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Stimulation by Chelerythrine of the Phosphorylation of the Amino Acid Serine in an ~20 kDa Protein Present in the Mitochondrial Fraction of the Rat Retina

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ABSTRACT. It has been reported that chelerythrine chloride, a benzophenanthridine alkaloid, with a wide variety of biologic effects stimulates the phosphorylation of an ~20 kDa protein present in the mitochondrial fraction of the rat retina. It has also been shown previously that both the serine and threonine resides in this phosphoprotein are phosphorylated when the retinal preparation is incubated in the presence of [γ-^{32}P]ATP. Phosphorylation of the serine residue(s) was determined to predominate over phosphorylation of the threonine residue(s). In the present investigation, it was demonstrated that chelerythrine stimulates the incorporation of radioactive phosphate into the serine residue(s), increasing the radioactivity in the phosphoserine/phosphothreonine ratio by 80%. This observation represents a novel and apparently contradictory effect for chelerythrine, which is used normally as a selective protein kinase C inhibitor. In addition to testing chelerythrine for its effects on the phosphorylation of the ~20 kDa protein, a number of other protein kinase inhibitors and activators were investigated. The results suggest that the enzyme responsible for the phosphorylation of the ~20 kDa protein is not a well-characterized or documented kinase. BIOCHEM PHARMACOL 52;2:253–257, 1996.

KEY WORDS. chelerythrine chloride; phosphorylation; amino acids; mitochondrial fraction; rat retina

Chelerythrine chloride a benzophenanthridine alkaloid, has been used widely as a selective inhibitor of PKC† [1]. Besides its well-known activity as a kinase antagonist, chelerythrine has a number of other biologic properties that are both independent and dependent upon its inhibitory effects on PKC [reviewed in ref. 2]. Previously, we reported a new and apparently contradictory role for chelerythrine in that chelerythrine stimulates the phosphorylation of an ~20 kDa protein present in the mitochondrial fraction of the rat retina [2]. Our laboratory has had an interest in this particular retinal protein since we initially reported in 1985 [3] and in subsequent reports [4–6] that taurine, 2-aminoethanesulfonic acid, which is present in the retina in high concentrations, inhibits its phosphorylation. Also, we found that when the mitochondrial fraction of the rat retina is incubated with [γ-^{32}P]ATP and then the ~20 kDa protein is isolated on one-dimensional polyacrylamide gel electrophoresis followed by hydrolysis in 6 N HCl and separation of the phosphoamino acids on two-dimensional flat plate electrophoresis, the incorporation of the radioactive phosphate moiety appears to be predominately in the serine residue(s) and only to a minor extent in the threonine residue(s) of the phosphoprotein [4].

In the present study, we have examined the stimulatory effect of chelerythrine on the phosphorylation of the ~20 kDa protein by determining which phosphoamino acid is affected by chelerythrine. Various other kinase inhibitors and activators were also tested for their effects on the phosphorylation of the ~20 kDa protein.

MATERIALS AND METHODS

Chemicals

Chelerythrine chloride, calphostin C, ISO-H7, H-7, HA1004, staurosporine, and W-7 were purchased from LC Laboratories, Woburn, MA. [γ-^{32}P]ATP (30 Ci/mmol) was obtained from New England Nuclear, Boston, MA.

Preparation of the Mitochondrial Subcellular Fraction of the Rat Retina

The preparation of the mitochondrial subcellular fraction of the rat retina was as described previously [2]. Briefly, for each experiment, twelve rats were killed (anesthetized with

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† *Abbreviations:* PKC, protein kinase C; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; cAMP, cyclic AMP; cGMP, cyclic GMP; PSER, phosphoserine; and PTHR, phosphothreonine.

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halothane and then decapitated), and the eyes were removed and placed in ice-cold 0.3 M mannitol, pH 7.0. Then the retinas were removed from the eyecup by scraping with a small spatula and placed in the mannitol solution. Rod outer segments were removed by vortex-mixing the retina for 6 sec, allowing the tissue to settle, and then aspirating the supernatant to eliminate the rod outer segments. The remaining tissue components were homogenized gently in a Potter–Elvehjem homogenizer in the mannitol solution. The supernatant was then placed on top of 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 mitochondrial fraction, which sedimented to the bottom of the gradient, was 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 [7].

Phosphorylation Assay and Polyacrylamide Gel Electrophoresis

The phosphorylation assay was as described previously [2]. Briefly, the incubation mixture contained bicarbonate buffer (above), mitochondrial fraction (0.1 mg protein), and chelerythrine (200 μM) when indicated (total volume 0.25 mL). Various kinase inhibitors were added as indicated in Table 2. The mixture was preincubated for 2 min at 37°, and then the reaction was initiated by adding 20 μCi of [γ-^{32}P]ATP (10 μM). The reaction was allowed to continue for an additional 6 min at which time 0.3 mL of gel electrophoresis sample buffer [60 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 2 mM mercaptoethanol, and 0.00125% bromophenol blue] was added and immediately boiled for 5 min. A total of five incubation reactions including sample buffer (total volume 2.75 mL) were pooled for the control sample and also for the chelerythrine-treated sample.

The pooled control or pooled chelerythrine-treated samples were subjected to one-dimensional polyacrylamide gel electrophoresis on 12% gels according to the method of Laemmli [8]. The samples were layered over a continuous stacking gel (no wells). When the kinase inhibitors or activators were tested, wells were utilized in the stacking gel.

Isolation and Hydrolysis of the ~20 kDa Phosphoprotein

After stopping the electrophoretic run, the gels were washed for 1 hr with running water, dried on a slab gel drier (Hoefer Pharmacia BioTech, San Francisco, CA), and exposed to X-ray film to visualize the location of the ~20 kDa protein. The ~20 kDa phosphoprotein was isolated from the one-dimensional gel as described [4]. The area on the gel corresponding to the ~20 kDa phosphoprotein was cut out and placed in 50 mM NH_4HCO_3 containing 0.15 mg of trypsin (the cellophane and paper backing were removed). The gel pieces were eluted for 24 hr at 37°. The phosphoprotein was then lyophilized to dryness and the residue hydrolyzed for 2 hr (110°) in 1.0 mL of 6 N HCl under vacuum (previously flushed with N_2). The hydrolysate was lyophilized to dryness and the residue taken up in 0.025 mL water.

High-voltage flat-plate electrophoresis (two-dimensional) was performed on cellulose mylar plates (20 × 20 cm) to separate the phosphoamino acids [4, 9]. Standards of PSER PTHR, and phosphotyrosine were applied in a single spot along with the unknown mixture. The first dimension consisted of formic acid:acetic acid:H_2O (1:10:89), pH 1.9, 600 V, 2.5 hr; the second dimension consisted of acetic acid:pyridine:H_2O (19:1:89), pH 3.5, 400 V, 2 hr. Ninhydrin (1% in acetone) was used to visualize the phosphoamino acids. Autoradiography using X-ray film was used to determine the location of the radioactive phosphoamino acids. Radioactivity in the PSER and PTHR residues was quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). When the kinase inhibitors or activators were tested, the amount of radioactive phosphate incorpo-

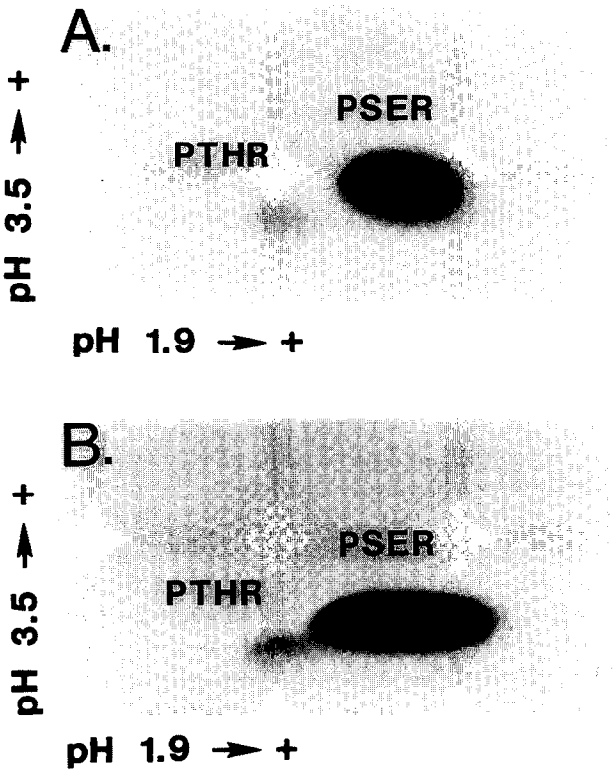

FIG. 1. Autoradiograms of the phosphoamino acid analyses of the ~20 kDa phosphoprotein obtained from the mitochondrial fraction of the rat retina (representative experiment). Panel A demonstrates the control pattern of radioactive phosphoamino acids, while panel B demonstrates the pattern after treatment with chelerythrine (200 μM). Isolation and hydrolysis of the ~20 kDa phosphoprotein and electrophoretic separation of the phosphoamino acids are described in Materials and Methods. Identification of the migratory pattern of standard phosphoamino acids [PSER, PTHR, and phosphotyrosine (not shown)] was determined initially by ninhydrin stain. Radioactivity in the PSER and PTHR position was indicated by autoradiography, using X-ray film.

TABLE 1. Effect of chelerythrine on the incorporation of radioactive phosphate into the serine and threonine residues in the ~20 kDa phosphoprotein present in a mitochondrial fraction of the rat retina

Addition	PSER/PTHR ratio	Percent
Control	8.79 ± 1.22 (4)	100
Chelerythrine (200 μM)	15.8 ± 1.3* (4)	180

Quantitation of the radioactivity in the PSER and PTHR residues was performed on a PhosphorImager. Data are presented as means ± SEM.
* $P < 0.05$ (Student's t-test).

rated into the ~20 kDa phosphoprotein was quantitated by densitometry (Bio-Rad scanning densitometer, model 1650, Hercules, CA).

RESULTS AND DISCUSSION

As previously reported [4], when radioactive ATP was incubated with the mitochondrial fraction of the rat retina containing the ~20 kDa protein, the phosphate label was found primarily in the PSER residue(s) with only a very minor amount detected in the PTHR residue(s). No radioactivity was observed to migrate with authentic phosphotyrosine.

In the present study, it was resolved to determine if the stimulation of phosphorylation by chelerythrine altered the quantity of the incorporated label in the PSER or in the PTHR residues. Representative autoradiograms (after two-dimensional high-voltage electrophoresis) demonstrating the location of the radioactive phosphoamino acids from the isolated and hydrolyzed ~20 kDa phosphoprotein are shown in Fig. 1. Panel A denotes the control pattern of radioactive phosphoamino acids obtained from the control phosphoprotein, while panel B shows the pattern of phosphoamino acids as a result of treatment with 200 μM chelerythrine. As can be observed from the autoradiograms, chelerythrine appeared to stimulate the incorporation of radioactive phosphate from [γ-^{32}P]ATP mainly into the serine residue(s) and only to a minor extent into the threonine residue(s). Verification of the phosphorylated amino acids as PSER and PTHR was confirmed by the comigration of the radioactive phosphate with standard phosphoamino acids during the high-voltage electrophoresis. As was observed in a previous report [4], radioactivity in the location of standard phosphotyrosine was not observed.

A summary of the PSER to PTHR ratios in both the control and chelerythrine-treated~20 kDa phosphoprotein is reported in Table 1. The addition of chelerythrine (200 μM) to the phosphorylation reaction increased the PSER to PTHR ratio by 80%, suggesting that chelerythrine primarily affects phosphorylation of only the serine residue(s).

The intriguing observation that chelerythrine stimulated the phosphorylation of the serine residue(s) is a unique finding for this compound and may have some use in studying other proteins that are also phosphorylated in the serine residue. The finding, previously reported [2], that a very high (37 μM) concentration of chelerythrine is necessary to stimulate the phosphorylation of the ~20 kDa protein by 50% is perhaps the reason that this observation was not noticed sooner. The usual application of chelerythrine is as a selective PKC inhibitor with an IC_{50} of 0.66 μM [1]. Much higher concentrations (100–170 μM) are required for the inhibition of other kinases such as PKA, calcium-calmodulin-dependent protein kinase, and tyrosine protein kinase [1].

In addition to the ~20 kDa protein present in the retina whose phosphorylation is stimulated by chelerythrine, an ~40 kDa protein present in a P_2 synaptosomal preparation of rat brain cortex is also stimulated by 100 μM chelerythrine [2]. However, when chelerythrine was tested in a mitochondrial fraction of the rat heart, only inhibitory effects on protein phosphorylation were observed [2,10].

Numerous compounds with known inhibitory activities directed towards the various classes of kinases were also tested for their effects on the phosphorylation of the ~20 kDa protein (Fig. 2 and Table 2). None of the antagonists

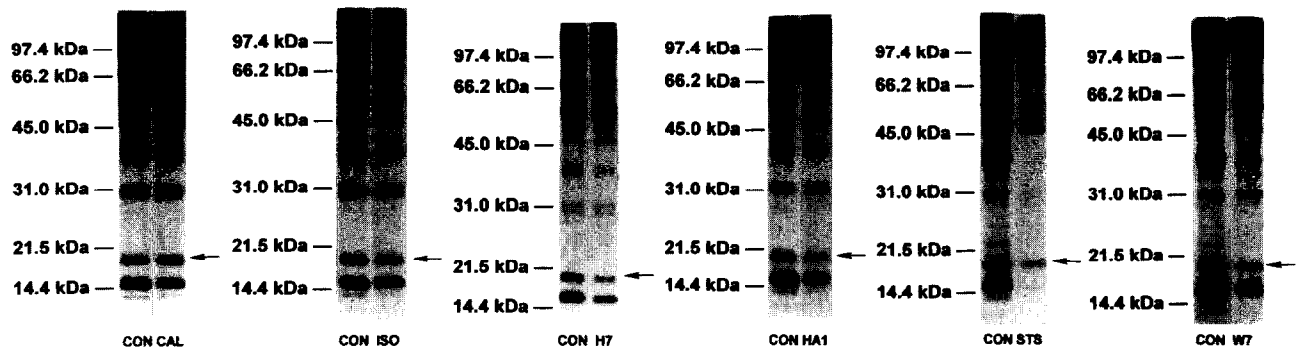

FIG. 2. Autoradiograms of the effects of various known kinase inhibitors on the phosphorylation of the ~20 kDa protein (representative experiment). The conditions of the phosphorylation assay, electrophoresis, and autoradiography are described in Materials and Methods. Marker proteins with molecular weights ranging from 14,400 to 97,400 are indicated. The arrows indicate the position of the ~20 kDa phosphoprotein. Abbreviations: CON, control; CAL, calphostin C; ISO, ISO-H7; H7, H-7; HA1, HA1004; STS, staurosporine; and W7, W-7.

TABLE 2. Effects of kinase inhibitors or activators on the phosphorylation of the ~20 kDa protein present in the mitochondrial fraction of the rat retina

Inhibitor	Activator	Maximum concn tested (μM)	Inhibition (%)	Activation (%)	Primary target
Calphostin C		50	−3.0 ± 5.7		PKC
ISO-H7		100	29.0 ± 3.1		PKC
H-7		100	34.4 ± 9.0		PKC, PKA, PKG
HA1004		25	34.6 ± 2.2		PKA, PKG
Staurosporine		10	67.0 ± 6.6		General inhibitor
W-7		400	39.7 ± 3.8		Calmodulin-dependent protein kinase
	cAMP	100		−15.4 ± 8.9	PKA
	cGMP	100		0.3 ± 2.6	PKG

Data are presented as means ± SEM (N = 3).

stimulated the phosphorylation of the ~ kDa protein even at very high concentrations. In addition, while some of the antagonists inhibited the phosphorylation of the ~20 kDa protein, the inhibitory effect, in general, was less than 50% and then only realized at extremely high concentrations, well above the reported inhibitory potency for each compound. For instance, as reported in Table 2, calphostin C, which mainly targets PKC with a reported IC_{50} of 0.05 μM [11], had no effect on the phosphorylation of the ~20 kDa protein at a maximum concentration of 50 μM. ISO-H7 when tested at 100 μM inhibited the phosphorylation of the ~20 kDa protein by 29%. ISO-H7 is reported to have an IC_{50} of 50 μM for PKC [12]. H-7, a reported inhibitor of PKC (K_i = 6.0 μM), PKA (K_i = 3.0 μM), and PKG (K_i = 5.8 μM) [13], inhibited the phosphorylation of the ~20 kDa protein by 34.4% only when tested at 100 μM. HA1004, a potent inhibitor of PKA (K_i = 2.3 μM) and PKG (K_i = 1.3 μM) [13], had only a 34.6% inhibitory effect on the phosphorylation of the ~20 kDa protein when tested at a concentration of 25 μM. Staurosporine, an extremely potent, general kinase inhibitor with an IC_{50} value of 2.7 nM for PKC [14], inhibited the phosphorylation of the ~20 kDa protein by 67% when tested at 10 μM. We observed an IC_{50} of 1.9 ± 0.8 μM for staurosporine. W-7, a calmodulin antagonist (IC_{50} = 28 μM) [15, 16], inhibited the phosphorylation of the ~20 kDa protein by 39.7% when tested at a concentration of 400 μM.

In addition, we previously reported [6] that when kinase activators such as phorbol ester (0.2 μM) plus phosphatidylserine (50 μg/mL) and calmodulin (50 units) were added to the phosphorylation incubation reaction, there was no effect on the phosphorylation of the ~20 kDa protein. cAMP (100 μM) and cGMP (100 μM) also had no significant effect on this protein (Table 2). Addition or deletion of calcium ions to the incubation mixture had no effect (data not shown). These data suggest the kinase responsible for the phosphorylation of the ~20 kDa protein is not likely to be PKC, PKA, PKG, or a calmodulin-dependent protein kinase.

The possibility that the stimulatory effect observed with chelerythrine on the phosphorylation of the ~20 kDa protein is due to an inhibitory effect of chelerythrine on phosphatase present in the retinal preparation has been examined previously [2]. When okadaic acid, a known phosphatase inhibitor, was tested up to a concentration of 20 μM, no change was observed in the phosphorylation of the ~20 kDa protein. Thus, while this result does not rule out definitively the action of chelerythrine on a phosphatase, it does suggest that this is probably not the mechanism of action.

To determine the physiologic significance of the stimulatory effect of chelerythrine on the phosphorylation of the serine residue(s) in the ~20 kDa protein, future studies will be necessary to identify this protein, confirm its cellular location, and determine its function and regulation in the retina.

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References

1. Herbert JM, Augereau JM, Gleye J and Maffrand JP, Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun* **172:** 993–999, 1990.
2. Lombardini JB, Paradoxical stimulatory effect of the kinase inhibitor chelerythrine on the phosphorylation of a ~20 K M_r protein present in the mitochondrial fraction of rat retina. *Brain Res* **673:** 194–198, 1995.
3. Lombardini JB, Effects of taurine on calcium ion uptake and protein phosphorylation in rat retinal membrane preparations. *J Neurochem* **45:** 268–275, 1985.
4. Lombardini JB, Partial characterization of an ~20 K M_r retinal protein whose phosphorylation is inhibited by taurine. *Biochem Pharmacol* **46:** 1445–1451, 1993.
5. Lombardini JB, Taurine: Retinal function. *Brain Res Rev* **16:** 151–169, 1991.
6. Lombardini JB, Effects of taurine on the phosphorylation of specific proteins in subcellular fractions of the rat retina. *Neurochem Res* **17:** 821–824, 1992.
7. Kuo C-H and Miki N, Stimulatory effect of taurine on Ca-uptake by disc membranes from photoreceptor cell outer segments. *Biochem Biophys Res Commun* **94:** 646–651, 1980.

8. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685, 1970.
9. Lombardini JB and Props C, Effects of cooling temperature on the separation of phosphoamino acids. *Anal Biochem* **227:** 339–400, 1995.
10. Lombardini JP and Props C, Effects of benzophenanthridine alkaloids on the phosphorylation of an ~44 kDa protein present in a mitochondrial fraction of the rat heart. *Biochem Pharmacol* **51:** 151–157, 1996.
11. Kobayashi E, Nakano H, Morimoto M and Tamaoki T, Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun* **159:** 548–553, 1989.
12. Quick J, Ware JA and Driedger PE, The structure and biological activities of the widely used protein kinase inhibitor, H7, differ depending on the commercial source. *Biochem Biophys Res Commun* **187:** 657–663, 1992.
13. Hidaka H, Inagaki M, Kawamoto S and Sasaki Y, Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23:** 5036–5041, 1984.
14. Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M and Tomita F, Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. *Biochem Biophys Res Commun* **135:** 397–402, 1986.
15. Itoh H and Hidaka H, Direct interaction of calmodulin antagonists with Ca^{2+}/calmodulin-dependent cyclic nucleotide phosphodiesterase. *J Biochem (Tokyo)* **96:** 1721–1726, 1984.
16. Hidaka H, Asano M and Tanaka T, Activity–structure relationship of calmodulin antagonists. *Molec Pharmacol* **20:** 571–578, 1981.