Pharmacology Biochemistry & Behavior, Vol. 18, pp. 949–952, 1983. © Ankho International. Printed in the U.S.A.

Effects of Experiences on Synaptic Protein Phosphorylation *in Vitro*[1]

BEVERLY D. LYN-COOK[2] AND JOHN ERIC WILSON

Department of Biochemistry and Nutrition, and The Neurobiology Program
University of North Carolina at Chapel Hill, Chapel Hill, NC 27514

Received 17 July 1982

LYN-COOK, B. D. AND J. E. WILSON. *Effects of experiences on synaptic protein phosphorylation* in vitro. PHARMACOL BIOCHEM BEHAV **18**(6) 949–952, 1983.—*In vitro* transfer of ^{32}P from [γ-^{32}P]-ATP into proteins of particulate fractions from osmotically shocked preparations enriched in rat brain synaptosomes was studied. Phosphate incorporation into protein bands of apparent molecular weights (MW) 44,000, 24,000, 21,000, and 19,000 was affected by the prior experiences of the rats from which the particulate fractions were prepared. Incorporation into all four proteins was increased in particulate fraction from previously naive rats that received active avoidance training. Handling of the subjects prior to training prevented the response of the 24,000 MW protein to training. Phosphate incorporation into 24,000 and 19,000 MW proteins was increased in preparations from previously naive rats that underwent a yoked experience, while incorporation into the 21,000 MW protein was slightly decreased. The yoked experience did not affect *in vitro* phosphate incorporation into any of these proteins in particulate fractions from previously handled rats.

Active avoidance Brain Synaptic phosphoproteins Handling Protein phosphorylation Footshock

VARIOUS experiences have been reported to affect the incorporation of radioactive phosphate into proteins of rodent brain, either *in vivo* during the experience [5, 9, 12–14, 16], or *in vitro* following the experience [3, 4, 7, 10, 15].

Both of the identified phosphoproteins that respond to experience are enzymes of carbohydrate metabolism. Phosphate incorporation into glucose 6-phosphatase (EC 3.1.3.9), an enzyme of the endoplasmic reticulum that accepts a phosphate from the substrate during catalysis, is increased in the brains of sleeping rats [1]; while phosphate incorporation from ATP into the α-subunit of the decarboxylase moiety (pyruvate:lipoamide oxidoreductase [decarboxylating], EC 1.2.4.1) of the pyruvate dehydrogenase complex is elevated in homogenates [11] and in particulate fractions of lysed P_2 pellet (crude mitochondria) [15] prepared from the brains of rats that have experienced passive avoidance training.

We now report that increased *in vitro* phosphate incorporation into pyruvate dehydrogenase is also evident in particulate fractions of synaptosome-enriched preparations from the brains of rats that have experienced active avoidance training. In addition, the *in vitro* phosphorylation of three other proteins in these preparations is also affected by experience.

METHOD

Subjects and Behavioral Procedures

Male rats of the Sprague-Dawley strain were used (weight 250–300 g; from Charles River Farms, Wilmington, MA).

The animals were trained to avoid foot-shock by stepping to a platform, as previously described [9]. Briefly, on each trial the subject was placed on a grid floor at the darkened end of a short alley and allowed 5 sec to step to a platform at the bright end of the alley before onset of footshock. After an escape or avoidance, it was allowed to remain on the platform for 15 sec before start of the next trial. Training lasted 15 min, during which the rats made an average of 25 avoidance responses in 30 trials. Yoked rats received the same amount of shock and handling at the same time as the trained rats, but access to the escape platform was blocked by a sheet of Plexiglas so that these animals had no means to escape or avoid the footshock. Both trained and yoked rats were compared with quiet (untrained) rats that remained in the home cage during the training session.

Experiments used both naive rats and rats that had been previously handled for 2 min per day for 2 days prior to training, as noted in the Results section. At the end of training, rats were decapitated and the brains (minus olfactory bulbs and cerebellum) were quickly removed and fractionated in the cold.

Preparation of Synaptic Particulate Fraction

A synaptosome-enriched fraction was prepared at 4°C from each whole brain by the method of Hajos [6], and a particulate fraction was prepared therefrom. The 0.8 M sucrose layer from the final step of the Hajos procedure was removed by pipette, diluted slowly with 1.2 vol ice-cold deionized water, and pelletted by centrifugation at 10,000 × g for 30 min (Sorval SS-34 rotor). Electron micrographs

[1]Supported by Grant NS-15334 from the U.S. Public Health Service.
[2]Postdoctoral Trainee of the Neurobiology Program, University of North Carolina at Chapel Hill.

showed that about 66% of the particles in the pellet were synaptosomes and about 25% were mitochondria. The pellet, enriched in synaptosomes, was then osmotically disrupted by suspension in 2 ml ice-cold water for 30 min and the suspension was centrifuged at 10,000 × g for 20 min. The resulting sediment (synaptic particulate fraction) was suspended in 1 ml 10 mM Na_2HPO_4/KH_2PO_4 (pH 7.4), 2 mM $MgSO_4$, 0.2 mM ethylenediamine tetraacetate. Protein concentration was determined by the method of Bradford [2].

Phosphorylation and Analysis on One-Dimensional Denaturing Gels

Particulate fraction (50 μl of suspension containing 0.5 mg protein per ml) was incubated for 15 sec at 30°C and the reaction was started by the addition of 10 μl of buffer containing [γ-^{32}P]ATP (final concentration 10 μM ATP, 5–10 μCi ^{32}P). After 20 sec the reaction was stopped by the addition of 60 μl dissociation buffer (2% sodium dodecyl sulfate, 5% β-mercaptoethanol) followed by heating in a 100°C bath for 2 min. Tracking solution (120 μl of 50% stacking gel buffer, 40% glycerol, 4% β-mercaptoethanol, 6% solution of 0.04% bromophenol blue) was added to the solubilized sample.

Samples were then fractionated by electrophoresis on 1.5 mm thick SDS-12% polyacrylamide gel slabs by a slight modification of the method of Laemmli [8]. Samples that were to be compared were always applied to the same gel. Following electrophoresis, gels were fixed overnight in 50% ethanol-10% acetic acid-5% trichloroacetic acid-0.025% Coomassie blue, stained for 2 hr in 0.025% Coomassie blue in 10% (v/v) acetic acid and destained in 10% acetic acid. For radioautography stained gels were dried and placed in contact with Kodak X-O-mat AR film for 3 days. After development the radioautographs were scanned on a Gilford microdensitometer.

Apparent molecular weights (M_R) were estimated by comparison with molecular weight standards: myosin (200×10^3), β-galactosidase (116.5×10^3), phosphorylase b (94×10^3), bovine serum albumin (68×10^3), ovalbumin (45×10^3) and soybean trypsin inhibitor (21×10^3).

MATERIALS

Isotopically labelled ATP was from New England Nuclear Co. (Boston, MA), acrylamide was from Bethesda Research Laboratories (Gaithersburg, MD), N,N'-methylene bisacrylamide and N,N,N',N'-tetramethylethylenediamine were from Eastman Kodak Co. (Rochester, NY), and molecular weight standards were from Bio-Rad Laboratories (Richmond, CA). Bromophenol blue, 0.04% in buffer, was from Fisher Scientific Co. (Pittsburgh, PA). Other chemicals were of reagent grade, from various suppliers.

RESULTS

Figure 1 shows a representative photograph of the Coomassie blue staining pattern of a gel containing electrophoretically separated particulate fraction, and an autoradiogram of part of a gel in which phosphorylated samples from naive quiet and trained rats were run side-by-side. Increased radioactivity in electrophoretically separated components of M_R 44,000 (44K), 24,000 (24K), 21,000 (21K) and 19,000 (19K) is apparent in the track of the fraction from the trained rat.

Table 1 summarizes the results of a dozen experiments in which ^{32}P incorporation into particulate fractions from trained previously naive rats was compared with incorporation into fractions from quiet naive rats. Significant increases were seen in phosphate incorporation into the four protein bands shown.

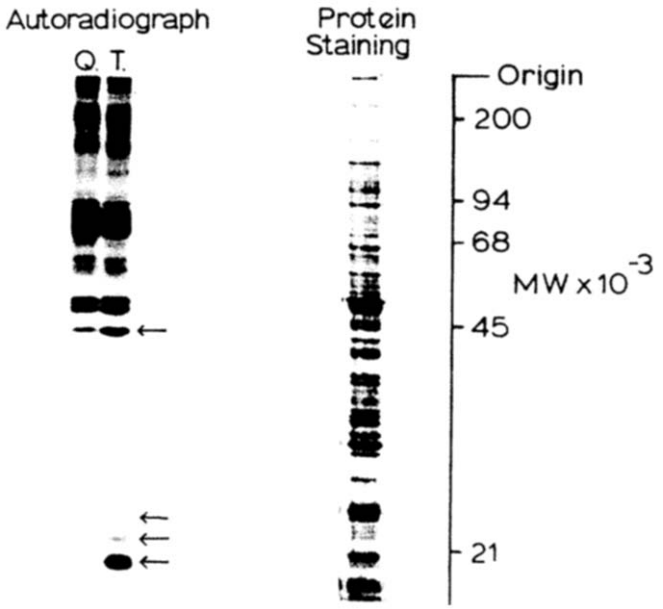

FIG. 1. On the right are shown the positions of molecular weight marker proteins adjacent to a Coomassie blue stained sodium dodecyl sulfate 12% polyacrylamide electrophoretic gel of rat brain synaptosomal particulate fraction. All preparations in this study yielded indistinguishable staining patterns. On the left is a radioautograph of two adjacent channels on an electrophoretic gel. Q=channel to which radioactive particulate fraction from a quiet naive rat was applied. T=channel to which radioactive particulate fraction from a trained, previously naive rat was applied. Arrows indicate (from top to bottom) positions of 44,000, 24,000, 21,000 and 19,000 M_R phosphoprotein bands.

To control for the effects of some of the experiences that are involved in training, a second series of experiments was done. Radioactive phosphoproteins of particulate fractions from both trained and yoked rats were compared with radioactive phosphoproteins of preparations from quiet rats. Naive rats were used in one subset of the second series and previously handled (2 min per day for 2 days) rats were used in the other subset.

The results of the second series of experiments are shown in Table 2.

DISCUSSION

The results presented here show that the immediate effects of active avoidance training on the *in vitro* phosphorylation of the 44K band are similar to the previously reported effects of passive avoidance training on protein F_2 of Routtenberg and his colleagues [10,15]. On the basis of its biochemical and electrophoretic properties, comparison with authentic mammalian pyruvate dehydrogenase, and its marked enrichment in mitochondrial preparations, we identify the 44K protein as the α-subunit of pyruvate dehydrogenase (Lyn-Cook, Ruder and Wilson, in preparation), i.e., it is identical to F_2. Our results, however, do not require that the animals be killed in liquid nitrogen for their demonstration [11]. This difference may be the consequence of different assay conditions, of different training paradigms or of

TABLE 1

EFFECTS OF ACTIVE AVOIDANCE TRAINING ON *IN VITRO* PHOSPHATE INCORPORATION INTO FOUR PROTEINS OF PARTICULATE FRACTION FROM SYNAPTOSOMES OF NAIVE RATS

M_R of phosphoprotein	Phosphorylation* Quiet	Trained	Mean % Increase	p†
44,000	4.58 ± 1.58	6.08 ± 1.61	33	0.016
24,000	2.62 ± 1.54	5.16 ± 1.72	97	<0.001
21,000	5.02 ± 2.54	8.15 ± 2.19	62	0.018
19,000	9.54 ± 1.58	11.79 ± 2.92	24	0.014

*Each value is the area under the densitometric tracing of the indicated peak, expressed as percent of the total area under the tracing of the radioautograph. Preparations from one quiet and one trained rat were resolved on the same gel. Values are means ± S.D. N=12 for each group.

†p calculated by a paired t-test.

TABLE 2

EFFECTS OF YOKED AND TRAINING EXPERIENCES ON *IN VITRO* PHOSPHATE INCORPORATION INTO FOUR PROTEINS OF PARTICULATE FRACTION FROM NAIVE AND PREVIOUSLY HANDLED RATS

M_R of phosphoprotein	Yoked % diff. from quiet*	p†	Trained % diff. from quiet*	p†
	Naive Rats			
44,000	− 5.4	0.078	+ 4.3	0.19
24,000	+48	0.038	+54	0.012
21,000	− 7.7	0.047	+32	0.003
19,000	+36	<0.001	+34	<0.001
	Previously Handled Rats			
44,000	− 6.5	0.29	+10.4	0.013
24,000	+ 2.8	0.56	+11	0.24
21,000	− 3.8	0.17	+56	0.003
19,000	+ 0.5	0.54	+83	0.024

*Values were calculated as in Table 1, and are expressed here as difference from values for quiet controls. Particulate fractions from one quiet, one yoked and one trained rat were resolved on the same gel. N=4 for each group.

†p calculated by a paired t-test.

using partially purified synaptosomes rather than a whole homogenate or a particulate fraction from crude mitochondria. If the explanation lies in the use of more purified synaptosomes, it would imply either that the effect is on synaptic mitochondria, or that effects in synaptic mitochondria differ from effects in other neuronal or glial mitochondria.

The effect of training on the *in vitro* phosphorylation of the 44K protein was most readily seen in preparations from rats that had been previously handled (cf., Tables 1 and 2). Whatever the behavioral trigger for this effect, it does not seem to be merely novelty of the training experience, since the effect was not observed in our preparations from yoked rats that had been previously handled, nor was it reported to occur in brain homogenates from previously handled rats that received footshocks alone [11].

For clarity, and as an aid in making comparisons, the results of the various sets of experiments are summarized in Table 3.

The *in vitro* phosphorylation of each of the phosphoproteins being considered was uniquely affected by the various experiences. Neither the yoked nor training experience, for example, seemed to affect phosphorylation of the 24K band in preparations from previously handled animals, while rather large effects were noted in preparations from previously naive animals that had undergone these experiences. It thus appears that neither footshock nor any unique aspect of training, such as acquisition of avoidance behavior, served as a trigger for the effects of experiences on *in vitro* phosphorylation of this protein in preparations from naive rats.

Phosphate incorporation into the 21K band, in contrast, was somewhat diminished in preparations from yoked naive

TABLE 3

SUMMARY OF EFFECTS OF EXPERIENCES ON ^{32}P INCORPORATION INTO PROTEINS OF SYNAPTIC PARTICULATE FRACTION

	Subjects			
	Naive		Pre-handled	
M_R of phosphoprotein	T*	Y	T	Y
44,000	↑↑	ns	↑	ns
24,000	↑↑	↑↑	ns	ns
21,000	↑↑	↓	↑↑	ns
19,000	↑↑	↑↑	↑↑	ns

*This column is based on Table 1. Other columns are from Table 2.
T = trained rats. Y = yoked rats.
Single arrow: <20% increase or decrease in ^{32}P incorporation.
Double arrow: >20% increase or decrease in ^{32}P incorporation.
ns: No significant effect of experience on ^{32}P incorporation.

rats, but was increased in preparations from trained rats regardless of whether they had been previously handled. The pattern of behavioral effects on this protein further differs from the pattern for the 44K protein in that significant increases in incorporation were more readily seen in preparations from both previously naive and previously handled rats that had been trained. It appears that increased phosphate incorporation into 21K and 44K bands is seen only as a consequence of the training experience.

The only group whose particulate fraction did not incorporate more ^{32}P into the 19K band after behavioral treatment consisted of previously handled yoked rats. The phosphorylation response to training was as great in preparations from previously handled animals as in preparations from naive animals (Table 2).

In summary, the effects of experience in the *in vitro* phosphorylation of the 24K band do not appear to be directly related to neural events uniquely required for associative learning or for the establishment of memory. It remains possible that one or more of the other biochemical effects described here are so related; although this seems improbable in the case of the 44K protein, which is located in the mitochondrial matrix and whose known function is as a catalyst in the production of cellular energy and acetyl CoA.

ACKNOWLEDGEMENTS

We are indebted to Daniel B. Hoch for helpful suggestions during the course of this work, and to Margaret A. Eastman for technical assistance.

REFERENCES

1. Anchors, J. M. and M. Karnovsky. Purification of cerebral glucose-6-phosphatase an enzyme involved in sleep. *J Biol Chem* **250:** 6408–6416, 1975.
2. Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
3. Conway, R. G. and A. Routtenberg. Endogenous phosphorylation in vitro: selective effects of sacrifice methods on specific brain proteins. *Brain Res* **139:** 366–373, 1978.
4. Ehrlich, Y. H., R. R. Rabjohns and A. Routtenberg. Experiential input alters the phosphorylation of specific proteins in brain membranes. *Pharmacol Biochem Behav* **6:** 169–174, 1977.
5. Glassman, E., W. H. Gispen, R. Perumal, B. Machlus and J. E. Wilson. The effect of short experiences on the incorporation of radioactive phosphate into synaptosomal and non-histone acid-extractable nuclear proteins from rat and mouse brain. In: *Pharmacology and the Future of Man*, vol 4, Proc. 5th Int. Congr. Pharmacol. Basel: Karger, 1973, pp. 14–17.
6. Hajos, F. An improved method for the preparation of synaptosomal fractions in high purity. *Brain Res* **93:** 485–489, 1975.
7. Holmes, H., R. Rodnight and R. Kapoor. Effect of electroshock and drugs administered *in vivo* on protein kinase activity in rat brain. *Pharmacol Biochem Behav* **6:** 415–419, 1977.
8. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685, 1970.
9. Machlus, B. J., J. E. Wilson and E. Glassman. Brain phosphoproteins: the effect of short experiences on the phosphorylation of nuclear proteins in rat brain. *Behav Biol* **10:** 43–62, 1974.
10. Morgan, D. G. and A. Routtenberg. Evidence that a 41,000 dalton brain phosphoprotein is pyruvate dehydrogenase. *Biochem Biophys Res Commun* **95:** 569–576, 1980
11. Morgan, D. G. and A. Routtenberg. Brain pyruvate dehydrogenase: phosphorylation and enzyme activity altered by a training experience. *Science* **214:** 470–471, 1981.
12. Perumal, R., W. H. Gispen, J. E. Wilson and E. Glassman. Phosphorylation of proteins from the brains of mice subjected to short-term behavioral experiences. In: *Hormones, Homeostasis and the Brain, Progress in Brain Research*, vol 42, edited by W. H. Gispen, Tj. B. van Wiemersma Greidanus, B. Bohus and D. de Wied. Amsterdam: Elsevier Scientific Publishing Co., 1975, pp. 201–207.
13. Reich, P., J. K. Driver and M. Karnovsky. Sleep: effects on incorporation of inorganic phosphate into brain fractions. *Science* **157:** 336–338, 1967.
14. Reich, P., S. J. Geyer, L. Steinbaum, M. Anchors and M. L. Karnovsky. Incorporation of phosphate into rat brain during sleep and wakefulness. *J Neurochem* **20:** 1195–1205, 1973.
15. Routtenberg, A. and G. E. Benson. In vitro phosphorylation of a 41,000 MW protein band is selectively increased 24 hr after footshock or learning. *Behav Neural Biol* **29:** 168–175, 1980.
16. Souza, D. O., E. Elisabetsky and I. Izquierdo. Effect of various forms of training and stimulation on the incorporation of ^{32}P into nuclear phosphoproteins of rat brain. *Pharmacol Biochem Behav* **12:** 481–486, 1980.