Biochemical Pharmacology, Vol. 55, pp. 557–565, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00502-9

Effect of Taurine on Chelerythrine Inhibition of Calcium Uptake and ATPase Activity in the Rat Retina

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ABSTRACT. Taurine potentiates calcium uptake in whole retinal homogenates as well as in rod outer segments and mitochondrial fractions. The aim of this study was to correlate taurine potentiation of calcium uptake with its effects on other cellular processes through the use of chelerythrine (CHT), a modulator of protein kinase C (PKC), ATPase activity, and, as recently shown, of retinal protein phosphorylation. CHT inhibited calcium uptake only when ATP was present, and inhibition increased significantly in conditions of ATP excess. Taurine potentiated ATP-dependent calcium uptake but decreased the potency of ATP to induce uptake activity. CHT inhibition of calcium uptake exhibited similar potencies in the presence and absence of taurine, and this inhibition seemed to be independent of PKC inhibition. Because of the ATP-dependence of the observed effect, total ATPase activity was studied using similar treatments. In the absence of taurine, CHT inhibited ATPase activity with the same potency (IC_{50} ~59.3 μM) as with calcium uptake inhibition (IC_{50} ~87.9 μM), presenting a possible mechanism of action of CHT. In the presence of taurine, no such correlation was observed, suggesting an ATPase-independent mechanism of action. In fact, taurine did not potentiate ATPase activity, but rather it decreased the potency of CHT inhibition of ATPase, effects incongruent with the effects of taurine on calcium uptake and on CHT inhibition of calcium uptake. Enzyme kinetic experiments provided more supporting data. Taurine was found to cause an increase in the affinity of the ATP substrate for the ATPase enzyme, contradicting the aforementioned effect of taurine to decrease the potency of ATP to induce calcium uptake. Thus, taurine seems to increase calcium uptake through a hitherto unreported mechanism distinct from its modulation of ATPase activity. BIOCHEM PHARMACOL **55**;5:557–565, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. rat retina; taurine; chelerythrine; calcium uptake; ATPase activity

Taurine (2-aminoethanesulfonic acid), an amino acid found in high but varying amounts in mammalian tissues, is the most abundant free amino acid in the retina [1]. Concentrations as high as 79 mM have been measured in the photoreceptor layer of the rat retina [2]. However, the exact physiologic role taurine performs in the retina has not been described clearly. Its effects, though, on various retinal processes have been demonstrated, the most important functional effect being the reversible electroretinogram deficits observed after taurine depletion in the cat, monkey, and rat [1]. This deficit is coincident with an increase in the phosphorylation of a specific ~20 K protein in the mitochondrial fraction of the rat retina. In earlier reports, the phosphorylation of this protein has been demonstrated to be inhibited by taurine *in vitro* [3]. However, the identity of the protein and its link to the functional deficit observed are unknown.

Taurine also has significant effects on *in vitro* calcium uptake in the retina where it potentiates the uptake increase caused by ATP [1]. Calcium plays a crucial role in the excitation and recovery of photoreceptor cells with light stimulation [4, 5]. Specifically, the closure of cyclic GMP (cGMP)-gated cation channels, the net extrusion of calcium, and the decrease in intracellular calcium that occur during photoexcitation lead to the activation of a calcium-sensitive guanylyl cyclase and to an increase in cGMP. The activation of cGMP-gated channels allows for the increased entry of calcium. The increased calcium level results in the inhibition of guanylyl cyclase and in the breakdown of cGMP by phosphodiesterase, allowing for recovery from the hyperpolarized state of the photoreceptor. Taurine most probably assists in this recovery, in light of its effects on calcium uptake, explaining in part the electrical deficits found in the retina after taurine depletion in the live rat.

CHT† is a benzophenanthridine alkaloid that exhibits several biological effects including the inhibition of PKC [6] and ATPase [7]. Previously, we demonstrated that CHT

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Received 14 May 1997; accepted 8 August 1997.

† *Abbreviations:* CHT, chelerythrine; PKC, protein kinase C; and SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase.

causes stimulation, both in the presence and absence of taurine, of the *in vitro* phosphorylation of the same ~20 K mitochondrial protein in the rat retina that taurine depletion stimulates [8, 9]. Half-maximal stimulation was estimated at approximately 37 μM.

The functional effects of taurine need to be better associated with the biochemical processes taurine modulates, such as protein phosphorylation. Given the effect of taurine and CHT on the phosphorylation of the ~20 K protein and the modulation of calcium uptake by taurine, the effect of CHT on calcium uptake was studied. Retinal calcium uptake was measured in the presence and absence of taurine with or without CHT present to examine the interaction of taurine with CHT. CHT was found to inhibit calcium uptake in both the presence and absence of taurine, at concentrations similar to the concentrations effective in stimulating the phosphorylation of the ~20 K protein. CHT inhibition was found to be dependent on the presence of ATP, and so the effects of both CHT and taurine on total ATPase activity were also studied. CHT was found to inhibit ATPase activity, and though taurine alone did not affect ATPase activity, taurine was found to decrease the inhibition of ATPase by CHT.

MATERIALS AND METHODS

Materials

Chelerythrine chloride and K252b were purchased from LC Laboratories, and thapsigargin was purchased from Sigma. [γ-^{32}P]ATP and $^{45}CaCl_2$ were obtained from New England Nuclear. Bicinchoninic acid (BCA) protein assay reagent was obtained from Pierce Chemicals.

Preparation of Membrane Homogenate

Adult Wistar rats were killed, and the eyes were removed from the animal immediately. The eyes were then stored at −80° until used. The eyes were thawed, and retinal tissue was teased out of the eye cup in 0.32 M sucrose while on ice. All subsequent procedures were done on ice to maintain a 2° temperature. The tissue was centrifuged for 15 min at 16,000 *g*, washed in 20 mM bicarbonate, recentrifuged as before, and then washed in sodium bicarbonate buffer [$NaHCO_3$, 50 mM; NaCl, 50 mM; KCl, 50 mM; KH_2PO_4, 1.2 mM; $MgCl_2$, 2 mM] with $CaCl_2$ added to a final concentration of 10 μM [10]. The tissue was recentrifuged, resuspended in sodium bicarbonate-$CaCl_2$ buffer, and homogenized gently.

Calcium Uptake Assay

The incubation system used sodium-bicarbonate buffer and was kept on ice until the start of the reaction. Reagents such as ATP, taurine, and chelerythrine were added in the appropriate concentrations, including identical amounts of $^{45}CaCl_2$ (400,000–500,000 dpm) in a final concentration of 10 μM $CaCl_2$. Then the reaction tubes were preincubated in a 37° water bath for 2 min. Retinal homogenate (100–300 μg) was added to start the reaction, making a final volume of 250 μL, and the mixture was incubated for an additional 2 min. The reaction was terminated by adding 3 mL of ice-cold sodium-bicarbonate buffer 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 retinal tissue was determined by subtracting the counts retained on the filter after a zero-time incubation with retinal preparation.

ATPase Assay

The ATPase assay was adapted from commonly used procedures [11], and the sodium-bicarbonate system was used for the calcium uptake assay. Briefly, all the appropriate reagents including 10 μM $CaCl_2$ were added as with the calcium uptake assay except for the $^{45}CaCl_2$. Instead, equal amounts of [γ-^{32}P]ATP (400,000–500,000 dpm) were added to each incubation mixture, and the reaction tubes were preincubated in a 37° water bath for 2 min. Then retinal homogenate was added as before, and the tubes were incubated for an additional 2 min. The reaction was terminated by adding 250 μL of 5% perchloric acid, and the radioactivity was extracted into *n*-butanol/benzene (1:1) from which an aliquot was taken and counted in a scintillation counter. Control tubes were incubated for the same length of time, and radioactivity was counted the same way, but the perchloric acid was added before preincubation and the addition of the retinal homogenate. ATPase activity was determined by subtracting experimental values from control counts.

Protein Assay

The amount of protein used was assayed using the BCA method. Briefly, standards and samples were mixed with a solution of BCA protein assay reagent and 4% copper II sulfate (50:1). The mixtures were incubated in a 37° water bath for 30 min and absorbance was read at 560 nm.

RESULTS

Effects of Taurine and CHT on Calcium Uptake

Figure 1 shows that basal calcium uptake in the absence of any stimulator was not affected by CHT up to 100 μM. The increase in calcium uptake with the addition of 1.2 mM ATP and the potentiation of this uptake by 32 mM taurine are also shown. Taurine did not have any effect when given without ATP (data not shown). CHT treatment resulted in the inhibition of calcium uptake with ATP only and with ATP and taurine together, with the estimated concentration producing half-maximal inhibition (IC_{50}) at approximately 59.3 and 22.7 μM, respectively (Table 1). This represented a marginally significant increase in CHT po-

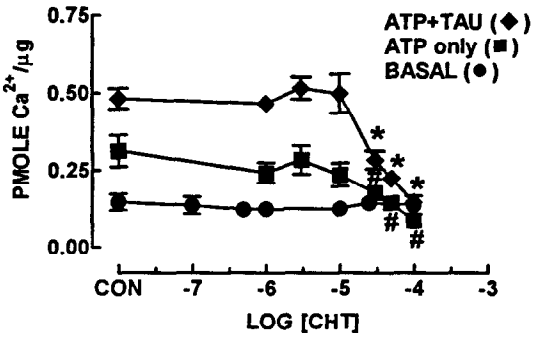

FIG. 1. Log concentration–response graph showing calcium uptake on the Y-axis and log molar concentration of CHT on the X-axis. ATP concentration was 1.2 mM; taurine concentration was 32 mM. An asterisk (*) or a pound sign (#) indicates a significant difference from their respective control (CON) values ($P < 0.05$) calculated by ANOVA and the Duncan's multiple range test (mean ± SEM, N = 5–6, each N being a determination from an independent experiment).

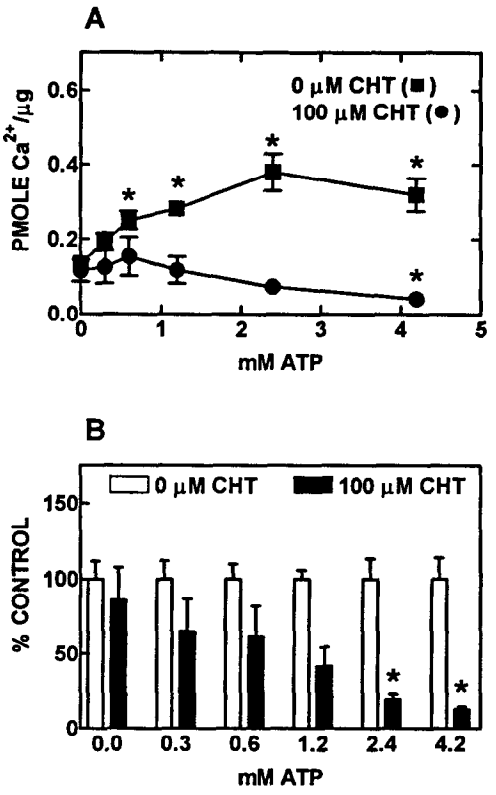

FIG. 2. ATP concentration–response showing the effects of increasing concentrations of ATP on calcium uptake, in the presence or absence of 100 µM CHT. (A) Calcium uptake activity as a function of increasing concentrations of ATP. (B) CHT inhibition at each ATP concentration presented as percent of control (see panel A for control values). An asterisk (*) indicates a significant difference from their respective control values (0 mM ATP) ($P < 0.05$) calculated by ANOVA and the Duncan's multiple range test (mean ± SEM, N = 4, each N being a determination from an independent experiment).

tency with the addition of taurine ($P < 0.07$, paired t-test). At 100 µM CHT, calcium uptake was inhibited to the same extent with both ATP and taurine as with ATP alone (70.5 and 72.0% inhibition), even though the former treatment produced almost twice the level of calcium uptake in control conditions.

Figures 2A and 3A show increasing calcium uptake with increasing ATP concentrations and inhibition of uptake by 100 µM CHT, in the absence and presence of 32 mM taurine, respectively. Taurine potentiated ATP-induced calcium uptake in control conditions as much as 4–5 times. At the same time, though, taurine was found to cause a decrease in the potency of ATP to induce calcium uptake. There was a significant increase in the ATP concentration estimated to produce half-maximal stimulation of calcium uptake (SC_{50}), from 0.30 ± 0.07 to 0.67 ± 0.07 mM (mean ± SEM).

In Figs. 2B and 3B, the data were regraphed to illustrate the degree of inhibition by 100 µM CHT at each ATP concentration used as percent of control calcium uptake. In the absence of taurine (Fig. 2B), it was found that at saturating levels of ATP (2.4 and 4.2 mM), CHT produced greater effects on calcium uptake (80–88% inhibition) than at non-saturating concentrations (15–60% inhibition). A similar trend was observed when ATP concentration was increased in the presence of 32 mM taurine (Fig. 3B). CHT inhibition of calcium uptake increased from 68–85 to 91–93% at saturating concentrations of ATP. In both conditions, calcium uptake did not increase any more

TABLE 1. Effect of taurine on the inhibitory potency of CHT against calcium uptake and ATPase activity in rat retinal homogenates

	CHT concentration producing half-maximal inhibition (IC_{50}) (µM)	
	Taurine	
	0 mM	32 mM
Calcium uptake (N = 6)	59.3 (34.2–102.56)	22.7 (17.8–29.1)
ATPase activity (N = 3)	87.9 (78.8–97.9)	141.7* (127.6–157.0)

The IC_{50} values were generated through non-linear regression analysis of log concentration–response curves using GraphPad Prism Software. Values are geometric means with the 95% confidence values given in parentheses. Each N represents an independent experiment comparing 0 vs 32 mM taurine and yielding a pair of independent IC_{50} values. Paired t-tests were done on the paired IC_{50} values within each assay system to analyze the effects of 32 mM taurine.

* $P < 0.05$, paired t-test, compared with 0 mM taurine.

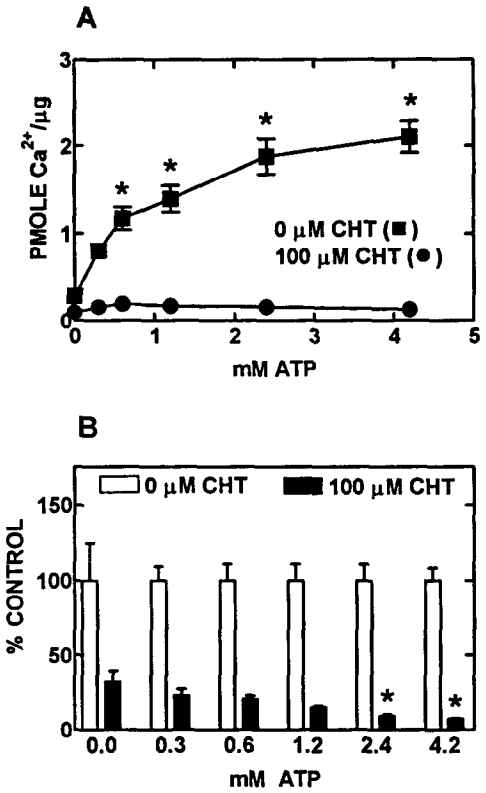

FIG. 3. ATP concentration–response showing the effects of increasing concentrations of ATP on calcium uptake, in the presence of 32 mM taurine, with or without 100 μM CHT. (A) Calcium uptake activity as a function of increasing ATP concentrations in the presence of 32 mM taurine. (B) CHT inhibition at each ATP concentration presented as percent of control (see panel A for control values). An asterisk (*) indicates a significant difference from their respective control values (0 mM ATP) ($P < 0.05$) calculated by ANOVA and the Duncan's multiple range test (mean ± SEM, N = 3, each N being a determination from an independent experiment).

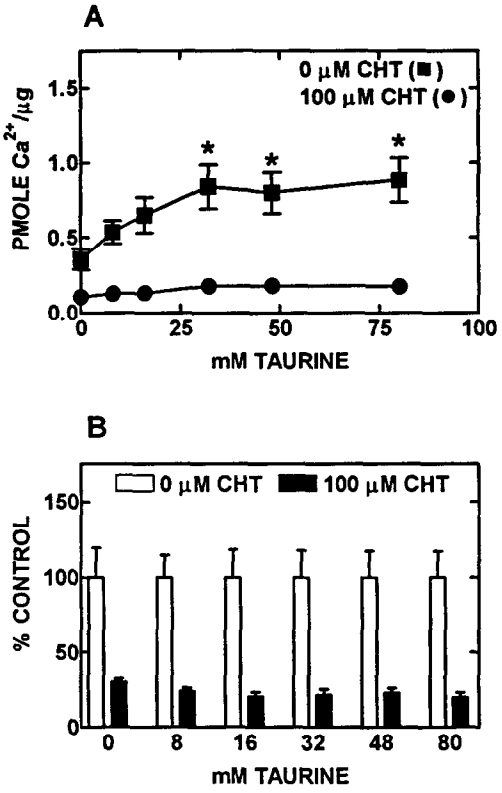

FIG. 4. Taurine concentration–response on calcium uptake, in the presence of a fixed concentration of ATP (1.2 mM), with or without 100 μM CHT. (A) Calcium uptake activity as a function of increasing concentrations of taurine. (B) CHT inhibition at each taurine concentration presented as percent of control (see panel A for control values). An asterisk (*) indicates a significant difference from the control value (0 mM ATP) ($P < 0.05$) calculated by ANOVA and the Duncan's multiple range test (mean ± SEM, N = 4, each N being a determination from an independent experiment).

at 2.4 and 4.2 mM ATP, at which points there was greater inhibition in terms of percent control uptake.

When taurine concentrations were increased in the presence of 1.2 mM ATP, the degree of inhibition of calcium uptake by 100 μM CHT remained constant (Fig. 4B). Calcium uptake increased with increasing taurine concentrations up to 32 mM, both in the presence and absence of 100 μM CHT, but no further change was noted at higher concentrations (Fig. 4A). Inhibition of calcium uptake by CHT (70–80%) did not change significantly at any of the taurine concentrations used.

K252b is a natural mold product and an indole-carbazole-containing compound [12]. It is a known inhibitor of PKC, which produces half-maximal inhibition at approximately 38.3 nM. Calcium uptake was measured in our system in the presence of different concentrations of K252b and of CHT (Fig. 5, A and B). At all concentrations of K252b used, no significant effect was observed in calcium uptake, whereas 50 and/or 100 μM CHT caused significant inhibition of calcium uptake with ATP only and with ATP and taurine together.

Effect of Taurine and CHT on ATPase Activity

Because CHT produced inhibition only in the presence of ATP, an effect that increased in conditions of ATP excess, ATPase activity was studied. Total ATPase activity was measured in the same ionic milieu used in the calcium uptake experiments with no distinction being given to the specific forms of ATPase affected.

First, the effect of taurine was studied, and it was found that taurine did not affect ATPase activity significantly (data not shown). Analogues of taurine such as guanidinoethanesulfonate (GES) (amino moiety replaced by a guanidino group) and β-alanine (β-ALA) (sulfonic acid moiety replaced by a carboxyl acid group) also did not have any effect on ATPase activity.

CHT was found to cause inhibition of ATPase activity in both the presence and absence of taurine (Fig. 6). Taurine, though, was found to cause a decrease in the potency of CHT, shifting the inhibition curve to the right. The IC_{50} value for CHT was found to increase significantly from 87.9 to 141.7 μM with the addition of taurine (Table 1).

Ouabain, a sodium-potassium ATPase (Na^+, K^+-

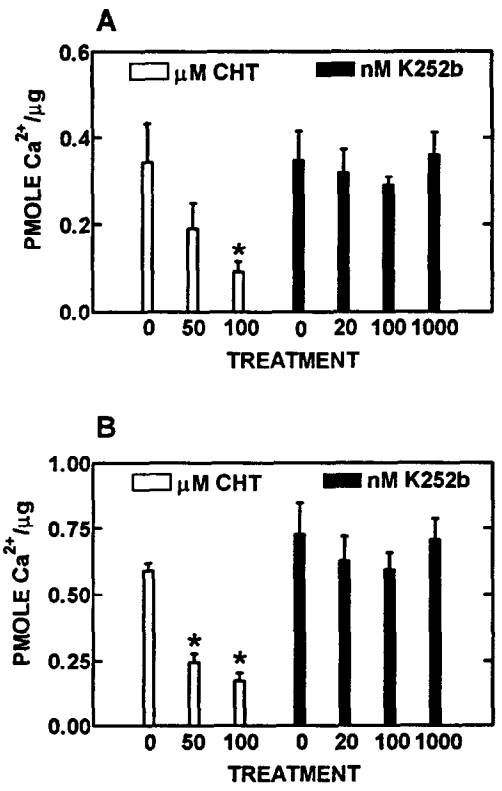

FIG. 5. Calcium uptake in the presence of the PKC inhibitor K252b and CHT. (A) K252b and CHT effects on calcium uptake with 1.2 mM ATP alone. (B) K252b and CHT effects on calcium uptake with both 1.2 mM ATP and 32 mM taurine present. An asterisk (*) indicates a significant difference from the control value (0 μM CHT) ($P < 0.05$) calculated by ANOVA and the Duncan's multiple range test (mean ± SEM, N = 3, each N being a determination from an independent experiment).

ATPase) inhibitor, did not cause a significant decrease in total ATPase activity at a concentration of 1 mM in either the presence or absence of taurine (Fig. 7). In the absence of taurine, CHT at 100 μM caused greater inhibition of the

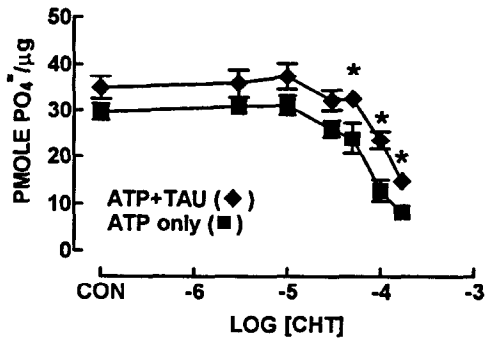

FIG. 6. Log concentration–response graph showing ATPase activity in the presence of ATP alone or with 32 mM taurine (ATP + TAU) inhibited by CHT. ATPase activity was measured as picomoles of inorganic phosphate released per microgram of protein. An asterisk (*) indicates a significant difference ($P < 0.05$, Student's t-test) between activity with ATP alone and with ATP plus taurine together (mean ± SEM, N = 3, each N being a determination from an independent experiment).

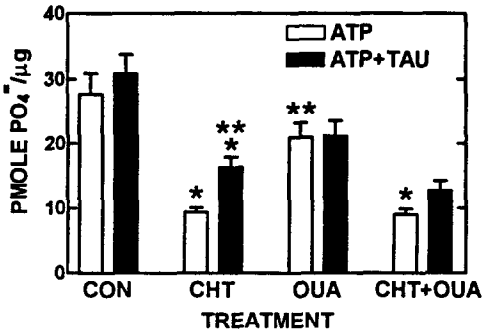

FIG. 7. ATPase activity in the presence of 100 μM CHT and 1 mM ouabain (OUA). ATPase activity was studied with 1.2 mM ATP alone or in the presence of 32 mM taurine. ATPase activity was measured as picomoles of inorganic phosphate released per microgram of protein. A single asterisk (*) indicates a significant difference ($P < 0.05$) when compared with their respective controls as calculated by ANOVA and the Duncan's multiple range test (mean ± SEM, N = 4, each N being a determination from an independent experiment). A double asterisk (**) indicates a significant difference ($P < 0.05$) when (1) ATPase activity in the presence of ATP and CHT was compared with activity in the presence of ATP plus taurine and CHT; and (2) when ATPase activity in the presence of ATP and ouabain was compared with activity in the presence of ATP and CHT.

ATPase activity than ouabain. This suggests that there is an inhibition of ouabain-insensitive ATPase activity by CHT. When taurine (32 mM) was present, CHT significantly inhibited ATPase activity compared with control but demonstrated no greater inhibition of ATPase activity than ouabain, suggesting some protective effect of taurine against the effects of CHT on ouabain-insensitive ATPase isoforms. Combining CHT and ouabain only produced inhibitory effects comparable to those seen with CHT alone.

ATPase activity with ATP alone and with ATP plus taurine together was found not to be affected by K252b, a PKC inhibitor, nor by thapsigargin [13, 14], a SERCA inhibitor (data not shown).

ATPase Enzyme Kinetic Experiments

Enzyme kinetic experiments were performed on the whole retinal homogenate to study the effects of taurine on ATPase activity and on CHT inhibition of ATPase activity. ATPase activity was measured with increasing concentrations of ATP in the presence or absence of a fixed concentration (100 μM) of CHT. Panels A and B of Fig. 8 show one of four experiments from which enzyme kinetic data were compiled, graphically illustrating the ATP concentration–response curves under the various conditions used and double-reciprocal analyses, respectively.

Taurine was found to cause a decrease in the K_m value (Table 2) for ATP in control experiments (from 1.23 ± 0.14 to 0.78 ± 0.09 mM ATP, mean ± SEM). This demonstrates for the first time the ability of taurine to increase ATP affinity for the ATPase enzyme. In contrast, there was no effect observed on the maximal enzyme

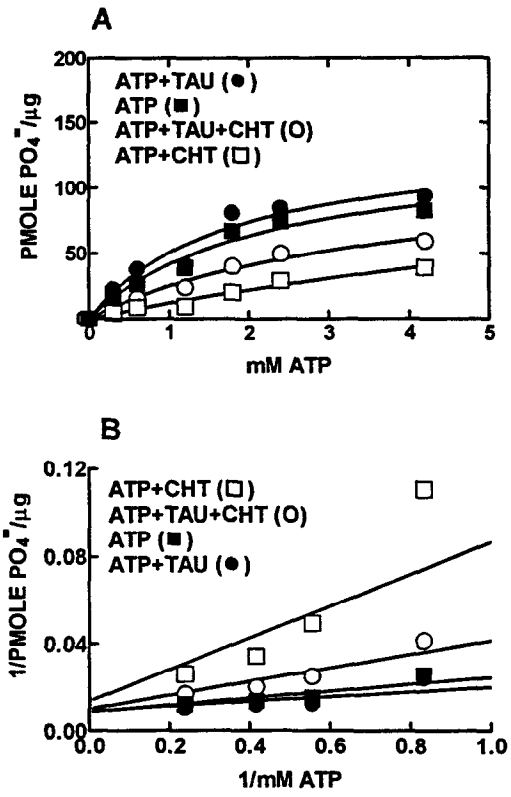

FIG. 8. ATPase activity as a function of the ATP concentration obtained under various experimental conditions (representative experiment). Activity was measured in the presence of one or more of the following: 1.2 mM ATP (ATP), 32 mM taurine (TAU), and 100 µM CHT (CHT). (A) Graphs of the ATPase activity were generated through non-linear regression analysis. (B) Reciprocal plots of the ATPase activity (1/pmol ATP/µg protein) versus the concentration of ATP (1/mM ATP). ATPase activity was measured as picomoles of inorganic phosphate released per microgram of protein. The linear regression lines shown were derived from all experimental points as in Fig. 8A, but the graph was truncated to magnify the Y-axis.

velocity (Table 2) after taurine was added (from 81.3 ± 11.2 to 74.6 ± 12.2 pmol $PO_4^=$/µg protein, mean ± SEM). This supports the previous data that demonstrated no significant changes in total ATPase activity with the addition of taurine.

CHT caused a decrease in the maximal velocity of the enzyme reaction, from 81.3 ± 11.2 to 37.3 ± 5.0 pmol $PO_4^=$/µg protein (mean ± SEM) in the absence of taurine, indicating a non-competitive interaction between ATP and CHT (Table 2). There was no significant change in the K_m value in this condition (from 1.23 ± 0.14 to 1.41 ± 0.19 mM ATP, mean ± SEM), indicating no changes in substrate–enzyme affinity. On the contrary, CHT caused no significant change in maximal velocity (from 74.6 ± 12.2 to 61.0 ± 6.7 pmol $PO_4^=$/µg protein, mean ± SEM) in the presence of taurine, suggesting a competitive type of inhibition. Under this condition (ATPase activity in the presence of both ATP and CHT), a significant increase in the K_m value was also observed, from 0.78 ± 0.09 to 1.32 ± 0.19 mM ATP (mean ± SEM), indicating decreased affinity of the ATP substrate to the ATPase enzyme. Thus, the presence of taurine radically changes the effects CHT has on ATPase enzyme activity, changing non-competitive inhibition to competitive inhibition and enabling CHT to affect substrate–enzyme affinity.

TABLE 2. Kinetic parameters calculated from reciprocal plots of the effects of taurine, CHT, and taurine plus CHT on ATPase activity in rat retinal homogenates

Conditions	V_{max} (pmol/µg)	K_m (mM)
No additions	81.3 ± 11.2^x	1.23 ± 0.14^b
CHT (100 µM)	37.3 ± 5.0^y	1.41 ± 0.19^b
Taurine (32 mM)	74.6 ± 12.2^x	0.78 ± 0.09^a
CHT + taurine	61.0 ± 6.7^x	1.32 ± 0.19^b

ATP was the variable substrate. ATPase activity was determined by measuring liberated $[^{32}P]PO_4^=$ from [γ-^{32}P]ATP as described in "Materials and Methods." Data are shown in graphical form in panels A and B of Fig. 8 (representative experiment). The X- and Y-intercepts were calculated from linear analysis utilizing GraphPad Prism software, and the data are presented as means ± SEM from 4 experiments (1/Y-intercept = maximum velocity; 1/X-intercept = ATP concentration at half-maximum velocity). Statistical differences were calculated by ANOVA and the Duncan's multiple range test. Means with different superscripts were significantly different from each other ($P < 0.05$).

DISCUSSION

The role of taurine in the function of the eye is woefully understudied, despite the clear functional deficits demonstrated in animals with artificially induced taurine deficiencies. Electroretinogram studies consistently show the inhibitory effects of taurine depletion in the amplitude of the b-wave in the rat [1, 9]. Electrical activity in the retina reflects the ability of the photoreceptor cells to recover from membrane hyperpolarization caused by photoexcitation, a recovery dependent on the flow of calcium into the cell at the appropriate speed and magnitude [4]. Calcium uptake in the retina has been shown to be potentiated by taurine at low calcium concentrations and only in the presence of bicarbonate [1, 15]. Inasmuch as taurine is present in the eye at high concentrations [2], it can be assumed that in the normal physiological state, calcium metabolism in the retina is modulated by taurine. In most of the experiments done with taurine, the concentration used (32 mM) was well within physiologically observed levels.

Aside from ERG measurements, protein phosphorylation in the retina was also shown to be affected by taurine depletion in the whole animal [9]. The phosphorylation of a specific ~20 K protein in the mitochondrial fraction was found to increase almost 100%. Conversely, taurine treatment causes inhibition of this phosphorylation *in vitro* [3]. Unfortunately, there is a lack of information on the identity and function of this retinal protein. It is known, though, that the phosphorylation of this protein is increased by CHT [8], a well-known PKC inhibitor, an effect independent of phosphatase inhibition. Previously, PKC activation

was shown to have no effect on the phosphorylation of the ~20 K protein [16], and so it was assumed that CHT was acting through some mechanism other than PKC inhibition to cause the increase in phosphorylation.

Given this background, CHT was prognosticated to also have an effect on calcium uptake. The demonstration of an effect would potentially shed more light on the possible link between the function of taurine, the ~20 K protein, and electrical activity in the retina. CHT was found to cause inhibition of calcium uptake, but inhibition was observed at magnitudes at least 20 times greater than what is needed for PKC inhibition. Analogous to the CHT stimulation of the phosphorylation of the ~20 K protein, there seems to be some kind of PKC-independent mechanism of action behind the CHT effects. K252b is a natural mold product known to inhibit PKC activity at nanomolar concentrations. K252b did not have any effect on calcium uptake (Fig. 5, A and B), strengthening this notion of an extra-PKC effect for CHT in the inhibition of calcium uptake.

Increasing the concentration of taurine did not attenuate CHT inhibition (fixed concentration of CHT, 100 μM) of calcium uptake due to ATP and taurine together, suggesting a non-competitive kinetic relationship between CHT and taurine (Fig. 4). In a reverse type of experiment with the ~20 K protein (i.e. varying CHT concentrations, fixed taurine), a similar relationship between CHT and taurine, which increase and inhibit phosphorylation, respectively, was observed [8]. Increasing the CHT concentration was found to be ineffective in reversing the taurine inhibition of phosphorylation. Thus, CHT seems to have the consistent action of antagonizing the effects of taurine in a non-competitive manner, whether in calcium uptake or in protein phosphorylation, through a mechanism independent of PKC activity.

CHT has inhibitory activity against Na^+, K^+-ATPase, producing 81.7% inhibition at 100 μM concentration [7], a concentration that produced very similar levels of calcium uptake inhibition in our system (approximately 70–72%). Apparently, CHT requires the presence of ATP to produce inhibition (Fig. 1), and, in fact, inhibition was potentiated by an excess of ATP (Figs. 2 and 3). It is clear that the inhibitory activity of CHT is dependent upon the presence and perhaps the hydrolysis of ATP by ATPase. Because of this, the effects of taurine and CHT on total ATPase activity were studied.

Total ATPase activity was found to be inhibited by CHT, with and without taurine present, with IC_{50} values at around 141.7 and 87.9 μM CHT, respectively. Taurine did not produce a significant effect on ATPase activity as was shown before [17], but it did decrease significantly the potency of CHT (Fig. 6). It is worthwhile to note at this point that with CHT inhibition of calcium uptake, taurine did not cause a significant change in the potency of CHT (Table 1).

In the retina, the total ATPase activity, as measured, is comprised of the activity of many different types of ATP-hydrolyzing enzymes. Among the subtypes found in the retina are Na^+, K^+-ATPase, Ca^{2+}-activated, Mg^{2+}-dependent ATPase (Ca^{2+}, Mg^{2+}-ATPase) and Mg^{2+}-ATPase [18, 19]. There is also evidence of bicarbonate-stimulated ATPase activity [20], but almost certainly other types of ATPase activity are present. Depending on the specific ions present in the buffer, the activity of these enzymes and the other ATPases that may be present may change. Therefore, total ATPase activity was measured under the same buffer conditions as with the calcium uptake assay, to make certain that the same types of ATPase activity are functional in both assays.

The calcium uptake and the ATPase enzyme kinetic data together serve to support the proposal of an ATPase-independent mechanism of action behind the stimulatory effects of taurine on calcium uptake. Taurine was found to cause a significant decrease in the potency of ATP to induce calcium uptake (compare Fig. 2 with Fig. 3). In contrast, the addition of taurine produced an increase in the affinity of ATP substrate for the ATPase enzyme (Table 2). If the concept that ATPase activity is positively linked to calcium uptake is taken as accurate, then taurine should increase the potency of ATP to induce calcium uptake because it increases ATP-ATPase affinity. But because an opposite taurine effect was observed (i.e. taurine decreased the potency of ATP to induce calcium uptake), it seems that taurine acts to increase calcium uptake through a mechanism of action independent of its effects on ATPase.

The calcium uptake and the ATPase data taken together support the same idea. It was found that in the absence of taurine, CHT acts to inhibit calcium uptake and ATPase activity with similar potencies (Table 1: IC_{50} = 59.3 vs 87.9 μM), suggesting that ATPase inhibition is causally linked to the decrease of ATP-dependent calcium uptake. On the other hand, no such relationship could be found between calcium uptake and ATPase activity in the presence of taurine (IC_{50} = 22.7 vs 141.7 μM). In fact, taurine seems to cause opposite effects, significantly decreasing CHT inhibitory potency with ATPase activity (IC_{50} = 87.9 vs 141.7 μM) but producing a small but statistically insignificant increase in potency with calcium uptake (IC_{50} = 59.3 vs 22.7 μM). It is thus very possible that CHT does not act through ATPase inhibition to cause the inhibition of taurine-potentiated calcium uptake, but rather through a hitherto unknown mechanism of action, maybe the same ATPase-independent mechanism that taurine uses to increase calcium uptake.

Though it was not the purpose of the study to delineate all of the different types of ATPase activity in the retina, some characterization of the total ATPase activity was done. ATPase activity was studied using pharmacological agents instead of alteration of the ionic composition of the buffer, as is usually done [18], as this would result in activity that might not be present in the original buffer conditions. Ouabain and thapsigargin were used to inhibit Na^+, K^+-ATPase and SERCA, respectively. Na^+, K^+-ATPase was first studied because of the known effects of CHT to inhibit its activity. Ouabain has been used to inhibit Na^+, K^+-

ATPase in the rat retina using a bicarbonate-free system and was found to cause half-maximal inhibition at around 20 μM [11]. In our system, ouabain at 1 mM caused a decrease in total ATPase activity that was not significantly different from that of the control. CHT (in the absence of taurine) consistently produced inhibition greater than ouabain and so is shown, for the first time, to inhibit ATPase activity other than ouabain-sensitive Na^+, K^+-ATPase. Because all of the components of the whole cell were present in the sample, it can be assumed that some level of Na^+, K^+-ATPase activity was actually present and was being inhibited by ouabain. This activity, though, is probably a small percentage of the total ATPase activity that is functional given the ionic composition of the bicarbonate buffer used, hence the lack of significant effect of ouabain. Also, the preparation of the membranes from the whole cell could have resulted in the formation of sealed vesicles that do not allow for optimal binding of sodium and potassium to the ATPase enzyme, thereby decreasing activity. In any case, these results differ from other reports that show measurable levels of Na^+, K^+-ATPase activity in rat retina, a difference probably borne out of the variation in the buffer composition.

A non-mitochondrial Ca^{2+}, Mg^{2+}-ATPase has been examined in the bovine retina [19], and CHT might be inhibiting this type of ATPase in the rat retina. SERCA probably comprises a part, if not all, of this specific ATPase activity, though present literature does not allow for any certainty on the matter. SERCA is potentially an important component in the mechanism of action of taurine in calcium uptake because it is involved in the active uptake of calcium into the endoplasmic reticulum. Thapsigargin has been used to inhibit SERCA activity in brain microsomes [13, 14], but in our system it was found to have no effect on total ATPase activity in the rat retina. Again, it can be assumed that SERCA is active in the whole cell preparation and that thapsigargin at the high concentration used is inhibiting its activity. It might just be that, like Na^+, K^+-ATPase, SERCA activity makes up only a small component of the total ATPase activity that functions in the bicarbonate buffer used for calcium uptake.

What type or types of ATPase activity actually function in our system and whether this activity is involved in calcium uptake is unknown and constitutes another interesting field of study. As mentioned before, some form of bicarbonate-activated ATPase is functional with a 25 mM bicarbonate concentration in the retina [20]. Our system uses 50 mM bicarbonate, which probably activates the enzyme more. It is possible that this is the ouabain-insensitive ATPase that is inhibited by CHT. It is intriguing to correlate the bicarbonate-dependency of this enzyme with the bicarbonate-dependency of the potentiating effects of taurine on calcium uptake, but our data are not extensive enough to support this concept.

The effects of taurine on the K_m of ATP and on the inhibition of ATPase activity by CHT are interesting (Table 2). Taurine caused a decrease in the K_m (from 1.23 ± 0.14 to 0.78 ± 0.09 mM), indicating increased substrate–enzyme affinity. It is known that, within a certain range of concentrations, increased calcium modulates the activity of ATPases, specifically increasing Ca^{2+}, Mg^{2+}-ATPase and decreasing Na^+, K^+-ATPase activity [21]. The effect of taurine on the K_m could simply be a result of its effects to increase calcium uptake, though the direct effect of taurine on the enzyme cannot be ruled out. In fact, its effects to change the nature of the inhibition by CHT point to a direct effect on the molecule. Taurine causes the non-competitive kinetic relationship between CHT and ATP to become competitive. A molecular model involving a conformational change in the ATPase molecule is presented here (Fig. 9), showing ATP and CHT actually making use of the other's binding site after taurine binding brings the ATP and the CHT binding sites closer together. ATP affinity is increased with the conformational change, and CHT inhibition is changed from a non-competitive type to a competitive one.

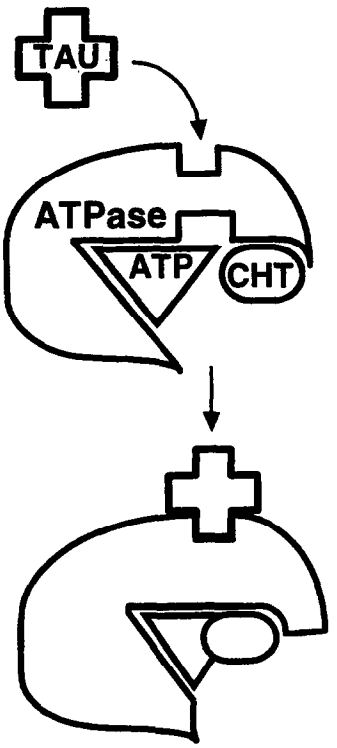

FIG. 9. Schematic diagram showing the model of taurine binding to the ATPase molecule and the change in the effect of CHT from a non-competitive to competitive type of inhibition.

This work was supported by the RGK Foundation of Austin, TX. We would like to thank the following for their gifts and assistance in procuring rat eyes: Dr. James C. Hutson, Dr. Howard K. Strahlendorf, Dr. John C. Fowler, Mrs. Janet Koss, and Dr. Ying Li. We would also like to thank Mrs. Josie Aleman for her assistance in preparing the manuscript.

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