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Experiential Input Alters the Phosphorylation of Specific Proteins in Brain Membranes[1]

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EHRLICH, Y. H., R. R. RABJOHNS AND A. ROUTTENBERG. *Experiential input alters the phosphorylation of specific proteins in brain membranes.* PHARMAC. BIOCHEM. BEHAV. 6(2) 169–174, 1977. – The effects of a training experience that involves foot shock on the endogenous phosphorylation of membrane-bound proteins from brain were studied. Crude membrane fractions were prepared from the cerebral cortex and neostriatum of animals that had been sacrificed by quick freezing. In vitro incubation of the membranes with gamma-^{32}P-ATP, followed by SDS-gel electrophoresis of the phosphorylated substrates, revealed that the phosphorylation of two protein components (designated F and H-I) increased in preparations from animals that were subjected to a training experience 24 hr prior to sacrifice. These effects were greater in preparations from the neostriatum than from the cerebral cortex, and were observed in experiments using both rats and mice. Although all trained animals showed a high phosphorylation of bands F and H-I, control animals showed a greater variability in the phosphorylation of these bands. The results indicate that the phosphorylation of specific proteins may play a mediatory role in the processing of experiential information.

Training experience Protein phosphorylation in vitro Brain-membranes Gamma-^{32}P-ATP
SDS-gel electrophoresis

NUMEROUS studies have demonstrated that metabolic alterations occur within brain cells consequent to various inputs from the environment [4, 7, 10]. Nevertheless, the mechanisms that link peripheral sensory stimulation to molecular events within cells of the central nervous system remain obscure. One means whereby environmental input can modify cellular metabolism appears to be mediated by protein phosphorylation systems that are activated by adenosine 3′, 5′ – monophosphate (cyclic AMP). On the basis of studies which have investigated the activity of these phosphorylation systems (for recent reviews see references [2, 22, 30]) a scheme that may help clarify the relation between experiential-input and biochemical changes can be suggested. Information provided by environmental stimuli is known to reach target cells in the brain by means of afferent input and in the form of neurotransmitters. The interaction of neurotransmitter molecules with receptors at the affected cells' surface activates a membrane-bound enzyme, adenyl cyclase. Its product, cyclic AMP, accumulates inside the cell and can act, therefore, as the intracellular messenger for information transmitted by neuronal activity. This information is then expressed and specified via the effects of cyclic AMP on the phosphorylation of specific proteins. The ability of cyclic AMP to produce rapid changes in the phosphorylation state of specific proteins in synaptic plasma membranes has been related to its postulated role in the formation of post synaptic potentials [12,25]. Such mechanisms may be involved in short-lived responses of the target cells to the evoking stimulus. In addition, phosphorylative modifications can play a role in long-term processes. Studies using the adrenal medulla [2,9] and cells grown in culture [23] as model systems have indicated that the transsynaptic induction of enzymes involved in the biosynthesis of neurotransmitters is mediated by phosphorylation mechanisms activated by cyclic AMP.

Several lines of investigation suggest that protein phosphorylation may be similarly involved in mediating input-dependent processes within the brain. Electrical [28] as well as neurohumoral [29] stimulation of cerebral cortex slices have been shown to increase the phosphorylation of membrane-bound proteins. Two specific proteins whose phosphorylation is cyclic AMP-regulated were identified in synaptic membrane fractions from the rat cerebrum

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[12,25]. Detailed characterization of the phosphorylative properties of several membrane-bound proteins from cerebral cortex indicated potential functional differences among these proteins [5,20]. Finally, recent reports have demonstrated increased incorporation of radioactive phosphate into membrane-bound [16] and nuclear [14,15] proteins in brain of mice undergoing training on a conditioned avoidance task.

Our previous studies [5, 6, 20] have identified several specific proteins in membrane preparations from the cerebral cortex and neostriatum of rats and mice which are phosphorylated and dephosphorylated during an in vitro incubation. The phosphorylative properties of these proteins suggested their potential for involvement in a sequence of time-dependent processes following input to the central nervous system. The present report is a demonstration that increased phosphorylation of specific protein components in brain membranes can be detected in vitro when rats or mice are subjected to a novel experience 24 hr prior to sacrifice by quick freezing. A preliminary report of this work has been presented [21].

METHOD

Male, adult albino rats (Holtzman, Madison, Wis.) weighing 80–120 g at time of sacrifice were used. In each experiment, 9 rats were equally divided among three treatment groups. Group 1 (controls) were handled, but were never exposed to footshock. Animals in Group 2 received inescapable footshock, while animals in Group 3 were permitted to escape the footshock by climbing on a platform. Rats of all three groups were handled for 1 min per day for at least four days prior to the treatment and sacrifice which occurred in the light portion of a 12 hr light (8:00 a.m. to 8:00 p.m.) to 12 hr dark cycle. In later experiments, inbred mice (Jackson Laboratory, Bar Harbor) weighing 20–25 g were used in the same experimental design. The training experience was similar to that used previously in studies of learning and electrical brain stimulation [1,18] and learning and brain chemistry [19]. Briefly, animals descending from a platform received 0.5 mA footshock, and typically descended 2 or 3 times before remaining on the platform. Animals of Group 3 spent the majority (more than 95%) of the test session (10–25 min) moving about on the platform. Animals in Group 2 received an equivalent duration of shock as those in Group 3, but were prevented from escaping the shock by a barrier placed between the platform and the floor shock grid. Most of the animals in this group remained motionless in the apparatus after receiving the shock. Control animals were placed in the apparatus but received no shock.

After handling (Group 1), shock (Group 2), and learned escape (Group 3) experience, all the animals were returned to their home cages. Twenty-four hours later they were sacrificed by whole body immersion in liquid nitrogen, in order to minimize the ischemic and anoxic effects which were reported to produce post-mortem changes in cerebral ATP [3] and cyclic nucleotides [8] after decapitation. Animals were then kept frozen at $-20^{\circ}C$ until tissue preparations were made, 7 days or less after the time of sacrifice. For purposes of dissection, animals were partially thawed in the cold room ($4^{\circ}C$). The cerebral cortex or caudate/putamen complex (neostriatum) from the three subjects in each of the three groups were pooled. The crude membrane fractions (P_2, osmotically shocked and washed three times in hypotonic solution to assure maximal removal of soluble components) were prepared as described previously [5,20].

Endogenous phosphorylation assays were carried out by incubating aliquots of the membrane preparations with gamma-^{32}P-ATP as described [5,20]. Each reaction mixture (0.06 ml) contained 0.66 mg/ml protein (determined by the Lowry method with bovine albumin as standard), 50 mM sodium acetate (pH 6.5), 10 mM magnesium chloride, 7.5 μM ATP and 5μM cyclic AMP. The gamma-^{32}P-ATP solution, used to initiate the reaction, was prepared by diluting commerically available radioactive ATP (ICN) with nonradioactive TRIS·ATP (Sigma) to give $10-20 \times 10^6$ CPM per n mole. The assay began by 5 min preincubation of each sample at 30°C and the reaction was initiated by adding the ATP. In order to compare phosphorylation patterns of specific proteins in the three experimental groups, standard reaction conditions were selected which consisted of two min incubation after the addition of the initiating ATP-gamma-^{32}P. This reaction time was chosen since the change with time in the amount of protein-bound phosphorus is minimal after two min reaction as compared to the extremely rapid changes that occur between 0 and 1 min [5,20]. Also, at 2 min reaction time the effects of preincubation time on phosphate incorporation are not significant [20]. The reactions were terminated and the membranes were solubilized by the addition of sodium dodecyl sulfate (SDS) to final concentration of 3% SDS, containing 2% beta-mercaptoethanol, 5 mM Tris-acetate (pH 8.0), 6% sucrose, and bromphenol blue (tracking dye) [5,20]. Electrophoresis was carried out using a 7–14% linear acrylamide gel gradient as previously described [20]. The gels were stained for protein with coomassie brilliant blue, destained, and subjected to autoradiography [20]. Quantitative analysis of incorporation of ^{32}P into specific bands was carried out by microdensitometry of the autoradiographs according to the recommendations of Ueda, *et al.* [25], since we have shown [5] that there is a high positive correlation (+0.87) between density and CPM from corresponding gel locations. Statistical analysis of the microdensitometric data collected from three separate experiments was carried out using a one-way analysis of variance [31] of the percent alteration of phosphorylation in Groups 2 and 3 relative to Group 1. The results of our assays provide a measure of the amount of radioactive phosphate in each band resolved by SDS-electrophoresis. Therefore, the terms phosphorylation and dephosphorylation are used only to indicate increments and decrements, respectively, in ^{32}P-content of specific bands in the gel, without reference to the capacity of specific enzymes. Over 80% of the phosphate incorporated under the standard assay conditions is bound to protein in a phospho-ester linkage [5,20].

RESULTS

The incorporation of ^{32}P-phosphate from ^{32}P-gamma-ATP into total proteins of crude membrane preparations obtained from rats that had experienced inescapable (Group 2) or escapable (Group 3) footshocks 24 hr prior to sacrifice by quick freezing was only slightly higher than that of similar preparations obtained from controls of Group 1. However, small differences in total incorporation were shown previously to be indicative of large and significant changes in the phosphorylation of specific, electrophoretically separated protein components [5, 12,

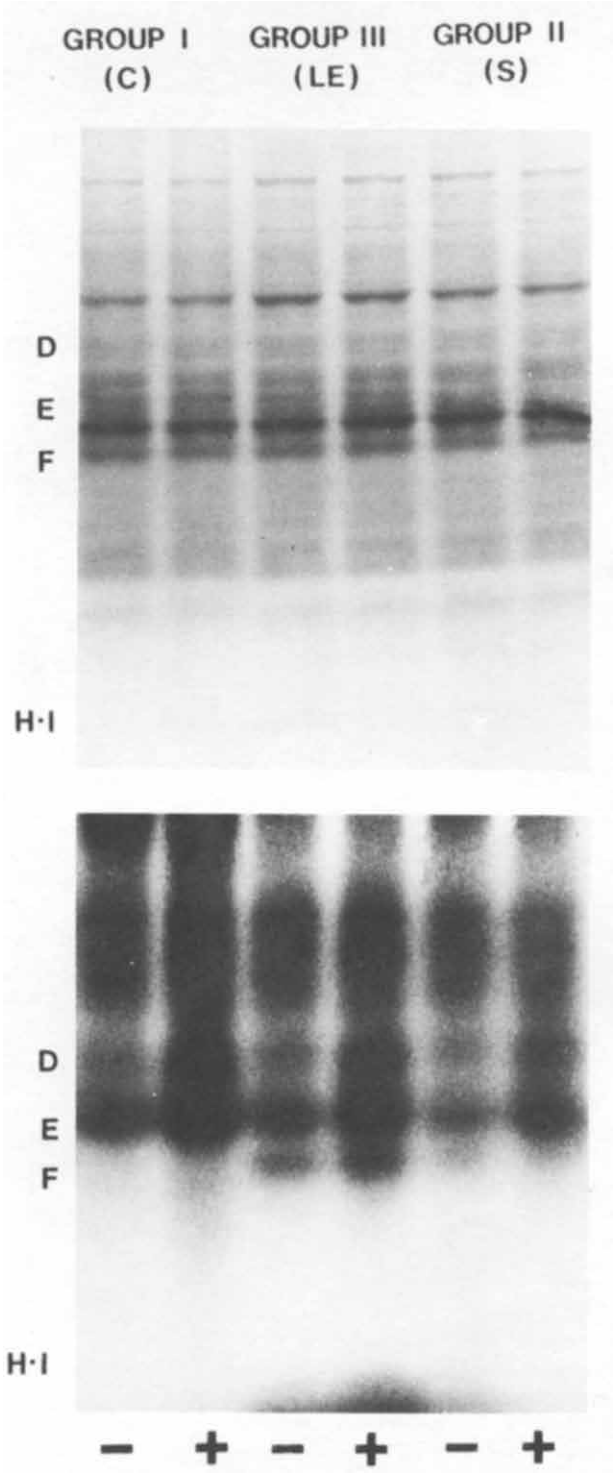

FIG. 1. Protein staining and autoradiograph of Group 1 (handled), Group 2 (shock only), and Group 3 (learned escape) of separated reaction products from caudate nucleus. Osmotically shocked crude membrane fractions were assayed as described in the text, solubilized in SDS and separated on a 7–14% linear acrylamide gel gradient to resolve the component proteins of the preparations. In the assay demonstrated here, the sp. act. of the [γ-^{32}P]-ATP was 14.8×10^6 cpm per nmole and 40 μg of protein was applied to each well of the gel. (+) and (−) refer to presence and absence of added cyclic AMP in the reaction mixture, respectively. Note that Group 3 is located between Group 1 and Group 2.

20, 22, 25]. Comparison of the electrophoretic patterns (Fig. 1, autoradiogram) indeed demonstrated that the phosphorylation of protein components F and H-1 in Groups 2 and 3 was substantially elevated compared to the amount of label that appeared in the corresponding bands obtained from the control animals (Group 1), while other bands showed only small differences between groups. On the other hand, there were no differences in protein profiles between the three groups (Fig. 1, coomassie staining). Confirming the reported finding [19] that the level of protein of each band, as detected by densitometry of the stained gels, is not affected by the conditions of training used in our studies.

A quantitative assessment (Fig. 2) of the phosphorylative changes observed revealed that shocked animals of Groups 2 and 3 showed a marked elevation of label in bands F and H-1 compared to controls (Group 1), and these effects were more pronounced in the caudate nucleus than in the cerebral cortex, $F(1,48) = 9.7$, $p<0.01$. When Group 3 only was considered, similar brain regional effects were observed, $F(1,22) = 6.1$, $p<0.01$. Although bands D and E also showed small effects of footshock and training in the caudate nucleus, band F showed a significantly greater phosphorylative effect than bands D and E, $F(1,7) = 10.8$, $p<0.01$. The effects of experiential-input on the phosphorylation of the band designated H-1 were very similar to those noted for band F. However, this band was found to be a complex that contained several bands which were difficult to resolve by the densitometric methods used. For this reason, quantitative analysis of the phosphorylation properties of band H was not included in our previous report [20]. The data provided for band H-1 here (Fig. 2) relate to the phosphate incorporated into the whole complex and in this respect, its quantitative assessment is less certain than that of band F. It should be noted, however, that as a complex, also the response of band H-1 to a variety of reaction conditions [20] was very similar to that of band F.

The quantitative analysis revealed that the phosphorylation of band F (75.0 ± 23.5; mean ± S.D. expressed in arbitrary density units, n = 3) from neostriatal membranes recovered from trained animals was higher than that of band E (65.0 ± 9.00) and D (30.3 ± 10.01). In control animals, however, the mean incorporation of phosphate into band F (20.3 ± 19.85) was lower than that incorporated into band E (55.0 ± 18.52) and close to that of band D (23.3 ± 11.06). Thus, the ratio of the amount of label incorporated into band F to that incorporated into band E appears to be a function of whether or not the animal had been exposed to footshock 24 hr prior to sacrifice.

A greater variability in the phosphorylation of band F in membrane preparations from controls (20.3 ± 19.85) compared to trained animals (75.0 ± 23.5) was observed. A similar difference in variability of H-1 was also noted (see Discussion).

The differences between Groups 2 and 3 were smaller than those observed between shocked animals on the one hand and nonshocked on the other. Nevertheless, it can be still seen (Fig. 2) that ^{32}P-phosphate incorporated into bands F and H-1 of preparations from rats that escaped the footshock (Fig. 2, Group 3) was higher than in corresponding bands from rats that experienced inescapable footshock (Fig. 2, Group 2).

The pattern of endogenously phosphorylated proteins in

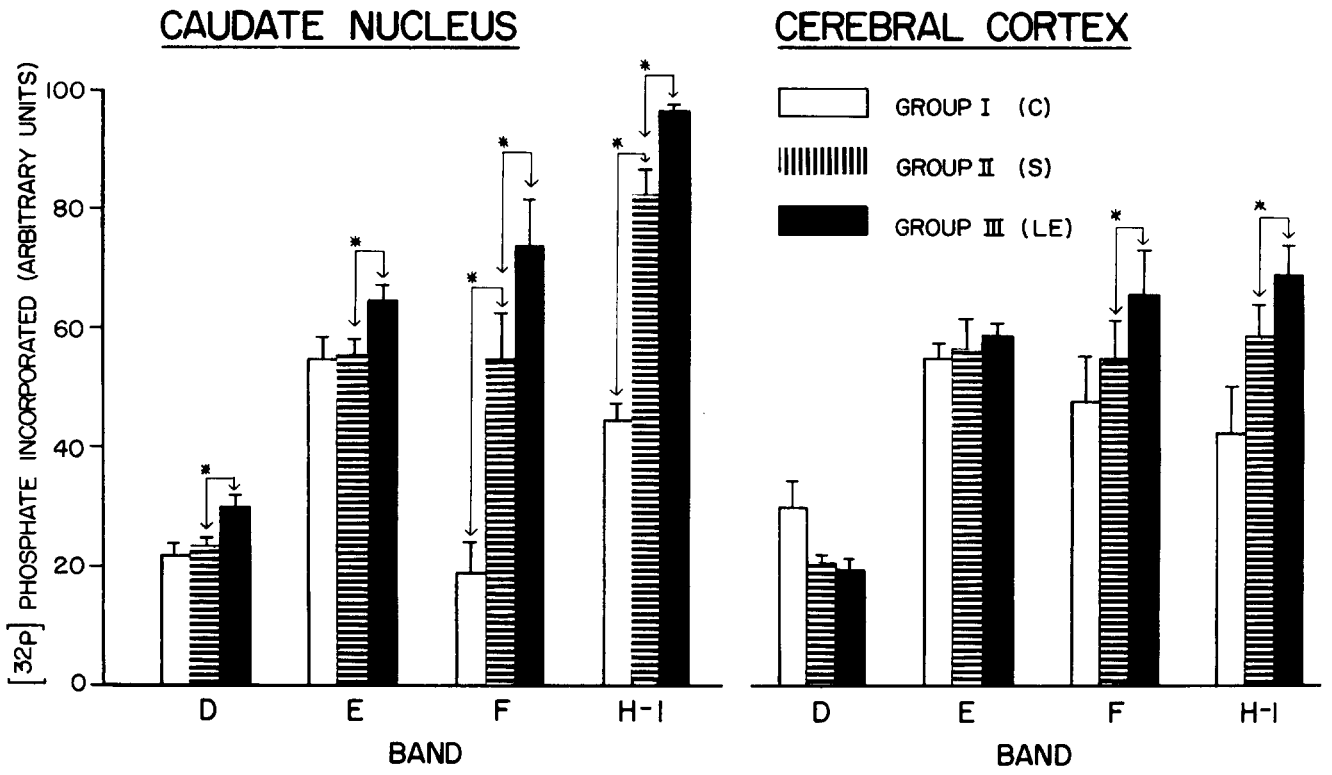

FIG. 2. Mean phosphate incorporated, in arbitrary units, into bands D, E, F or H-1 as a function of brain location and training conditions (+ SEM). Reactions were carried out under conditions described in Methods with 7.5 μM ATP and 5 μM cyclic AMP included in the reaction mixture. Each point is the mean of three assays in each of which brain tissue from three animals was pooled prior to membrane preparation. Since the specific activity of the gamma-^{32}P-ATP batches used in these three experiments was very similar, the results could be analyzed without normalization of the data [20]. Arrows between groups and asterisk denote no overlapping values ($U = 0$, $n_1 = 3$, $p<0.05$) C=control, S=shocked only, LE=learned escape.

membrane preparations obtained from mice resembles that obtained using corresponding preparations from rat brain [6]. It was found that membrane preparations from the caudate nucleus-putamen (neostriatum) of mice showed specific differences in phosphorylation pattern as a consequence of training that were similar to those observed in the rat (Fig. 3). These effects on bands F and H-1 were apparent in two experiments in which both DBA/2J and C57BL/6J strains of mice were used.

DISCUSSION

Previous studies [16] have shown that an increase in the total incorporation of radioactive phosphate into proteins of brain membranes occurs in vivo during training on an active avoidance task. The present study has demonstrated that modifications in phosphorylative activity can be detected in an in vitro assay after a training experience although 24 hr had elapsed between the presentation of the stimuli and sacrifice. Several advantages are provided by the present experimental approach. First, specific phosphoprotein substrates which were particularly reactive to experiential input were identified. Second, it was not necessary to determine the specific activity of the immediate precursor (gamma-^{32}P-ATP), as is required in in vivo experiments [14–16]. Third, the observed effects are likely to be a consquence of biochemical events rather than secondary changes that are related to blood flow or other complications inherent in in vivo studies which require the introduction of radioactive precursors into the intact animal.

The specificity of the phosphorylative modifications induced by footshock was reflected by the fact that the specific band F and the composite band H-1 (estimated molecular weights 47,000 and 10–18,000, respectively [20]) were significantly more reactive than the other phosphoprotein components. The membrane-bound protein components F and H-1 differ from the other phosphoprotein bands in that they achieve high levels of phosphorylation in vitro in the absence of added cyclic AMP and that they are minimally stimulated by the addition of cyclic AMP [5,20]. The phosphorylation of such proteins in vitro may depend on the presence of endogenous cyclic AMP in the preparation [26], and/or on the presence of cyclic AMP-independent phosphorylative activity, which may have been translocated intracellularly via a cyclic AMP activated process [13,24]. Since increased cyclic AMP levels and subsequent translocation of protein kinase were reported recently to be induced by hormonal [23] and by environmental [9] inputs, such mechanisms may be operative also in bringing about the effects observed in this study. In addition, increased phosphate incorporation in vitro may be a reflection of decreased phosphorylation that had occurred in vivo [27]. Since our assays only measure the net result of the activity of phosphoprotein kinase(s) and phosphatase(s) in the membrane preparation [5], the relative contribution of each of these enzymes to

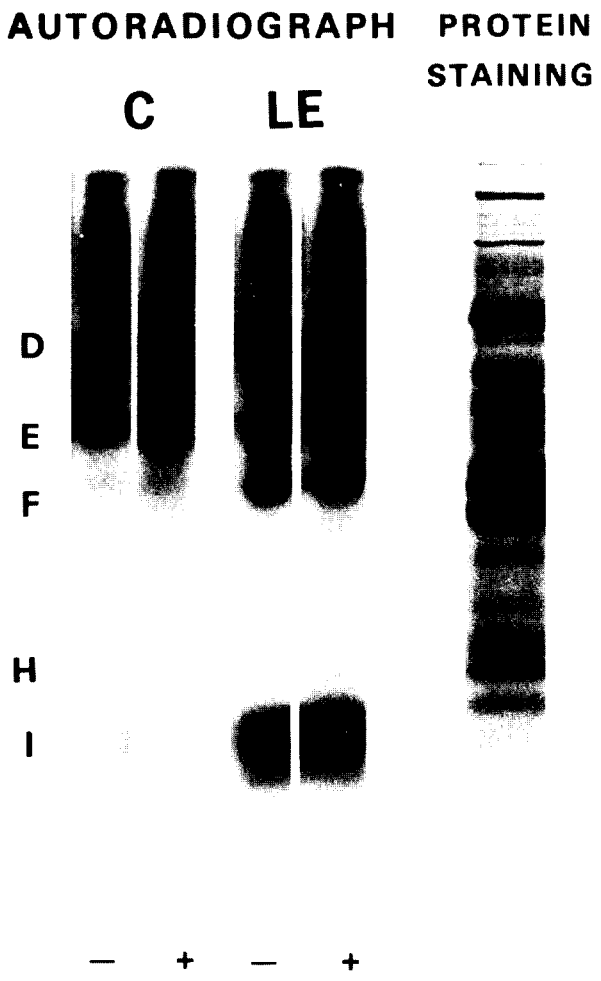

FIG. 3. Phosphorylation pattern of separated reaction products from the caudate nucleus of handled (C) and learned-escaped (LE) DBA/2J mice. Training and reaction conditions were as described in the text and in the legend to Fig. 1. For electrophoretic separation, 10 cm long gels were used instead of the 6 cm long gels used in Fig. 1. The resolution of more than one band in the composite band H-1 was first noted under these conditions. (+) and (−) refer to the presence and absence of added cyclic AMP in the reaction mixture. Protein staining was the same for all the separated samples.

mechanisms underlying the observed effects can not as yet be evaluated. However, the likelihood that the observed changes in phosphorylation pattern reflect changes in enzymatic activity is supported by the observation (Fig. 1 and [19]) that the protein profiles of the assayed preparations were not altered by the conditions of training.

The phosphoprotein bands D and E were shown to have similar properties to the proteins designated I and II, respectively, by Ueda *et al.* [25]. It was suggested that the phosphorylation of these bands is related to changes in membrane permeability [12,25]. The phosphorylation of these bands has shown only small, or no changes in preparations from animals sacrificed 24 hr after training. We have also identified another band, designated G, which was characterized as being highly sensitive to the addition of cyclic AMP [20]. Under the standard assay conditions used here (2 min reaction), however, phosphorylation of band G was barely detectable (see also Fig. 2 in Reference 20).

The high levels of phosphorylation of bands F and H-1 observed consequent to footshock may be a general phenomenon associated primarily with stress [17]. However, the observation that phosphorylation in membranes from animals that had escaped the footshock was greater than that in animals that experienced inescapable footshock (Fig. 2) suggests the possible involvement of these phosphoproteins in some aspects of memory mechanisms. The detection of the phosphorylative changes in membrane preparations from animals that were sacrificed 24 hr after the novel experience supports this contention. Previous studies in our laboratory have indeed shown that rats which experience escapable footshock (Group 3) demonstrate retention by avoiding the shock 24 hr later [11, 18, 19]. In the same context it may be worthwhile noting the differential responsiveness of the neostriatum and cerebral cortex observed in the present study. This observation supports the views concerning the role of the neostriatum and its dopaminergic afferent, the substantia nigra, pars compacta, in stimulus-response integration [18,19].

Previous studies [14–16] have indicated that the past experience and mode of handling of animals will affect protein phosphorylation in the brain in vivo. The greater variability in the in vitro phosphorylation of bands F and H-1 in the controls as compared to trained subjects may reflect differences in the quality of past experience among the control animals. In trained animals, however, the novelty of an experience that involves footshock may have caused changes in the metabolism of bands F and H-1 that supersede and mask any differences in phosphorylative activity for bands F and H-1 that are inherent in control animals.

Quantitative evaluation of the results of the in vitro phosphorylation assay is dependent on the specific activity of the gamma-^{32}P-ATP that is used to initiate the reaction [20]. The analysis presented in Fig. 2 could be performed without normalization of the data, since the specific activity of the gamma-^{32}P-ATP used in these three experiments was very close. It should be noted that additional experiments with minor modifications in behavioral and biochemical procedures and using gamma-^{32}P-ATP with higher or lower specific activities yielded results that were similar to those shown in Figs. 2 and 3. Again, phosphorylation of bands F and H-1 in control animals showed a greater variability than that observed in trained animals. In several cases, the phosphorylation of bands F and H-1 from pooled control animals was as high as that found in samples obtained from animals that had received footshock. The incidence of control samples that gave a phosphorylation pattern which was similar to that of trained subjects was particularly high when samples from individual animals were assayed without pooling (Ehrlich and Brunngraber, unpublished observations). Such findings support the assumption that past experience affects the phosphorylation of bands F and H-1 and serve to emphasize that the past experience of animals to be used in phosphorylative studies should be carefully controlled.

Several studies have implicated phosphorylative activity as a mediating step in the series of events that link external stimuli to intracellular responses [2, 9, 13, 23, 24, 28, 29]. Such mechanisms may operate within the central nervous system, and proteins of bands F and H-1 may be the specific substrates whose phosphorylation is involved in the central processing of experiential information.

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