Immunological Approach to the Detection of Taurine and Immunocytochemical Results

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Abstract: An immunological approach to the detection of taurine resulted in antibodies specific enough to be used for immunocytochemical studies. The experimental conditions were similar to those previously described for raising antibodies against some small-sized neurotransmitter molecules: antisera were obtained from rabbits immunized with taurine conjugated to carrier proteins via glutaraldehyde and purified by adsorption on the glutaraldehyde-treated protein carriers. Antibody affinity and specificity were determined in competition experiments between conjugated taurine and other conjugated amino acids or derivatives by enzyme-linked immunosorbent assay. The resulting cross-reactivity ratios, calculated at half-displacement, showed conjugated taurine to be the best recognized compound. Given the molecular struc-

ture of taurine and the method used to prepare the conjugate, it seemed necessary to perform an oxidation step. However, adsorption of antisera on reoxidized or non-reoxidized taurine conjugates suggested that reoxidation did not make a significant difference. Immunocytochemical application of the sera revealed populations of strongly immunopositive nerve cells in the cerebellum, striatum, and septum. The results confirmed that anti-*taurine* antibodies can be used as specific tools for a better understanding of the role of taurine in the central nervous system. **Key Words:** Taurine—Hypotaurine—Antibodies—Specificity—Affinity—Immunocytochemistry. **Campistron G. et al.** Immunological approach to the detection of taurine and immunocytochemical results. *J. Neurochem.* 46, 862–868 (1986).

According to several publications (Curtis and Watkins, 1960; Jasper and Koyama, 1969; Guidotti et al., 1972), taurine (2-aminoethanesulfonic acid) may play a role in neuronal activity, acting as an inhibitory neurotransmitter released from nerve terminals (Bradford et al., 1976; Greuner et al., 1976; Mandel and Pasantes-Morales, 1978), or as a membrane stabilizer (Barbeau and Donaldson, 1974; Greuner et al., 1976). In mammalian nervous tissues, taurine has a number of biosynthetic routes: the major two involve the chemical transformation of methionine to cysteine, the subsequent formation of cysteic acid or cysteine-sulfinic acid, and their conversion into taurine. The decarboxylation of either cysteine-sulfinic acid or cysteic acid into hypotaurine or taurine (Jacobsen and Smith, 1968) is catalyzed by a single enzyme, the cysteine sulfinic acid decarboxylase (EC 4.1.1.29). Responsible for taurine synthesis in the brain, this protein is distinct

from L-glutamic acid decarboxylase (GAD; EC 4.1.1.15) which allows the synthesis of γ -aminobutyric acid (GABA). Antibodies raised against each enzyme show no cross-reactivity (Wu, 1982). Until now, putative taurine-containing cells have been visualized in the CNS by use of antibodies directed against cysteine sulfinic acid decarboxylase (Chan-Palay et al., 1982a,b).

Some previous work has been carried out to visualize amino acids and derivatives (Storm-Mathisen et al., 1983). The chemical manipulation of some neurotransmitter-like molecules, belonging to the indolealkylamine, catecholamine, and amino acid families, enabled us to raise antisera specific enough to be used for immunocytochemical studies (Geffard et al., 1984, 1985a,b; Seguela et al., 1984). The immunological approach to taurine was carried out under the same experimental conditions. The present report relates the raising and characteriza-

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Abbreviations used: β -Ala, β -alanine; BSA, bovine serum albumin; Cys, cysteine; ELISA, enzyme-linked immunosorbent assay; G, glutaraldehyde; GABA, γ -aminobutyric acid; Glu, glutamate; Gly, glycine; HSA, human serum albumin; OPD, *o*-phenylenediamine; PL, poly-L-lysine; TH, thyroglobulin.

tion of anti-aurine antibodies as well as their immunocytochemical applications to a molecular detection of taurine in rat brain.

MATERIALS AND METHODS

Reagents

Glutaraldehyde (25% in aqueous solution), hydrogen peroxide (30% in aqueous solution), aspartate (Asp), glutamate (Glu), and glycine (Gly) were obtained from Merck. GABA, cysteine (Cys), β -alanine (β -Ala), hypotaurine, taurine, bovine serum albumin (BSA), poly-L-lysine (PL; MW 60,000), porcine thyroglobulin (TH), and *o*-phenylenediamine (OPD) were from Sigma. Human serum albumin (HSA) came from the Centre de Transfusion Sanguine of Bordeaux.

Synthesis of the immunogens

Five milligrams of taurine were dissolved in 1 ml of a 1.5 M sodium acetate solution, pH 8. The pH was adjusted to 7.6. Two microliters of [3 H]taurine (sp act, 20–40 Ci/mmol, New England Nuclear) were added. Five hundred microliters of a 0.5 M glutaraldehyde solution and 1 ml of 15 mg BSA, or HSA, or PL or TH dissolved in a 1.5 M sodium acetate solution were rapidly and successively added. The reaction time lasted 3 min at room temperature. Glutaraldehyde was chosen for its high yield of coupling with the amino group residues. A yellow color indicated the coupling. Then, 1 ml of a 0.1 M sodium borohydride solution (Fluka) was added to saturate the double bonds. The mixture became nearly colorless, indicating that the saturation was completed. The reduction step could partly provoke the transformation of the sulfoxide group of taurine into a sulfhydryl group. Therefore, the conjugated taurine was reoxidized. After 1 day of dialysis at 4°C, the pH of each taurine-G-polypeptide solution was adjusted to 5 with a 0.1 M acetic acid solution. Then, 300 μ l of a 30% hydrogen peroxide solution was added. The mixture was allowed to stand for 16 h at 4°C and redialyzed against distilled water for 1 day at 4°C. Insoluble material was removed by centrifugation at 10,000 g for 15 min. β -Radioactivity was determined on a 100- μ l aliquot, both before and after dialysis to calculate the concentration of conjugated taurine. After weighing the lyophilized immunogen from 1 ml of the solution, the molar coupling ratios were calculated. Depending on the carrier chosen, they varied as follows: taurine-G-BSA, 70; taurine-G-HSA, 65; taurine-G-PL, 150; and taurine-G-TH, 350.

Synthesis of the amino acid and derivative conjugates

GABA, Asp, Glu, Gly, Cys, β -Ala, and hypotaurine (Fig. 1) were conjugated via glutaraldehyde to PL under the same experimental conditions as those used for conjugated taurine. The use of tritiated GABA, Asp, Glu, Gly, Cys, Tau, and β -Ala allowed to calculate the concentration of the conjugates after purification. All these conjugates were reduced by a sodium borohydride solution. A reoxidation step was performed for taurine and hypotaurine conjugates. Part of these latter conjugates was neither reduced nor reoxidized, giving taurine = G = PL and hypotaurine = G = PL.

TAURINE	$\text{SO}_3^- - (\text{CH}_2)_2 - \text{NH}_3^+$	M.W. : 125
HYPOTAURINE	$\text{SO}_2\text{H} - (\text{CH}_2)_2 - \text{NH}_3^+$	M.W. : 109
CYSTEINE	$\text{SH} - \text{CH}_2 - \underset{\text{COO}^-}{\text{CH}} - \text{NH}_3^+$	M.W. : 121
β -ALANINE	$\text{COO}^- - (\text{CH}_2)_2 - \text{NH}_3^+$	M.W. : 89
GABA	$\text{COO}^- - (\text{CH}_2)_3 - \text{NH}_3^+$	M.W. : 103
GLUTAMATE	$\text{COO}^- - (\text{CH}_2)_4 - \underset{\text{COO}^-}{\text{CH}} - \text{NH}_3^+$	M.W. : 147
ASPARTATE	$\text{COO}^- - \text{CH}_2 - \underset{\text{COO}^-}{\text{CH}} - \text{NH}_3^+$	M.W. : 133
GLYCINE	$\text{COO}^- - \text{CH}_2 - \text{NH}_3^+$	M.W. : 75

FIG. 1. Structural relationships between amino acid and derivative neurotransmitters. Each molecule bearing a free amino group differs from the others by one, two, or three chemical groups.

Immunization

Two rabbits were immunized according to our previously described protocol (Geffard et al., 1984). Every 10 days, they were alternately injected, either subcutaneously or intramuscularly, with 500 μ g of immunogen emulsified in 500 μ l of 0.15 M NaCl and 1 ml of Freund's complete adjuvant (Difco). They received in rotation: taurine-G-HSA, taurine-G-PL, and taurine-G-TH. The antisera were collected 7 days after each injection and tested to determine the anti-aurine antibody affinity and specificity.

Purification of the antisera

Antisera were purified by adsorption with the carrier proteins, previously treated with glutaraldehyde. A solution containing 5 mg of lyophilized glutaraldehyde-treated proteins was mixed with 1 ml of crude antiserum for 18 h at 4°C. Then, the serum was centrifuged at 10,000 g for 10 min to discard the pellet containing the immune complexes. The purified supernatant was used to study the antibody specificity and affinity by enzyme-linked immunosorbent assay (ELISA).

ELISA

Tests of antibody affinity and specificity were performed on ELISA plates (Engvall and Perlmann, 1972; Butler et al., 1978; Geffard et al., 1985a). Polystyrene well plates (Nunc) were coated with conjugates of BSA, since the rabbits were not immunized with such. The well plates were filled up with 200 μ l of either a solution of taurine-G-BSA or G-BSA at 1 μ g/ml in 0.05 M carbonate buffer, pH 9.6, for 16 h at 4°C. After this period, they were filled with phosphate-buffered saline (PBS) containing 0.05% Tween-20, plus 1% bovine serum and left for 1 h at 37°C, to saturate fully the well plates and to prevent the nonspecific binding of the antisera. The

well plates were then rinsed three times with PBS-Tween. Purified antisera incubated for 1 h at 37°C with or without amino acid compounds, conjugated or not, were diluted in PBS-Tween containing 1% bovine serum and applied onto well plates coated with taurine-G-BSA or G-BSA for 2 h at 37°C. The final dilution of the antiserum was 1:20,000. Again, the well plates were washed three times with PBS-Tween and filled up with 200 μ l of 1:10,000 goat anti-rabbit labelled with horseradish peroxidase (Miles) diluted in PBS-Tween containing 1% bovine serum. After incubation at 37°C, the wells were rewashed three times with PBS-Tween. Peroxidase was assayed by incubating with an 0.4 mg/ml OPD solution in 0.1 M citrate-0.2 M phosphate buffer, pH 5, containing 1% hydrogen peroxide for 10 min in darkness. The reaction was stopped by addition of 50 μ l of a 4 M H_2SO_4 solution per well. The optical density at 492 nm was read with a multiskan Titertek apparatus. Experimental values were corrected by deducting blank values from well plates coated with G-BSA. Blank values were <5% as compared to experimental values.

Immunocytochemical procedure

Male 200–220 g Wistar rats WU:Cpb from TNO Zeist (The Netherlands) were anesthetized with sodium pentobarbital 0.1 mg/100 g body weight, and perfused intracardially with 0.9% saline followed by 5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.5. The brains were dissected and immersed in the same fixative for 1 h at 4°C, after which 50- μ m thick transverse or sagittal sections were cut with a Lancer Vibratome. The sections were incubated in a 0.1 M borohydride solution for 5 min and then in 3% H_2O_2 for 16 h. The free-floating sections were washed in buffer (0.05 M Tris-HCl containing 0.9% NaCl, pH 7.6) and subsequently incubated in taurine antiserum diluted from 1:1,000 to 1:5,000 in buffer plus 0.5% Triton overnight at 4°C. Then, the sections were washed and incubated in goat anti-rabbit IgG 1:50 for 60 min; washed and incubated in peroxidase anti-peroxidase 1:1,000 for 60 min; washed and incubated with 0.05% diaminobenzidine in Tris-NaCl, pH 7.6, with 0.01% H_2O_2 for 15 min; and finally washed again. The sections were mounted on glass slides, dried, and coverslipped.

RESULTS

Titration of the anti-taurine antibody

After 1 month of immunization, the ELISA tests showed a quite high antibody titer. Figure 2 shows two peaks, separated by 1 month. After 3 months of immunization, the antibody titer plateaued, though the rabbits were still injected.

Evaluation of antibody specificity

The antibody specificity was tested by the ELISA method, with competition experiments. A decrease in the optical density (B) was observed as the result of a competition between taurine-G-BSA coated on well plates, and conjugated or non-conjugated amino acids or derivatives, preincubated with the antibody dilution. B_0 was the optical density corresponding to the antibody binding without competition. The value of B_0 was adjusted to approximately 1 at 492 nm by varying the dilution

FIG. 2. Antibody titer and time course of immunization.

of the taurine antiserum and of the anti-rabbit antiserum labeled with peroxidase. The ratio B/B_0 allowed us both to analyze the results more precisely than with absorbance alone and to calculate the average of three experiments.

Antibody specificity for the analogues of taurine

The most immunoreactive compound was taurine-G-PL, synthesized with or without performing the reoxidation step (Fig. 3). The other conjugates, less well recognized than taurine-G-PL, were: GABA-G-PL, β -Ala-G-PL, Asp-G-PL,

FIG. 3. Displacement curves obtained with ELISA tests from competition experiments between Tau-G-BSA coated at 1 μ g/ml and each of the conjugated amino acids and derivatives, previously incubated with anti-taurine antibodies for 16 h at 4°C: (1) Tau-G-PL (unreoxidized Tau-G-PL); (2) rTau-G-PL (reoxidized Tau-G-PL); (3) GABA-G-PL; (4) β -Ala-G-PL; (5) Asp-G-PL; (6) Gly-G-PL; (7) Cys-G-PL; (8) Glu-G-PL. Antibody dilution was 1:20,000. B/B_0 is the ratio between the absorbances with competition (B) and without (B_0). Each point drawn for the displacement curves represents the average of three experiments. For ease in reading, standard deviations are not shown. C, Concentration of competitor conjugate.

TABLE 1. Specificities of anti-aurine antibodies

Compounds	Cross-reactivity ratio ^a
Tau-G-PL, reoxidized or not	1
GABA-G-PL	1:560
β -Ala-G-PL	1:1,860
Asp-G-PL	1:5,040
Gly-G-PL	1:14,200
Cys-G-PL	1:>20,000
Glu-G-PL	1:>20,000

^a Concentration of reoxidized or nonreoxidized Tau-G-PL/concentration of conjugated amino acids and derivatives at half displacement (Fig. 3).

and Gly-G-PL (Table 1). Only slight antibody affinity was found for Cys-G-PL and Glu-G-PL. The cross-reactivity ratios were calculated from the displacement curves at half-displacement ($B/B_0 = 0.5$) as follows: the concentration of the best recognized conjugate was divided by the concentration of each other conjugate. When the displacement curves did not reach a B/B_0 of 0.5, the cross-reactivity ratios were calculated after extrapolation of the curves. This always indicated a weak immunoreactivity.

Antibody recognition for taurine and hypotaurine, free or conjugated

Hypotaurine is the proposed precursor of taurine

FIG. 4. Displacement curves obtained with ELISA tests from competition experiments between Tau-G-BSA coated at 1 μ g/ml and each of the following conjugates, reduced or not, and reoxidized or not: (1) Tau-G-PL (unreoxidized Tau-G-PL); (2) rTau-G-PL (reoxidized Tau-G-PL); (3) Hypotau-G-PL; (4) Tau=G=PL; (5) Hypotau=G=PL; (6) nonconjugated Tau. The nonreduced conjugates are written as Tau=G=PL and the reduced ones as Tau-G-PL. Antibody dilution was 1:20,000. B/B_0 is the ratio between the absorbances with competition (B) and without (B_0). Each point drawn represented the average of three experiments.

(Hope, 1955; Peck and Awapara, 1964; Guion-Rain et al., 1975; Barbeau and Huxtable, 1978; Mandel and Pasantes-Morales, 1978) (Fig. 1).

Displacement curves were established between coated taurine-G-BSA and either hypotaurine-G-PL, or both nonreduced taurine and hypotaurine conjugates (taurine=G=PL and hypotaurine=G=PL) and nonconjugated taurine (Fig. 4). The most immunoreactive conjugate, taurine-G-PL, reoxidized or not, showed a displacement between 10^{-9} and 10^{-6} M, indicating a quite good antibody affinity. As previously described (Geffard et al., 1984, 1985a,b), the nonconjugated taurine exhibited a slight immunoreactivity. Taurine=G=PL was 6,700 times less well recognized than taurine-G-PL and hypotaurine=G=PL was not recognized at all (Table 2). The most immunoreactive compound, after taurine-G-PL, was hypotaurine-G-PL. The cross-reactivity ratio was 15 (Table 2). It was about the same as that calculated between taurine=G=PL and hypotaurine=G=PL, indicating that the slight difference in molecular structure, i.e., SO_3^- for taurine and SO_2 for hypotaurine, was recognized by antibodies.

In conclusion, the best antibody recognition was for conjugated taurine, provided the reduction step was performed before the reaction with the antibody.

Antibody recognition of the chemical form of sulfur in taurine residues

The presence of SO_3^- group in taurine and the reduction step carried out during the immunogen synthesis suggested that a reoxidation step should be performed to preserve the original molecular structure of taurine. To evaluate the amount of immunoglobulins directed against either the SO_3^- group of conjugated taurine or against SO_2 and SH groups of reduced taurine conjugates, taurine antiserum, previously purified on protein carriers, was adsorbed either on reoxidized or nonreoxidized conjugates (Tau-G-BSA). At a concentration of 5×10^{-5} M of conjugated taurine, the antibody

TABLE 2. Antibody specificity for taurine and hypotaurine conjugates

Compounds	Cross-reactivity ratios ^a
Tau-G-PL, reoxidized or not	1
Tau=G=PL ^b	1:6700
Hypotau-G-PL	1:15
Hypotau=G=PL ^b	1:>40,000
Tau	1:>40,000

^a Concentration of reoxidized or nonreoxidized Tau-G-PL/concentration of conjugated amino acids and derivatives at half displacement (Fig. 4).

^b The nonreduced conjugates are written as Tau=G=PL and the reduced ones as Tau-G-PL.

FIG. 5. Displacement curves obtained with ELISA tests from competition experiments between Tau-G-BSA coated at $1 \mu\text{g/ml}$ and each of the following conjugates reduced or not, and reoxidized or not: (1) Tau-G-PL (unreoxidized Tau-G-PL); (2) rTau-G-PL (reoxidized Tau-G-PL); (3) Hypotau-G-PL; (4) Tau=G=PL; (5) Hypotau=G=PL. Antibody was dilution 1:15,000. Antisera were successively adsorbed with protein carriers treated with glutaraldehyde and partially on nonreoxidized (A) or reoxidized (B) Tau-G-BSA before performing the competition experiments. Conjugated taurine concentration used for partial adsorption was 10^{-5} M .

binding was fully abolished. Consequently, a partial adsorption of 10^{-5} M was found sufficient to evaluate the importance of the reduction step. Competition experiments were performed between coated taurine-G-BSA and taurine and hypotaurine conjugates, reduced or not, and reoxidized or not (Fig. 5; Table 3). At half-displacement, we noticed that: (a) For antisera partially adsorbed on reoxidized conjugates (Fig. 5A), reoxidized taurine conjugates

TABLE 3. Antisera adsorbed both on protein carriers and on taurine conjugates reduced or reduced and reoxidized

Compounds	Cross-reactivity ratio ^a	
	Reduced	Reduced and reoxidized
Tau-G-PL	1	1
Hypotau-G-PL	1:11	1:15
Tau=G=PL	1:3500	1:80000
Hypotau=G=PL	1:>100000	1:>100000
Hypotau=G=PL ^b	1:50	1:40

For adsorption of antisera, refer to Materials and Methods and to Fig. 5.

^a Concentration of reoxidized or nonreoxidized Tau-G-PL/concentration of taurine or hypotaurine conjugates at half displacement.

^b Concentration of nonreduced conjugated taurine (i.e., Tau=G=PL)/concentration of nonreduced conjugated hypotaurine (i.e., Hypotau=G=PL) at half displacement.

were as well recognized as the nonreoxidized ones. Taurine=G=PL was 3,500 times less well recognized than taurine-G-PL. Hypotaurine-G-PL was 11 times less well recognized than taurine-G-PL and hypotaurine=G=PL was not recognized at all.

(b) For antisera partially adsorbed on nonreoxidized conjugates (Fig. 5B), reoxidized taurine conjugates were again as well recognized as the nonreoxidized ones. Taurine=G=PL was 80,000 times less well recognized than taurine-G-PL. Hypotaurine-G-PL, reoxidized or not, was 15 times less well recognized than taurine-G-PL. Hypotaurine=G=PL was not recognized at all.

These results suggest that the reoxidation step, performed during the synthesis of taurine conjugates, was less important than previously thought and that the SO_3^- group of taurine seemed to be chemically stable.

Immunocytochemical results

Incubation with taurine antibodies resulted in staining in many brain regions. The positive reaction disappeared after adsorption of the antibody with reoxidized or nonreoxidized taurine conjugates (concentration of conjugated taurine: $5 \times 10^{-5} \text{ M}$). Adsorption with GABA, aspartate, and glutamate conjugates did not result in any diminishment of the staining.

Immunopositive neurons were visualized in many parts of the CNS but most preeminent in the cerebellum, striatum, and septum. In the cerebellum, many Purkinje cells were stained with their axons and dendrites (Fig. 6). In the striatum (Fig. 7), many positive cell bodies were present with a dense fiber network, whereas in the lateral septum cell bodies and their processes were stained. Apart from in neurons, staining was also observed in oligodendrocyte-like cell bodies.

FIG. 6. Sagittal sections of the rat cerebellum stained for taurine. **A:** Low magnification view ($80\times$) in which can be seen that most Purkinje cells (P) are stained with their dendrites extending into the molecular layer (m). Apart from an occasional Golgi cell (arrow) in the granular layer (g) no other positive neurons can be observed. The axons of the Purkinje cells in the white matter (w) can be more clearly seen at higher magnification ($200\times$) in **(B)** (small arrows). The large arrow indicates the presence of unstained Purkinje cells. Scale bar = $100\ \mu\text{m}$.

DISCUSSION

The results obtained with anti-aurine antibodies illustrate the remarkable discriminating power of these molecules which can take into account the slight structural difference between the SO_3^- group of taurine and the SO_2 group of hypotaurine (Fig. 1). These antisera can distinguish between also other conjugated amino acids and derivatives that differ only slightly in structure (Table 1). GABA-G-PL, β -Ala-G-PL, and Asp-G-PL are poorly recognized. Gly-G-PL, Cys-G-PL, and Glu-G-PL are virtually not recognized. When the difference between the molecular structures of the various conjugates is large, the cross-reactivity ratio is low. In analogy with previously raised antisera (Geffard et al., 1984, 1985a,b; Seguela et al., 1984), anti-aurine antisera show very low affinity for the nonconjugated amino acid (Table 2).

The use of sodium borohydride during the reduction step of the immunogen synthesis was expected to transform the SO_3^- group into SO_2 or/and SH groups. However, partial adsorption of the anti-serum suggested that only a small amount of im-

munoglobulins is directed against conjugated taurine, the SO_3^- group of which is transformed (Fig. 5A and B; Table 3). Consequently, the reoxidation step appears less important than expected.

In agreement with our previous results, anti-aurine antibodies have a weak affinity for nonreduced conjugates which are 3,500 times less well recognized than the reduced ones (Fig. 4; Table 2). During the immunocytochemical applications, the borohydride treatment, in combination with the peroxide oxidation, gave the most intense staining. This indicates that the ELISA system used may indeed predict how the given antibody will react with the antigens fixed in the tissue. The immunocytochemical results obtained in this study indicate that taurine can be demonstrated specifically in the CNS. The localization of taurine in cerebellar Purkinje cells is in good agreement with the results obtained by Chan-Palay et al. (1982a,b) demonstrating [^3H] taurine uptake and the presence of a taurine-synthesizing enzyme in cerebellar Purkinje cells.

The use of specific anti-aurine antibodies in immunocytochemistry should contribute to the clarification of the role of taurine in the CNS.

FIG. 7. Transverse section of the rat striatum (200 $\times$) stained for taurine. cc, Corpus callosum. Note the presence of many taurine-containing fibers and the presence of small taurine-positive neurons in the striatum (arrows). Scale bar = 100 μ m.

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