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CYCLIC TAURINE ANALOGS

SYNTHESIS AND EFFECTS ON ATP-DEPENDENT Ca^{2+} UPTAKE IN RAT RETINA

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Abstract—Syntheses of (±)*trans*- and (±)*cis*-2-aminocyclohexane sulfonic acid (TAHS and CAHS) and (±)*trans*-2-aminocyclopentane sulfonic acid (TAPS) were achieved. In solution, the preferred conformations of TAHS and CAHS have been determined by high field NMR to be diequatorial and equatorial (sulfonic acid moiety)-axial (amino moiety) respectively. When these agents were evaluated as cyclic analogs of taurine in rat retina, TAHS and TAPS inhibited ATP-dependent calcium uptake in the micromolar range, whereas CAHS stimulated calcium uptake in the millimolar range in a manner similar to taurine. TAHS and TAPS are the most potent inhibitors of ATP-dependent calcium uptake in the rat retinal preparation yet reported.

High levels of taurine are present in the retina of a variety of animal species, including humans [1]. The greatest concentrations of taurine (50–80 mM) are found in the photoreceptor cell layer of the retina of the rat. While the precise function of taurine in the retina is unknown, three functions have been postulated: a putative neurotransmitter [1, 2] or neuromodulator [38]; a membrane stabilizer [1, 4]; and a modulator of calcium ion uptake [5–7] by regulating protein phosphorylation [8].

Pathological conditions attributed to a taurine deficiency have been documented in a variety of species, including monkey and human [9–12]. Moreover, a reversible photoreceptor degeneration has been demonstrated in the cat [13], which may serve as a model for retinitis pigmentosa [14]. However, inability to reproduce this retinal degeneration in other species has limited its applicability.

The actions of taurine in the retina and other excitable tissues are possibly related to a single phenomenon: the regulation of the excitation threshold by modulating calcium fluxes. Thus, by altering calcium mobilization during depolarization, taurine may exert a membrane-stabilizing effect. In the perfused heart, experimental conditions that modify calcium fluxes result in extensive cellular and functional damage. Addition of taurine to the perfusion medium protects against some of the deleterious effects resulting from the abnormal calcium fluxes [15]. In the retina there is an active ATP-dependent calcium uptake mechanism that has been demonstrated to be stimulated by taurine [6, 7, 16]. Other observations in the cat and the rat indicate that taurine is necessary for photoreceptor structural integrity, which can be disrupted by taurine depletion or illumination [4, 17–19]. These reports add support to the early suggestions by Huxtable and Bressler [20] and Gruener *et al.* [21] that taurine may be a membrane stabilizer.

Since the amino and sulfonic acid moieties of taurine have free rotation around the central meth-

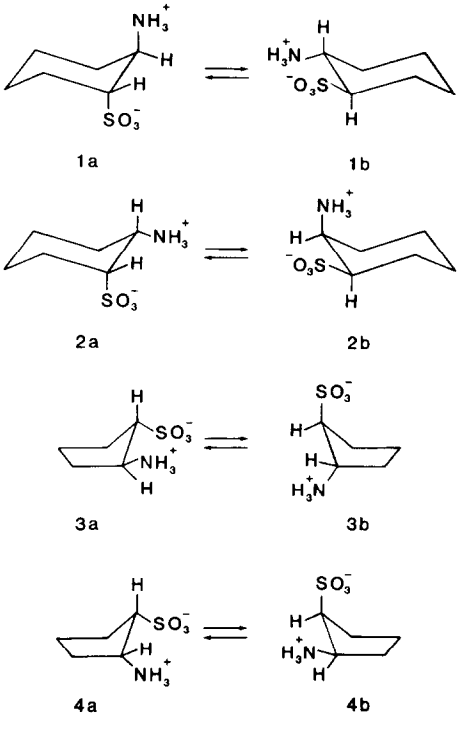

Fig. 1. Structures of the diastereomeric racemates of 2-aminocyclohexane sulfonic acid and 2-aminocyclopentane sulfonic acid.

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ylene linkage, the configuration of the taurine molecule at the biological active site is unknown. Thus, the syntheses of diastereomeric racemates 1 and 2 of 2-aminocyclohexane sulfonic acid and diastereomeric racemates 3 and 4 of 2-aminocyclopentane sulfonic acid (Fig. 1), which contain the taurine structure within a ring configuration, were attempted. These cyclic analogs, although conformationally mobile, are more restricted than taurine and approximate gauche conformations of taurine.

In this report, the syntheses of compounds 1, 2 and 3 are described, and the preferred conformations of 1 and 2 in solution are reported. In addition, biological activity concerning the effects of these compounds on ATP-dependent calcium ion uptake in rat retina is presented.

MATERIALS AND METHODS

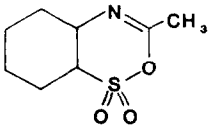
Materials. Taurine was obtained from the Aldrich Chemical Co. and was recrystallized twice from water. TAG (6-aminomethyl-3-methyl-4*H*-1,2,4-benzothiadiazine-1,1-dioxide hydrochloride) was a gift from Dr. George Yarbrough of Merck Sharp & Dohme Research Laboratories, West Point, PA. Guanidinoethanesulfonic acid (GES) was synthesized according to the procedure of Huxtable *et al.* [22].

Spectroscopy. Infrared spectra were recorded on a Perkin–Elmer 1330 instrument. Proton NMR spectra were determined on either a Varian EM390 or a Nicolet NT-200 instrument. DSS [3-(trimethylsilyl)-1-propane sulfonic acid] was used as an internal reference with D_2O samples. Mass spectra were recorded on a Dupont 21-491 spectrometer. Analyses were obtained from Atlantic Microlabs, Atlanta, GA. Simulated NMR spectra were generated using an IBM-PC-XT operating with RACCOON-2, written by Paul F. Schatz and obtained from project SEPHRAIM, NSF-Science Education, Eastern Michigan University, Department of Chemistry, Ypsilanti, MI 48197.

*Synthesis of (±)*trans-2-*aminocyclopentane sulfonic acid*. The synthesis of (±)*trans*-2-aminocyclopentane sulfonic acid, 3 (TAPS; Fig. 1), was achieved in the following manner: a solution of acetonitrile (19.7 g, 0.48 mol) in ethylene dichloride (15 ml) was added dropwise at −30° to a stirred solution of liquid sulfur trioxide (19.3 g, 0.24 mol) in ethylene dichloride (75 ml). Stirring at this temperature was continued for 30 min whereupon a solution of cyclopentene (32.7 g, 0.48 mol) in ethylene dichloride (20 ml) was added. The reaction mixture was warmed to room temperature, and stirring was continued overnight. Excess reagents and solvents were removed *in vacuo*. To the light brown residue, 10% aqueous methanol (100 ml) was added, and the mixture was refluxed for 2 hr. The methanol was removed *in vacuo*, 10% HCl (50 ml) was added, and the mixture was refluxed for 4 hr. The cooled solution was decolorized through charcoal and removal of the solvent *in vacuo* afforded 15.2 g of a yellowish precipitate. Recrystallization from H_2O-ethanol yielded 4.0 g (10%) of pure 3 as white crystals: m.p. 330° dec. (All melting points are uncorrected and were determined on a Mel-Temp capillary melting point apparatus.) Concentration of the mother liquor gave 3 contaminated with inorganic sulfate salts that could not be purified by fractional recrystallization. An additional 1.15 g of pure 3 was isolated by low pressure reverse phase (C-18) liquid chromatography (mobile phase: H_2O-*n*-propanol; 1:9). NMR (D_2O)δ 1.71–2.02 (m, 4H), 2.17–2.30 (m, 2H), 3.82 (q, 1H), 3.39 (q, 1H); IR (KBr) 1040, 1160–1220, 3050–3180 cm^{-1}; MS (70 eV), *m/e* 156 (M^+). Anal. ($C_5H_{11}NO_3S$)C,H,N.

*Synthesis of (±)*trans-2-*aminocyclohexane sulfonic acid*. The synthesis of (±)*trans*-2-aminocyclohexane sulfonic acid, 1 (TAHS; Fig. 1), was achieved in a manner analogous to that of 3 by addition of cyclohexene to a mixture of sulfur trioxide and acetonitrile in ethylene dichloride at −30°. The reaction mixture was stirred overnight at room temperature after which the solvent and excess reagents were removed *in vacuo*. The residue was mixed overnight with 10% aqueous methanol to yield 2-acetamidocyclohexanesulfonic acid. Subsequent hydrolysis of the amide group with 10% HCl yielded TAHS in 41% overall yield. Analytical purity was obtained by recrystallization from aqueous ethanol: m.p. 410° dec. [lit. 23, m.p. 410° dec.]; NMR (D_2O) δ 1.27–1.61 (m, 4H), 1.81–1.87 (m, 2H), 1.12–1.31 (M, 2H), 2.95 (td, 1H), 3.40 (td, 1H); IR (KBr) 1030, 1140–1240, 3020–3240, cm^{-1}; MS (70 eV), *m/e* 179 (M^+). Anal. ($C_6H_{13}NO_3S$)C,H,N.S.

The isolation of 4-alkyl-6-methyl 1,2,5-oxathiazine-2,3-dioxides from the reaction mixture prior to methanolysis has been reported [24] when long chain olefins, e.g. 1-*n*-hexadecene, have been employed. However, attempts to isolate the 1,2,5-oxathiazine-2,2-dioxides 5 and 6 were unsuccessful. Efforts to



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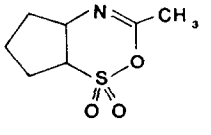

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synthesize TAHS and TAPS utilizing chlorosulfonic acid instead of sulfur trioxide, as has been reported [23], were also unsuccessful.

*Synthesis of (±)*cis-2-*aminocyclohexane sulfonic acid*. The synthesis of (±)*cis*-2-aminocyclohexane sulfonic acid, 2 (CAHS; Fig. 1), was achieved by catalytic reduction of aniline-2-sulfonic acid [25]. Aniline-2-sulfonic acid (3.0 g, 17.3 mmol) in H_2O (100 ml) was hydrogenated with 5% rhodium on carbon (5.0 g) at 50° and 1600 psi for 48 hr. The catalyst was filtered and the solvent removed *in vacuo*. The yellowish precipitate was recrystallized from methanol to afford 2.9 g (94%) of 2 as white crystals: m.p. 305° dec. [lit. 25, m.p. 300–301°]; NMR (D_2O) δ 1.35–2.10 (m, 8H), 3.18 (dt, 1H), 3.98 (q, 1H). Anal. ($C_6H_{13}NO_3S$)C,H,N.

Preparation of membrane homogenate. Adult Wistar rats (175–225 g; 6- to 8-weeks-old), which were fed Purina rat chow and water *ad lib.* were used in all experiments. The animals were housed in the vivarium and placed on a 12-hr light–dark cycle (light cycle starting at 7:00 a.m.). In the following experi-

ments all animals were killed between 8:00 and 9:00 a.m. by anesthetizing with ether and then decapitating. The eyes were immediately removed from the animal and placed in 0.32 M sucrose (2°). In all subsequent procedures the retinal tissue was maintained at 2° except for the final incubation. The retinal tissue was teased out of the eye cup with a small spatula and placed in 0.32 M sucrose and then gently homogenized. The homogenate was centrifuged for 20 min at 16,000 *g*. The resulting pellet was washed in 20 mM bicarbonate and recentrifuged as above. The retinal membranes located in the pellet were then washed in bicarbonate buffer, pH 7.4 [lit. 6, $NaHCO_3$, 50 mM; KCl, 50 mM; NaCl, 50 mM; KH_2PO_4, 1.2 mM; $MgCl_2$, 2 mM; ouabain, 50 μM], recentrifuged, and finally homogenized in a glass–glass homogenizer in the bicarbonate buffer.

Preparation of P_1 subcellular fraction of the retina. The retina was fractionated into a P_1 subcellular component according to the procedure of Redburn and Thomas [26]. Briefly, rat retinas from fifteen rats were suspended in 10 ml of 0.32 M sucrose (2°) and gently vortexed for 10 sec. The suspension was allowed to stand for approximately 10 min, and the supernatant fraction which contained the rod outer segments was removed and discarded (procedure repeated two additional times). The retinal fragments were then hand-homogenized in a Potter–Elvehjem homogenizer (five strokes) and centrifuged at 150 *g* for 10 min to remove a pre-P_1 pellet. The supernatant fraction was then re-centrifuged at 800 *g* for 10 min to produce a P_1 pellet consisting of photoreceptor cell synaptosomes. The P_1 subcellular fraction was characterized for its synaptosomal composition by its ability to take up [^{14}C]glutamic acid according to the procedures of Thomas and Redburn [27]. Similar kinetic constants were obtained for our rat preparations (homogenate: $K_m = 1.54\ \mu M$, $V_{max} = 118$ pmol/mg/4 min; P_1 fraction: $K_m = 0.71\ \mu M$, $V_{max} = 333$ pmol/mg/4 min) as were reported for the rabbit homogenate and P_1 fraction [27]. The glutamate uptake activity associated with our rat P_1 preparation demonstrates the presence and enrichment of the expected structural entities (synaptosomes) and their functional integrity.

Calcium ion uptake assay. The incubation system contained the above bicarbonate buffer, $^{45}CaCl_2$ (0.5 μCi), 10 μM $CaCl_2$, 1.2 mM ATP, and retinal preparation (crude retinal homogenate, ~0.25 mg protein; P_1 fraction ~0.03 mg). Glass test tubes treated with Prosil-28, a surface treating agent for preparation of a water-repellent surface, were used for the incubations. The reaction mixture minus the retinal preparation was preincubated for 2 min at 37°. The retinal preparation was then added, and the system was incubated for an additional 2 min at 37°. The reaction was terminated by adding 3 ml of ice-cold bicarbonate buffer to the incubation system and immediately filtering on a millipore glass fiber filter (Whatman GF/B filter). The filter was washed three times with 3 ml of the above bicarbonate buffer and then counted for radioactivity with Aquasol scintillation fluid. The amount of $^{45}Ca^{2+}$ taken up by the membrane preparations was determined by subtracting the radioactivity retained on the filter after a zero-time incubation with retinal preparation.

Protein estimations. Protein concentrations were determined by the method of Lowry *et al.* [28] with bovine serum albumin utilized as the standard.

RESULTS

NMR spectroscopy. The proton NMR spectrum (200 MHz, D_2O) of TAHS exhibits two triplets of doublets at δ 2.95 and δ 3.40 relative to DSS for the C*H* adjacent to the sulfur and nitrogen atoms respectively (Fig. 2a). The proton NMR spectrum (200 MHz, D_2O) of CAHS exhibits a doublet of triplets at δ 3.18 and a quartet at δ 3.65 relative to DSS for the C*H* adjacent to the sulfur and nitrogen atoms respectively (Fig. 2b). The proton NMR spectrum (D_2O) of TAPS (Fig. 2c) shows two apparent quartets at δ 3.39 and δ 3.82 relative to DSS for the C*H*

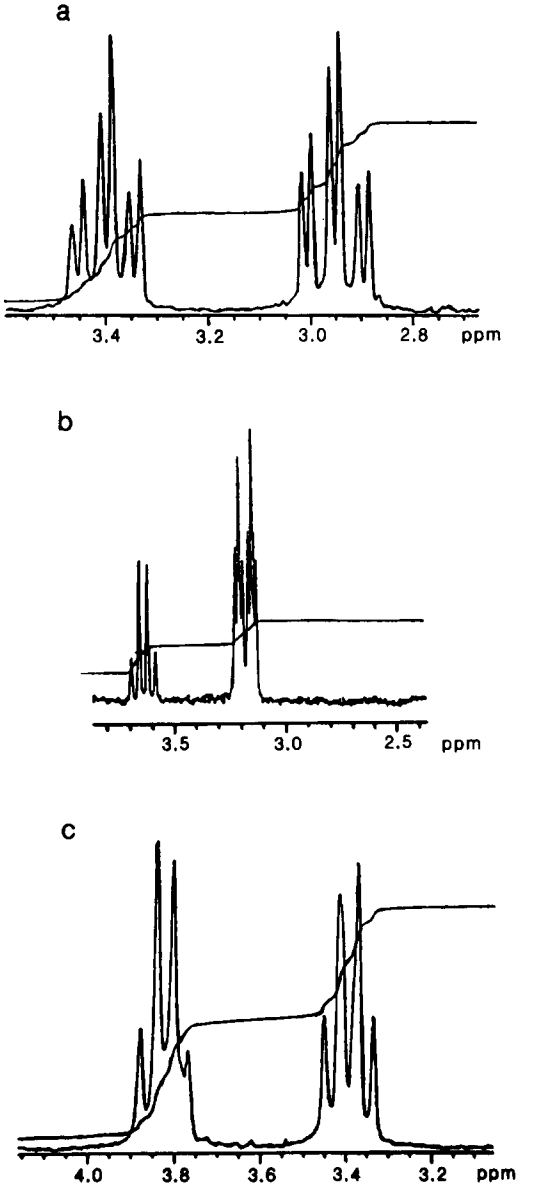


Fig. 2. ^{1}H-NMR spectra of taurine analogs in D_2O relative to DSS at 200 MHz. Key: (a) TAHS; (b) CAHS; and (c) TAPS.

Table 1. Observed and calculated chemical shifts for selected coupled protons in TAHS and CAHS substructures

	J (Hz)			
	TAHS		CAHS	
Coupled protons	obs	calc	obs	calc
H^1–H^2	11.22	12.35	3.44	4.68
H^1–H^5	3.93	4.68	3.39	4.68
H^1–H^6	11.34	12.35	11.77	12.35
H^2–H^3	4.35	4.68	3.40	4.68
H^2–H^4	11.08	12.35	3.50	4.68
H^3–H^4		0		0
H^5–H^6		0	0	0

adjacent to the sulfur and nitrogen atoms respectively. To ascertain the preferred conformation that TAHS, CAHS and TAPS assume in solution, simulated NMR spectra were generated and compared to spectrometer obtained spectra. Low temperature NMR spectra could not be obtained due to the low solubility of the compounds in solvents other than water or dimethyl sulfoxide. Solvent combinations (i.e. DMSO-$CHCl_3$) were also ineffective for solvolysis.

For TAHS and CAHS, spectra were generated for the six spin AA'BB'MX system $-CH_2$-$CH(SO_3H)$-$CH(NH_2)$-CH_2. Vicinal coupling constant (J) values were predicted for the Karplus [29] equations:

$$J = J^{\circ} \cos^2 \phi - C \; (0^{\circ} \leqq \phi \leqq 90^{\circ})$$

$$J = J^{180} \cos^2 \phi - C \; (90^{\circ} \leqq \phi \leqq 180^{\circ})$$

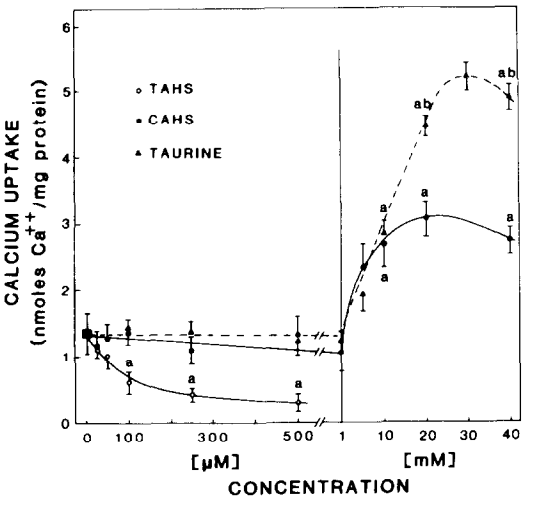


Fig. 3. Effects of various concentrations of TAHS, CAHS, and taurine on ATP-dependent calcium ion uptake in rat retinal membrane preparations. Retinal membrane preparations and the calcium ion uptake assay are described in Materials and Methods. Data are presented as means ± SEM; each point represents the mean of three experiments performed in duplicate. Analysis of variance and Duncan's multiple-range test were used to determine significant differences ($P < 0.05$). Means with superscript "a" are significantly different from control values (absence of taurine or taurine analog). Calcium uptake in the presence of 20 and 40 mM taurine is significantly greater than calcium uptake in the presence of 20 and 40 mM CAHS (designated by superscript "b").

where J°, J^{180} and C are constants and have the values of 8.5 Hz, 9.5 Hz and −0.3 Hz respectively. Geminal coupling between A and A' and B and B' were assumed to be zero Hz. Spectra were generated for the diaxial (1a) and diequatorial (1b) conformers of TAHS and for axial-equatorial (2a) and equatorial-axial (2b) conformers of CAHS (Fig. 1). The generated spectra for the M and X protons of 1b and 2b were consistent with the spectrometer-obtained spectra of TAHS and CAHS respectively. Table 1 gives the observed and calculated coupling constants for TAHS and CAHS.

Attempts to generate the observed spectrum of TAPS were unsuccessful. Disubstituted cyclopentanes have smaller energy differences between conformations as compared to cyclohexane systems. Twist and half-chair conformations are also more often observed in cyclopentane systems [30]. The observed spectrum of TAPS most probably represents an average spectrum of a mixture of conformations of which 3b may be the predominant form.

Pharmacology. A stimulatory effect on ATP-dependent calcium ion uptake, similar to that observed for taurine, was observed for CAHS (Fig. 3). CAHS, however, was less potent than taurine in stimulating calcium ion uptake. In the micromolar concentration range, CAHS had no effect on ATP-dependent calcium ion uptake (Fig. 3).

Unlike CAHS, TAHS did not stimulate ATP-dependent calcium ion uptake in the rat retina but, rather, inhibited calcium uptake. This effect is illustrated in Figs. 3 and 4. TAHS inhibited calcium ion uptake activity by 50% at 100 μM. TAPS was a more potent inhibitor of ATP-dependent calcium ion uptake than TAHS in whole rat retina homogenates (Fig. 4) as well as in a P_1 subcellular fraction of the rat retina which consists of photoreceptor cell

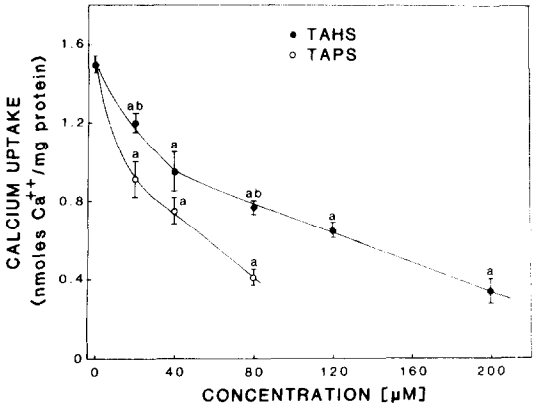


Fig. 4. Effects of various concentrations of TAPS and TAHS on ATP-dependent calcium ion uptake in rat retinal membrane preparations. Retinal membrane preparations and the calcium ion uptake assay are described in Materials and Methods. Data are presented as means ± SEM; each point represents the mean of three experiments performed in duplicate. Analysis of variance and Duncan's multiple-range test were used to determine significant differences ($P < 0.05$). Means with superscript "a" are significantly different from control values (absence of taurine analog). Means with superscript "b" are significantly different from the means at the same concentration of taurine analog.

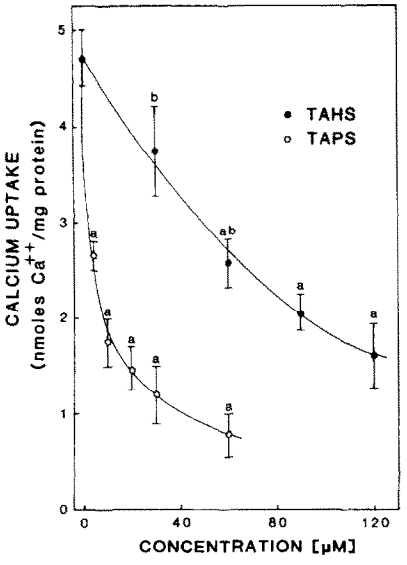


Fig. 5. Effects of various concentrations of TAPS and TAHS on ATP-dependent calcium ion uptake in the P_1 subcellular fraction of the rat retina. Preparations of the P_1 fraction and the calcium uptake assay are described in Materials and Methods. Data are presented as means ± SEM; each point represents the mean of three experiments performed in duplicate. Analysis of variance and Duncan's multiple-range test were used to determine significant differences ($P < 0.05$). Means with superscript "a" are significantly different from control values (absence of taurine analog). Means with superscript "b" are significantly different from the means at the same concentration of taurine analog.

synaptosomes (Fig. 5). TAPS and TAHS were tested in the P_1 fraction of the retina because of its higher specific activity for ATP-dependent calcium ion uptake.

The effects of TAPS in combination with taurine are shown in Fig. 6. Taurine half-maximally stimulated calcium ion uptake at approximately 10 mM. The presence of 40 μM TAPS lowered the calcium ion uptake by approximately 50% at all taurine concentrations. Guanidinoethanesulfonate (GES), a taurine transport inhibitor and a structural analog of taurine that decreases taurine levels in the retina [31, 32], was also tested over a wide concentration range (0–120 mM) to determine whether increasing ionic strength might have any effect on calcium ion uptake. The results in Fig. 6 demonstrate that GES had no effect on calcium ion uptake and that 40 μM TAPS maintained its inhibitory effect in the presence of GES.

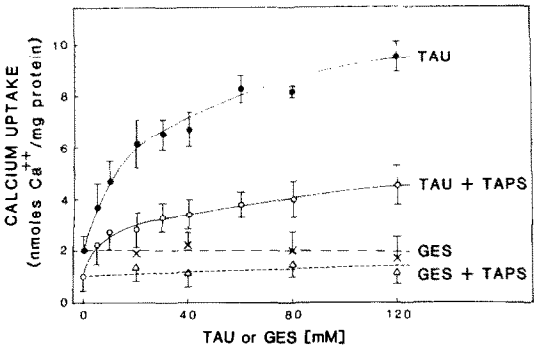
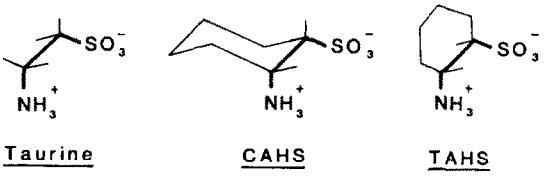


Fig. 6. Effects of taurine (TAU), guanidinoethanesulfonate (GES), taurine plus 40 μM (±)*trans*-2-aminocyclopentanesulfonic acid (TAPS), and GES plus 40 μM TAPS on ATP-dependent calcium uptake in rat retinal membrane preparations. Data are presented as means ± SEM; each point represents the mean of three experiments performed in duplicate.

DISCUSSION

The ATP-dependent calcium ion uptake in the rat retina is stimulated by 20 mM taurine at low calcium ion concentrations [6, 7, 16]. Since the structure of taurine has no conformational restrictions, the preferred conformation that taurine assumes when it exerts its stimulatory effect on calcium ion uptake is unknown. Because of the lack of rigidity in the taurine molecule, the following analogs of taurine, CAHS, TAHS and TAPS, were synthesized, as probes to determine the conformation of taurine at its biologically active site. It is noteworthy that these three analogs do not display the same pharmacological profile. Both taurine and CAHS stimulated calcium ion uptake, suggesting that the amino and sulfonic acid moieties assume a gauche conformation approximate to that shown below when



Taurine CAHS TAHS

stimulating calcium ion uptake. In contrast, micromolar concentrations of TAHS and TAPS inhibited ATP-dependent calcium ion uptake, suggesting that accommodation of the cycloalkyl moiety by the biological receptor is different for the *cis versus* the *trans* isomer. This is reasoned on the basis that the interatomic distances between the functional moieties within CAHS and TAHS both approximate 3Å, and the dihedral angles of each approximates 60°. The most apparent structural dissimilarity between CAHS and TAHS is the position of the cyclohexane ring relative to the functional groups. TAHS has a more planar overall geometry as a result of the preferred diequatorial conformation, whereas CAHS has an axial amino group out of the plane of the cyclohexane ring. The syntheses of other cyclic taurine analogs are ongoing to further explore these structure–activity relationships.

The combined antagonistic effect of TAPS and agonist effect of taurine on calcium ion uptake (Fig. 6) demonstrate a non-competitive relationship since increasing the concentration of taurine did not overcome the inhibitory effect of TAPS. These results thus suggest that taurine and TAPS affect the calcium ion uptake system at different sites and thus by different mechanisms.

TAG has been characterized as a taurine antagonist based upon its ability to antagonize the inhibitory effects of taurine and alanine on rat somato-

sensory cerebral cortical neurons and cerebellar Purkinje neurons [33]. Since TAG was reported to be a taurine antagonist, it was of interest to compare its effects on ATP-dependent calcium ion uptake to TAPS and TAHS. In the rat retinal preparation described, TAG had no effect on ATP-dependent calcium ion uptake [control = 1.88 ± 0.32 nmol/mg protein; plus TAG = 1.91 ± 0.28 nmol/mg protein (N = 3)].

In the retina, taurine produced an increase in ATP-dependent calcium uptake. TAPS and TAHS are the first reported agents to inhibit ATP-dependent calcium ion uptake in rat retina.

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