Brain Pyruvate Dehydrogenase: Phosphorylation and Enzyme Activity Altered by a Training Experience

Abstract. *The active portion of the alpha subunit of pyruvate dehydrogenase in rat frontal cortex was elevated after a training experience. No change in total pyruvate dehydrogenase activity was observed. The phosphorylation in vitro of pyruvate dehydrogenase (band F-2) was also elevated after training. Since activation of pyruvate dehydrogenase requires its dephosphorylation, the following sequence is proposed. Training alters frontal cortex activity and reduces the phosphate content of pyruvate dehydrogenase in vivo; this leads to enzyme activation and an increase in back-titration of sites available for phosphorylation in vitro.*

The phosphorylation states of brain proteins are altered in the rat by pharmacological (*1*) and electrophysiological (*2*) treatments and by behavioral treatments such as the induction of avoidance responses (*3*). We have shown that the phosphorylation in vitro of a 41,000-dalton phosphopeptide, band F-2, was elevated in the frontal cortex in subjects trained on a step-down avoidance task (*4*), and that band F-2 is the alpha subunit of pyruvate dehydrogenase (PDH) (*5*). To determine whether the training-induced increase in band F-2 phosphorylation (*4*) corresponds to a change in PDH activity, we evaluated the effect of training on the enzyme activity and phosphorylation state of this enzyme in the frontal cortex.

Beginning 4 days after their arrival, 16 male albino rats of the Holtzman strain (200 to 230 g) were weighed and handled for 2 minutes per day for 5 days and were then assigned to one of four groups. Group 1 received passive avoidance training (*6*) for the next 5 days (one trial each day); group 2 was weighed and handled for four more days and subjected to passive avoidance training on day 10. These two groups were compared to evaluate the effects of overtraining. Group 3 was given noncontingent footshocks on day 10, but was otherwise treated in the same fashion as group 2; group 4 was weighed and handled for five additional days. Immediately after treatment on day 10, all animals were killed by immersion in liquid nitrogen for 90 seconds (*7*) and stored at −20°C.

We dissected the frozen brains in a cold room (2° to 4°C), homogenized the frontal cortex minus white matter, and extracted the PDH complex (*8*). Portions of the extract were removed for assays of PDH activity (Table 1) and protein phosphorylation in vitro and for the determination of protein (*9*). These portions were rapidly frozen and stored at −20°C.

Pyruvate dehydrogenase in brain and other tissues is active or inactive, depending on the state of phosphorylation. The phosphorylated (inactive) enzyme can be dephosphorylated (activated) in vitro by incubation in the presence of magnesium and calcium, which stimulate the PDH phosphatase. Thus, the active portion (*A*) and the total PDH activity (*T*) can be measured independently in vitro by conducting the assay before and after incubation in magnesium and calcium ions. The ratio of these values (*A*/*T*) is an index of the proportion of the PDH molecules that are dephosphorylated and thus enzymatically active (*10*).

In trained animals, both the active portion of PDH and the *A*/*T* ratio were greater than the corresponding values in untrained subjects (Table 1). The total PDH activity did not differ in the trained and untrained groups, indicating that passive avoidance training did not cause a net increase in synthesis or a net decrease in degradation.

Training also caused an increase in PDH (band F-2) phosphorylation. Both the absolute densitometric area of band F-2 and the percentage of the total radioactive phosphate bound to protein in band F-2 were increased (Table 1). Neither the total ^{32}P incorporation nor phosphoprotein bands other than band F-2 were affected by the training experience (*10*). Thus, the training-induced increase in band F-2 phosphorylation was not due to a general increase in phosphorylation, but rather was specific to this phosphopeptide.

Groups 3 and 4, which were shocked only and handled only, respectively, had similar PDH activities (3.2 and 3.1 nmole/min per milligram of protein), as well as similar band F-2 phosphorylation (240 and 245 mm^2). The animals trained for 1 day had a somewhat greater response to the learning task than those trained for 5 days (PDH, 4.9 compared to 4.2 nmole/mg-min; and band F-2, 340 compared to 270 mm^2). No statistically significant differences among these groups ($.1 > P > .05$) were observed with a one-way analysis of variance or a Newman-Keuls test for individual comparisons. The correlation between band F-2 phosphorylation and PDH activity is +.679 for *A*/*T* and F-2 area and +.647 for active pyruvate dehydrogenase and

Table 1. Effects of training on relative pyruvate dehydrogenase activity and band F-2 phosphorylation. Frontal cortex homogenates were assayed for both active and total PDH activity by a modification of the methods of Cremer and Teal (*19*). For the measurement of initial activity, frozen homogenates were thawed at 30°C for 1 minute, and 250 μl was transferred to the center well of the decarboxylase assay vessel (*20*). The homogenate was incubated for 2 minutes in the assay mixture; final concentrations were potassium phosphate, 30 m*M* (*p*H, 7.8); EDTA, 1 m*M*; dithiothreitol, 2 m*M*; magnesium chloride, 4 m*M*; thiamine pyrophosphate, 0.5 m*M*; coenzyme A, 1.5 m*M*; NAD^+, 6 m*M*; lactate dehydrogenase, 50 μg/μl; and phosphotransacetylase, 5 μg/μl; [1-^{14}C]pyruvate was then added in a volume of 20 μl to produce a final concentration of 0.5 m*M* (specific activity, ~1000 cpm/nmole). The reaction was continued for 5 minutes at 30°C and then 100 μl of 6*N* sulfuric acid was added. Incubation was continued for 1 hour to trap the evolved CO_2 in 0.3 ml of solubilizer (Nuclear Chicago). The amount of CO_2 evolved was calculated as nanomoles per minute per milligram of protein and represents the active portion of the PDH activity. The total PDH activity was determined after an 18-minute incubation in 10 m*M* $MgCl_2$ and 0.5 m*M* $CaCl_2$ to dephosphorylate the enzyme (*21*). The assay was conducted as above, with the $MgCl_2$ content of the reaction mix adjusted to a final concentration of 4 m*M*. The frontal cortex homogenates were also phosphorylated in vitro (*4*, *5*), in a phosphate buffer. The densitometric area of band F-2 was divided by the total integrated area of the sample to produce the percentage of total measure. Total incorporation of ^{32}P was determined from trichloroacetic acid precipitates (*4*, *5*). Values are means ± the standard error. Statistical analyses were performed with Student's *t*-test ($N = 8$ for trained and untrained groups).

PDH activity			Phosphorylation in vitro		
			Band F-2		
Active (nmole/mg-min)	Total (nmole/mg-min)	*A*/*T*	Area (mm^2)	Percentage of total area	Total (pmole/mg)
Trained					
4.62 ± 0.26	7.54 ± 0.54	0.64 ± 0.06	307.0 ± 27.7	1.3 ± 0.1	69.5 ± 5.1
Not trained					
3.18 ± 0.47*	7.18 ± 0.35	0.43 ± 0.05*	242.0 ± 9.7*	0.97 ± 0.07*	65.9 ± 4.9

*$P < .05$.

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F-2 area ($P < .01$ for both correlations).

Increased PDH activity results from a decreased phosphorylation of the enzyme (*11*); we have demonstrated this relationship in the brain (*12*). With precautions taken to preserve the phosphorylation in vivo and the enzyme activity state (*7, 8*), increased phosphorylation of PDH (band F-2) in vitro in trained animals reflects a reduced phosphorylation of PDH in vivo. We conclude that the training-induced increase in frontal cortex PDH activity occurs after a dephosphorylation of PDH in vivo.

Training-induced alterations in brain PDH may regulate synaptic function. This could involve synthesis of transmitters such as acetylcholine (via acetyl coenzyme A) and glutamate (via the tricarboxylic acid cycle) (*13*) in response to synaptic activation after repetitive stimulation (*14*). Moreover, insulin, which is localized to presynaptic terminals (*15*), can induce an increase in PDH activity (*16*), by stimulating PDH phosphatase through a peptide intermediary (*17*). It is possible that such peptide second messengers alter the brain PDH activity observed 24 hours after training (*3*).

These considerations suggest that brain PDH, through a phosphorylation-dephosphorylation cycle, is sensitive to manipulations of brain activity and may initiate or participate in the biochemical response of the cells involved in the neuronal plasticity of learning and memory (*18*).

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References and Notes

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6. ———, *Brain Res.* **179**, 329 (1979). Groups 3 and 4 (shocked only and handled only) are referred to here as not trained, although some learning, not related to step-down training, occurred.
7. Liquid nitrogen immersion prevents the rapid dephosphorylation of PDH observed after decapitation [R. Jope and J. P. Blass, *J. Neurochem.* **26**, 709 (1976)]. We have never observed a training effect on band F-2 after decapitation, probably because of this rapid dephosphorylation. For a comparison of the liquid nitrogen and decapitation methods, see R. G. Conway and A. Routtenberg [*Brain Res.* **139**, 366 (1978)]. In a recent study (*22*), dephosphorylation at 0°C was less than 10 percent, even after 1 hour. Although more rapid methods are available for brain enzyme fixation [J. V. Passonneau, R. A. Hawkins, W. D. Lust, F. A. Welsh, *Cerebral Metabolism and Neural Function* (Williams & Wilkins, Baltimore, 1980) pp. 10–19], these are not compatible with brain dissection and regional analysis. With adult rats frozen in liquid nitrogen, the frontal cortex would attain 0°C within 5 to 30 seconds, depending on the cortical layer.
8. The frontal cortex anterior to the caudate nucleus was removed bilaterally and homogenized, while still frozen, in 20 volumes of 30 m*M* potassium phosphate buffer (2°C, *p*H 7.2) with 1 m*M* EDTA to inhibit the PDH phosphatase. The homogenate was frozen in liquid nitrogen and thawed in the cold room to extract the PDH complex. The total time in a fluid state between homogenization and transfer to assay and reaction tubes was less than 5 minutes. These precautions were taken to minimize dephosphorylation of the PDH complex. Proteolysis was evaluated and not observed with five different protease inhibitors.
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Questions About Spatial Adaptation of Short-Wavelength Pathways in Humans

Stromeyer *et al.* (*1*) have reported color-selective spatial adaptation of the blue-sensitive visual pathway. They found that a violet adapting grating superposed on a circular yellow-green adapting field of uniform luminance decreased the detectability of violet (but not of red) test gratings of the same orientation and spatial frequency. This effect was strongly monocular. They tested two subjects under dichoptic conditions, one with alternating adapting and test gratings and the other with a continuously presented adapting grating. The first subject showed only slight (less than 10 percent) interocular transfer and the second showed none.

Stromeyer *et al.*'s study contains a flaw with regard to postreceptoral adaptation. Specifically, their subjects fixated the adapting field (presumably at its center, since no explicit fixation target is described) (*2*). This procedure almost certainly produced substantial patterned adaptation of the short-wavelength cones themselves (*3*). Such adaptation is expected to be selective for wavelength, spatial frequency, and orientation, just as Stromeyer *et al.* found.

The dichoptic results cannot be fully explained by either local retinal adaptation, which is strictly monocular, or by cortical spatial adaptation, which predicts strong interocular transfer. An alternative hypothesis is that the negative afterimage of the adapting grating in the alternating grating condition raised the test grating threshold by means of dichoptic opponent color cancellation (*4*). Under this hypothesis, the other subject would not have seen a comparable effect because the continuous adapting grating produced no negative afterimage.

Retinal adaptation, unlike central adaptation (*5*), depends on the relative spatial phases of the test and adapting gratings. Stromeyer *et al.* could therefore have checked for retinal artifacts by shifting the phase of the test grating by, say, 180°. Their figure 1, however, suggests that they used only test and adapting gratings of equal phase, the condition expected to produce the maximum retinal effect.

Whereas color-sensitive spatial adaptation of central visual pathways remains possible, Stromeyer *et al.*'s results can be easily explained without it.

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