neurophysin has a role in anterior pituitary function. Its presence in high concentration in plasma from long portal vessels supports the concept that the contents of secretory granules containing neurophysin and vasopressin are secreted in the median eminence. Since oxytocin also has an intragranular association with neurophysin (*21*), it too may be secreted into the portal circulation.

These data provide evidence that vasopressin and neurophysin reach the anterior pituitary gland via the portal circulation. Collection of blood from individual portal vessels in primates and radioimmunoassay estimates of neurohypophyseal peptides in portal blood have not been previously reported. On the basis of our studies and those of others, we propose two distinct secretory pathways and two hormonal functions for vasopressin: One, the well-established secretion into the general circulation from the posterior pituitary gland for antidiuretic action in the kidney; and the other, secretion into the hypophyseal portal circulation to influence the secretion of tropic hormones.

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References and Notes

1. L. Martini, in *The Pituitary Gland*, G. W. Harris and B. T. Donovan, Eds. (Univ. of California Press, Berkeley, 1966), vol. 3, pp. 535–577.
2. A. V. Schally, A. Arimura, C. Y. Bowers, A. J. Kastin, S. Sawano, T. W. Reading, *Recent Prog. Horm. Res.* **24**, 497 (1968).
3. R. E. Blackwell and R. Guillemin, *Annu. Rev. Physiol.* **35**, 357 (1973).
4. V. Meyer and E. Knobil, *Endocrinology* **79**, 1016 (1966).
5. F. C. Greenwood and J. Landon, *Nature (Lond.)* **210**, 540 (1966).
6. S. M. McCann, *Endocrinology* **60**, 664 (1957); J. R. Tucci, E. A. Espiner, P. I. Jagger, D. P. Lauler, G. W. Thorn, *Ann. Intern. Med.* **69**, 191 (1968).
7. G. W. Clayton, L. Librik, R. L. Gardner, R. Guillemin, *J. Clin. Endocrinol. Metab.* **23**, 975 (1963).
8. F. E. Yates, S. M. Russell, M. F. Dallman, G. A. Hedge, S. M. McCann, A. P. S. Dhariwal, *Endocrinology* **88**, 3 (1971).
9. E. Scharrer and B. Scharrer, *Recent Prog. Horm. Res.* **10**, 183 (1954); H. Kobayashi, *Int. Rev. Cytol.* **29**, 281 (1970); W. Doepfner, in *Neurohypophyseal Hormones and Similar Peptides*, B. Berde, Ed. (Springer-Verlag, New York, 1968), pp. 625–654.
10. A preliminary report of this work has appeared (E. A. Zimmerman, P. W. Carmel, M. K. Husain, M. Ferin, A. G. Frantz, A. G. Robinson, program of the 55th meeting of the Endocrine Society, Chicago, 1973, p. A-85).
11. E. A. Zimmerman, K. C. Hsu, A. G. Robinson, P. W. Carmel, A. G. Frantz, M. Tannenbaum, *Endocrinology* **92**, 931 (1973).
12. A. G. Robinson and E. A. Zimmerman, *J. Clin. Invest.* **52**, 1260 (1973).
13. K. M. Knigge and D. E. Scott, *Am. J. Anat.* **129**, 223 (1970).
14. H. B. Parry and B. G. Livett, *Nature (Lond.)* **242**, 63 (1973).
15. S. Oyama, A. Kagan, S. M. Glick, *J. Clin. Endocrinol. Metab.* **33**, 739 (1971).
16. M. K. Husain, N. Fernando, M. Shapiro, A. Kagan, S. M. Glick, *ibid.* **37**, 616 (1973).
17. J. C. Porter, R. S. Mical, J. G. Ondo, I. A. Kamberi, *Karolinska Symp. Res. Methods Reprod. Endocrinol.* **4**, 249 (1971).
18. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
19. H. Tuppy, in *Neurohypophyseal Hormones and Similar Peptides*, B. Berde, Ed. (Springer-Verlag, New York, 1968), pp. 67–129.
20. If 1 international unit (I.U.) equals 2.5 μg of AVP, then, for a 70-kg man with 3 liters of plasma, 5 I.U. or 12.5 μg of AVP distributed initially in 3000 ml gives a concentration of 4000 pg/ml.
21. C. R. Dean, D. B. Hope, T. Kazic, *Brit. J. Pharmacol.* **34**, 192 (1968).
22. We thank J. C. Porter for advice on portal blood collection; K. C. Hsu for help in developing immunoperoxidase techniques; S. Glick for antiserum to AVP; M. Salcman for preparing glass pipettes, and S. Rosario for technical assistance. Supported in part by PHS grants NS 11,008, NS0561805, AM11294, AM5397, CA11704, and CA13696; and by grants from the John A. Hartford Foundation, Ford Foundation, Health Research and Services Foundation of Pittsburgh (0–62), and the Population Council of New York (M72.074C).

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Phosphorylation of Muscle Membranes: Identification of a Membrane-Bound Protein Kinase

Abstract. *A membrane-bound protein kinase occurs in membranes derived from rat skeletal muscle and appears limited to a surface membrane fraction. The enzyme is magnesium dependent, is only minimally stimulated by cyclic nucleotides, and phosphorylates serine and to a lesser extent threonine residues of three membrane proteins with molecular weights of less than 30,000.*

Soluble protein kinases in various eukaryotic tissues participate in the regulation of cellular metabolism (*1*). Isolated membranes from various tissues contain endogenous protein kinases that phosphorylate membrane proteins and may participate in the regulation of membrane ion-binding and permeability (*2–5*). While such membrane phosphorylation may be of significance in many excitable tissues, only the membrane-bound protein kinase of brain has been studied (*2, 3, 6, 7*). We now report on a particulate protein kinase not previously described in skeletal muscle.

Our study of muscle membrane protein kinase is a result of our demonstration (*8*) of altered activity of membrane-bound protein kinase of frozen aged erythrocytes from patients with myotonic dystrophy, a systemic disorder that involves the membranes of many excitable tissues including skeletal muscle. The membrane-bound protein kinase reported here in skeletal muscle phosphorylates three membrane proteins whose molecular weights are less than 30,000. This phosphorylation in vitro occurs only in a muscle membrane subfraction that has several characteristics of surface membrane.

Muscle membrane isolation and subfractionation were performed as described (*9, 10*). Rat hind limb muscles were homogenized; the nuclei and mitochondria were removed by centrifugation. The resulting supernatant was centrifuged at 100,000*g*, and the sediment was extracted sequentially with lithium bromide, potassium chloride, and deionized water and then centrifuged on continuous density sucrose gradients that ranged from 15 to 35 percent sucrose. The lighter density membranes which failed to penetrate 19 percent sucrose contained three markers for surface membranes. These were a Na,K-stimulated, Mg-dependent adenosine triphosphatase, sialic acid–containing macromolecules, and components of the intact tissue which could be iodinated by extracellular lactoperoxidase and ^{125}I. Higher density membranes which penetrated 19 percent sucrose contained peak specific activities of Ca- or Mg-dependent adenosine triphosphatase, the membrane marker for cytoplasmic membranes, or sarcoplasmic reticulum (*9*).

Muscle membrane subfractions were examined for endogenous protein kinase activity by two techniques. After brief incubation with [γ-^{32}P]adenosine triphosphate (ATP) under the appropriate ionic and *p*H conditions the phosphorylated proteins were separated from free [γ-^{32}P]ATP either by repetitive washing with trichloroacetic acid and hydroxylamine (*6*) or by solubilization in 1 percent sodium dodecyl sulfate (SDS) and subsequent electrophoresis in 7.5 percent polyacrylamide gels. No significant endogenous protein kinase could be demonstrated in the water washings before the gradient centrifugation, or in the membranes derived from the nuclear or mitochondrial fractions. The endogenous protein kinase was present only in membranes derived from the supernatant from which the mitochondria had been removed.

The peak specific activity of membrane phosphorylation (353 pmole per milligram of membrane protein per minute) occurred in the light membranes with the aforementioned characteristics of surface membrane.

The degree of phosphorylation in several controls (zero time, no magnesium, no membrane, or boiled control) was insignificant (Table 1). Incorporation of phosphate into endogenous substrate under appropriate conditions increased linearly with membrane protein concentration. The amount of phosphate incorporated increased linearly with time for less than 15 seconds and leveled off after 2 minutes. Magnesium was required for phosphorylation. Millimolar concentrations of calcium inhibited the reaction although the marked inhibition by ethylenebis(oxyethylenenitrilo)tetraacetate which binds calcium suggests that calcium may be required in micromolar concentrations. The *p*H optimum was higher than that described for various soluble kinases. Both cyclic adenosine monophosphate (cyclic AMP) and cyclic guanosine monophosphate (cyclic GMP) gave only minimal, although reproducible, stimulation. The lack of significant activation by cyclic AMP may be related to the extensive exposure of the membrane subfraction to 0.3*M* lithium bromide and 0.6*M* KCl. Alternatively the endogenous protein kinase may be unresponsive to cyclic nucleotide stimulation.

The phosphorylated product was particulate and nondialyzable and it could be solubilized by detergents such as sodium octyl sulfate or SDS. It was precipitated by trichloroacetic acid and

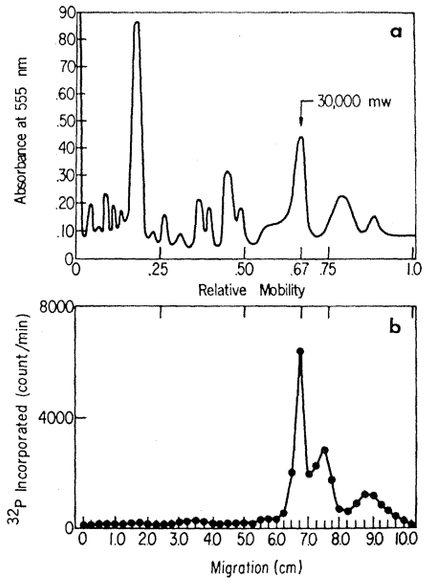

Fig. 1. (a) Absorbance scan of SDS polyacrylamide gel electrophoresis of 100 μg of the light density membrane subfraction. (b) Corresponding radioactivity of ^{32}P phosphorylated product. The endogenous protein kinase was from 100 μg of muscle membrane. Reaction conditions are as in Table 1; *mw*, molecular weight.

was insoluble in ether. When extracted with an acidified mixture of chloroform and methanol, it was found at the chloroform and methanol interface. The phosphate bond was stable to acidified hydroxylamine and hot acetic acid, but was effectively cleaved by brief exposure to hot alkali (Table 1). Limited acid hydrolysis followed by high voltage electrophoresis (*11*) indicated that the ^{32}P was present in phosphoserine (90 percent of radioactivity) and phosphothreonine (10 percent).

Table 1. Conditions that affect the endogenous muscle membrane protein kinase and its phosphorylated product. Numbers represent the mean of three assays (standard errors of the mean are at most 5 percent). The phosphorylated proteins are separated from free [^{32}P]ATP by washing with trichloroacetic acid and hydroxylamine. EGTA, ethylenebis(oxyethylenenitrilo)tetraacetate.

Assay condition	Specific activity (pmole/min per mg of protein)
Complete reaction*	353
Zero time	0
Boiled before reaction	8
Without $MgCl_2$	16
Sodium acetate buffer (10 m*M*), *p*H 6.5	123
With EGTA (5 m*M*)	139
With $CaCl_2$ (6 m*M*)	285
With cyclic AMP (10^{-6}*M*)	383
With cyclic GMP (10^{-6}*M*)	393
NaOH after reaction (2*N*, 95°C, 3 minutes)	0

* Membrane protein (100 μg of the light density subfraction) was incubated for 20 seconds at 25°C, in the presence of 12 m*M* $MgCl_2$, 24 m*M* tris(hydroxymethyl)aminomethane-HCl, *p*H 7.5, 10^{-5}*M* ATP (1500 to 2000 count/min per picomole).

These data provide cogent evidence for identity of the phosphorylated product as membrane phosphoprotein, a conclusion confirmed by SDS gel electrophoresis. The gel profile of the surface membrane fraction revealed 16 membrane polypeptides ranging in molecular weight from a few thousand to several hundred thousand (Fig. 1). The highest molecular weight ^{32}P-phosphorylated product migrated as a single band which absorbed at 280 nm in the ultraviolet, and could be stained with the protein stain Coomassie blue. Its relative mobility of 0.67 corresponds to a molecular weight of 30,000. Control gels (zero time or no magnesium) were devoid of radioactivity in this band. The endogenous muscle membrane protein kinase did not phosphorylate any membrane polypeptides with molecular weights higher than 30,000. In contrast, the endogenous protein kinase of red cell membranes (*5*) and the soluble protein kinase of muscle (*12*) both phosphorylate higher molecular weight proteins.

The functional significance of this muscle surface membrane protein phosphorylation is unknown. The experiments of Greengard and colleagues (*13*), plus our observations in synaptosomes of rats subjected to electroconvulsive shock (*14*), would suggest a role for this reaction in ion permeability. Whether the same reactions are specifically involved in ion conductances of muscle membranes in normal states, after denervation, or in myotonic muscular dystrophy remains to be determined.

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References and Notes

1. D. A. Walsh, J. P. Perkins, E. G. Krebs, *J. Biol. Chem.* **243**, 3763 (1968); J. F. Kuo and P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.* **64**, 1349 (1969); T. A. Langan, *J. Biol. Chem.* **244**, 5763 (1969); M. Tao, M. Salas, F. Lipmann, *Proc. Natl. Acad. Sci. U.S.A.* **67**, 408 (1970).
2. H. Maeno, E. M. Johnson, P. Greengard, *J. Biol. Chem.* **246**, 134 (1971).
3. S. Gaballah and C. Popoff, *J. Neurochem.* **18**, 1795 (1971).
4. S. Lemaire, G. Pelletier, F. Labrie, *J. Biol.*

Chem. **246**, 7303 (1971); M. A. Kirchberger, I. L. Schwartz, R. Walter, *Proc. Soc. Exp. Biol. Med.* **140**, 657 (1972); T. P. Dousa, H. Sands, O. Hechter, *Endocrinology* **91**, 757 (1972); C. S. Rubin, J. Erlichman, O. M. Rosen, *J. Biol. Chem.* **247**, 6135 (1972); C. E. Guthrow, J. E. Allen, H. Rasmussen, *ibid.*, p. 8145.
5. A. D. Roses and S. H. Appel, *J. Biol. Chem.* **248**, 1408 (1973); C. S. Rubin and O. M. Rosen, *Biochem. Biophys. Res. Commun.* **50**, 421 (1973).
6. E. M. Johnson, H. Maeno, P. Greengard, *J. Biol. Chem.* **246**, 7731 (1971).
7. E. M. Johnson, T. Ueda, H. Maeno, P. Greengard, *ibid.* **247**, 5650 (1972).
8. A. D. Roses and S. H. Appel, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1855 (1973).
9. C. G. Andrew and S. H. Appel, *J. Biol. Chem.* **248**, 5156 (1973).
10. R. J. Boegman, J. F. Manery, L. Pinteric, *Biochim. Biophys. Acta* **203**, 506 (1970).
11. S. E. Allerton and G. E. Perlman, *J. Biol. Chem.* **240**, 3892 (1965).
12. V. L. Seery, E. H. Fischer, D. C. Teller, *Biochemistry* **6**, 3315 (1967).
13. R. J. DeLorenzo, K. G. Walton, P. F. Curran, P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 880 (1973).
14. S. H. Appel and C. Locher, *Neurology* **23**, 410 (1973).
15. Supported by PHS grants NS07872 and GM00233 and grant 558-C-4 from the National Multiple Sclerosis Society. We thank W. Clingenpeel for technical assistance.

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Muscle Carnitine Palmityltransferase Deficiency and Myoglobinuria

Abstract. *Muscle carnitine palmityltransferase activity, measured by three different methods, was very low (0 to 20 percent of controls) in a patient with a familial syndrome of recurrent myoglobinuria. Long-chain fatty acyl CoA synthetase activity was normal; acetylcarnitine transferase activity was decreased by 40 percent, and carnitine content was 1.7 times higher than the mean control value. Utilization of palmitate by isolated mitochondria was more impaired than utilization of palmitylcarnitine, suggesting a more severe defect of carnitine palmityltransferase I than transferase II. Thus, myoglobinuria may be due to a genetic defect of lipid metabolism in skeletal muscle.*

In 1970 Engel *et al.* (*1*) described a pair of identical twin girls, 18 years old, with intermittent "cramps" and myoglobinuria, frequently but not always related to exercise. The symptoms were also induced by fasting or a high fat diet. On the basis of metabolic studies, they suggested a defect in the utilization of long-chain fatty acids by skeletal muscle. Long-chain fatty acyl coenzyme A (CoA) synthetase was present in the muscle of these patients, but the activity of carnitine palmityltransferase was not investigated. We now report a defect of carnitine palmityltransferase in muscle of a patient with similar symptoms.

A 29-year old man had episodic muscle "cramps" and pigmenturia for 16 years. The symptoms were often, but not always, related to physical exertion and generally followed the exercise by several hours. Prolonged rather than strenuous exercise appeared to cause symptoms. The urinary pigment was identified as myoglobin on several occasions, and one attack was accompanied by renal shutdown. The patient's only brother had identical symptoms. Between attacks examination was normal. Ischemic work was followed by a normal rise of venous lactate, and contracture was not induced. Ultrastructural study of muscle showed no remarkable abnormality; there was no accumulation of lipid or glycogen. The clinical and morphological investigations have been described (*2*).

To exclude glycogen storage diseases causing myoglobinuria, glycogen metabolism was investigated in a muscle biopsy. The glycogen content was 1.15 percent [1.08 ± 0.04 (S.E.) in 33 controls]; phosphorylase and phosphofructokinase activities were normal.

Carnitine content, measured in frozen muscle (*3*), was 3.25 μmole per gram of tissue. In 13 controls, carnitine concentration was 1.92 ± 0.18 μmole per gram of tissue. The activity of the long-chain fatty acid activating enzyme, palmityl-CoA synthetase, measured in homogenates of frozen muscle (*4*), was normal (Table 1).

The short-chain fatty acyl carnitine transferase, or acetylcarnitine transferase, was determined in muscle extracts by a spectrophotometric method (*5*). Protein concentration was measured by the procedure of Lowry *et al.* (*6*). Acetylcarnitine transferase was about 40 percent less active in the patient's muscle than in six controls (Table 1). Carnitine palmityltransferase was measured by three different methods: (i) in the direction of palmityl-CoA formation (hydroxamate method) (*7*); (ii) by the "isotope exchange" method (*8*); and (iii) by a "forward reaction," in the direction of palmitylcarnitine formation, with DL-[^{14}C]carnitine and palmityl-CoA as substrates (*9*).

Carnitine palmityltransferase was studied in both crude muscle extracts and in mitochondrial fractions isolated from frozen muscle (*10*). In view of the predominant or exclusive mitochondrial localization of carnitine palmityltransferase (*11*), we isolated mitochondrial fractions to increase the sensitivity of the assay. Mitochondrial preparations from frozen muscle were considered satisfactory for this purpose, because the specific activity of the enzyme was about six times higher than that in whole homogenates and about ten times higher than in supernatants of the homogenates (Table 1). The mean values obtained for each assay were comparable in preparations from fresh and frozen muscles.

Table 1. Activities of palmityl CoA synthetase, carnitine acetyltransferase and carnitine palmityltransferase in human muscle. The activities are expressed as nanomoles of product formed per minute per milligram of protein (± S.E.). Control muscles were from six normal volunteers (vastus lateralis) and from non-weak patients undergoing operations (pectoral and gastrocnemius). Number of controls is in brackets. The values for the patient refer to two biopsies (right and left vastus lateralis).

Enzyme	Preparation	Enzyme activities	
		Controls	Patient
Palmityl CoA synthetase	Homogenate	2.77 ± 0.17 [9] (2.4–3.9)	2.21
Carnitine acetyltransferase	1000*g* supernatant	20.9 ± 1.6 [6] (16.0–27.2)	12.0
Carnitine palmityltransferase			
Hydroxamate	Homogenate	2.3 ± 0.2 [14] (1.2–3.8)	0; 0; 0
	Mitochondria	13.6 ± 1.0 [13] (8.6–19.0)	0.98; 1.20; 2.66
Isotope exchange	1000*g* supernatant	0.72 [3]	0; 0.17
	Mitochondria	6.8 ± 1.21 [8] (3.6–12.5)	0.64; 1.07
"Forward" radioactive	Mitochondria	16.6 ± 2.4 [5] (11.3–23.7)	3.3