preparations. However the relative differences within these experiments were highly reproducible, irrespective of the preparation used.

Protein determination. Protein was determined according to the method of Lowry *et al.* (20).

RESULTS

Inhibition of GABA Transport by ACHC and β-Alanine. The inhibition of sodium- and chloride-coupled GABA transport by two substrate analogues is illustrated in Fig. 1. ACHC as well as β-alanine inhibit the initial rate of GABA transport in crude synaptosomal membrane vesicles of rat brain in the lower micromolar range, up to about 50%. At higher concentrations there is a second phase of inhibition causing an additional inhibition of about 20%. This second phase is probably nonspecific. In any case, even at a concentration of 1 mM, inhibition by either of the two substrate analogues is only partial. For a comparison the potent competition of the process by unlabeled GABA is illustrated (Fig. 1). At 5 μM labeled GABA transport is 65% inhibited and at 50 μM it is fully suppressed. This is in accord with the previously published values for K_m of 2–4 μM (7, 19, 21). Although either of the two analogues gives only a partial inhibition, when both of them are added together a single phase of inhibition—essentially going to completion—is observed (Fig. 1). It should be noted that, with both analogues present, the indicated concentration is the sum of the concentrations of the two analogues (i.e., each is present at half the indicated concentration). Thus in the lower micromolar range the inhibitory effect of the two analogues is additive, as if two types of GABA transporters are present in the membrane vesicles, one sensitive to ACHC, the other to β-alanine.

When the crude synaptosomal vesicles are fractionated on a Ficoll gradient it can be seen that GABA transport in the lighter vesicles is somewhat more sensitive to β-alanine than to ACHC as compared to the crude vesicles (Table 1). It should be noted that both inhibitors were used at the relatively low level of 20 μM to ensure their specific action (Fig. 1). On the other hand, the heavier vesicles display GABA transport which is clearly more sensitive to ACHC than to β-alanine. This is paralleled by the distribution of β-[^{3}H]alanine transport (Table 1). This activity is highest in the light fraction (2–8% Ficoll interface). In the heavy fraction (12–16% Ficoll interface) it is about 15% of what it is in the crude synaptosomal membrane vesicles.

Transport of β-Alanine. The correlation between the distribution of β-alanine-sensitive GABA transport and that of β-alanine transport suggests that the two activities are manifestations of the same phenomenon. GABA transport is absolutely dependent on external sodium and chloride (6). If β-alanine transport is catalyzed by one of the subtypes of GABA transporters, it too should be absolutely dependent on these ions. This is borne out by the results presented in Fig. 2. GABA transport is electrogenic (6, 19) and the same is true for that of β-alanine. The potassium-specific ionophore valinomycin, which under the experimental conditions—an outward-directed potassium gradient—increases the interior negative membrane potential, stimulates transport (Fig. 2). This indicates that—as with GABA transport—positive charge moves in during the sodium- and chloride-dependent β-alanine influx. The ionophore nigericin, which under these conditions will effectively exchange external sodium for internal potassium, will dissipate the electrochemical sodium gradient, one of the driving forces for the uptake process. As shown in Fig. 2, a time-dependent inhibition by nigericin is observed, resulting in a virtually complete inhibition at later times.

Our hypothesis is that β-alanine transport is catalyzed by the GABA transporter sensitive to β-alanine, and ACHC interacts with another GABA transporter. This hypothesis leads us to predict that the rate of β-[^{3}H]alanine transport will be sensitive to β-alanine and GABA, but not to ACHC. The data presented in Fig. 3 indicate that this is precisely what

Table 1. Distribution of ACHC- and β-alanine-sensitive GABA transport on Ficoll gradients

Fraction	GABA transport, pmol·min^{-1} per mg protein			β-Alanine transport, pmol·min^{-1} per mg protein
	No inhibitor	ACHC	β-Alanine	
Crude membrane vesicles	86.3 ± 3.6	60.2 ± 4.2 (30%)	55.3 ± 2.4 (36%)	1.46 ± 0.05
2–8% Ficoll interface (I)	101.7 ± 4.8	78.4 ± 60.0 (23%)	64.1 ± 3.2 (37%)	1.67 ± 0.08
8–12% Ficoll interface (II)	91.3 ± 2.4	57.5 ± 1.5 (37%)	70.8 ± 3.0 (23%)	1.00 ± 0.04
12–16% Ficoll interface (III)	36.8 ± 3.2	20.0 ± 1.6 (46%)	31.6 ± 1.0 (14%)	0.22 ± 0.04

Crude membrane vesicles and the Ficoll gradient fractions were isolated and GABA and β-alanine transport rates (mean ± SD, n = 3) were determined at 60- and 30-sec time points, respectively. Inhibitors were present at final concentrations of 20 μM. Percentage of inhibition is indicated in parentheses. The following amounts of protein (μg) were used for measurement at each time: crude membrane vesicles, 130; fractions I, II, and III, 48, 60, and 48, respectively.

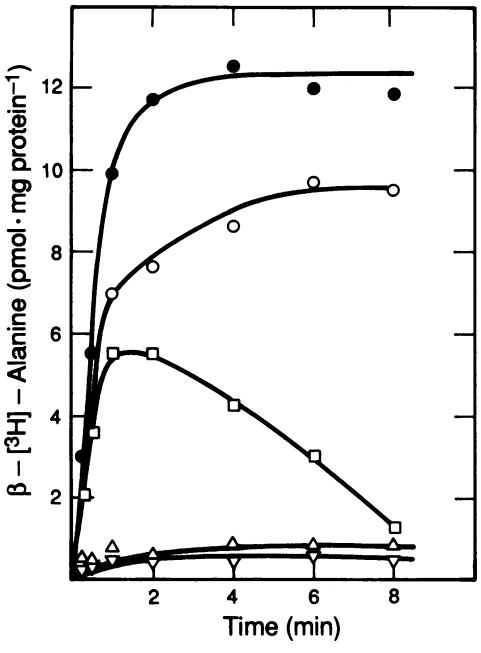

FIG. 2. Effect of external ions and ionophores on β-[^{3}H]alanine transport. Transport of β-alanine was measured for the indicated times, using crude synaptosomal membrane vesicles at 120 μg of protein per time point. In each reaction mixture 0.5 μCi of β-[^{3}H]alanine was present. The composition of the external medium was 0.15 M NaCl (○, ●, □), 0.15 M choline chloride (▽), or 0.15 M sodium isethionate (△). The ionophores tested were valinomycin (●) and nigericin (□) at final concentrations of 2.5 and 5 μM, respectively.

happens. Unlabeled β-alanine inhibits this process fully, with a half-maximal effect at about 50 μM. GABA is even more potent than β-alanine, but ACHC hardly inhibits at all. At those concentrations of ACHC where its specific inhibition of GABA transport takes place, below 100 μM (Fig. 1), β-alanine transport is only slightly inhibited (Fig. 3). The interaction of GABA and β-alanine with the β-alanine transporter—or the β-alanine-sensitive GABA transporter—is a direct one since it is competitive (Fig. 4). The K_m for

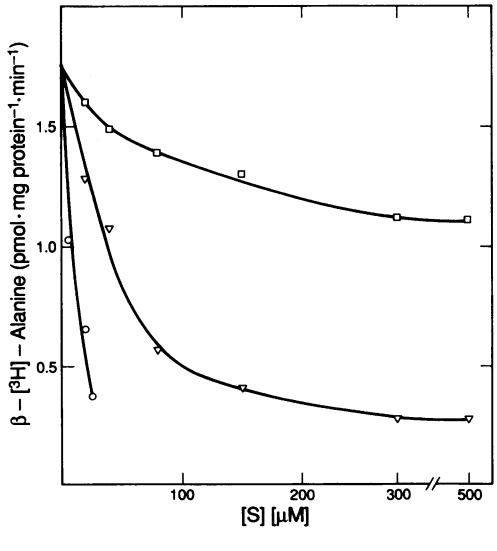

FIG. 3. Effect of substrate analogues on β-[^{3}H]alanine transport. Transport reactions proceeded for 1 min in the presence of the indicated final concentration of substrate analogues, using 120 μg of crude synaptosomal membrane vesicle protein per time point and 0.5 μCi of β-[^{3}H]alanine. The analogues added were GABA (○), ACHC (□), and β-alanine (▽).

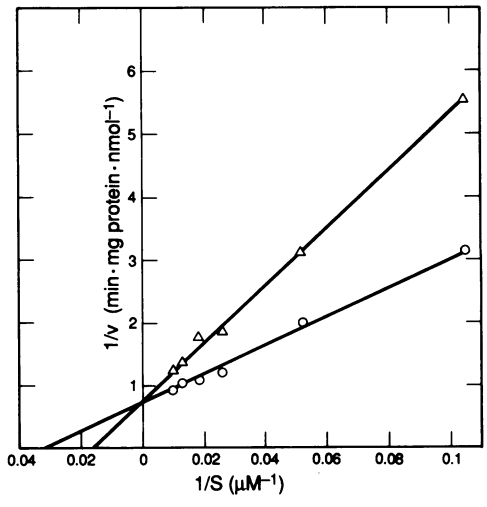

FIG. 4. Kinetics of β-[^{3}H]alanine transport in crude synaptosomal membrane vesicles. Transport was measured by using 58 μg of membrane protein per time point with 2.5 μCi of β-[^{3}H]alanine and unlabeled β-alanine between 10 and 100 μM. Triplicate 1-min measurements were taken. For some of the concentrations 30-sec measurements were taken as well to ensure the linearity of the reactions. ○, Control; △, +3 μM unlabeled GABA. In this experiment the K_m for β-alanine is 32 μM and V_{max} is 1.37 nmol·min^{-1} per mg of protein. The K_i for GABA is 3.1 μM.

β-alanine was found to be 44.3 ± 9.8 μM and V_{max} was 1.2 ± 0.25 nmol·min^{-1} per mg of protein (mean ± SD, n = 3). The K_i for GABA was found to be 3.3 ± 1.0 μM, in excellent

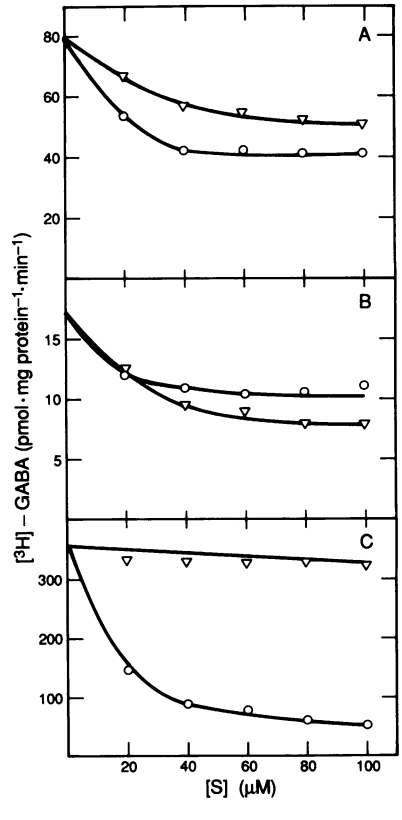

FIG. 5. Inhibitor sensitivity of GABA transport in reconstituted preparations. The cholate extract (*A*), 50 p (*B*), and 70 p$_I$ (*C*) were reconstituted and transport was measured. The amount of protein used for each assay (2 min) was 17.5 (*A*), 20 (*B*), and 7 (*C*) μg, and 1 μCi of [^{3}H]GABA was used per 360-μl influx solution, yielding a final concentration of 0.057 μM. The inhibitors ACHC (○) and β-alanine (▽) were added at the indicated concentrations.

agreement with the published values (2–4 μM) for its K_m for transport (7, 19, 21).

Inhibitor Sensitivity of GABA Transport after Solubilization and Fractionation. The above data indicate that it is possible to study the β-alanine-sensitive GABA transporter, without interference of its ACHC-sensitive counterpart, by monitoring sodium- and chloride-dependent β-alanine transport. Since radioactive ACHC is not commercially available, we chose another approach to monitor the ACHC-sensitive GABA transporter. This is to solubilize the transporters, fractionate them, and reconstitute the two subtypes. For this, GABA transport activity is solubilized from the membrane with the detergent cholate and fractionated with ammonium sulfate. The various fractions are reconstituted and sodium- and chloride-dependent GABA transport is measured in the absence and the presence of the two inhibitors ACHC and β-alanine. As shown in Fig. 5*A*, the GABA transport activity, reconstituted from the membrane proteins solubilized by cholate, is sensitive to ACHC and to a lesser extent to β-alanine. One of the key steps in the purification of the GABA transporter (19) is the observation that while most proteins solubilized by cholate are precipitated by 50% saturated ammonium sulfate (50 p), most of the GABA transport activity is not. The latter is precipitated at a higher degree of saturation of the ammonium sulfate, namely 70% (70 p_I). The latter fraction therefore shows a 4- to 5-fold-increased specific activity of GABA transport over that of the solubilized fraction. It can be seen in Fig. 5*C* that this GABA transport activity is fully inhibited by ACHC and not at all by β-alanine. The β-alanine-sensitive activity, not well resolved from the ACHC-sensitive transport, is recovered in the 50 p fraction (Fig. 5*B*). Thus, the 70 p_I fraction enables us to measure the ACHC-sensitive transporter in isolation from the β-alanine-sensitive subtype. Using this fraction, we have determined the affinity of this transporter for GABA and ACHC (Fig. 6). The apparent K_m for GABA was found to be 2.5 ± 0.2 μM (mean ± SD). This is similar to the β-alanine-sensitive subtype (Fig. 4) and in excellent agreement with published values for GABA transport (7, 9, 21). The V_{max} is 9.9 ± 3.1 nmol·min^{-1} per mg of protein (n = 3), 8- to 12-fold higher than that of the starting membranes, in accord with the purification achieved. ACHC competitively inhibits GABA transport with a K_i of 6.8 ± 1.3 (n = 3). This indicates a direct interaction of the substrate analogue ACHC with the transporter at the GABA binding site.

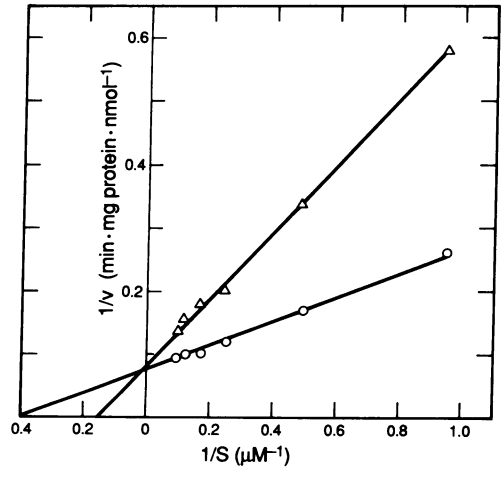

FIG. 6. Kinetics of [^{3}H]GABA transport in proteoliposomes incorporating the 70 p_I fraction. Triplicate samples were taken at 1 min to assay GABA transport in the proteoliposomes incorporating the 70 p_I fraction (9 μg of protein per assay). Each reaction mixture contained 1 μCi of [^{3}H]GABA and various concentrations of unlabeled GABA (1–10 μM). Additional measurements were taken at several time points for each concentration to ensure the linearity of the reaction. ○, Control; △, +30 μM unlabeled ACHC. In this experiment the K_m for GABA is 2.5 μM and V_{max} 13.3 nmol·min^{-1} per mg of protein. The K_i for ACHC is 7.5 μM.

DISCUSSION

The observations documented in this paper indicate the presence of two subtypes of GABA transporter. Two inhibitors, ACHC and β-alanine, inhibit sodium- and chloride-dependent GABA transport only partly—even at exceedingly high concentrations. However, when both inhibitors are added together, their effect is additive at low concentrations and a full inhibition of GABA transport is attained (Fig. 1). Both are competitive inhibitors (Figs. 4 and 6). Thus they exert their effect by interacting with the substrate binding site of their respective subtypes. We have been able to measure the transport activity of the two subtypes individually. Uptake of the β-[2,3-^{3}H]alanine serves as an indicator for the B-subtype, which is sensitive to β-alanine. This is valid for the following reasons: (*i*) cofractionation of the two activities (Table 1); (*ii*) identical mechanistic properties, including an absolute sodium and chloride dependence and electrogenicity (Fig. 2); and (*iii*) potent competitive inhibition by GABA (Figs. 3 and 4) and virtually no inhibition by ACHC (Fig. 3). The mechanistic properties of β-[2,3-^{3}H]alanine transport are in excellent agreement with those found in membrane vesicles from adult rat brain (22, 23), except for the lower K_i for GABA (3 vs. 19 μM) and the higher V_{max} (1.3 vs. 0.07 nmol·min^{-1} per mg of protein) we find (Fig. 4 and refs. 22 and 23). These differences may be due to an age difference of the rats (3 weeks vs. adult). The ACHC-sensitive subtype (A) could be resolved from subtype B upon solubilization by cholate and fractionation with ammonium sulfate (Fig. 5). As an alternative to the physical separation of the two subtypes, it can also be argued that the B subtype is more sensitive in detergent solution than its counterpart. This is certainly possible even though in the presence of the same detergent and salt the β-alanine-sensitive activity was observed to be with the bulk of the proteins (Fig. 5*B*). Whichever of the two explanations is correct, from both it follows that we are dealing here with two distinct proteins. The GABA transporter we have recently purified to virtual homogeneity (24) is derived from the 70 p_I fraction (19, 24), which exclusively exhibits A-type activity (Fig. 5). Therefore, it must be of this subtype.

In slices from tissue where GABA transport is either predominantly neuronal or glial, ACHC and β-alanine, respectively, seem to be selective inhibitors (9–11). Therefore, it is tempting to speculate that the A subtype is neuronal and the B subtype is glial. However, additional experimentation will be required to substantiate this idea. One approach could be to isolate antibodies (monoclonal or polyclonal after the purification of the B subtype) specific to the various subtypes, and to perform immunocytochemistry.

Both subtypes are very similar in their ionic requirements—both are absolutely dependent on sodium and chloride (Fig. 2 and refs. 6, 19 and 24). This indicates that in the membrane vesicle preparation we have used, the sodium-independent GABA transporter from the synaptic vesicles (25, 26) is below detectable levels. The two subtypes appear to differ only in their substrate binding site. Molecular cloning of the GABA transporter gene(s) and deduction of their protein sequences could disclose which amino acids are involved in the GABA binding sites.

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