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Effects of Benzophenanthridine Alkaloids on the Phosphorylation of an ~44 kDa Protein Present in a Mitochondrial Fraction of the Rat Heart

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ABSTRACT. Chelerythrine and sanguinarine, benzophenanthridine alkaloids that are known to have a wide variety of biologic actions including inhibitory activity against the phosphorylation of proteins, were tested for their effects on the phosphorylation of a specific ~44 kDa protein present in the mitochondrial fraction of the rat heart. The concentrations required for 50% inhibition were determined to be 90.3 and ~200 μM for chelerythrine and sanguinarine, respectively, while the median-effect concentrations were 71 and 98 μM for chelerythrine and 186 μM for sanguinarine. The combination index values, determined from median-effect plots, for the combination of chelerythrine and taurine in a ratio of 1:100 were greater than 1, which indicates that chelerythrine plus taurine is antagonistic. Both chelerythrine and sanguinarine had biphasic (i.e. stimulation and inhibition) effects on the phosphorylation of the ~44 kDa protein. It was determined that the biphasic effect for chelerythrine depended upon the time of preincubation at 37° of chelerythrine with the mitochondrial preparation. Preincubation times of 0.5 and 1 min produced 70 and 82% stimulation, while longer preincubation times of 2–22 min resulted in inhibition of the phosphorylation reaction by 40–95%. Dithiothreitol (DTT), a reducing agent, prevented the inhibitory effect of chelerythrine. Glutathione was less effective in protecting the phosphorylation of the ~44 kDa protein. It is suggested that the iminium bond of chelerythrine reacts with the thiol group on DTT, thereby preventing chelerythrine from reacting with thiol groups on the kinase responsible for phosphorylating the ~44 kDa protein. The inhibitory effects of taurine were only partially eliminated by DTT. BIOCHEM PHARMACOL 51;2:151–157, 1996.

KEY WORDS. chelerythrine; sanguinarine; taurine; protein phosphorylation; rat heart; mitochondria

Chelerythrine and sanguinarine, benzophenanthridine alkaloids isolated from a number of rutaceous and papaveraceous plants, have been studied extensively in a wide variety of biological systems. It has been demonstrated previously that both compounds have antimicrobial activity [1, 2], anti-inflammatory effects [3], and inhibitory activity against liver alanine aminotransferase [4] and brain Na^+, K^+-ATPase [5]. In addition, chelerythrine has been shown to be a potent and selective inhibitor of protein kinase C with an IC_{50} of 0.66 μM [6]. It has also been suggested that some of the other biologic properties of chelerythrine such as an inhibitor of [^{3}H]thymidine incorporation into DNA of fetal rat brain cell aggregates [7], cytoxicity to L-1210 tumor cells [6], anti-platelet activity [8], and its ability to reduce fever induced by lipopolysaccharide in rats [9] may be based on its inhibitory mechanism of action on protein kinase C.

Recently, we reported that chelerythrine stimulates the phosphorylation of an ~20 kDa protein present in the mitochondrial fraction of the rat retina [10]. The unique stimulatory action of chelerythrine on the phosphorylation of this protein could be eliminated by the addition of 10 mM DTT† to the incubation system [10]. Previously, we also observed that physiologic concentrations of exogenous taurine (34 mM) inhibit the phosphorylation of this protein by 50% [11]. The present studies were undertaken to test the effects of chelerythrine and sanguinarine on the phosphorylation of an ~44 kDa protein present in the mitochondrial fraction of the rat heart. The phosphorylation of this particular protein also appears to be regulated by taurine [12–14].

MATERIALS AND METHODS

[γ-^{32}P]ATP (30 Ci/mmol) was purchased from New England Nuclear, Boston, MA. Chelerythrine chloride was purchased from LC Laboratories, Woburn, MA. Sanguinarine chloride was obtained from the Sigma Chemical Co., St. Louis, MO.

Preparation of the Mitochondrial Subcellular Fraction of the Rat Heart

A mitochondrial subcellular fraction of rat heart was prepared as follows. Four grams of rat hearts were homogenized with a

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† *Abbreviations:* DTT, dithiothreitol; and GSH, glutathione.

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Polytron homogenizer (6 sec) in a Krebs-bicarbonate buffer (NaCl, 118 mM; KCl, 4.7 mM; $MgSO_4$, 1.17 mM; KH_2PO_4, 1.2 mM; and $NaHCO_3$, 25 mM; pH 7.4, 2°). Cell debris was removed by centrifuging at 400 *g* (2200 rpm) for 10 min. The supernatant was centrifuged at 2700 *g* (5000 rpm) for 10 min to collect the mitochondria. The mitochondrial pellet was then washed in the above buffer and recentrifuged at 2700 *g*. The mitochondrial pellet was suspended in the Krebs-bicarbonate buffer.

Phosphorylation Assay

The phosphorylation assay was performed in a total volume of 0.25 mL, which contained Krebs-bicarbonate buffer (as above), cardiac mitochondria (~1 mg), and alkaloid or taurine as designated. The incubation system was normally preincubated for 2 min at 37° in a shaking water bath, and the reaction was initiated with the addition of 10 μM (20 μCi) [γ-^{32}P]ATP. The incubation was continued for an additional 6 min at which time the reaction was stopped by the addition of 0.5 mL of gel electrophoresis sample buffer [50 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 2 mM mercaptoethanol, and 0.00125% bromophenol blue] and immediately boiled for 5 min.

PAGE and Autoradiography

Aliquots of the incubation mixture were subjected to one-dimensional PAGE on 12% gels according to the procedures of Laemmli [15]. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R, destained overnight, and dried. X-ray film was placed on the dried gels to visualize the incorporation of radioactive phosphate into the various proteins. The film was developed after 1–2 days at –80°. The amount of radioactive phosphate in the ~44 kDa phosphoprotein was quantitated by densitometry (Bio-Rad scanning densitometer, model 1650).

Dose–Effect Analysis

The median-effect equations and their plots derived by Chou and Talalay [16, 17] were used to calculate the median dose–effects and the combination index values of the benzophenanthridine alkaloids and taurine on the phosphorylation of the ~44 kDa protein. Briefly, the equations are as follows:

$$\frac{fa}{fu} = \left(\frac{D}{D_m}\right)^m \tag{1}$$

or

$$\log(fa/fu) = m \log D - m \log D_m$$

where *fa* and *fu* are the decimal fractions of activities of the phosphorylation of an ~44 kDa protein affected and unaffected by a dose (*D*) of a drug. D_m is the median-effect dose (e.g. IC_{50}) and *m* signifies the shape of the dose–effect curve ($m = 1$, >1, and <1 indicate hyperbolic, sigmoidal, and negative sigmoidal curves, respectively). A plot (the median-effect plot) of $y = \log(fa/fu)$ versus $x = \log(D)$ gives a slope of *m*, and the antilog of the x-intercept gives the D_m value. The *m* and D_m parameters for each drug and their mixtures are substituted into the multiple drug effect equation [16, 17] for mutually exclusive inhibitors:

$$\frac{(fa)_{1,2}}{(fu)_{1,2}} = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} \tag{2}$$

where $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and drug 2 that produce x% inhibition and at which $(D)_1$ and $(D)_2$ in combination also inhibit by x%. By definition, $fa + fu = 1$, ($fu = 1 - fa$). In the special circumstance in which $(fa)_{1,2} = 0.50$, equation (2) simplifies to the following equation:

$$\frac{(fa)_{1,2}}{(fu)_{1,2}} = \frac{(D)_1}{(D_m)_1} + \frac{(D)_2}{(D_m)_2} \tag{3}$$

and, therefore

$$\frac{(D)_1}{(D_m)_1} + \frac{(D)_2}{(D_m)_2} = 1 \tag{4}$$

Equation 4 is the classical isobologram for ED_{50}, where $(D)_1$ and $(D)_2$ are fractions of the median-effect dose (50% inhibition). For a general case, the combination index (CI) is defined by:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} \tag{5}$$

where CI = 1, <1, and >1, indicating summation, synergism, and antagonism, respectively. The calculation of CI is carried out as follows. Rearrangement of Equation 1 gives

$$D = D_m [fa/(1 - fa)]^{1/m} \tag{6}$$

Thus, when the parameters D_m and *m* are determined from the median-effect plot (see above), the dose (*D* or D_x) required for any degree of effect (*fa*) can be calculated. These calculated doses are then used in Equation 5 for the calculation of CI to determine summation, synergism, or antagonism.

RESULTS

Effects of Chelerythrine and Sanguinarine on the Phosphorylation of the ~44 kDa Protein Present in a Mitochondrial Fraction of the Rat Heart

A representative autoradiogram obtained from one-dimensional PAGE demonstrates the inhibitory effect of various concentrations of chelerythrine on the phosphorylation of the ~44 kDa protein present in the mitochondrial subcellular fraction of the rat heart (Fig. 1). The top panel of Fig. 2 shows the concentration–response effects of chelerythrine and sanguinarine, while the bottom panel depicts the median-effect plots for both alkaloids. The concentrations of chelerythrine and sanguinarine required to inhibit the phosphorylation of the ~44 kDa protein by 50% (IC_{50}) were 90.3 ± 13.1 and ~200 μM,

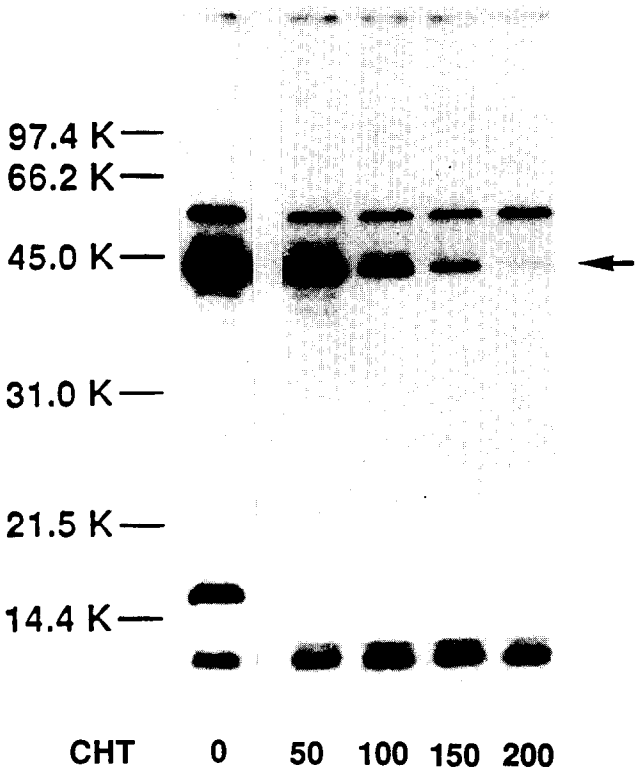

FIG. 1. Concentration–response of chelerythrine (CHT) on the phosphorylation of an ~44 kDa protein present in a mitochondrial fraction of the rat heart (representative autoradiogram). The concentrations of CHT were varied from 0 to 200 μM. Conditions of the phosphorylation assay, one-dimensional polyacrylamide electrophoresis, and autoradiography are described in Materials and Methods. The mitochondrial preparation was preincubated with CHT for 2 min. Location of the ~44 kDa phosphoprotein is designated by the arrow. Marker proteins with molecular weights ranging from 14.4 to 97.4 kDa are indicated.

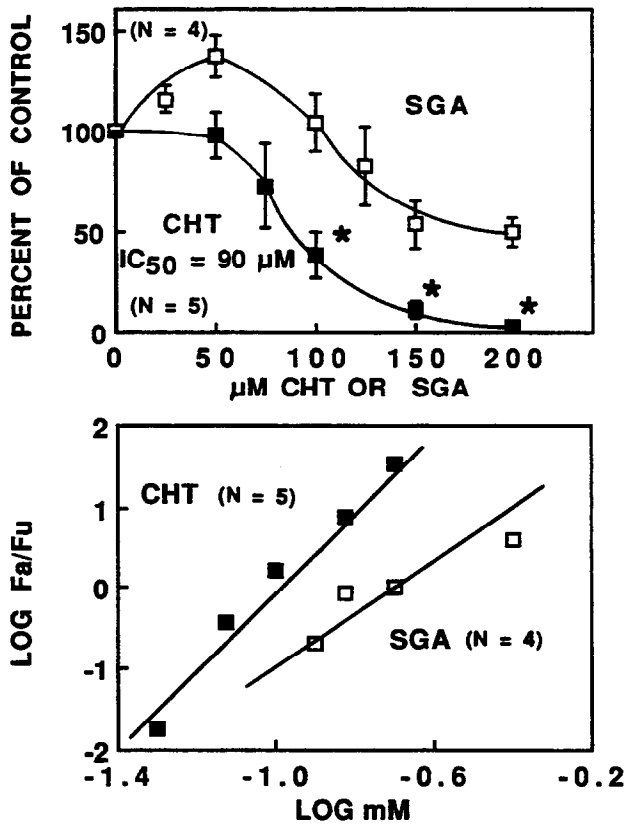

FIG. 2. Effects of chelerythrine (CHT) and sanguinarine (SGA) on the phosphorylation of an ~44 kDa protein present in the mitochondrial fraction of the rat heart. Data generated by densitometry measurements of autoradiographs are presented as means ± SEM. Top panel: concentration–response relationships of the effects of CHT and SGA. The concentration necessary to inhibit the phosphorylation of the ~44 kDa protein was determined to be 90.3 ± 13.1 μM. Bottom panel: median-effect plot for the effects of CHT and SGA. The number of experiments is designated in the parentheses. The mitochondrial preparation was preincubated with CHT or SGA for 2 min. Statistical differences (designated by asterisks: $P < 0.05$) between the control (0 CHT) and various concentrations of CHT were determined by ANOVA and the Duncan's multiple range test.

respectively (Table 1). At low concentrations (≤50 μM), sanguinarine appeared to stimulate the phosphorylation of the ~44 kDa protein.

Median-effect concentrations (D_m) for both chelerythrine and sanguinarine were also calculated from the median-effect equation and are reported in Table 1. In two series of experiments (N = 5 and 4), the D_m for chelerythrine was calculated to be 98 and 71 μM.

Combined Effects of Taurine and Chelerythrine on the Phosphorylation of the ~44 kDa Protein

Median-effect plot parameters and combination index values for the combined effects of taurine and chelerythrine were also determined by analysis of the concentration–effect relationships. The median-effect plots of the effects of chelerythrine, taurine, and a constant ratio of chelerythrine and taurine (1:100) are shown in Fig. 3. The plot parameters and combination index values calculated from the median-effect plot and accompanying formulas that were developed by Chou and Talalay [16, 17] are reported in Table 1. The combination index values for 30, 50, and 70% saturation are greater than 1 (2.36, 2.33, and 2.91), which is indicative of an antagonistic relationship.

Effects of Reducing Agents in Combination with Chelerythrine on the Phosphorylation of the ~44 kDa Protein

The effects of chelerythrine, DTT and the combined effects of chelerythrine and DTT on the phosphorylation of the ~44 kDa protein are shown in Fig. 4. DTT had no effect on the phosphorylation reaction. However, DTT completely blocked the inhibition produced by 100 μM chelerythrine. In this study, the phosphorylation reaction was initiated after a 2-min preincubation. In contrast, the data presented in Fig. 5 show the effects of chelerythrine, DTT, and GSH after a preincu-

TABLE 1. IC_{50} Values (concentrations required to inhibit by 50%), median-effect plot parameters, and combination index values for the benzophenanthridine alkaloids and the interactions of taurine and chelerythrine on the phosphorylation of the ~44 kDa protein present in a rat heart mitochondrial fraction

Compounds	IC_{50} (μM)	Median-effect plot parameters*			Combination index values†		
		Median-effect concentration (D_m) (μM)	Slope (m)	Linear correlation coefficient (r)	30% Saturation	50% Saturation	70% Saturation
Chelerythrine (CHT)	90.3 ± 13.1	98	5.235	0.981			
Sanguinarine (SGA)	~200	186	3.117	0.713			
Taurine (TAU)	9100 ± 3500	7800	0.683	0.964			
Chelerythrine (CHT)		71	2.699	0.981			
CHT + TAU (1:100)		8760	0.973	0.321	2.36	2.33	2.91

Values were calculated from pooled data obtained from four or five experiments. Where ± values are given, they represent means ± SEM.

* Median-effect plot parameters, D_m, m, and r, signify the potency, the shape of the dose-effect curve, and the applicability of the median-effect principle, respectively, as described by Chou and Talalay [16, 17].

† Combination index values <1, =1, and >1 indicate synergism, summation, and antagonism, respectively.

bation of 22 min. In this series of experiments, chelerythrine inhibited the phosphorylation reaction by 95%. Again, DTT alone had no effect while completely blocking the effects of chelerythrine. DTT in the presence of chelerythrine slightly increased the phosphorylation of the ~44 kDa protein over the control value. GSH was not as effective in restoring the activity of the phosphorylation reaction against the inhibitory activity of chelerythrine.

Biphasic Effects of Chelerythrine on the Phosphorylation of the ~44 kDa Protein

Because of our previous published findings that chelerythrine stimulates the phosphorylation of an ~20 kDa protein present in the mitochondrial fraction of the retina [10], we were interested in determining if there were any conditions that would produce the same stimulatory results in the mitochondrial preparation of the rat heart. Unexpectedly, when we exposed the heart mitochondrial preparation to chelerythrine (100 μM) for short preincubation times of 0.5 and 1 min, we noted that the phosphorylation reaction was stimulated by 70 and 82% (Fig. 6). Longer preincubation times of 2–22 min inhibited the phosphorylation by 40–95% (Fig. 6).

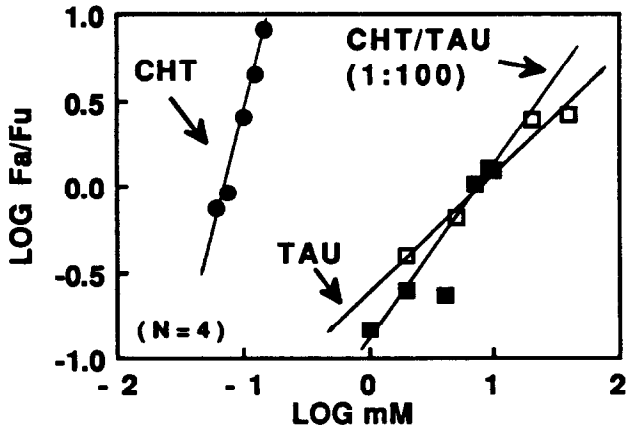

FIG. 3. Median-effect plots of chelerythrine (CHT), taurine (TAU), and the combination of CHT and TAU (1:100) on the phosphorylation of an ~44 kDa protein present in a mitochondrial fraction of the rat heart. The mitochondrial preparation was preincubated with CHT, TAU, and CHT plus TAU for 2 min. The number of experiments is designated in the parentheses.

Effects of DTT on the Inhibition by Taurine of the Phosphorylation of the ~44 kDa Protein

A 20 mM concentration of taurine inhibited the phosphorylation of the ~44 kDa protein by 65% (Fig. 7). The inhibitory effect of taurine could only be partially blocked by 10 mM DTT.

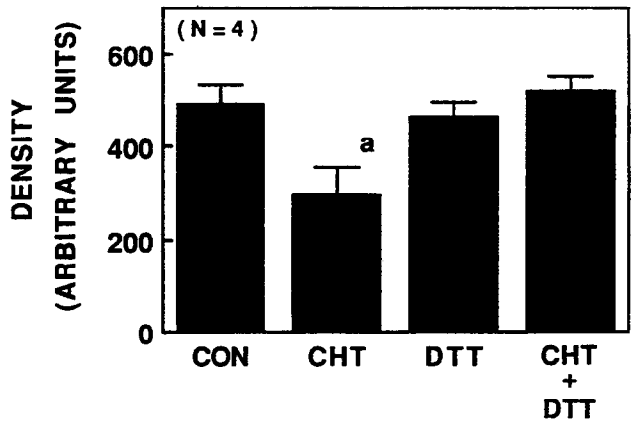

FIG. 4. Effect of DTT in combination with CHT on the phosphorylation of an ~44 kDa protein present in the mitochondrial fraction of the rat heart. CHT (100 μM), DTT (10 mM), and CHT (100 μM) plus DTT (10 mM) were preincubated with the mitochondrial preparation for 2 min at 37°. Data are presented as means ± SEM. The number of experiments is designated in parentheses. Statistical differences were determined by ANOVA and the Duncan's multiple range test. The mean with a different superscript (a) was significantly different from the other means ($P < 0.05$).

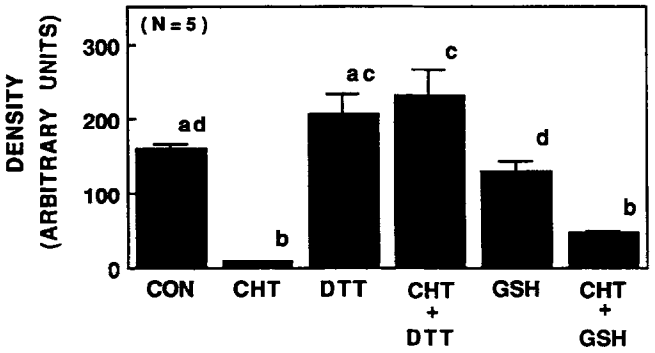

FIG. 5. Effects of reducing agents in combination with CHT on the phosphorylation of an ~44 kDa protein. CHT (100 μM), DTT (10 mM), GSH (10 mM), and CHT plus DTT or GSH were preincubated with the mitochondrial preparation for 22 min at 37°. Data are presented as means ± SEM. The number of experiments is designated in parentheses. Statistical differences were determined by ANOVA and the Duncan's multiple range test. Means with different superscripts were significantly different from each other ($P < 0.05$).

DISCUSSION

The effects of taurine on protein phosphorylation were first observed in 1985 [18]. In that report, it was demonstrated that approximately 30 mM taurine inhibits by 50% the phosphorylation of an ~20 kDa protein present in a mitochondrial fraction of the rat retina. In addition to the effect of taurine on protein phosphorylation in the retina, our laboratory has demonstrated in a P_2 fraction prepared from the cortex of the rat brain that taurine inhibits a Ca^{2+}-dependent, protein kinase C-catalyzed phosphorylation of an ~20 kDa protein [19–21]. It should be noted that the ~20 kDa phosphoprotein described for the brain appears to be a different protein than that reported for the retina. Sturman and Gargano [22] reported in 1990 that taurine also affects the *in vitro* phosphorylation of cat cortical membranes during development.

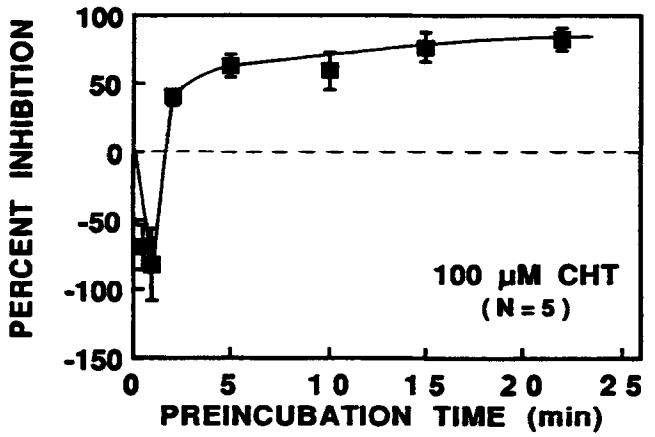

FIG. 6. Biphasic effect of CHT on the phosphorylation of an ~44 kDa protein present in the mitochondrial fraction of the rat retina. The heart mitochondrial fraction was preincubated with CHT (100 μM) for 0–22 min. Data are presented as means ± SEM. The number of experiments is designated in parentheses.

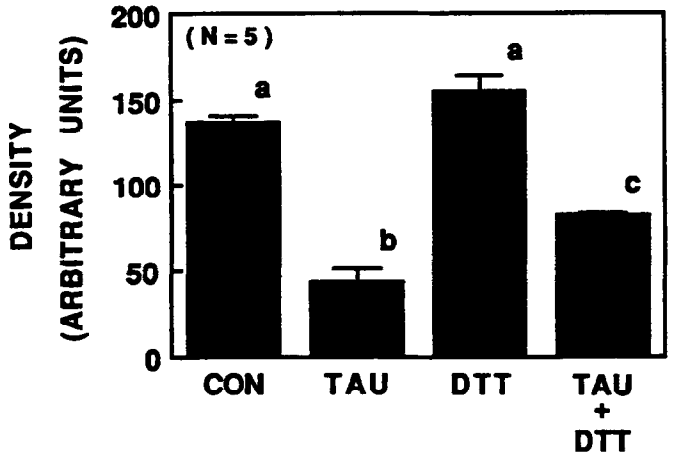

FIG. 7. Effects of TAU (20 mM), DTT (10 mM), and the combination of TAU (20 mM) plus DTT (10 mM) on the phosphorylation of the ~44 kDa protein present in a mitochondrial fraction of the rat heart. TAU, DTT, and TAU plus DTT were preincubated with the mitochondrial preparation for 2 min at 37°. Experimental conditions are described in Materials and Methods. Data are presented as means ± SEM. The number of experiments is denoted in parentheses. ANOVA and Duncan's multiple range test were used to determine significant differences between means. Means with different superscripts were significantly different from each other ($P < 0.05$).

In subsequent reports, we have shown that taurine (approximately 10 mM) inhibits the phosphorylation of an ~44 kDa protein present in the mitochondrial fraction of the rat heart [12–14, 23]. In this regard, Schaffer and colleagues [24] have also described a potential regulatory role for taurine as an inhibitor of an ~44 kDa protein (also ~59 kDa and ~190 kDa proteins) present in a rat heart sarcolemmal preparation. However, in the sarcolemmal preparation, the phosphorylation of the ~44 kDa protein appears to be regulated by calmodulin, while in our heart mitochondrial fraction calmodulin had no effect (data not shown). Instead, the ~44 kDa protein that we are studying was both inhibited and stimulated by chelerythrine.

Walterová and colleagues [4] initially reported that chelerythrine and sanguinarine were enzyme inhibitors, specifically of liver alanine aminotransferase, and that the inhibitory activity depended upon the reaction of the iminium bond of the quaternary cation on the alkaloids with the thiol groups of the enzyme. In addition, they demonstrated that reducing agents with thiol groups such as thioethanol and GSH partially protected the enzyme against the inhibitory action of chelerythrine and sanguinarine. These results suggested that the iminium bond of the alkaloids most likely reacts with the thiol group of the reducing agents rather than the thiol group of the enzyme. In our studies, we also demonstrated that chelerythrine and sanguinarine have an inhibitory effect on enzyme activity—presumably a kinase present in the mitochondrial fraction of the rat heart that phosphorylates the ~44 kDa protein. In addition, we observed that chelerythrine can stimulate the phosphorylation of the ~44 kDa protein as was reported recently for an ~20 kDa protein present in a mitochon-

drial fraction of the rat retina [10]. The biphasic effect depended upon the length of time that chelerythrine was incubated at 37° with the heart mitochondrial preparation. Short incubation times of 0.5–1 minute produced 70–82% stimulation while longer times of 2–22 min resulted in 40–95% inhibition. DTT completely blocked the inhibitory action of chelerythrine presumably by the same mechanism as suggested by Walterová and colleagues [4] for alanine aminotransferase, i.e. by reacting with the iminium group of chelerythrine.

The biphasic effect of chelerythrine on the phosphorylation of the ~44 kDa protein, i.e. stimulatory effect up to 2 min of preincubation and inhibitory effect from 2 min to longer time periods, is difficult to explain. However, it is possible that there are two sites for the binding of chelerythrine to the kinase responsible for the phosphorylation of the ~44 kDa protein—a regulatory site that is stimulatory and a catalytic side that is inhibitory. In this hypothesis, the inhibitory effect with chelerythrine binding to the inhibitory (catalytic) site predominates with time.

Alternatively, there may be only one binding site for chelerythrine with a requirement for a finite period of time to pass before the chelerythrine binds covalently to the responsible kinase and modifies the enzyme in an irreversible manner. Preincubation of chelerythrine with the kinase for short time periods alters the kinase in such a manner as to produce a stimulatory effect.

The physiological function of the inhibitory effect of taurine on protein phosphorylation is only speculation at this time. However, phosphorylation (or dephosphorylation) of specific proteins has been recognized as a final common pathway of signal transduction. Therefore, modulation of the phosphorylation of a particular protein, such as the ~44 kDa protein present in the mitochondrial fraction of the rat heart, could result in modification of cellular responses regulated through a specific pathway.

The results of the present studies support a regulatory role for taurine in excitable tissues, such as the heart. Further studies are obviously warranted to define the physiologic meaning of the effects of taurine on the phosphorylation of specific proteins in the rat heart. The precise molecular explanation must await complete purification of both the ~44 kDa protein and the kinase responsible for its phosphorylation.

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