The Substrates of a Sodium- and Chloride-Coupled γ-Aminobutyric Acid Transporter Protect Multiple Sites throughout the Protein against Proteolytic Cleavage†

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ABSTRACT: Fragments of the (Na^+ + Cl^-)-coupled $GABA_A$ transporter were produced by proteolysis of membrane vesicles and reconstituted preparations from rat brain. The former were digested with Pronase, the latter with trypsin. Fragments with different apparent molecular masses were recognized by sequence-directed antibodies raised against this transporter. When GABA was present in the digestion medium, the generation of these fragments was almost entirely blocked. At the same time, the neurotransmitter largely prevented the loss of activity caused by the protease. The effect was specific for GABA; protection was not afforded by other neurotransmitters. It was only observed when the two cosubstrates, sodium and chloride, were present on the same side of the membrane as GABA. The results indicate that the transporter may exist in two conformations. In the absence of one or more of the substrates, multiple sites located throughout the transporter are accessible to the proteases. In the presence of all three substrates—conditions favoring the formation of the translocation complex—the conformation is changed such that these sites become inaccessible to protease action.

High-affinity neurotransmitter uptake is thought to terminate the overall process of synaptic transmission (Iversen, 1971, 1973; Bennett et al., 1974). γ-Aminobutyric acid (GABA)[1] is the predominant inhibitory neurotransmitter in the mammalian brain. Using pharmacological and biochemical criteria, at least two transporter subpopulations for this neurotransmitter can be distinguished in rat brain. One—the $GABA_A$ transporter—is preferentially inhibited by ACHC and another—the $GABA_B$ transporter—by β-alanine (Kanner & Bendahan, 1990). These transporters catalyze cotransport of sodium, chloride, and GABA (Kanner, 1978; Keynan & Kanner, 1988). The $GABA_A$ transporter has been purified to near-homogeneity using a rapid reconstitution assay (Radian & Kanner, 1985; Radian et al., 1986), and is an 80-kDa glycoprotein which represents 0.1% of the membrane protein. Two apparent affinities for the neurotransmitter are exhibited by the $GABA_A$ transporter: a high-affinity site corresponding to the binding site facing the outside, and a low-affinity site facing the inside (Mabjeesh & Kanner, 1989). This transporter was the first neurotransmitter transporter to be cloned and expressed (Guastella et al., 1990). The deduced protein has 599 amino acids with a molecular weight of 67 000 (Guastella et al., 1990), which agrees with the apparent molecular mass of the deglycosylated $GABA_A$ transporter (Kanner et al., 1989). Hydropathy analysis suggests the presence of 12 putative transmembrane α-helices (Guastella et al., 1990). Cloning of this transporter led to the discovery of a neurotransmitter transporter superfamily. Many members of this superfamily have been cloned (Uhl, 1992; Schloss et al., 1992), and their number is rising. Today, four different GABA transporters have been identified (Guastella et al., 1990; Clark et al., 1992; Borden et al., 1992; Liu et al., 1993). The $GABA_A$ transporter, encoded by GAT-1, appears to be the most abundant of these four (Borden et al., 1992; Liu et al., 1993; Lopez-Corcuera et al., 1992). Although their amino acid sequences were deduced, the molecular mechanism by which transport proteins translocate solutes across the plasma membrane is not well understood.

Conformational changes of these transporters should accompany the various steps leading to the formation of the translocation complex as well as the translocation step itself. Indeed, in some cases, such changes have been demonstrated in ATPases (Jorgensen, 1975; Jackson et al., 1983) and glucose transporters (Peerce & Wright, 1984; Holman & Rees, 1987).

In this study, we have monitored the conformational changes of the (Na^+ + Cl^-)-coupled $GABA_A$ transporter associated with the formation of the translocation complex. The susceptibility of the transporter to proteases—in the presence and absence of its substrates—was monitored using sequence-directed antibodies. Interestingly, it appears that the simultaneous presence of all the three substrates (Na^+, Cl^-, and GABA) affords protection against proteolytic cleavage at multiple sites throughout the protein.

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]GABA (47.6 Ci/mmol) was obtained from the Nuclear Research Center, Negev. [^{125}I]Protein A (30 mCi/mg) was obtained from Amersham. Standard proteins for Tricine–SDS–PAGE were obtained from Pharmacia LKB Biotechnology Inc. Pronase E (protease type XXV, from *Streptomyces*

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[1] Abbreviations: GABA, γ-aminobutyric acid; GAT-1, cDNA encoding GABA transporter 1; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; P_{COOH}, peptide corresponding to residues 571–586 of the $GABA_A$ transporter, IQPSEDIVRPENGPEQ; $P_{189-206}$, peptide corresponding to residues 189–206, VVEFWERNMHQMTDGLDK; P_{NH_2}, peptide corresponding to residues 6–19, SKVADGQISTEVSE; ACHC, *cis*-3-aminocyclohexanecarboxylic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PBS, phosphate-buffered saline.

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griseus), trypsin (type XI, from bovine pancreas, DPCC-treated), trypsin inhibitor (type 1-S, from soybean), valinomycin, asolectin (soybean phospholipids, catalogue no. P. 5038), cholic acid, and Tricine were purchased from Sigma. Nigericin was purchased from Calbiochem. Affi-gel 15 was obtained from Bio-Rad. Nitrocellulose membranes (0.2 μm) were purchased from Hoefer Scientific Instruments. Tween 20 was purchased from J. T. Baker Inc. All other reagents were analytical grade. Asolectin was purified by acetone extraction (Kagawa & Racker, 1971), crude bovine brain lipids were extracted as described (Folch et al., 1957), and cholic acid was recrystallized from 70% ethanol (Kagawa & Racker, 1971).

Methods

Preparation of Crude Brain Membrane Vesicles. Membrane vesicles from rat brain were prepared as described (Kanner, 1978) except that the crude mitochondrial pellet was not fractionated by Ficoll gradient centrifugation, but was directly subjected to osmotic shock as described (Kanner, 1978). The membrane vesicles were washed and resuspended in 90 mM NaP_i, pH 7.4, 10 mM Tris–SO_4, pH 7.4, 0.5 mM EDTA, and 1 mM $MgSO_4$ at a protein concentration 15–20 mg/mL. The vesicles were frozen in aliquots at −70 °C.

Proteolytic Digestion of Membrane Vesicles. Crude membrane vesicles were rapidly thawed at 37 °C in a water bath, and loaded with loading buffer (150 mM NaCl and 10 mM Na-Hepes, pH 7.4) as described (Kanner, 1978).

Membrane vesicles (50 μL, 200–500 μg of protein) were diluted in 500 μL of loading buffer with or without 1 mM GABA, and incubated at 37 °C for 10 min. Subsequently, 1 mg of Pronase was added. After incubating at 37 °C for 2 h, the reaction was terminated by diluting the mixture with 10 mL of ice-cold loading buffer containing 1% bovine serum albumin. Zero time points were performed by adding the ice-cold stop solution prior to the Pronase. After centrifugation at 27000*g* for 15 min, the pellet was washed with 10 mL of ice-cold loading buffer, centrifuged, and resuspended in 50 μL of the same buffer. The membrane suspension either was subjected to Tricine–SDS–PAGE and immunoblotted or was solubilized, reconstituted, and assayed for ($Na^+ + Cl^-$)-coupled GABA transport (see below).

Proteolytic Digestion of Proteoliposomes. Crude membrane vesicles (15–20 mg/mL) were solubilized with 2% sodium cholate in the presence of 10% ammonium sulfate (Radian & Kanner, 1985). The mixture was centrifuged for 20 min at 12000*g*. Aliquots of the supernatant (35 μL) were mixed with phospholipids (85% asolectin and 15% bovine brain lipids) and reconstituted with 10 mM Na-Hepes, pH 7.4, and 150 mM NaCl as the internal medium as described (Radian & Kanner, 1985). Proteoliposomes (200 μL; 350–500 μg of protein) were incubated with either 20 μg or 100 μg of trypsin at 37 °C for 1 or 2 h, respectively. The reaction was stopped by adding trypsin inhibitor (5:1 trypsin inhibitor:trypsin ratio, w/w). The mixture was delipidated prior to Tricine–SDS–PAGE or re-reconstituted for transport assay (see below).

Preparation and Purification of Antibodies. Antibodies were raised in rabbits. Antisera against peptides corresponding to residues 189–206 ($P_{189-206}$, part of the loop connecting transmembrane α-helices 3 and 4; VVEFWERNMHQMTDGLDK) and to residues 571–586 (P_{COOH}, part of the carboxy terminal; IQPSEDIVRPENGPEQ) of the $GABA_A$ transporter from rat brain (Guastella et al., 1990) were a generous gift from Dr. Reinhard Jahn (Yale University Medical School, New Haven, CT). The antisera were characterized against the DEAE peak, a purified preparation of the GABA transporter (about 10% pure) (Radian et al., 1986). In all cases, only the 80-kDa band lit up in Western blots. Furthermore, in immunoblots against synaptic plasma membranes, the 80-kDa band was a major band, although others were visualized. However, only in the case of the 80-kDa band was the immunoreactivity abolished by the peptides used to raise the antibodies (data not shown).

Prior to use for the experiments described in this paper, all antibodies were affinity-purified by binding to an Affi-gel 15 column carrying the partially purified transporter as described (Radian et al., 1990).

GABA Transport. Pronase-treated or control (incubated without the protease) membranes (80 μL, 2 mg/mL) were solubilized with 2% sodium cholate in the presence of ammonium sulfate as described (Radian & Kanner, 1985). Aliquots of the solubilized membranes (35 μL) were reconstituted into liposomes (85% asolectin and 15% brain lipids) suspended in K-medium (120 mM KP_i, pH 6.8, 1% glycerol, 10 mM Tris–SO_4, pH 7.4, 0.5 mM EDTA, and 1 mM $MgSO_4$). Trypsin-treated or control proteoliposomes were reconstituted again as follows: to aliquots of proteoliposomes (200 μL) were added 1.0% sodium cholate and 350 mM NaCl (final concentration) and incubated for 10 min on ice. Subsequently, the mixture was applied onto a dried Sephadex minicolumn equilibrated with K-medium, as described (Radian & Kanner, 1985).

The proteoliposomes (20 μL) were assayed for ($Na^+ + Cl^-$)-coupled GABA transport (Radian & Kanner, 1985; Radian et al., 1986).

Electrophoresis and Immunoblotting. Membrane suspensions were solubilized by adding an equal volume of 4% SDS, 20% glycerol, and 125 mM Tris-HCl, pH 6.8, and boiled for 5 min. Proteoliposomes were delipidated as described (Barzilai et al., 1987). The delipidated proteins were dissolved in 2% SDS, 10% glycerol, and 62.5 mM Tris-HCl, pH 6.8, and boiled for 5 min. In order to obtain a resolution of small proteins as well as large ones, the samples were analyzed by discontinuous one-dimensional Tricine–SDS–PAGE (4% stacking gel and 10% separating gel) using the method of Schagger and Von Jagow (1987). Standard proteins for SDS–PAGE were as follows: phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa), and myoglobin residues 1–153 (17.2 kDa), residues 1–131 (14.6 kDa), residues 56–131 (8.24 kDa), residues 1–55 (6.38 kDa), and residues 132–153 (2.56 kDa).

The proteins from the gel were transferred to 0.2-μm nitrocellulose paper using the method of Towbin et al. (1979). The nitrocellulose paper was washed and incubated with the diluted affinity-purified antibodies in PBS containing 3% bovine serum albumin. After washing, incubation with [^{125}I]-protein A, and further washing (all in PBS containing 0.2% Tween 20), the radioactivity was visualized by autoradiography. Densitometry of the films was done using a Quick Scan R & D (Helena Laboratories).

Protein Determination. Protein determination was performed using the method of Bradford (1976).

RESULTS

Protection of the GABA Transporter against Proteolysis. Membrane vesicles from rat brain were incubated with Pronase for various times. After stopping the reaction and washing, the membrane proteins were separated on Tricine–SDS–PAGE and transferred to nitrocellulose. The transporter

peptides were visualized with anti-P_{COOH}, a polyclonal antibody directed against a peptide located in the carboxy-terminal part of the (Na^+ + Cl^-)-coupled $GABA_A$ transporter. The 80-kDa band of the $GABA_A$ transporter (Radian et al., 1986) was the only immunoreactive species observed in the absence of protease (Figure 1A, lanes 1 and 2). The intensity of this band decreased with Pronase incubation (Figure 1A, lanes 3–7). The major immunoreactive degradation product generated is a species with an apparent molecular mass of about 10 kDa. In addition, small amounts of 50- and 30-kDa bands are generated (Figure 1A, lanes 4–7). However, these species do not appear to be precursors of the 10-kDa band. Strikingly, when proteolysis is carried out in the presence of 1 mM GABA, the formation of the immunoreactive 10-kDa band is largely prevented (Figure 1A, lanes 9–12). This protection was also observed for the 50- and 30-kDa bands, albeit to a lesser extent. In a control experiment, we established that the Pronase activity (Narahashi, 1970) is not affected by GABA and NaCl (data not shown).

In order to quantify the phenomenon, the autoradiographs were analyzed by densitometry at the linear part of their exposure (Figure 1B). In this particular experiment, the ratio of the intensities of the 10- and 80-kDa bands from the same lane (I_{10}/I_{80}) were 0.45, 0.9, and 1.58 upon Pronase treatment for 30, 60, and 120 min, respectively. The corresponding values in the presence of 1 mM GABA were 0.02, 0.06, and 0.37. Since the accuracy of the measurement was much greater at longer time intervals, subsequent experiments used 2 h of incubation with Pronase. It is of interest to note that the sum of the intensities of the bands from the proteolyzed preparation exceeds that of the control. Possible reasons for this phenomenon are more efficient transfer and better recognition by the antibody of the small fragments.

In parallel, we examined whether or not the apparent protection at the structural level was also manifested functionally. We used reconstitution to express all the potentially active transporters in the membrane vesicle population after incubation with Pronase. The vesicles were solubilized with cholate and immediately reconstituted using a mixture of asolectin and brain lipids (Radian & Kanner, 1985), and an internal medium containing potassium phosphate. The reconstituted proteoliposomes were diluted into the transport medium containing sodium chloride, [^{3}H]GABA, and the potassium-selective ionophore valinomycin. These conditions (inwardly directed sodium and chloride ion gradients and an internal negative membrane potential) generated the maximal driving force for GABA accumulation. It can be seen in Figure 1C that digestion with Pronase caused a 65% inhibition of GABA transport. When 1 mM GABA was added together with Pronase, this inhibition was almost completely prevented (Figure 1C). There is an apparent delay in the formation of the 10-kDa band, relative to the disappearance of reconstitutable transport activity or the broad 80-kDa band (see Discussion).

The digestion of membrane vesicles with Pronase revealed a slight degradation (about 25%) of the 80-kDa band (Figure 1A). The digestion pattern did not change when incubation was extended beyond 2 h or when the amount of Pronase was increased. No digestion at all was observed with proteases cleaving at defined sites, such as trypsin (data not shown). A possible explanation could be the presence of entrapped membranes protecting a large fraction of the transporter against proteolysis. In order to expose all transporter molecules to the protease, we used reconstitution. Membrane vesicles were solubilized and reconstituted (see Experimental Procedures). Proteoliposomes were subjected to tryptic digestion

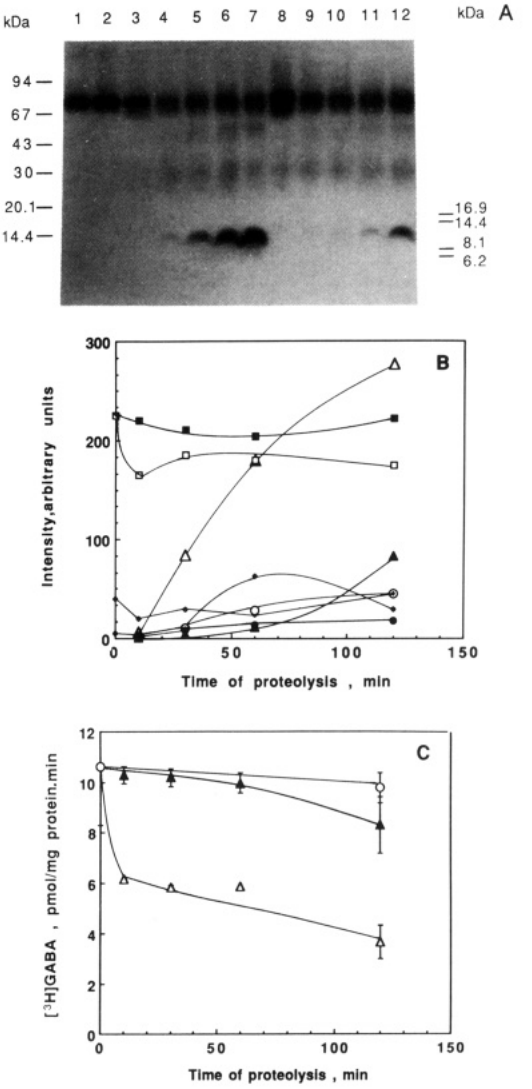

FIGURE 1: Immunoreactivity and GABA transport of the Pronase-treated membrane vesicles. Membrane vesicles were loaded with 150 mM NaCl/10 mM Na-Hepes, pH 7.4, and digested with Pronase for the indicated times (Experimental Procedures). (A) Lanes 1 and 2 represent membrane vesicles analyzed in the absence of Pronase immediately or after 2-h incubation at 37 °C, respectively. Lanes 3–7 and 8–12 represent Pronase-treated membranes in the absence or presence of 1 mM GABA in the digestion medium, respectively. The proteolysis was stopped at (minutes) 0 (lanes 3 and 8), 10 (lanes 4 and 9), 30 (lanes 5 and 10), 60 (lanes 6 and 11), and 120 (lanes 7 and 12). The membranes were analyzed by Tricine–SDS–PAGE and immunoblotted with the diluted (1:100) affinity-purified anti-P_{COOH} antibody. Each lane was loaded with 100 μg of membrane protein. (B) The autoradiogram presented in (A) was analyzed and quantitated by densitometry. The intensities of the 80-kDa band (□, ■), 50-kDa band (○, ●), 30-kDa band (◇, ◆), and 10-kDa band (△, ▲) were plotted as a function of the time of proteolysis in the absence (open symbols) or presence (closed symbols) of 1 mM GABA. (C) The proteolyzed membranes in the absence (△) or presence of 1 mM GABA (▲) were solubilized and reconstituted into liposomes for [^{3}H]GABA uptake. Control membranes were processed without Pronase (○) and reconstituted as well. The external medium (360 μL) contained 150 mM NaCl, 1% glycerol (v/v), 2 mM $MgSO_4$, 2.5 μM valinomycin, and 1 μCi of [^{3}H]GABA. The influx of [^{3}H]GABA into proteoliposomes was carried out in duplicate for 3 min. Twenty microliters of liposomes (12 μg of protein) was used for each time point.

(Figure 2). After stopping the reaction and delipidation, the proteolytic products were analyzed by immunoblotting using the anti-P_{COOH} antibody (Figure 2A). Two bands with apparent molecular masses of 37 and 25 kDa were recognized by the antibody when proteolysis was performed with low

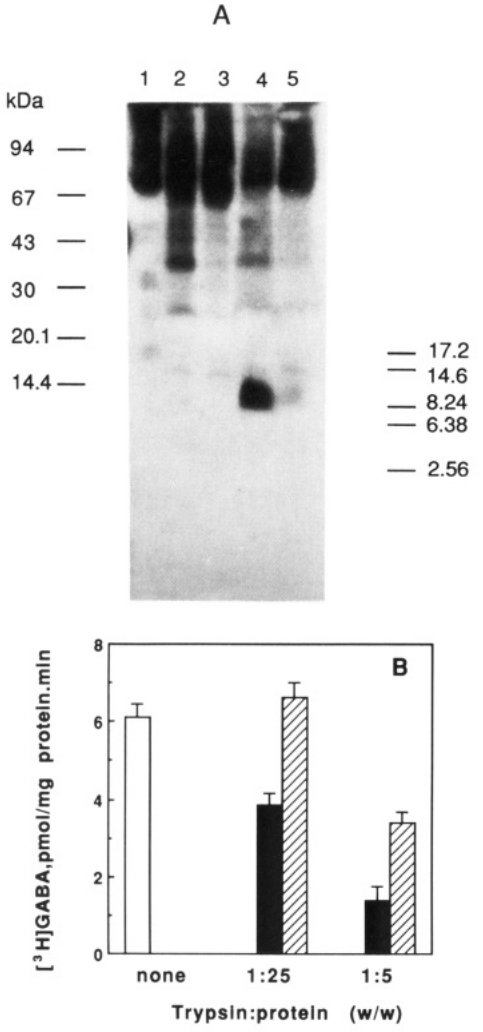

FIGURE 2: Immunoreactivity and GABA transport of trypsin-treated proteoliposomes. Proteoliposomes were digested with trypsin (Experimental Procedures). (A) The proteoliposomes were delipidated, analyzed by Tricine–SDS–PAGE, and immunoblotted with the anti-P_{COOH} antibody. Low (1:25 trypsin:protein ratio, w/w; lanes 2 and 3) and high trypsin (1:5 trypsin:protein ratio, w/w; lanes 4 and 5) were used. GABA (1 mM) was added to the digestion medium in lanes 3 and 5. (B) The digested proteoliposomes were re-reconstituted, and [^{3}H]GABA transport was carried out in triplicate for 3 min. Open bars, proteoliposomes processed without trypsin; closed bars, digestion in the absence of 1 mM GABA; hatched bars, digestion in the presence of 1 mM GABA.

trypsin levels (1:25 trypsin:protein ratio, w/w) (Figure 2A, lane 2). Treatment with high trypsin levels (1:5 trypsin:protein ratio, w/w) gave rise to a highly immunoreactive 10-kDa band (Figure 2A, lane 4). When GABA was present during proteolysis, the generation of these fragments was significantly reduced, while higher levels of the 80-kDa band were observed (Figure 2A, lanes 3 and 5). Trypsin-treated proteoliposomes were re-reconstituted such that they contained an internal potassium phosphate medium. They were subsequently assayed for their ability to transport [^{3}H]GABA (Figure 2B). It can be seen that the protection by GABA against tryptic digestion is manifested on the functional level as well.

Characterization of Pronase and Tryptic Fragments. It is necessary to verify that all bands visualized are digestion products of the intact transporter. This was established by competition with homologous peptides (peptides which were used to produce the antibody). Figures 3A,B shows that the immunoreactivity of the Pronase and tryptic fragments (lane 2 of panels A and B) was completely abolished when a 10 μM aliquot of the homologous peptide, P_{COOH}, was incubated

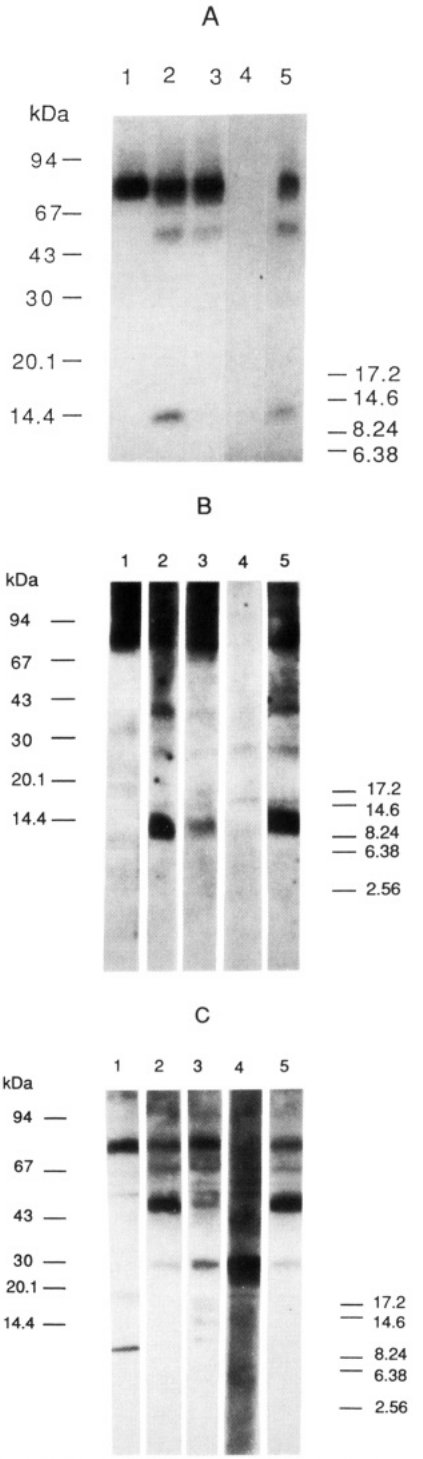

FIGURE 3: Specificity and protection of proteolytic fragments of the GABA transporter. Membrane vesicles were treated with Pronase, analyzed by Tricine–SDS–PAGE, and immunoblotted with the anti-P_{COOH} antibody (A). Proteoliposomes were digested with trypsin (1:6 trypsin:protein ratio, w/w), delipidated, analyzed by Tricine–SDS–PAGE, and immunoblotted with the anti–P_{COOH} antibody (B) or the anti-$P_{189-206}$ antibody (C). Digested preparations are illustrated in lanes 2–5 with the following additions: none (lane 2); 1 mM GABA (lane 3); 10 μM homologous peptide—P_{COOH} in (A) and (B) and $P_{189-206}$ in (C) (lane 4)—and 10 μM heterologous peptide—P_{NH_2} in (A) and (B) and P_{COOH} in (C) (lane 5). Control preparations are shown in lane 1.

together with the antibody (lane 4, panels A and B). As a control, a 10 μM sample of the heterologous peptide, P_{NH_2}, was used (lane 5, panels A and B). Another sequence-directed antibody, anti-$P_{189-206}$, was used to examine the generation of other tryptic fragments, not necessarily containing the carboxy

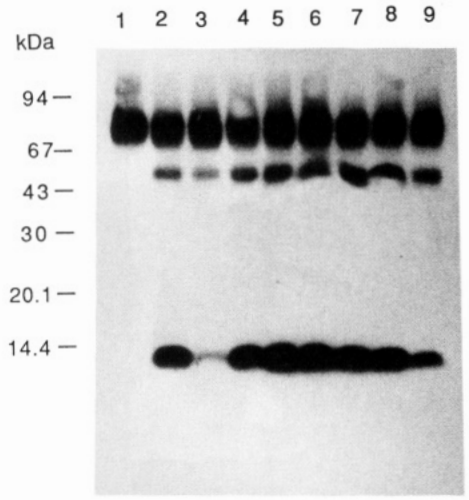

FIGURE 4: Specificity of protection of the GABA transporter against proteolysis. Membrane vesicles were digested with Pronase. The membranes were analyzed by Tricine–SDS–PAGE and immunoblotted with the anti-P_{COOH} antibody. The digestion was carried out in the presence of 1 mM aliquots of the following: none (lane 2), GABA (lane 3), glycine (lane 4), L-glutamate (lane 5), norepinephrine (lane 6), taurine (lane 7), β-alanine (lane 8), and ACHC (lane 9). Control (undigested) membrane vesicles are presented in lane 1.

terminal of the transporter. The anti-$P_{189-206}$ antibody recognized the intact transporter in untreated proteoliposomes (Figure 3C, lane 1). Upon trypsin treatment, the antibody recognized two immunoreactive species of bands with apparent molecular masses of 50 and 30 kDa (Figure 3C, lane 2). Strikingly, the formation of the 50-kDa band was completely blocked when proteolysis was carried out in the presence of GABA (Figure 3C, lane 3). This was not true for the 30-kDa band (Figure 3C, lane 3). Furthermore, the immunoreactivity of the 30-kDa band was not abolished in the presence of 10 μM homologous peptide, $P_{189-206}$. On the other hand, it completely abolished the immunoreactivity of the 50-kDa band as well as the residual undigested transporter (Figure 3C, lane 4). The heterologous peptide, P_{COOH}, fails to inhibit the immunoreactivity of the 50-kDa band recognized by anti-$P_{189-206}$ (Figure 3C, lane 5). Hence, the 50-kDa fragment is a tryptic product of the intact transporter, and its formation is prevented by GABA. It is noteworthy that the 50-kDa band is not recognized by the anti-P_{COOH} antibody upon tryptic digestion.

Characteristics of Protection. The specificity of the protective effect of GABA is shown in Figure 4. The neurotransmitters glycine, L-glutamate, and norepinephrine and also the analogue taurine failed to protect against Pronase digestion (lanes 4–7). Moreover, ACHC, a specific substrate of the $GABA_A$ transporter (Kanner & Bendahan, 1990), did protect (Figure 3, lane 9), but β-alanine, which interacts only weakly with the $GABA_A$ transporter, did not (Figure 3, lane 8). The specificity for GABA protection was also observed in proteoliposomes treatedwith trypsin (data not shown).

The GABA transporter exhibits a high-affinity site toward the outside of the cell (K_m = 2 μM), and a 50–100-fold lower affinity site facing the cytosol (Mabjeesh and Kanner, 1989). In order to examine if the protection against proteolysis afforded by GABA agrees with one of these sites, the concentration dependence of its protection was measured using the anti-P_{COOH} antibody (Figure 5). At increasing concentrations, GABA became more effective (Figure 5A). The half-maximal effect was observed at about 100–200 μM (Figure 5B), in good agreement with the affinity of the transport site facing the cytosol.

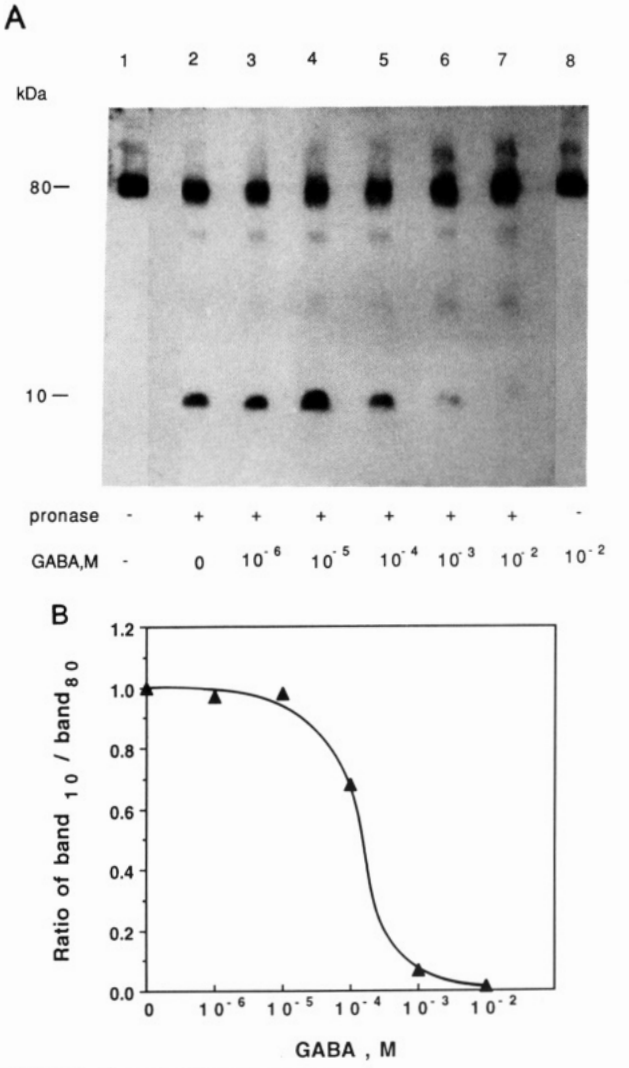

FIGURE 5: Concentration dependence of the protection by GABA. (A) The digestion of membrane vesicles by Pronase was performed in the presence of increasing GABA concentrations (0–10 mM) as indicated in lanes 2–7. Control membranes without (lane 1) or with 10 mM GABA (lane 8) are presented. The blots were analyzed with the anti-P_{COOH} antibody. (B) The ratio of the intensities of the 10-kDa band and 80-kDa band is plotted as a function of GABA concentration. This ratio was calculated from densitometric values measured in the linear range.

GABA is cotransported with sodium and chloride (Kanner, 1978; Keynan & Kanner, 1988). The effect of the presence of the cosubstrates on the ability of GABA to protect is illustrated in Figure 6. It can be seen that only in the simultaneous presence of sodium and chloride is the effect observed (Figure 6A). In the absence of either sodium (Figure 6B) or chloride (Figure 6C), GABA was completely ineffective in protecting the transporter against proteolysis by Pronase. Similarly, protection of the transporter against proteolysis by trypsin was also completely dependent on the simultaneous presence of sodium and chloride (data not shown).

Internal GABA per se Is Not Required for Protection. One way to explain the above data would be that internal GABA per se is sufficient to elicit the protection. The sodium and chloride dependence of the effect could be explained by the requirement of these two ions to translocate the neurotransmitter to the inside via the transporter. Vesicles were loaded with GABA by including it during the osmotic shock of synaptosomes, a critical step in their preparation (Kanner, 1978). We believe these vesicles indeed contained the neurotransmitter because they demonstrated counterflow

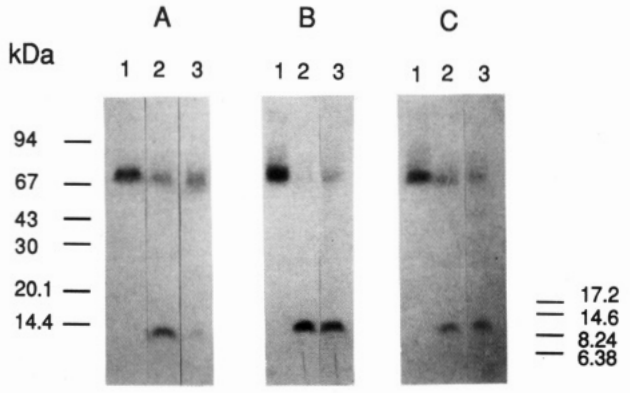

FIGURE 6: Effect of the ion composition on protection. Membrane vesicles were loaded with 150 mM NaCl/10 mM Na-Hepes, pH 7.4 (A), 150 mM choline chloride/10 mM Tris-HCl, pH 7.4 (B), or 100 mM NaP_i, pH 7.4 (C). The membranes were proteolyzed with Pronase in the absence (lane 2) or presence (lane 3) of 1 mM GABA. Lane 1 shows control membrane vesicles processed without protease. After the proteolysis was terminated, the membranes were analyzed by Tricine–SDS–PAGE and immunoblotted with the anti-P_{COOH} antibody.

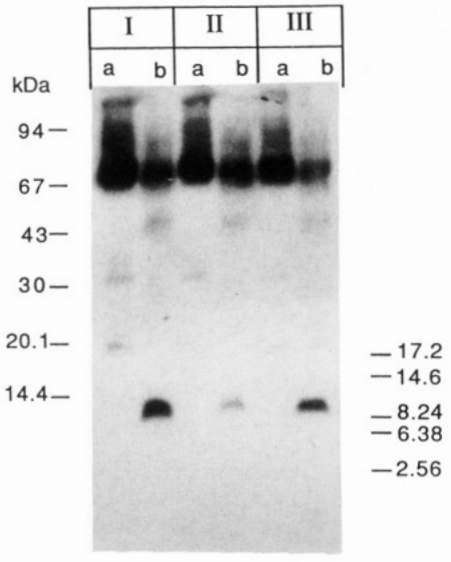

FIGURE 7: Effect of internal GABA on the Pronase-treated GABA transporter. Membrane vesicles were loaded by including 10 mM GABA during the osmotic shock (Kanner, 1978). After being washed with 135 mM KCl, 10 mM KP_i, pH 6.9, and 100 mM GABA, the membranes were resuspended in the same buffer at a protein concentration of 16 mg/mL and frozen in small aliquots at –70 °C. Before use, membranes (0.4 mL) were thawed rapidly at 37 °C and washed 3 times with 12 mL of 135 mM KCl/10 mM KP_i, pH 6.9, and then resuspended in the same washing buffer. Vesicles containing GABA were diluted in 150 mM NaCl/10 mM Na-Hepes, pH 7.4 (I), or in the same plus 1 mM GABA (II) or 5 μM nigericin (III). They were incubated with (b lanes) or without (a lanes) Pronase and immunoblotted with the anti-P_{COOH} antibody.

(Mabjeesh and Kanner, unpublished data), the ability of the transporter to take up substrate by means of an exchange mechanism under conditions where net flux is not operative [e.g., see Pines and Kanner (1990)]. To prevent efflux of internal GABA, vesicles containing KCl (rather than NaCl) with and without GABA were used to determine if the presence of internal GABA would be sufficient to protect the transporter against cleavage by Pronase. It is very likely that under these conditions, internal GABA concentrations are high enough to saturate the internal transport site. As can be seen in Figure 7, where an NaCl-containing external medium was used, the characteristic 10-kDa band was generated by Pronase (lane Ib), unless GABA was added from the outside (lane IIb). Nigericin is an ionophore which under the conditions used here will exchange the internal potassium with external sodium. Since chloride and GABA were already present on the inside, the sudden rise of the internal sodium concentration enables the formation of the translocation complex from the inside. This is promoted by the ionophore, and under these conditions, a partial protection was observed (lane IIIb). Thus, protection against proteolysis is observed when a translocation complex can be formed from either side of the membrane.

DISCUSSION

The data presented in this paper document that when membrane preparations from rat brain are digested with proteases, the (Na^+ + Cl^-)-coupled $GABA_A$ transporter is cleaved into several fragments (Figure 3). The transportable substrate, GABA, protects against this cleavage at multiple sites (Figures 1, 2, and 3). This protection is dependent on the presence of both cosubstrates, sodium and chloride (Figure 6). The protection was afforded only by GABA and to some extent also by ACHC (Figure 4). The latter is a selective substrate of the $GABA_A$ transporter which has a lower affinity than GABA (Kanner & Bendahan, 1990).

Other neurotransmitters and analogues, including β-alanine, the selective substrate of the $GABA_B$ transporter, were ineffective (Figure 4). The above-mentioned protection is manifested on the functional level as well (Figure 1C and Figure 2B).

Digestion of membrane vesicles by the nonselective protease Pronase removes around 25% of the $GABA_A$ transporter (Figure 1A,B), while the decrease in GABA transport activity was about 60% of the control (Figure 1C). Moreover, the formation of the major 10-kDa band is slower than the loss of transport activity. Therefore, it appears that there are cleavage events which impair transport, but do not give rise to the generation of the 10-kDa band containing the P_{COOH} epitope. This loss of transport activity could be due to the cleavage of transporter molecules residing in inside-out vesicles. Since the P_{COOH} epitope is predicted to be located in the cytoplasm (Guastella et al., 1990), it should face the outside of these vesicles and be destroyed by the Pronase. The 10-kDa fragment containing the epitope is expected to result from cleavage of right-side-out vesicles as the proteases employed are membrane-impermeant (Capasso et al., 1992; Johnston et al., 1989). It is not unreasonable to assume that the kinetics of these two processes are different.

The GABA transporter present in membrane vesicles was completely resistant against trypsin, and only a small fraction of it was digested by Pronase (Figure 1). We wanted to find out if the protection afforded by GABA was due to a small subpopulation of transporters, or if it is associated with conformational changes of the majority of transporters. Upon solubilization and reconstitution, the majority of the transporters became sensitive to cleavage by trypsin (Figure 2). Possibly, in the synaptic plasma membrane, part of the transporters are located in small vesicles entrapped in larger ones or else could be stabilized by association with other proteins (e.g., structural elements involved in the maintenance of the synaptic structure). In any case, it is clear that the protection by GABA is a phenomenon intrinsic to most, if not all, of the transporter molecules.

It is well-known that hydrophobic proteins run on SDS–PAGE with different mobilities than those of standard proteins of the same size (Tagaki, 1991). The hydrophobic nature of the fragments (containing transmembrane α-helices) and the fact that the proposed model (Guastella et al., 1990) has not

been proven experimentally yet do not allow us to deduce the location of the cleavage sites. This will require isolation of the fragments and determination of their sequence.

While external GABA protects in the presence of sodium and chloride, it appears that internal GABA alone does not protect (Figure 7). Only when sodium and chloride are present on the inside does internal GABA protect (Figure 7, panel III). This suggests that it is the formation of the translocation complex which induces a conformational change, causing the transporter to be relatively resistant to digestion by proteases.

The concentration of GABA where a half-maximal effect is observed corresponds to the low-affinity transport site—that facing the cytosol. This can be explained by assuming that the translocation complex needs to be maintained throughout the protease treatment. If not, the transporter will be digested. If the translocation complex is formed from the outside, GABA can be released, after translocation, on the inside. Only at concentrations high enough to saturate the low-affinity site will the translocation complex be stable.

The use of sequence-directed antibodies to monitor epitopes of transporter molecules involved in conformational changes accompanying the transport process is likely to be of general applicability. Indeed, in parallel with this work, a similar approach has been applied on the glucose transporter which invokes a substrate-induced conformational change of the loop connecting helices 6 and 7 (Asano et al., 1992). It should be considered that the phenomenon observed here may be a general one occurring in a wide variety of cotransporters.

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REFERENCES

Asano, T., Katagiri, H., Tsukuda, K., Lin, J.-L., Ishihara, H., Inukai, K., Yazaki, Y., & Oka, Y. (1992) *FEBS Lett. 298*, 129–132.

Barzilai, A., Spanier, R., & Rahamimoff, H. (1987) *J. Biol. Chem. 262*, 10315–10320.

Bennett, J. P., Jr., Mulder, A. H., & Snyder, S. M. (1974), *Life Sci. 15*, 1046–1056.

Borden, L. A., Smith, K. E., Hartig, P. R., Branchek, T. A., & Weinshank, R. L. (1992) *J. Biol. Chem. 267*, 21098–21104.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Capasso, J. M., Hoving, S., Tal, D. M., Goldshleger, R., & Karlish, S. J. D. (1992) *J. Biol. Chem. 267*, 1150–1158.

Clark, J. A., Deutch, A. Y., Gallipoli, P. Z., & Amara, S. G. (1992) *Neuron 7*, 337–348.

Folch, J., Lees, M., & Sloane, G. H. (1957) *J. Biol. Chem. 226*, 497–509.

Guastella, J., Nelson, N., Nelson, H., Czyzyk, L., Keynan, S., Miedel, M. C., Davidson, N., Lester, H. A., & Kanner, B. I. (1990) *Science 249*, 1303–1306.

Holman, G. D., & Rees, W. D. (1987) *Biochim. Biophys. Acta 897*, 395–405.

Iversen, L. L. (1971) *Br. J. Pharmacol. 41*, 571–591.

Iversen, L. L. (1973) *Br. Med. Bull. 29*, 130–135.

Jackson, R. J., Mendlein, J., & Sachs, G. (1983) *Biochim. Biophys. Acta 731*, 7–15.

Johnston, P. A., Jahn, R., & Sudhof, T. C. (1989) *J. Biol. Chem. 264*, 1268–1273.

Jorgensen, P. L. (1975) *Biochim. Biophys. Acta 401*, 399–415.

Kagawa, Y. & Racker, E. (1971) *J. Biol. Chem. 246*, 5477–5487.

Kanner, B. I. (1978) *Biochemistry 17*, 1207–1211.

Kanner, B. I., & Bendahan, A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2550–2554.

Kanner, B. I., Keynan, S., & Radian, R. (1989) *Biochemistry 28*, 3722–3727.

Keynan, S., & Kanner, B. I. (1988) *Biochemistry 27*, 12–17.

Liu, Q.-R., Lopez-Corcuera, B., Mandiyan, S., Nelson, H., and Nelson, N. (1993) *J. Biol. Chem. 268*, 2106–2112.

Lopez-Corcuera, B., Liu, Q.-R., Mandiyan, S., Nelson, H., & Nelson, N. (1992) *J. Biol. Chem. 267*, 17491–17493.

Mabjeesh, N. J., & Kanner, B. I. (1989) *Biochemistry 28*, 7694–7699.

Narahashi, Y. (1970) *Methods Enzymol. 19*, 651–664.

Peerce, B. E., & Wright, E. M. (1984) *J. Biol. Chem. 259*, 14105–14112.

Pines, G., & Kanner, B. I. (1990) *Biochemistry 29*, 11209–11214.

Radian, R., & Kanner, B. I. (1985) *J. Biol. Chem. 260*, 11859–11865.

Radian, R., Bendahan, A., & Kanner, B. I. (1986) *J. Biol. Chem. 261*, 15437–15441.

Radian, R., Ottersen, O. P., Storm-Mathisen, J., Castel, M., & Kanner, B. I. (1990) *J. Neurosci. 10*, 1319–1330.

Schagger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368–379.

Takagi, T. (1991) *Adv. Electrophor. 4*, 391–406.

Towbin, H., Stahelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

Schloss, P., Mayser, W., & Betz, H. (1992) *FEBS Lett. 307*, 76–80.

Uhl, G. R. (1992) *Trends Neurosci. 15*, 265–268.