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PARTIAL CHARACTERIZATION OF AN ~20 K M_r RETINAL PROTEIN WHOSE PHOSPHORYLATION IS INHIBITED BY TAURINE

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Abstract—It has been demonstrated previously that taurine (2-aminoethanesulfonic acid) is an inhibitor of protein phosphorylation in a mitochondrial fraction of the rat retina. It appears that taurine is most effective in inhibiting the phosphorylation of an ~20 K apparent molecular weight (M_r) protein found in the retinal tissue. This study further characterizes the location of the ~20 K phosphoprotein by phase separation using Triton X-114 and also characterizes the nature of the phosphate bond by various solvent extractions and by exposure to acid and base conditions. Triton X-114 experiments indicated that the ~20 K phosphoprotein is located in the aqueous phase and, consequently, is probably not an integral protein of the mitochondrial membranes. Treatment of the phosphoprotein with solvents, acid, and/or base determined the phosphate linkage to be through a phosphoester bond rather than an acylphosphate bond. The ~20 K M_r phosphoprotein was also isolated from one-dimensional polyacrylamide gels and subsequently digested with trypsin and hydrolyzed with 1 M HCl to break all peptide bonds. Analysis of the phosphoamino acids by two-dimensional high voltage electrophoresis on cellulose plates revealed that it is both the serine and threonine residues that are phosphorylated. However, phosphorylation of the serine residue(s) is predominant.

While the physiological actions of taurine (2-aminoethanesulfonic acid) in the retina and other excitable tissues such as brain and heart are myriad [1, 2], the exact mechanism(s) of action of this ubiquitous sulfur compound in mammalian tissues is unknown. However, one possible mechanism of action for taurine at the molecular level is its inhibitory effects on the phosphorylation of specific proteins found in the retina [3–5], brain [6–8], and heart [9–11].

Recent studies have demonstrated that taurine inhibits the phosphorylation of an ~20 K apparent molecular weight (M_r)† protein present in the mitochondrial fraction of the rat retina [12]. We have also reported previously that taurine inhibits the phosphorylation of an ~20 K M_r protein present in the rat cortex [6–8]. However, the two phosphoproteins in the retina and brain appear not to be identical based on slightly different electrophoretic patterns, different isoelectric points, and differences in the effects of kinase activators [12]. In addition, taurine inhibits the phosphorylation of a series of proteins found in cardiac tissue with apparent molecular weights other than ~20 K [9, 10].

A second potential mechanism of action for taurine is its role in Ca^{2+} modulation. In general, it has been demonstrated that taurine has a biphasic effect on Ca^{2+} uptake, i.e. increases Ca^{2+} uptake at low Ca^{2+} concentrations [13] but prevents Ca^{2+} accumulation in situations of high Ca^{2+} concentrations in the retina [14] and in the heart as in the Ca^{2+} paradox model [15, 16]. Furthermore, we have suggested, at least for the retinal and brain tissues, that the stimulatory effects of taurine on Ca^{2+} uptake and the inhibitory effects of taurine on protein phosphorylation may be causally related [5, 8].

The present studies partially characterize the ~20 K M_r protein found in a mitochondrial fraction of the rat retina whose phosphorylation is inhibited by taurine. Experiments utilizing Triton X-114 were performed to determine if the ~20 K M_r phosphoprotein is an integral membrane protein. Also, the ~20 K M_r phosphoprotein and the labeled phosphate moiety within the ~20 K M_r protein were subjected to various solvent extractions, digestive enzymes, and exposure to acid and base conditions (both at 2° and 100°) to determine if the phosphate is indeed incorporated into protein and if the linkage is through either a phosphoester bond or an acylphosphate bond. Identification of the specific phosphorylated amino acids has also been ascertained.

MATERIALS AND METHODS

Chemicals. Taurine, pronase, and ribonuclease A were purchased from the Sigma Chemical Co. [γ-^{32}P]ATP (30 Ci/mmol) was obtained from New England Nuclear. Cellulose chromatography sheets (20 × 20 cm) were purchased from the Eastman Kodak Co.

Preparation of the mitochondrial subcellular

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† Abbreviations: M_r, apparent molecular weight; ROSs, rod outer segments; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; and TCA, trichloroacetic acid.

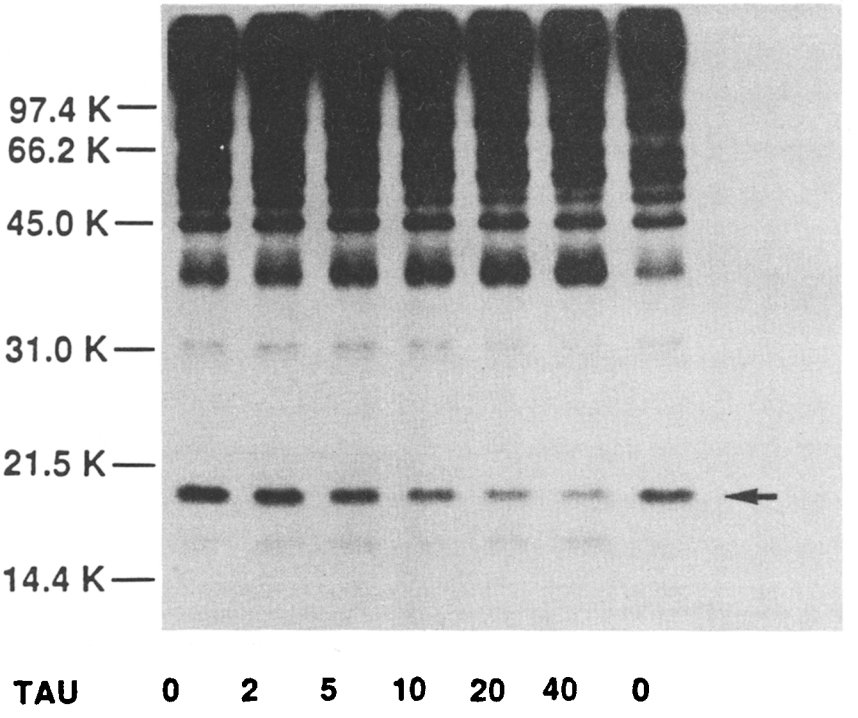

Fig. 1. Autoradiographs of the concentration–response relationship of the effect of taurine on the phosphorylation of an ~20 K M_r protein in a mitochondrial preparation of the rat retina (representative experiment). The concentration of taurine was varied from 0 to 40 mM. (Lane 7 is an additional 0 taurine control.) Conditions of the phosphorylation assay and polyacrylamide gel electrophoresis are described in Materials and Methods. Location of the ~20 K M_r phosphoprotein is designated by the arrow. Marker proteins with molecular weights ranging from 14.4 to 97.4 K are indicated. TAU = taurine.

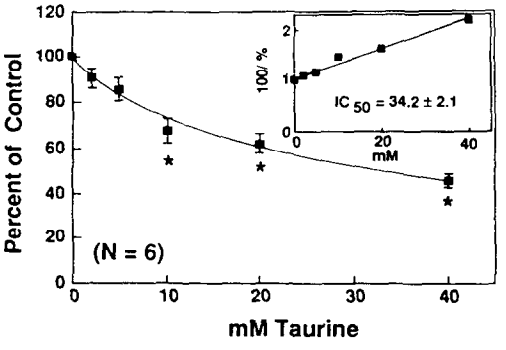

Fig. 2. Concentration–response relationship of the effect of taurine on the phosphorylation of an ~20 K M_r protein in a mitochondrial preparation of the rat retina. Data generated by densitometry measurements of the autoradiographs are presented as means ± SEM and represent 6 experiments. The inset contains the data plotted as the reciprocal of the per cent of control (×100) vs the concentration of taurine (mM). The concentration of taurine necessary to inhibit the phosphorylation of the ~20 K M_r protein by 50% was calculated to be 34.2 ± 2.1 mM. Statistical differences (designated by asterisks) between the control (0 taurine) and various concentrations of taurine were determined by non-parametric Kruskal–Wallis one-way analysis of variance ($P < 0.05$).

fraction. The mitochondrial fraction of the retinas was prepared as previously described [17]. Briefly, 6–10 adult Wistar rats (175–225 g) were killed by decapitation. The eyes were immediately removed and placed in ice-cold 0.3 M mannitol, pH 7.4. The retinal tissue, maintained at ice temperatures for this and all subsequent procedures, was teased out of the eye cup and placed in the mannitol solution. The rod outer segments (ROSs) were removed by vortex-mixing the retina for 6 sec, allowing the tissue to settle, and then decanting (and discarding) the supernatant which contained the ROS. The remaining tissue components were gently hand-homogenized with 10 up-and-down strokes in a Potter–Elvehjem homogenizer and centrifuged at 150 *g* for 15 min to remove cell debris. The supernatant was centrifuged at 12,500 *g* for 15 min and the pellet was suspended in the mannitol solution. The tissue preparation (minus the ROSs) was then layered on a discontinuous Ficoll gradient (8, 16, and 20% in 0.3 M mannitol) and centrifuged at 63,000 *g* for 1 hr in a swinging bucket rotor. The pellet at the bottom of the gradient contained mitochondria. The mitochondria were suspended in a bicarbonate buffer ($NaHCO_3$, 50 mM; NaCl, 50 mM; KCl, 50 mM; KH_2PO_4, 1.2 mM; $MgCl_2$, 2 mM; $CaCl_2$, 10 μM, pH 7.4) as described by Kuo and Miki [18].

Phosphorylation assay and polyacrylamide gel electrophoresis (PAGE). The incubation system

utilized to phosphorylate the proteins of the mitochondrial fraction of the rat retina is described in Ref. 3. The incubation mixture containing bicarbonate buffer, mitochondrial fraction (~0.1 mg protein), and taurine when indicated was pre-incubated for 2 min in a shaking water bath at 37°, and the phosphorylation reaction was started by the addition of [γ-^{32}P]ATP (20 μCi, 10 μM). The system was incubated for 6 min which was determined to be in the linear time range for phosphorylation and the optimal time for the inhibitory effect of taurine. The reaction was then stopped by adding 0.5 mL of gel electrophoresis sample buffer [60 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 2 mM mercaptoethanol, and 0.00125% bromophenol blue] and immediately boiling for 5 min or the reaction was stopped as described below. Aliquots of the incubation mixture were subjected to PAGE on 12% gels according to the method of Laemmli [19]. The gels were dried and exposed to X-ray film to visualize the incorporation of radioactive phosphate into the various proteins.

Triton X-114 phase separation. Phosphorylation of the mitochondrial ~20 K M_r protein was accomplished as described above. The reaction was stopped by adding 0.1 M nonradioactive ATP and 0.3 M EDTA (final concentrations 0.01 and 0.03 M, respectively). Phase separation of the ~20 K M_r phosphorylation was accomplished by following the procedure of Bordier [20] with modifications. Briefly, the retinal mitochondrial preparation was phosphorylated as described above. The protein in the incubation mixture was then solubilized with Triton X-114 (TX-114, 2%; NaCl, 300 mM; and Tris–HCl, 10 mM, pH 7.4) at 2° for 5 min during which a faint precipitate could be observed. This detergent-insoluble precipitate was removed by centrifuging at 40,000 *g* for 15 min at 2° and saved. The supernatant was placed over a cushion of 0.25 M sucrose containing Triton X-114 (0.06%), NaCl (150 mM), and Tris–HCl (5 mM, pH 7.4). The sample and the sucrose cushion were maintained at 30° for 3 min and then centrifuged at 2500 *g* for 5 min. The supernatant (above the sucrose cushion) was designated the aqueous phase, and the oily residue (below the sucrose cushion) was designated the detergent phase. The aqueous phase, detergent phase, and original detergent-insoluble precipitate were boiled in gel electrophoresis sample buffer for 5 min prior to PAGE.

Characterization of the phosphate bond in the ~20 K M_r phosphoprotein. Phosphorylation of the mitochondrial ~20 K M_r protein was accomplished as described above. A series of duplicate incubation systems containing bicarbonate buffer and the mitochondrial fraction were phosphorylated with [γ-^{32}P]ATP. The reaction was stopped with 10% trichloroacetic acid (TCA), and the incubation systems were maintained at 2° for 20 min. The incubation systems were then centrifuged for 10 min at 10,000 *g*. The treatments to determine if radioactive phosphate was incorporated into the ~20 K M_r protein and to characterize the type of phosphate bond that was formed are described by Moore *et al.* [21]. Briefly, the TCA precipitates were subjected to one of five treatments as follows: (a) extracted three times with acetone; (b) extracted three times with chloroform:methanol (2:1) followed by acetone extraction as above; (c) suspended in 0.5 M NaOH (2°) for 5 min, re-precipitated with TCA, centrifuged, and the precipitate extracted with acetone; (d) suspended in 0.5 M NaOH and boiled for 1 min, 4 min at 2°, re-precipitated with TCA, centrifuged, and the precipitate extracted with acetone; (e) suspended in 10% TCA and boiled for 1 min, 4 min at 2°, centrifuged, and the precipitate extracted with acetone. After these treatments the precipitates were boiled in PAGE sample buffer for 5 min. In addition, two of the original TCA precipitates were treated as follows: extracted with acetone (three times), PAGE sample buffer added, incubated with either pronase (0.04 mg) or ribonuclease A (0.05 mg) for 10 min at 37°, and then boiled for 5 min. All samples were subjected to one-dimensional PAGE.

Phosphoamino acid analysis. Phosphorylation of the mitochondrial ~20 K M_r protein was accomplished as described above. Analysis of the specific phosphorylated amino acid was determined by the procedure of Lau [22]. The ~20 K M_r phosphoprotein was isolated from one-dimensional PAGE gels by cutting the gel in the region that corresponds to the radioactivity and placing this portion of the gel in 50 mM NH_4HCO_3 containing 0.15 mg of trypsin. This mixture was then incubated for 24 hr at 37° to elute the ~20 K M_r radioactive phosphoprotein from the gel and for partial digestion. The mixture was subsequently lyophilized and then hydrolyzed (2 hr, 110°) with 1 mL of 6 N HCl under vacuum. The hydrolysate was lyophilized to dryness and the residue taken up in 0.02 mL H_2O. Two-dimensional high voltage electrophoresis was performed on cellulose mylar plates (20 × 20 cm) to separate the phosphoamino acids. The first dimension consisted of formic acid : acetic acid : H_2O (1 : 10 : 89), pH 1.9, 600 V, 3 hr; the second dimension consisted of acetic acid : pyridine : H_2O (19 : 1 : 89), pH 3, 600 V, 2 hr. Standard phosphoamino acids (phosphoserine, phosphothreonine, and phosphotyrosine) were detected with 1% ninhydrin in acetone. Autoradiography using X-ray film was used to determine the location of the radioactive phosphoamino acids.

RESULTS AND DISCUSSION

The original observation in 1985 that taurine inhibits the phosphorylation of specific proteins in a rat retinal preparation [3] has led us and others [10] to pursue this phenomenon as a possible role for taurine in excitable tissues. We have reported previously that 20 mM taurine inhibits the phosphorylation of an ~20 K M_r protein present in a mitochondrial fraction of the rat retina [12]. In the present studies a concentration–response relationship was determined for taurine to calculate the concentration of taurine necessary to inhibit the phosphorylation of the ~20 K M_r protein by 50%. Experiments were performed with Triton X-114 to determine if the ~20 K M_r phosphoprotein present in the mitochondrial fraction is an integral membrane protein or a soluble protein. The nature of the phosphate bond was analyzed by exposure of the

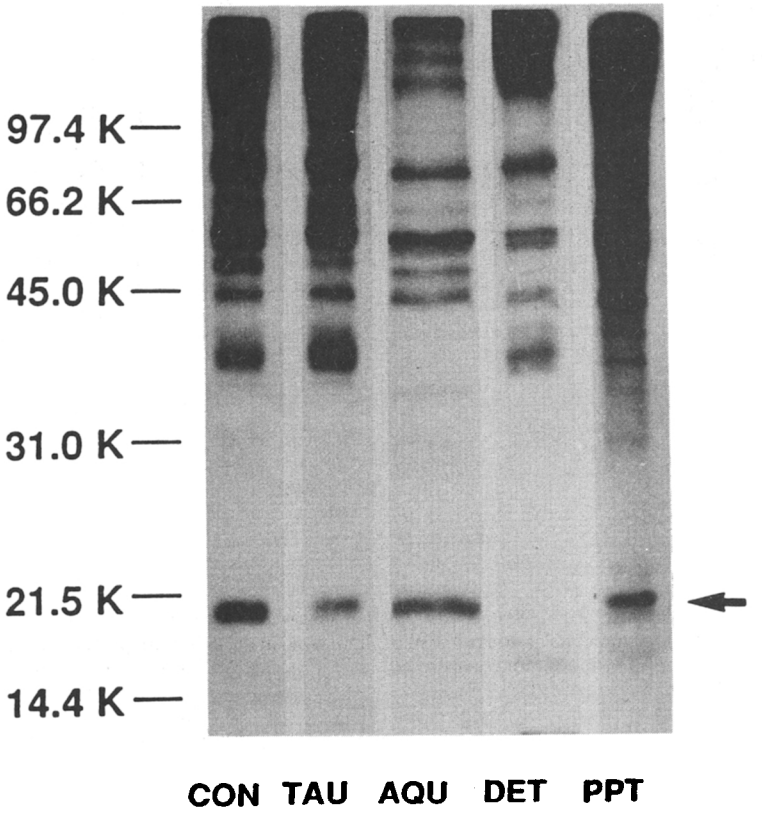

Fig. 3. Autoradiographs of a polyacrylamide gel electrophoretic analysis of proteins from a mitochondrial preparation of rat retina after phase separation with Triton X-114 (representative experiment). The location of the ~20 K M_r phosphoprotein is designated by the arrow. The samples in lanes 1 and 2 were obtained from incubation systems that were stopped by boiling directly in SDS sample buffer. (The conditions of lanes 1 and 2, i.e. ±taurine, were included in the experiment as controls to ensure that the inhibitory effect of taurine was present.) The samples in lanes 3–5 were obtained from incubation systems that were stopped by the addition of 0.1 M ATP and 0.3 M EDTA as described in Materials and Methods. Marker proteins with molecular weights ranging from 14.4 to 97.4 K are indicated. Abbreviations: CON = control, TAU = taurine (20 mM), AQU = aqueous phase, DET = detergent phase, and PPT = detergent-insoluble precipitate.

~20 K M_r phosphoprotein to organic solvents, hot and cold base and acid, and enzymes that degrade protein and ribonucleic acid. Also, the specific phosphorylated amino acid was determined after isolation of the ~20 K M_r phosphoprotein and hydrolysis of the peptide bonds.

Concentration–response relationship for taurine. The concentration–response relationship for the effect of taurine on the phosphorylation of the ~20 K M_r protein is shown in a representative autoradiograph (Fig. 1). The concentration of taurine required to inhibit the phosphorylation of the ~20 K M_r protein by 50% (IC_{50}) was 34.2 ± 2.1 mM (Fig. 2). This value for the IC_{50} was calculated from the reciprocal plot of the inhibitory activity versus the concentration of taurine as shown in the inset (Fig. 2).

Phase separation of the ~20 K M_r *phosphoprotein.* Phosphorylation of the ~20 K M_r phosphoprotein in the absence of taurine is shown in lane 1 (Fig. 3) and in the presence of 20 mM taurine is shown in lane 2. Phase separation with TX-114 indicated that the ~20 K M_r phosphoprotein is a soluble protein found in the aqueous fraction (lane 3, Fig. 3). No evidence was observed to indicate that any of the ~20 K M_r phosphoprotein partitioned into the detergent phase (lane 4), and thus these data initially suggest that the ~20 K M_r phosphoprotein is not an integral membrane protein in the mitochondrial preparation.

The ~20 K M_r phosphoprotein was also present in the detergent-insoluble precipitate (lane 5, Fig. 3). It has been reported previously that membrane proteins are not solubilized completely with Triton X-114 [23, 24]. For example, Bürgisser and Matthieu [24] noted that 33% of rabbit myelin basic protein remained in the insoluble fraction when phase separation experiments were performed. Accordingly, the results of our partition experiments with the retinal ~20 K phosphoprotein do not differ from published observations on other proteins in that our phosphoprotein is also not completely extracted into the aqueous or detergent phase but an appreciable portion remains in the detergent-insoluble precipitate.

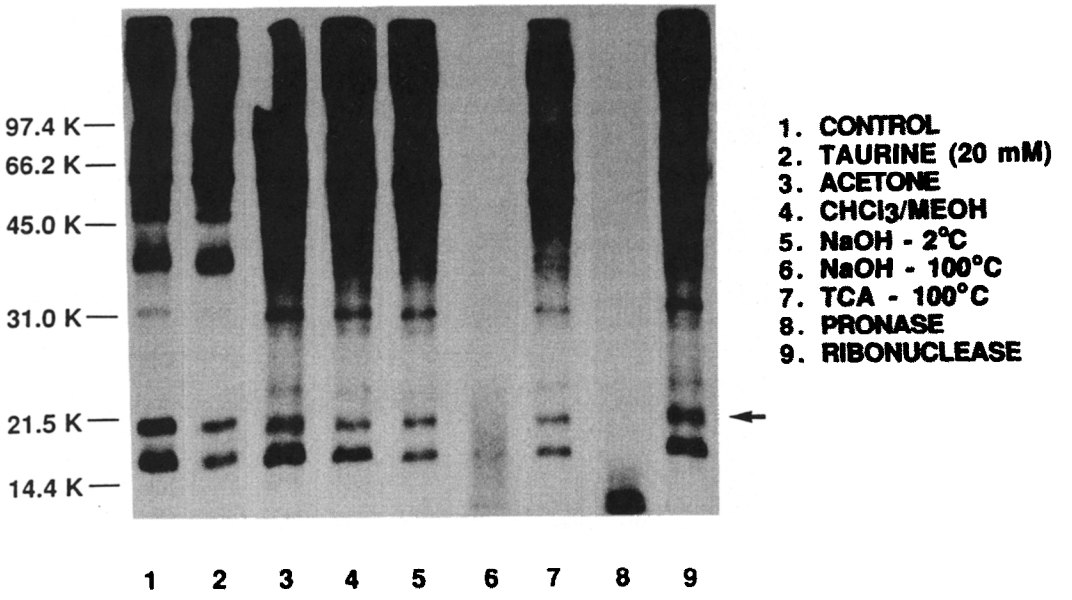

Fig. 4. Autoradiographs of phosphorylated proteins in a mitochondrial preparation of rat retina after various treatments to determine the nature of the phosphate bond in the ~20 K M_r protein (representative experiment). The samples in lanes 1 and 2 were obtained from incubation systems that were stopped by boiling directly in SDS sample buffer. (The conditions of lanes 1 and 2, i.e. ±taurine, were included in the experiment as controls to ensure that the inhibitory effect of taurine was present.) The samples in lanes 3–9 were obtained from incubation systems that were stopped by the addition of 10% TCA as described in Materials and Methods. Location of the ~20 k M_r phosphoprotein is designated by the arrow. Marker proteins with molecular weights ranging from 14.4 to 97.4 K are indicated.

Table 1. Characterization of the phosphate bond in the ~20 K M_r phosphoprotein present in a mitochondrial preparation of rat retina

Treatment	Densitometry measurement (arbitrary units)
Acetone (control)	1094 ± 71
Chloroform : methanol	723 ± 86
NaOH—2°	660 ± 46
NaOH—100°	0
TCA—100°	671 ± 84
Pronase (0.01 mg)	0
Ribonuclease (0.01 mg)	633 ± 112

Data were calculated from densitometry tracings of autoradiographs from one-dimensional (PAGE) gels and are presented as means ± SEM of 3 experiments.

Characterization of the phosphate bond in the ~20 K M_r *phosphoprotein.* The inhibitory effect of taurine (20 mM) on the phosphorylation of the ~20 K M_r phosphoprotein is shown in lane 2 (Fig. 4) and is compared with the non-inhibited phosphorylation in lane 1.

The phosphate bond in the ~20 K M_r protein was characterized by exposure to a number of different treatments such as acetone (lane 3) and chloroform : methanol (lane 4) extractions, exposure to cold (lane 5) and hot (lane 6) NaOH, exposure to hot TCA (lane 7), and incubation with pronase (lane 8; protease) and ribonuclease A (lane 9) (Fig. 4; Table 1). The effects of the treatments on the ~20 K M_r phosphoprotein in the retinal preparation displayed in lanes 4–9 are compared with the effects of acetone extraction shown in lane 3. Boiling the TCA precipitate in NaOH or incubation with pronase completely removed the [^{32}P]phosphate label from the gel. The treatments had a 30–40% effect on the [^{32}P]phosphate label in the ~20 K M_r phosphoprotein. [A reduction of 30–40% of the amount of radioactivity in the ~20 K M_r phosphoprotein was not considered significant due to unavoidable and uneven loss of TCA precipitate when the precipitate was handled and extracted multiple times with organic solvents after and in addition to acetone extraction (Fig. 4, lanes 3–9; Table 1). The incubation systems for which data (Fig. 4) are presented in lanes 1 (control) and 2 (20 mM taurine) were stopped by boiling directly in SDS sample buffer. Since there were no TCA precipitation steps in this treatment, there was no loss of the ~20 K M_r phosphoprotein as was observed in varying degrees in lanes 3–9.] These data suggest that the incorporation of phosphate was into protein rather than into either lipid or nucleic acid. Furthermore, the absence of a major effect upon treatment with cold NaOH and hot TCA indicates that the ~20 K M_r protein contains a phosphoester bond rather than an acylphosphate bond.

Phosphoamino acid analysis. The phosphoamino acids of the ~20 K M_r phosphoprotein were analyzed by two-dimensional high voltage electrophoresis

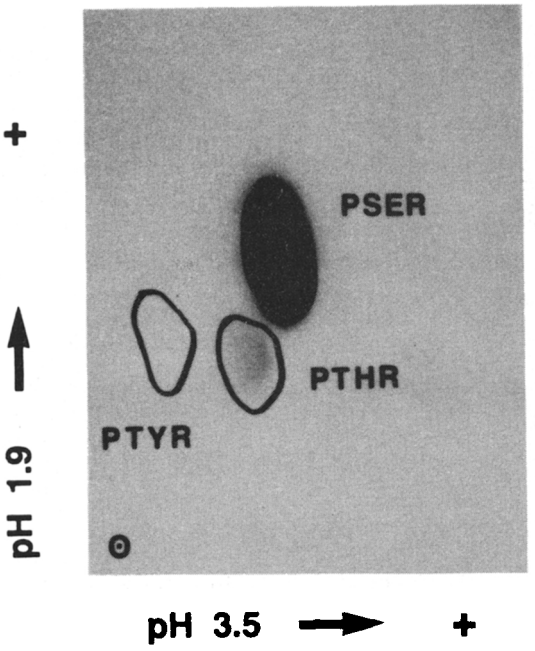

Fig. 5. Autoradiograph of the phosphoamino acid analysis of the ~20 K M_r phosphoprotein obtained from the mitochondrial fraction of the rat retina. Isolation and hydrolysis of the ~20 K M_r phosphoprotein are described in Materials and Methods. Locations of the standard phosphoamino acids, phosphoserine (PSER), phosphothreonine (PTHR), and phosphotyrosine (PTYR), were determined by ninhydrin stain and are indicated by circles. Radioactivity in the labeled phosphoserine and phosphothreonine positions is indicated by autoradiography.

using cellulose mylar plates (Fig. 5). Serine and threonine were both phosphorylated by [γ-^{32}P]-ATP. However, phosphorylation of serine was predominant over threonine (Fig. 5).

Effects of taurine on the phosphorylation of proteins other than the ~20 K M_r protein in the mitochondrial preparation can also be observed in Figs. 1, 3 and 4. In Fig. 1 taurine appears to inhibit the phosphorylation of proteins with approximate molecular weights of 31 and 48 K while stimulating the phosphorylation of a phosphoprotein with an approximate weight of 40 K. In Fig. 3 (lanes 1 and 2), taurine appears to stimulate the phosphorylation of two proteins with molecular weights of approximately 40 and 44 K and to inhibit the phosphorylation of a protein at ~48 K. In Fig. 4 (lanes 1 and 2), taurine appears to inhibit proteins with molecular weights of approximately 16, 31, and 48 K while again stimulating the phosphorylation of a protein at ~40 K. The effects of taurine on the phosphorylation of these additional proteins are of potential interest; however, only limited studies have been considered thus far to investigate and characterize these other proteins or their phosphorylation reactions. The primary reasons to date for less effort in studying the proteins whose phosphorylation is affected by taurine other than the ~20 K M_r protein are (1) some of these other phosphoproteins are in regions of the gel in which separation by PAGE is less defined, and (2) the phosphorylation reaction of some of the other proteins is not consistent in all mitochondrial preparations as viewed by autoradiography.

In addition to the effect of taurine on protein phosphorylation in the retina, our laboratory has demonstrated that in a P_2 fraction prepared from the cortex of the rat brain taurine inhibits a Ca^{2+}-dependent, protein kinase C-catalyzed phosphorylation of an ~20 K M_r protein [7]. It should be noted that the ~20 K M_r phosphoprotein described for the retina appears to be a different protein than that reported for the brain. This conclusion was drawn from (a) slight differences observed in the molecular weights of the proteins when the two tissues were subjected to side-by-side electrophoresis, (b) differences in the isoelectric points, and (c) differences in kinase activators that stimulate the phosphorylation [11]. In addition, results obtained from two-dimensional PAGE suggest that the retinal ~20 K M_r phosphoprotein is composed of a family of three proteins [11], while only one phosphoprotein was demonstrated to be present in the brain [6, 11].

Interestingly, the effects of taurine on the ~20 K M_r phosphoprotein in the rat brain were only observed if a mitochondrial fraction from the cortex was present with the cytosol. These data led to speculation that taurine may be stimulating Ca^{2+} uptake into the mitochondria of the cortex and thus lowering the optimal cytosolic Ca^{2+} levels required for protein phosphorylation [8]. It has also been reported that taurine inhibits both the total incorporation of phosphate into membrane proteins and the phosphorylation of specific proteins found in the cat cortex [25].

In the heart there is evidence that taurine inhibits the phosphorylation of specific proteins with apparent molecular weights ranging from 15 K to 190 K depending upon the cardiac preparation [9–11]. Schaffer and colleagues [10] have suggested that the phosphorylation of three proteins (190 K, 59 K, and 44 K) found in a rat heart sarcolemmal preparation may be mediated through calmodulin. We have also observed in our laboratory that calmodulin stimulates the phosphorylation of an ~44 K M_r protein found in a rat heart mitochondrial fraction. However, we noted that phosphorylation of the ~44 K M_r protein appeared to be inhibited by calcium ions [11]. Thus, the stimulatory effect of calmodulin on its phosphorylation may be due to the ability of calmodulin to bind Ca^{2+} and, therefore, remove free Ca^{2+} from the incubation medium [11].

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