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EFFECTS OF AMINOCYCLOALKANESULFONIC ACID ANALOGS OF TAURINE ON ATP-DEPENDENT CALCIUM ION UPTAKE AND PROTEIN PHOSPHORYLATION

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Abstract—A cyclopentane analog of taurine [(±)*cis*-2-aminocyclopentanesulfonic acid] (CAPS) was synthesized, and its effects on ATP-dependent calcium ion uptake and protein phosphorylation in rat retina were investigated along with other cyclic analogs of taurine, (±)*trans*-2-Aminocyclopentanesulfonic acid (TAPS) is the most potent aminocycloalkanesulfonic acid inhibitor of ATP-dependent calcium ion uptake in retinal homogenates [S. M. Leibowitz, J. B. Lombardini and P. S. Salva, *Biochem. Pharmac.* **36**, 2109 (1987)], eliciting its effects in the micromolar range ($I_{50} = 39 \pm 5\ \mu M$). CAPS was found to be a less potent aminocycloalkanesulfonic acid inhibitor ($I_{50} = 1780 \pm 400\ \mu M$) of ATP-dependent calcium ion uptake in retinal homogenates. Taurine inhibited phosphate incorporation into rat retinal proteins, whereas TAPS, TAHS and CAPS stimulated incorporation.

Taurine (2-aminoethanesulfonic acid), which is the end product of methionine and cysteine metabolism, is a ubiquitous amino acid in the animal kingdom [1]. In the retina of vertebrates, including humans, taurine is the most abundant free amino acid, ranging from 10 to 52 μmol/g tissue [2]. The importance of taurine as an essential amino acid for the integrity of the retina in the cat has been recognized for the past 10 years [3–5]. Without taurine in the diet, the electroretinogram of the cat becomes nonrecordable, and a severe degeneration of the photoreceptors develops which ultimately leads to blindness. However, the nutritional requirement of taurine for humans has been established only recently by Geggel and colleagues [6] who examined electroretinograms of children receiving long-term parenteral nutrition that contained no taurine. These children had reduced fasting plasma taurine levels and abnormal electroretinograms, both of which reverted to normal in approximately 50% of the children after taurine was added to the intravenous solutions.

While the function of taurine is not well understood, there is evidence that taurine affects specific molecular processes in the retina such as calcium fluxes and phosphorylation of retinal proteins. It has been demonstrated repeatedly that taurine stimulates ATP-dependent calcium ion uptake at low calcium ion concentrations and inhibits ATP-dependent calcium ion uptake at high calcium ion concentrations [7–13]. At low calcium ion concentrations taurine also inhibits the phosphorylation of specific membrane proteins in the rat retina [14, 15].

Previous studies have indicated that there are specific structural requirements for taurine which are necessary for it to exert its biological activity in the ATP-dependent calcium ion uptake system [13, 16, 17]. Thus, in an attempt to probe further the structural requirements for taurine, we explored the structure–activity relationships among a series of aminocycloalkanesulfonic acids which are structural, conformationally restricted, cyclic analogs of taurine. In this study we present evidence that the cyclic analogs of taurine have dissimilar effects on ATP-dependent calcium ion uptake, that is, that certain analogues are inhibitors while one is a stimulator. In addition, the cyclic analogs demonstrated inverse relationships in their effects on ATP-dependent calcium ion uptake and protein phosphorylation.

MATERIALS AND METHODS

Materials. Adenosine-5′-triphosphate[γ-^{32}P] was purchased from ICN Radiochemicals (25 Ci/mmol). Calcium chloride ($^{45}Ca^{2+}$) was purchased from Amersham (6.7 mCi/mg). (±)*trans*-2-Aminocyclohexanesulfonic acid (TAHS), (±)*cis*-2-aminocyclohexanesulfonic acid (CAHS), and (±)*trans*-2-aminocyclopentanesulfonic acid (TAPS) were synthesized in our laboratory [18].

Synthesis of (±)cis-2-*aminocyclopentanesulfonic acid (CAPS).* A solution of cyclopentene oxide (8.41 g, 0.1 mol), NH_4Cl (8.02 g, 0.15 mol) and NaN_3 (9.75 g, 0.15 mol) in 70% ethanol was refluxed until the complete disappearance of oxide was noted by thin-layer chromatography. The mixture was diluted with an equal volume of water and extracted with ethyl acetate. The combined organic extracts were washed with brine, dried ($MgSO_4$), and concentrated *in vacuo*. Distillation of the concentrated organic layer produced 10.8 g (85%) of pure *trans*-1-azido-2-hydroxycyclopentane, **1**; b.p. 48° (0.5 mm). A solu-

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tion of triphenylphosphine (2.62 g, 0.01 mol) and diisopropylazodicarboxylate (1.74 g, 0.01 mol) in tetrahydrofuran (10 ml) was stirred for 30 min at 0°. To this solution was added a solution of **1** (0.64 g, 5 mmol) in tetrahydrofuran (2 ml) dropwise at 0°; the mixture was stirred for 30 min. Next was added a solution of thiolacetic acid (0.76 g, 5 mmol) in tetrahydrofuran (2 ml) at 0°. Upon concentration of the reaction mixture, the resultant residue was treated with 50 ml of dry hexane and agitated, and the resultant precipitate was filtered and washed with dry hexane (2 × 25 ml). The combined hexane solution was concentrated *in vacuo*, and the resultant crude oil was vacuum distilled to produce 2.86 g (87%) of analytically pure (±)*cis*-acetylthio-2-azidocyclopentane, **2**: b.p. 96° (2.5 mm Hg). Anal. Calc. for $C_7H_{11}N_3OS$: C,H,N.

To a degassed solution of azido thioester **2** (0.55 g, 2.97 mmol) in acetic acid (5 ml) at 60° was added a solution of 70% hydrogen peroxide (0.5 g, 14.8 mmol) in acetic acid (5 ml). After 3 hr the reaction mixture was concentrated, and the residue was subjected to high vacuum (0.001 mm Hg) for 2 hr at room temperature, to yield 0.57 g (100%) crude (±)*cis*-2-azidocyclopentanesulfonic acid **3**. Without further purification, **3** was hydrogenated with 5% palladium on carbon (0.5 g) in dry ethanol (15 ml) (60 psi, room temperature, 2 days). The infrared spectra revealed complete disappearance of azide absorption. The catalyst was filtered and washed (ethanol), and the combined ethanol layer was concentrated *in vacuo*. Recrystallization of the precipitate from methanol–isopropanol afforded 480 mg (87%) of pure CAPS: m.p. 260° (dec.) Anal. Calc. for $C_5H_{11}NO_3S$: C, H, N. Correct stereochemistry was assigned based upon high field NMR and the nuclear Overhauser effect compared with TAPS.

Preparation of membrane homogenate. Retinal membrane homogenates from adult Wistar rats weighing 175–225 g were prepared by the method of Lombardini [14]. The retinal membranes were homogenized in bicarbonate buffer, pH 7.4 [8] ($NaHCO_3$, 50 mM; KCl, 50 mM; NaCl, 50 mM; KH_2PO_4, 1.2 mM; $MgCl_2$, 2 mM; ouabain, 50 μM) and utilized in the calcium ion uptake and phosphorylation experiments.

Calcium uptake assay. The uptake of $^{45}Ca^{2+}$ by the retinal homogenate was measured as previously described by Lombardini [14]. The incubation system (0.5 ml) contained bicarbonate buffer, $^{45}CaCl_2$ (0.5 μCi), 10 μM $CaCl_2$, 1.2 mM ATP, and retinal preparation (~0.2 mg protein). Uptake was terminated by rapid dilution with buffer and immediate filtration with suction on Whatman GF/B filters.

Phosphorylation incubation system. Quantitation of the incorporation of $^{32}PO_4^{2-}$ from [γ^{32}P]ATP into the retinal membranes was described previously in detail by Lombardini [14]. In brief, the incubation system (0.25 ml) contained bicarbonate buffer, [γ-^{32}P]ATP (20 μCi), taurine and analogs when appropriate, and retinal membrane homogenate (~1 mg protein). After preincubation for 2 min at 37°, [γ-^{32}P]ATP was added and the membranes were further incubated for 10 min. The incubation was terminated by adding gel electrophoresis sample buffer (1 ml) and boiling in a water bath for 5 min. Incorporation of $^{32}PO_4^{2-}$ into the retinal membranes was determined by quantitating the amount of radioactivity in trichloroacetic acid precipitates.

Sodium dodecyl sulfate–Polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography. The phosphorylated proteins were subjected to SDS–PAGE on a 1.5 mm thick slab gel. The 12% polyacrylamide gel system was prepared according to the procedures of Laemmli [19]. The gels were stained for proteins with Coomassie Blue R-250, destained, dried on a Hoefer Scientific Instruments slab gel dryer, and exposed to Kodak diagnostic X-ray film GBS-2 as described by Lombardini [14]. The X-ray film containing the images of the phosphorylated proteins was photographed at two different time exposures in order to enhance specific regions. During the development process, the region between molecular weights 24,000 and 40,000 was exposed to 50% more light than the rest of the film.

Protein estimations. Protein concentrations were determined with bovine serum albumin utilized as the standard [20].

RESULTS

Structures of TAPS, CAPS, TAHS, and CAHS are shown in Figs. 1 and 2.

The approximate interatomic distance between the nitrogen and sulfur atoms was determined by Dreiding stereomodels to be 4.0 Å for TAPS and 2.7 Å for CAPS. The distances of the proton attached to the carbon atoms adjacent to each heteroatom were determined by nuclear Overhauser enhancement (NOE) in a high field (360 MHz) nuclear magnetic resonance spectrometer. The average NOE for TAPS was found to be 2%, corresponding to a distance between vicinal protons of 2.7 Å. The average NOE for CAPS was 5%, corresponding to a distance between vicinal protons of 2.3 Å. These data confirm the *trans* and *cis* orientations. In comparison, the intraatomic distances between heteroatoms in TAHS and CAHS were found to be 3.1 and 3.0 Å respectively. These distances are based upon the preferred conformations of each molecule as determined by NMR spectroscopy.

The effects of taurine and the cyclic taurine analogs on ATP-dependent calcium ion uptake in rat retinal preparations are reported in Table 1 as the concentrations necessary to produce either 50% stimulation or 50% inhibition. Taurine stimulates ATP-dependent calcium ion uptake [18]. Also, as observed previously [18], CAHS had a similar effect on ATP-dependent calcium ion uptake as does taurine in that the concentration necessary for 50% stimulation was the same (taurine, 8.1 ± 1.4 mM; CAHS, 6.5 ± 3.2 mM). Of the aminocycloalkanesulfonic acids tested (Table 1), TAPS is the most potent inhibitor of ATP-dependent calcium ion uptake in rat retinal tissue.

The effects of taurine and taurine analogs on phosphate incorporation into rat retinal membrane preparations are reported in Table 2. Taurine had no effect on phosphate incorporation at 2 mM but was inhibitory at 20 mM (33%). However, CAHS had no significant inhibitory effect at 20 mM. Conversely, TAPS and TAHS stimulated phosphate incor-

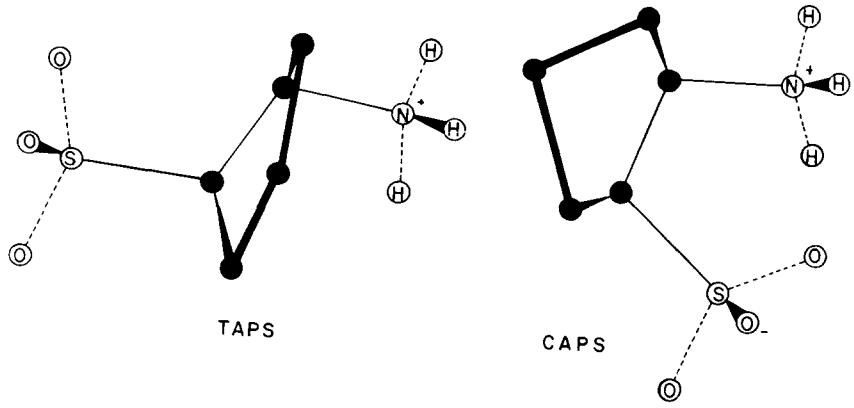

Fig. 1. Structures of TAPS and CAPS.

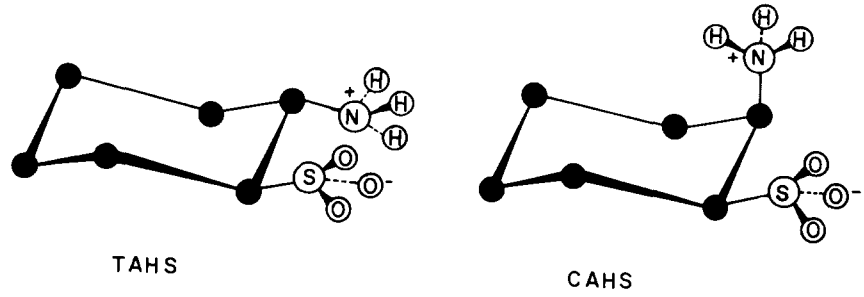

Fig. 2. Structures of TAHS and CAHS.

poration at 2 mM (31 and 28% respectively), whereas CAPS had no effect at 2 mM. At 20 mM CAPS was stimulatory (219%).

Autoradiograms of rat retinal membrane proteins that were incubated with [γ-^{32}P]ATP in the presence or absence of taurine or the aminocycloalkanesulfonic acid analogs of taurine (TAPS, CAPS, TAHS, CAHS), solubilized, and then subjected to SDS-PAGE are shown in Fig. 3. Autoradiography of dried gels revealed that numerous proteins were phosphorylated in the absence of taurine or taurine analogs (CON), in the presence of taurine (TAU), and in the presence of aminocycloalkanesulfonic acids (TAPS, TAHS, CAPS, and CAHS). Some of the more prominent differences in the phosphorylation pattern were as follows. In the presence of 20 mM taurine, a band of protein(s) with a molecular weight of approximately 20,000 (labeled a) incorporated less radioactive phosphate than control. All four of the aminocycloalkanesulfonic acid analogs of taurine also inhibited the incorporation of radioactive phosphate into this band. Taurine and the aminocycloalkanesulfonic acid analogs also demonstrated similar effects on the phosphorylation of a band of protein(s) designated "d". The phosphorylation of a band of protein(s) labeled "b" was also slightly reduced in the presence of taurine and CAHS, but was stimulated markedly in the presence

Table 1. Effects of taurine and cyclic taurine analogs on ATP-dependent calcium ion uptake by rat retinal membrane preparations

Compound	Concentration required for 50% inhibition (μM)	Concentration required for 50% stimulation (mM)
Taurine		8.1 ± 1.4 (7)
TAPS	39 ± 5* (5)	
CAPS	1775 ± 403† (4)	
TAHS	86 ± 13‡ (6)	
CAHS		6.5 ± 3.2 (4)

Details of the calcium ion uptake assay are described in Materials and Methods. Data are presented as means ± SEM. The number of experiments is given in parentheses. Analysis of variance and Duncan's multiple-range test were used to determine significant differences between the values.

*–‡ Means with different superscripts are significantly different from each other ($P < 0.05$).

Table 2. Effects of taurine and taurine analogs on incorporation of radioactive phosphate into rat retinal membrane proteins

Compound	Phosphate incorporation (pmol/mg protein/10 min)	% of Control
Control	3.22 ± 0.13 (21)	100
Taurine [2 mM]	2.95 ± 0.25 (5)	92
TAPS [2 mM]	4.21 ± 0.32* (8)	131
TAHS [2 mM]	4.13 ± 0.15* (8)	128
CAPS [2 mM]	3.63 ± 0.35 (7)	113
CAHS [2 mM]	3.01 ± 0.16 (5)	93
Taurine [20 mM]	2.16 ± 0.09* (14)	67
CAPS [20 mM]	7.05 ± 0.48* (6)	219
CAHS [20 mM]	2.73 ± 0.15 (11)	85

Details of the radiolabeling of the retinal membrane proteins with [^{32}P]phosphate are described in Materials and Methods. Final concentrations of taurine and the taurine analogs in the incubation systems are indicated in brackets. Data are presented as means ± SEM. The number of experiments is given in parentheses.

* Analysis of variance and Duncan's multiple-range test were used to determine significant differences ($P < 0.05$) between the control values and the values obtained in incubation systems that contained taurine or taurine analogs.

of CAPS. At a molecular weight of approximately 30,000, a band of protein(s) (labeled c) was phosphorylated in the control and in the presence of taurine and CAHS, but was less phosphorylated in the presence of TAPS, TAHS, and CAPS. The two bands designated "e" appeared to be less phosphorylated in the presence of CAPS. Finally, a band of protein(s) with a molecular weight of approximately 46,000 (labeled f) was phosphorylated to a greater extent in the control (CON) and in the presence of taurine (TAU) and CAHS than in the presence of TAPS, TAHS, and CAPS.

Densitometry tracings of the SDS–polyacrylamide gel autoradiograms, obtained in the presence and absence of taurine and the aminocycloalkanesulfonic acids, are shown in Fig. 4.

DISCUSSION

The stimulation of ATP-dependent calcium ion uptake in retinal membrane preparations by taurine is well documented [8–13]. However, the mechanism by which taurine produces the stimulation is not known. A relationship between taurine and ATP is evident in the ATP-dependent calcium ion uptake system since, in the absence of ATP, taurine does not stimulate calcium ion uptake. Furthermore, the stimulation appears to be specific for taurine and close structural analogs [13]. It therefore seemed reasonable to postulate that rotationally restricted analogs of taurine of relatively low molecular weight would have similar effects on calcium ion uptake.

Previous studies have demonstrated that the structural, conformationally restricted, cyclic analog of taurine, CAHS, is a stimulator of ATP-dependent calcium ion uptake [18]. We also have reported [18] that two other cyclic analogs of taurine, TAPS and TAHS, demonstrate inhibitory effects on calcium ion uptake. In the present study we have reported the effect of a fourth cyclic analog of taurine, CAPS, on ATP-dependent calcium ion uptake. In addition, the effects of all four of the cyclic analogs on phosphorylation of retinal membrane proteins are reported.

The *trans* isomer of the cyclopentane analogs, TAPS, is the most potent inhibitor of ATP-dependent calcium ion uptake of the four compounds that were tested. TAPS exerts its inhibitory action in the low micromolar concentration range [18]. In addition, its configurational isomer, CAPS was also an inhibitor in the calcium ion uptake system, but was the least potent isomer, requiring millimolar concentrations in order to exert its effect. The cyclohexane analog of taurine, TAHS, inhibits calcium ion uptake in the micromolar range [18], but it is clearly less potent than TAPS but more potent than CAPS. Conversely, CAHS, the isomeric analog of TAHS, has a stimulatory effect on ATP-dependent calcium ion uptake, as does taurine, although the maximum stimulatory effect of taurine is greater than CAHS [18].

When these cyclic analogs of taurine were tested in the protein phosphorylation system, opposite effects were observed. As reported previously [14], taurine and 2-aminoethanehydrogen sulfate stimulated ATP-dependent calcium ion uptake and inhibited phosphate incorporation. In the present studies this inverse relationship is, in general, maintained for the aminocycloalkanesulfonic acid analogs of taurine. The inverse relationship is observed for TAPS and TAHS which inhibit ATP-dependent calcium ion uptake but stimulate phosphorylation at 2 mM. However, while CAHS stimulates ATP-dependent calcium ion uptake [18] it does not significantly inhibit phosphate incorporation. In the present studies, it was also determined that CAPS was a weak inhibitor of calcium ion uptake and stimulated phosphate incorporation (219%) at a concentration of 20 mM but not at 2 mM. It was not possible to study the effects of TAPS and TAHS on phosphate incorporation at concentrations greater than 2 mM

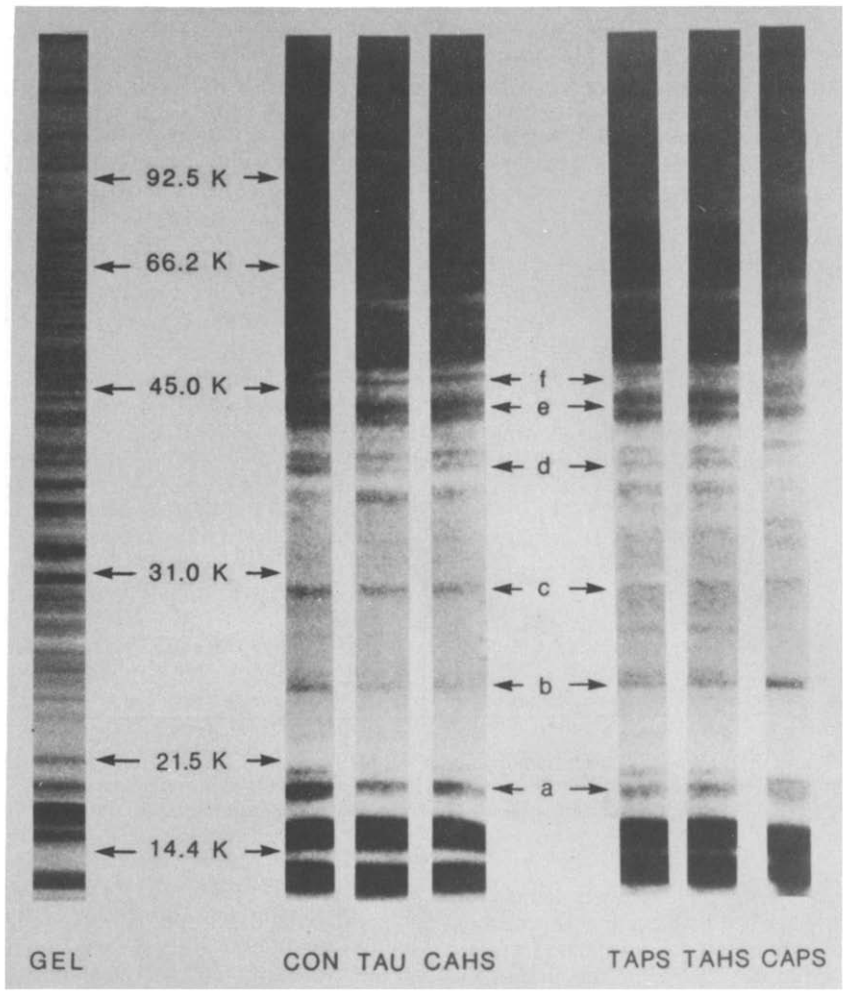

Fig. 3. SDS–PAGE and autoradiography of phosphorylated membrane proteins obtained from rat retinal homogenates. The phosphorylation reaction was carried out in incubation systems containing bicarbonate buffer, 1.7 mg of protein, 20 μCi [γ-^{32}P]ATP, and taurine or taurine analog as described in Materials and Methods. The well labeled GEL is a pattern of the retinal proteins stained with Coomassie Blue after electrophoresis. The autoradiographs are labeled as followed: CON = control (absence of taurine or taurine analog); TAU = 20 mM taurine; CAHS = 20 mM (±)*cis*-2-aminocyclohexanesulfonic acid; TAPS = 2 mM (±)*trans*-2-aminocyclopentanesulfonic acid; TAHS = 2 mM (±)*trans*-2-aminocyclohexanesulfonic acid; and CAPS = 20 mM (±)*cis*-2-aminocyclopentanesulfonic acid. Each well for the polyacrylamide gel electrophoresis contained equal quantities of radioactivity (12,700 cpm), as determined by TCA precipitation, but unequal quantities of protein. Marker proteins with molecular weights ranging from 14,400 to 92,500 are indicated. Letters a–f indicate the major phosphorylated protein bands with molecular weights of approximately 20,000 to 46,000 that are affected by taurine or the aminocycloalkanesulfonic acids. The results presented represent a single typical experiment of a total of three experiments.

because they are relatively insoluble in water. Thus, calcium ion uptake and protein phosphorylation were inversely related. Other taurine analogues that do not affect ATP-dependent calcium ion uptake also do not affect phosphate incorporation [14].

Autoradiograms indicated that TAPS, TAHS, and CAPS affected phosphorylation of specific proteins in the retina differently than did taurine and CAHS. A phosphorylated protein band(s) with a molecular weight of approximately 46,000 (designated e) was present in control and incubation systems that contained either taurine or CAHS. However, this band(s) was absent or much reduced in the presence of TAPS, TAHS, and CAPS, all of which stimulated retinal protein phosphorylation, as measured by phosphate incorporation into TCA precipitates. The cyclic analogs, including taurine, diminished the phosphorylation of a protein band(s) with an approximate molecular weight of 20,000 (designated a).

Of the various retinal proteins, the phosphorylation of which was affected by taurine or the aminocycloalkanesulfonic acids, band "a" contained the highest quantity of protein, as observed in the

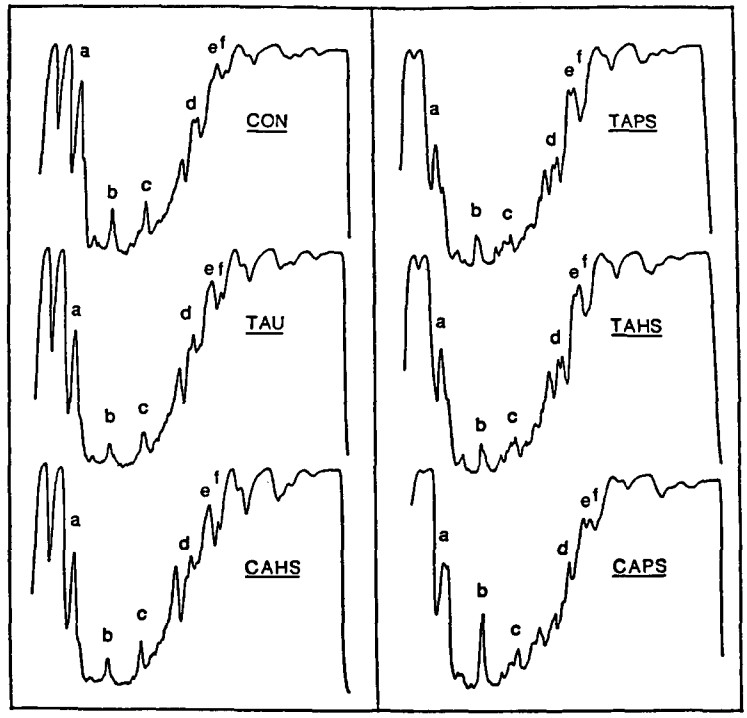

Fig. 4. Densitometry tracings of autoradiograms of the SDS–polyacrylamide gel electrophoretograms of the rat retinal membrane proteins. Letters a–f indicate major bands of phosphorylated proteins that were affected by taurine or the aminocycloalkanesulfonic acids.

Coomassie Blue stained SDS–polyacrylamide gel (Fig. 3, GEL). This band of protein(s) will most likely be the easiest to isolate if future studies warrant such an approach to determine the function of this protein(s).

The effects of these compounds on calcium ion uptake activity and protein phosphorylation cannot be explained solely on the basis of the intra-atomic distance between nitrogen and sulfur atoms within each of the molecules. The distances in TAPS and TAHS, clearly the most potent inhibitors of calcium ion uptake and stimulators of protein phosphorylation, were 4.0 and 3.1 Å respectively. This assumes a nontwist conformation for TAPS with the functional groups of both molecules in an equatorial–equatorial conformation. When the functional groups of TAHS were positioned in a diaxial orientation, the intra-atomic distance between heteroatoms increased to 4.0 Å. CAHS, however, did not alter the intra-atomic distance between heteroatoms as dramatically by reorientating from the preferred axial amino-equatorial sulfonate conformation (3.0 Å) to the equatorial amino-axial sulfonate conformation (3.1 Å). CAPS, which had an intra-atomic distance between heteroatoms of 2.7 Å, was therefore not predicted to have inhibitory action on calcium ion uptake, but rather stimulatory activity similar to CAHS. Clearly, the biological action of these isomers is not dependent solely on the intra-atomic distance between the heteroatoms within each molecule. The size, or most probably the positioning of the alkyl moiety, also must contribute significantly to biological activity.

The inverse effects on ATP-dependent calcium ion uptake and phosphorylation suggest different binding site tolerance for the positioning and/or size of the hydrocarbon bulk. The abilities of these compounds to distinguish these sites differently than taurine does indicate that they may be useful as biochemical probes. Further studies that isolate the specific proteins involved in the reactions influenced by the aminocycloalkanesulfonic acids and that probe the relationships among the aminocycloalkanesulfonic acids, protein phosphorylation, and calcium ion fluxes may be warranted.

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