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Alteration of Hippocampal B50 Phosphorylation After Adrenalectomy

LINDA A. DOKAS

Departments of Neurosciences and Biochemistry, Medical College of Ohio, Toledo, OH 43699

HENK ZWIERS AND WILLEM H. GISPEN

Division of Molecular Neurobiology, Rudolf Magnus Institute for Pharmacology and Institute of Molecular Neurobiology, University of Utrecht, The Netherlands

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DOKAS, L. A., H. ZWIERS AND W. H. GISPEN. *Alteration of hippocampal B50 phosphorylation after adrenalectomy.* BRAIN RES BULL **13**(1) 33–38, 1984.—Adrenalectomy alters endogenous phosphorylation of the presynaptic protein, B50, in the hippocampus. Three and four days following adrenalectomy decreases are seen, relative to control values, in the *in vitro* phosphorylation of B50 when a synaptic plasma membrane fraction from the hippocampus is incubated with [γ-^{32}P]ATP. At four days post-adrenalectomy, the percent decrease in B50 phosphorylation is $-49.8 \pm 6.8\%$. No alteration is seen in the level of B50 phosphorylation when comparing hippocampal membrane preparations from sham-operated and intact animals. Fourteen days following adrenalectomy, hippocampal B50 phosphorylation was restored to normal levels. Hypophysectomy did not alter the degree of *in vitro* B50 phosphorylation, but the effect of adrenalectomy occurred in hypophysectomized rats. Following adrenalectomy, no differences are seen in the phosphorylation of any hippocampal cytosolic proteins. Changes in B50 phosphorylation seen in hypothalamic synaptic plasma membranes are likely due to the effects of sham-operation. The results indicate that a transient neurochemical or neuroendocrine event following adrenalectomy modulates *in vivo* the degree of B50 phosphorylation in hippocampal synaptic membranes.

Membrane phosphorylation Adrenalectomy B50 Hippocampus

A calcium-dependent, phospholipid-activated protein kinase is found in high amounts in brain synaptic plasma membranes [1,10]. The major endogenous synaptic substrate for this enzyme is the protein B50, with a molecular weight of 48,000 daltons and an isoelectric point of 4.5 [25]. The presynaptic localization of B50 [20], sensitivity of B50 protein kinase to Ca^{2+} and phospholipids and the reciprocal relationship that exists between levels of B50 phosphorylation and the activity of membrane-bound diphosphoinositide (DPI) kinase [9] suggest that the phosphorylated state of B50 is related functionally to the process of neurotransmitter release or presynaptic transmitter receptor activation [9,25].

Yet, little is known regarding the *in vivo* modulation of B50 kinase. Zwiers *et al.* [22] have shown that ACTH administered *in vivo* will alter the phosphorylated state of B50. This observation is consistent with a large body of evidence demonstrating that ACTH acts *in vitro* to inhibit the activity of B50 kinase [23,24]. Effectiveness of the peptide ACTH *in vivo* suggests that other neuroendocrine mechanisms may influence endogenous B50 phosphorylation. To test this hypothesis, we have examined the effect of adrenalectomy on B50 phosphorylation in hippocampal synaptic plasma membranes. Because of its large population of glucocorticoid receptors [15], it was felt that the effects of diminished endogenous glucocorticoid levels would be most apparent in the hippocampus. In addition, VanDijk *et al.* [21] have shown that ACTH levels in the hippocampus are decreased at short times following adrenalectomy. This observation allows the opportunity to test if hormonal manipulation of the ACTH content of the hippocampus would result in alterations of B50 phosphorylation. Finally, it is known that hippocampal synaptic plasma membranes contain large amounts of B50 and B50 kinase compared to other brain regions [11]. Our results demonstrate that adrenalectomy alters endogenous phosphorylation of B50 in hippocampal membranes, but with characteristics that suggest mechanisms other than long-term depletion of adrenal steroids are responsible.

METHOD

Animals and Surgery

Adrenalectomized and hypophysectomized male Sprague-Dawley rats were obtained from Blue Spruce Farms, Altamont, NY. Some intact and hypophysectomized rats were bilaterally adrenalectomized in the authors' laboratories under ether anesthesia using the dorsal approach. Sham-operated rats were anesthesized and the adrenals were located through the incision, but not removed. Adrenalectomized animals were maintained on 0.9% saline following surgery, while sham-operated and intact rats received water. Rats were sacrificed by decapitation. Each animal was checked for the absence of residual adrenal tissue. In some

cases, trunk blood was collected into heparinized tubes and the plasma content of glucocorticoid hormones was measured using the protein binding assay of Murphy [18]. All animals were found to be free of adrenal tissue and were shown to have negligible levels of circulating adrenal steroids.

Brain Dissection and Tissue Fractionation

Hippocampal or hypothalamic tissue was dissected by the method of Gispen *et al.* [8]. Tissue from three or four intact, sham-operated or adrenalectomized animals was combined and homogenized in 10 volumes of 0.32 M sucrose. Each homogenate was spun at 1000×g in a Sorvall SS34 rotor. The resultant supernatants were spun at 10,000×g and the post-mitochondrial supernatants (PMS) were used as the "cytosolic fraction." Light synaptic plasma membranes were prepared as described by Dokas *et al.* [6]. This fraction is known to be free of significant contamination by myelin or mitochondria and to be enriched in presynaptic elements [2]. Briefly, the crude mitochondrial-synaptosomal pellet (P2) was lysed in distilled water and applied to a discontinuous sucrose gradient of 1.0 M sucrose overlaid with 0.4 M sucrose. After a centrifugation at 100,000×g for 80 min in a Beckman SW27 rotor, the synaptic plasma membrane (SPM) fraction was collected from the 0.4–1.0 M interface. Protein was collected by recentrifugation for 30 min in 10 mM sodium acetate—10 mM magnesium acetate—1 mM calcium acetate, pH 6.5 (10:10:1). Each final SPM pellet was resuspended in 200 μl of 10:10:1. Protein content of all subcellular fractions was determined by the method of Lowry *et al.* [12] with bovine serum albumin as a standard.

Endogenous Phosphorylation Assays

Endogenous protein kinase activity in cytoplasmic fractions or SPM preparations was assayed for 20 sec. following a 5 min preincubation at 30°C. The total assay volume was 25 μl of 10:10:1, containing 25 μg of PMS or SPM protein and 2 μCi of [γ-^{32}P]ATP (Specific activity between 10–40 Ci/mmole from New England Nuclear Corp, Boston, MA). The stock [γ-^{32}P]ATP was adjusted to a concentration of 37.5 μM with cold ATP in 10:10:1 and 5 μl of this solution was used per assay to give a final concentration of 7.5 μM. Assays were terminated by the addition of 12.5 μl of a stop mix to produce a final concentration of 62.5 mM Tris-HCl, pH 6.5, 2% sodium dodecyl sulfate, 10% glycerol, 0.001% bromphenol blue and 5% 2-mercaptoethanol.

Proteins were separated by slab gel electrophoresis for 2 hours at 40 mA. The acrylamide concentration of the running gel was 11% and of the stacking gel was 3%. Proteins were stained in a 0.1% solution of Fast Green in 50% methanol—10% acetic acid and destained overnight in the same solution without Fast Green. The gel was dried down under vacuum and labeled proteins were visualized by autoradiography or scintillation counting of excised bands [6]. Where radioactivity as ^{32}P-cpm was converted to fmoles/μg protein, the conversion factor was 1 cpm=0.04 fmoles phosphate.

RESULTS

Effect of Adrenalectomy on Hippocampal Protein Phosphorylation

The pattern of phosphorylated proteins of the rat hippocampal synaptic plasma membrane fraction used in these studies is shown in Fig. 1. The major phosphorylated protein labeled under the assay conditions employed (7.5 μM [γ-^{32}P]ATP, 1 mM Ca^{2+}) is the protein B50 with a molecular weight of 48,000 daltons. Adrenalectomy produces a loss of labeling of this protein band from [γ-^{32}P]ATP, as measured in an *in vitro* protein kinase assay. The results of six separate experiments done four days post-adrenalectomy show the average decrease in hippocampal B50 phosphorylation to be −49.8±6.8%. Less incorporation of radioactivity is also seen in the three low molecular weight bands (>20,000 daltons). Phosphorylation of the heterogeneous proteins of molecular weights greater than B50 appears to be less affected by prior adrenalectomy. In particular, phosphorylation of the protein band migrating just above B50 (52,000 daltons) seems to be resistant to any effects of removal of the adrenals. No alterations were seen in the staining of any synaptic plasma membrane protein following adrenalectomy.

Figure 2 shows quantitation of the effect of four days of adrenalectomy on subsequent phosphorylation of B50 from [γ-^{32}P]ATP. In this experiment, the B50 band was excised and radioactivity was measured by scintillation counting. Adrenalectomy decreases the phosphorylation of B50 to 4.1±0.1 fmoles/μg protein from a control value of 6.2±0.3 fmoles/μg protein ($p<0.005$). Sham adrenalectomy has no effect on subsequent phosphorylation of B50 (6.1±0.3 fmoles/μg protein) as compared to control values. The control values for phosphorylation of B50 in hippocampal membranes (6.2±0.3 fmoles/μg protein) are in agreement with those (7.17±0.86 fmoles/μg protein) previously reported by Kristjansson *et al.* [11].

To determine the subcellular specificity of the effect, protein phosphorylation was also examined in the hippocampal cytosolic fraction four days following adrenalectomy. For these experiments, the post-mitochondrial supernatant (from a 10,000×g centrifugation) was used as the source of cytosolic protein kinase and substrate. The post-mitochondrial supernatant was used since the pattern of phosphorylation and amount of protein kinase activity is the same in this fraction as in a 100,000×g cytosolic fraction (data not shown). Figure 1 shows the pattern of phosphorylated proteins seen in the cytosolic fraction when 25 μg of protein is incubated with 2 μCi of [γ-^{32}P]ATP for 20 seconds. The major phosphorylated protein is of molecular weight 52,000 daltons. Adrenalectomy (four days) produces no effect on the labeling from [γ-^{32}P]ATP of any of the cytosolic proteins. In addition, no change in the amount of staining of any protein band was observed.

Regional Specificty of the Effect of Adrenalectomy on B50 Phosphorylation

The effect of adrenalectomy (four days) on B50 phosphorylation was also examined in the synaptic plasma membrane fraction from the hypothalamus. Previous localization of B50 in nervous tissue has shown hypothalamic membranes to have approximately one-half of the amount of phosphorylatable B50 as do hippocampal membranes [11]. Consistent with this observation, less phosphorylation of B50 was noted in the hypothalamic membrane fraction from intact rats as compared to hippocampal synaptic plasma membranes. In addition, the degree of *in vitro* B50 phosphorylation seen in hypothalamic membranes following adrenalectomy was more variable and more dependent upon surgical manipulation of the animal. B50 phosphorylation in hypothalamic synaptic plasma membranes was decreased (−59.6±9.6%; N=3) when comparing preparations from ad-

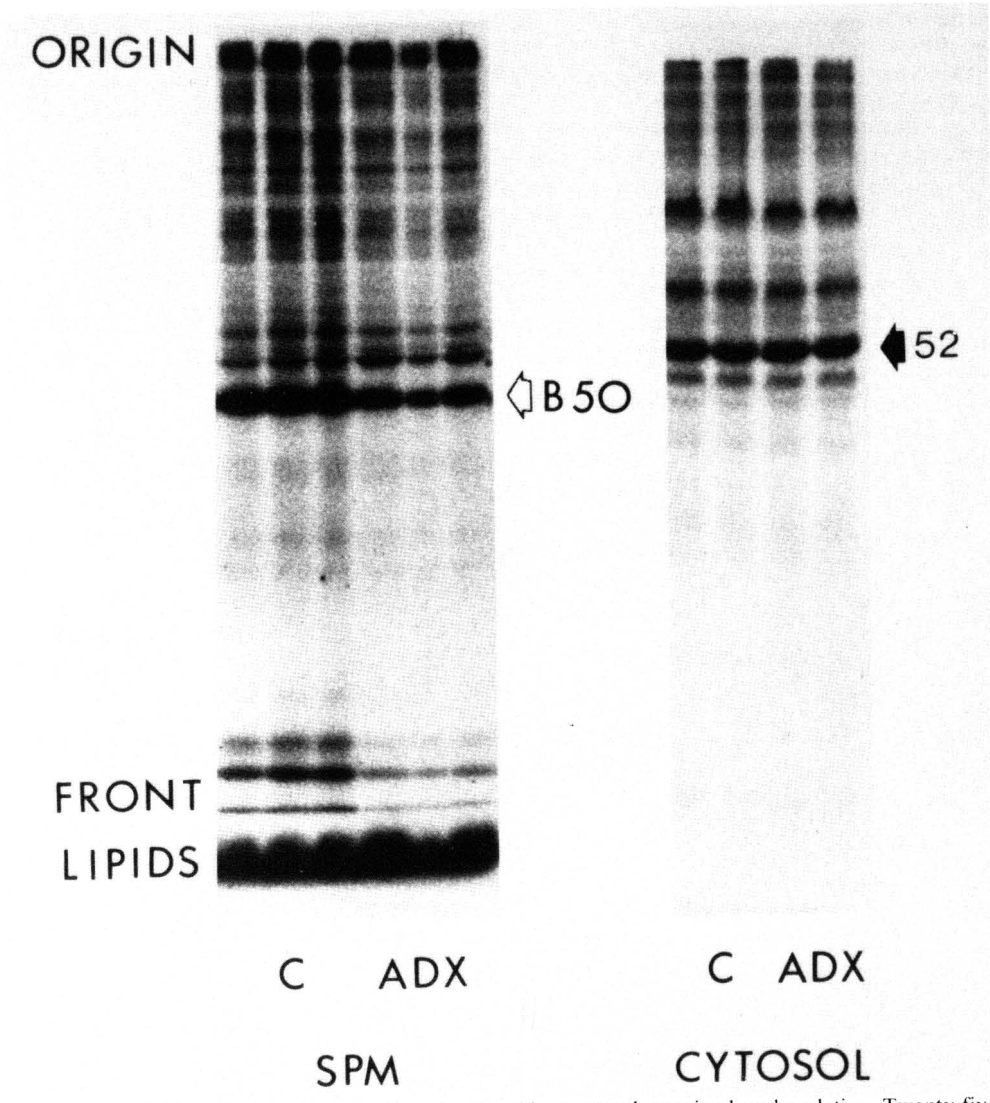

FIG. 1. The effect of adrenalectomy (four days) on hippocampal protein phosphorylation. Twenty-five μg of hippocampal synaptic plasma membranes or of the cytosolic fraction (post-mitochondrial supernatant) from intact or adrenalectomized rats were incubated with 2 μCl of [γ-^{32}P]ATP. After gel electrophoresis, labeled proteins were visualized by autoradiography. Abbreviations used: SPM, synaptic plasma membrane fraction; CYTOSOL, cytosolic fraction; C, assays done with preparations from control rats; ADX, assays done with preparations from adrenalectomized rats; B50, position of the presynaptic protein, B50 (molecular weight, 48,000 daltons); 52K, position of the major phosphorylated protein of the cytosolic fraction (molecular weight, 52,000 daltons).

renalectomized (four days) rats to those from intact rats. But, when comparing B50 phosphorylation in hypothalamic preparations of adrenalectomized rats to those of sham-operated rats, an increase in B50 phosphorylation was seen in three of four experiments. This is in contrast to the hippocampus where decreases in B50 phosphorylation *in vitro* are seen whether membranes from adrenalectomized rats are compared to those from either intact or sham-operated rats (Fig. 2).

Time Course of the Effect of Adrenalectomy on Hippocampal B50 Phosphorylation

In vitro phosphorylation of B50 in hippocampal membranes was examined at various times following adrenalectomy. At three days following adrenalectomy, a decrease of $-19.7\pm5.5\%$ (N=4) was seen in endogenous B50 phosphorylation from [γ-^{32}P]ATP. Four days after surgery, the average decrease in hippocampal B50 phosphorylation was $-49.8\pm6.8\%$ (N=6). No change in B50 phosphorylation was found at eight, ten or fourteen days following adrenalectomy. A comparison of the effect of adrenalectomy after four days or fourteen days on hippocampal B50 phosphorylation is shown in Fig. 3. Decreased phosphorylation of B50 is not seen fourteen days after surgery. Rather, a small increase in *in vitro* B50 phosphorylation is noted ($+19.9\pm11.6$; N=3) relative to levels seen in control hippocampal membrane preparations. These results show the effect of adrenalectomy on subsequent B50 phosphorylation to be transitory in nature.

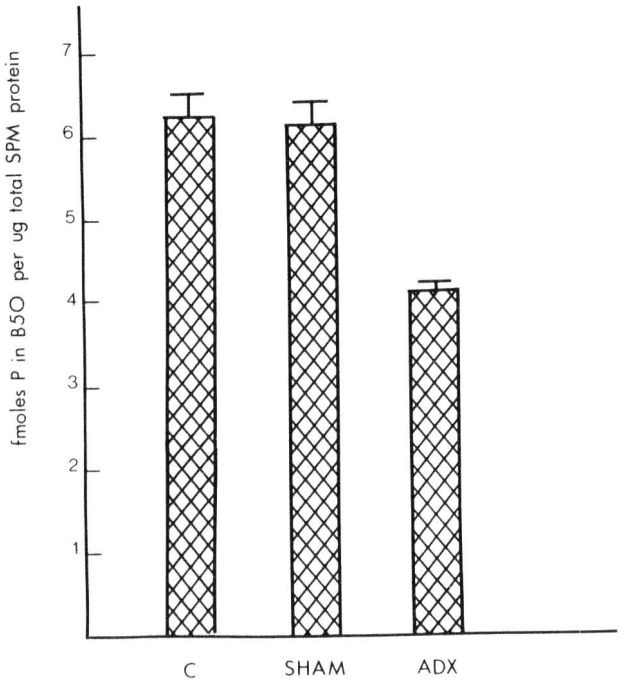

FIG. 2. Quantitation of the effect of adrenalectomy (four days) on B50 phosphorylation. The labeled B50 protein band was excised from gels as shown in Fig. 1 and counted for radioactivity. To convert cpm to fmoles phosphate, the conversion factor of 1 cpm=0.04 fmoles phosphate was used. The labeling of B50 was determined in hippocampal membranes from control (C), sham-operated (SHAM) and four days-adrenalectomized (ADX) rats.

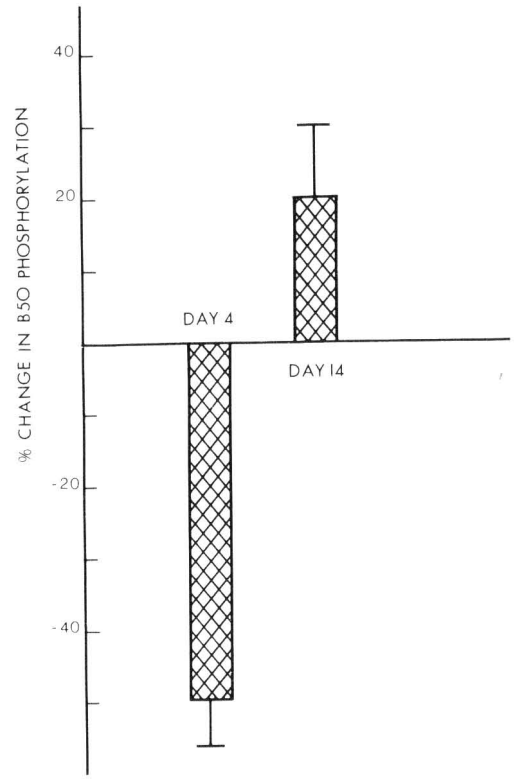

FIG. 3. Comparison of the effects of adrenalectomy at four and fourteen days on hippocampal B50 phosphorylation. Control levels of B50 phosphorylation were as shown in Fig. 1.

Effect of Hypophysectomy on B50 Phosphorylation in the Hippocampus

Unlike adrenalectomy, hypophysectomy has no effect on hippocampal B50 phosphorylation at short times following surgery. To ensure that effects of pituitary removal were being measured directly, B50 phosphorylation in membranes from hypophysectomized rats were compared to those of sham-operated animals. Three days following hypophysectomy, *in vitro* B50 phosphorylation is decreased by only $-2.8 \pm 8.2\%$ (N=3), an insignificant effect (Table 1). Moreover, prior hypophysectomy (four days) does not interfere with the effect seen four days after secondary adrenalectomy. Hippocampal B50 phosphorylation is diminished in synaptic plasma membranes from hypophysectomized and adrenalectomized rats by -47.6%, a value similar to that seen in the hippocampal membranes from rats which had been adrenalectomized only for the same period of time (Figs. 1 and 2).

TABLE 1

B50 PHOSPHORYLATION IN THE RAT HIPPOCAMPUS AFTER HYPOPHYSECTOMY AND ADRENALECTOMY

Condition	% Change in B50 Phosphorylation
Hypophysectomy	-2.8 ± 8.2
Hypophysectomy and Adrenalectomy	-47.6

Hippocampal membranes were prepared from rats hypophysectomized three or four days previously or from hypophysectomized rats that were subsequently adrenalectomized for four days before use. B50 phosphorylation was measured by densitometric scanning of autoradiograms after incubation of membranes with [γ-^{32}P]ATP and gel electrophoresis. The percent change in B50 phosphorylation in hypophysectomized rats was determined relative to that seen after sham operation and the effect of hypophysectomy and adrenalectomy is expressed relative to that of hypophysectomy alone.

DISCUSSION

The hippocampus is an appropriate model system in which to examine neuroendocrine modulation of synaptic plasma membrane protein phosphorylation. Not only do hippocampal membranes contain relatively high amounts of the major presynaptic phosphoprotein, B50, and B50 kinase [11], but it is a brain region subject to known endocrine regulation. The presence in the hippocampus of a large population of glucocorticoid receptors [15] and the known mechanism of action for steroids [19] suggests that steroid-determined proteins must be present in this brain region [5,7]. Loss of B50 phosphorylation in the hippocampus following adrenalectomy would result if either B50 or B50 protein kinase is synthesized in response to glucocorticoids in the hippocampus.

However, characteristics of the effect of adrenalectomy on B50 phosphorylation preclude the possibility that deple-

tion of adrenal glucocorticoids is the responsible factor. Most relevant to this conclusion is the time course of the effect of adrenalectomy. Decreases in *in vitro* B50 phosphorylation are seen only at short times (three and four days) following surgery. Long-term depletion of adrenal glucocorticoids (fourteen days) produces no subsequent loss of phosphorylation. The lack of any alterations in protein staining patterns following adrenalectomy also indicates that significant changes in the synthesis of hippocampal synaptic plasma membrane or cytosolic proteins do not occur following removal of the adrenals. In addition, no changes are seen in the phosphorylation of hippocampal cytosolic proteins at times when maximal decreases are seen in the phosphorylation of membrane-bound B50. Synaptic membrane-bound B50 kinase shares characteristics with and co-purifies with protein kinase C [1]. Since a significant fraction of total protein kinase C activity is found in the cytoplasm [10], a deficit in the synthesis of this enzyme in response to depletion of glucocorticoids should then coincide with diminished phosphorylation of proteins in both the cytosolic and synaptic plasma membrane fractions. Yet, no alterations are seen in the phosphorylation of cytosolic proteins after adrenalectomy.

Other reports in the literature describe neuroendocrine effects in the hippocampus that are maximal at short times after adrenalectomy. Miller *et al.* [17] have shown that the Vmax of the GABA uptake system in hippocampal membranes is increased twenty-four hours to one week following adrenalectomy, with a decrease toward control values by two weeks. Adrenalectomy potentiates the increase in ornithine decarboxylase seen in the contralateral hippocampal lobe after unilateral removal of the dorsal hippocampus [4]. The elevation of ornithine decarboxylase activity is seen at five, but not ten days of adrenalectomy. Van Dijk *et al.* [21] have shown that hippocampal ACTH content is transiently lowered following adrenalectomy. It is this observation that correlates most closely with the effect of adrenalectomy on B50 phosphorylation.

First, both the loss of hippocampal ACTH and the lowering of B50 phosphorylation in synaptic plasma membranes are seen only at three and four days after adrenalectomy. Neither effect is noted two weeks after surgery. Secondly, similar effects are not seen in each regard in hypothalamic membranes when comparing sham-operated and adrenalectomized animals. Finally, hypophysectomy does not mimic the effect of adrenalectomy on either variable, although the effect of adrenalectomy persists in the presence of prior hypophysectomy.

Lowering of hippocampal ACTH levels *in vivo* could lead to a subsequent reduction of *in vitro* B50 phosphorylation. It is known that ACTH inhibits the activity of B50 kinase [23,24]. If so, it can be assumed that reduction of ACTH levels in the hippocampus following adrenalectomy should relieve some endogenous inhibition of B50 kinase. Then, as hippocampal synaptic plasma membranes are isolated from adrenalectomized rats, they would be more extensively phosphorylated than those from intact rats. Being covalently bound, the phosphate attached to B50 would remain incorporated through the preparation of membranes, whereas any ACTH bound reversibly to hippocampal membranes would be lost. The result would be a greater number of sites on B50 molecules for phosphorylation in control membranes when [γ-^{32}P]ATP is added in a post-hoc phosphorylation assay than would be available in membranes from adrenalectomized rats. Evidence that alteration of ACTH levels *in vivo* can be reflected in the phosphorylated state of B50 exists since Zwiers *et al.* have shown that administration of ACTH *in vivo* leads to subsequent stimulation of incorporation of labeled phosphate from [γ-^{32}P]ATP *in vitro* into a synaptic plasma membrane protein with the molecular weight of B50 from subcortical (including hippocampal) tissue [22].

Certain aspects of the decreased phosphorylation of B50 phosphorylation seen in hippocampal membranes following adrenalectomy, however, do not coincide with the findings of Van Dijk *et al.* [21]. Most notably, they report a decrease of hippocampal ACTH content in sham-operated rats as compared to control rats that is at least equally as large as the depletion seen when comparing hippocampal levels of the peptide between adrenalectomized and sham-operated rats and greater than that seen when comparing hypothalamic preparations from sham-operated to control animals. Yet, no alterations in B50 phosphorylation are seen in hippocampal membranes when comparing sham-operated and intact rats (Fig. 2). It may be that multiple factors act upon the B50 kinase system of the hippocampus following adrenalectomy to determine its total activity, only one of which is depletion of hippocampal ACTH. Possibilities include glial cell-neuronal interactions and glucocorticoid-independent mechanisms.

The hippocampus possesses multiple cell types that accumulate and respond to adrenal steroids [3,16]. Both neurons and glia are responsive and it may be that multiple effects following adrenalectomy are segregated into one or the other of these cell types. It is unlikely that the loss of B50 phosphorylation following adrenalectomy occurs in glial cells, since B50 is known to be a presynaptic protein [20]. But, this does not mean that alterations in glial cell function following depletion of glucocorticoid receptor occupancy in response to adrenalectomy could not alter the function of associated neurons. If glial cells responded to the loss of glucocorticoids with transient alterations in their metabolism or physiology, a pattern of response such as is observed regarding B50 phosphorylation might be seen.

While characterizing the depletion of ACTH levels in the rat hippocampus after adrenalectomy, Van Dijk *et al.* [21] found that administration of corticosterone (which would associate with either neuronal or glial cell cytoplasmic receptors) could not reverse the effect of adrenalectomy. This implies that completely glucocorticoid-independent mechanisms must also be considered to explain reduction of peptide levels and the loss of B50 phosphorylation, if the two effects are causally related. Loss of other adrenal factors, namely adrenalmedullary hormones or aldosterone, might be involved. Adrenal demedullation does modulate the learning response of rats to enkephalin peptides and ACTH [14]. It might also influence functioning levels of brain peptides including ACTH, particularly in brain regions such as the hippocampus, known to be critical for acquisition and consolidation of learned behaviors [13].

At a minimum, this work has shown that levels of B50 phosphorylation in the hippocampus are responsive to *in vivo* manipulation of hormone levels. An array of factors, distinguishable by their time courses and dependence upon adrenal steroids, may act upon neurons in the hippocampus of adrenalectomized animals to regulate synaptic function. The total set of variables may include loss of adrenal steroids themselves, secondary responses to the lack of glucocorticoids and cellular interactions between different populations of steroid-sensitive neurons and glial cells.

NOTE IN PRESS

Borrell *et al.* (*Behav Neural Biol* **39:** 241–258) have recently reported impaired retention of an inhibitory avoidance response in rats following adrenalectomy. The deficit is seen up to 5 days after adrenalectomy, but not at 10 days. Corticosterone replacement did not improve the retention impairment, although administration of adrenal catecholamines did. These studies are consistent with our own with regard to the time course of the effects. Deficits in avoidance behavior could be correlated with alterations in hippocampal metabolism such as we report here. Both studies indicate the lack of adrenal glucocorticoids are not critical. Analysis of the influence of adrenal catecholamines on hippocampal protein phosphorylation seems warranted.

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