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# DEFECTIVE PROTEIN PHOSPHORYLATION IN RENAL MEDULLA OF VASOPRESSIN-RESISTANT MICE

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SUMMARY: Protein phosphorylation was compared in renal medulla of normal mice and a strain with hereditary vasopressin-resistant diabetes insipidus (DI mice). The phosphorylation of a specific pair of proteins ($M_r$ 185,000 and 175,000) in the particulate fraction was found to be impaired in DI mice in a fashion that correlated with the severity of the concentrating defect. Defective protein phosphorylation in DI mice may be related to the pathogenesis of cellular unresponsiveness to vasopressin.

## INTRODUCTION

Cyclic AMP[3] mediates the effect of vasopressin on the water permeability of responsive renal epithelia (1). By analogy with other systems, it has been proposed that the nucleotide does so by stimulating the phosphorylation or dephosphorylation of specific proteins (2). However, the proteins involved in the regulation of water permeability have not been identified with certainty. We have examined protein phosphorylation in a strain of mice with hereditary vasopressin-resistant diabetes insipidus (DI mice). Since it appears that a specific defect in the cellular action of vasopressin in affected mice results in decreased urinary concentrating ability (3), a comparison of protein phosphorylation in renal medullary extracts of DI and normal mice was undertaken to identify proteins whose phosphorylation might

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[3]Abbreviation: Cyclic AMP, adenosine-3',5'-monophosphoric acid.

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be directly related to the control of water permeability. We report here an impairment of the phosphorylation of a specific pair of proteins.

## METHODS

Urinary osmolality was measured by freezing point depression on random urine samples obtained while mice had free access to food and water, either without treatment or 18 hours after subcutaneous injection of 0.5 U Pitressin tannate in oil (Parke Davis, Detroit, MI). Mice were killed by cervical dislocation at least 48 hours after vasopressin testing. Two renal medullae from each mouse were removed by dissection and homogenized at 2° in 1 ml of 0.05 M sodium phosphate, pH 7, containing 0.25 M sucrose (10 strokes, 7 ml motor-driven Teflon-glass homogenizer). Protein concentration was measured (4) and the extracts were adjusted with the phosphate buffer so that equal amounts of protein from each were added to the phosphorylation mixture.

Mixtures (90 µl) containing 40 mM sodium phosphate, pH 7, 10 mM NaF, 0.1 mM isobutylmethylxanthine, 5 mM $MgCl_2$, 10 µM [$^{32}$P]-ATP (30 Ci/mMol), and homogenates or subcellular fractions were incubated at 30°C. Incubations were stopped by the addition of 30 µl containing 4% sodium dodecyl sulfate (SDS), 120 mM dithiothreitol, 0.25 M Tris HCl, pH 6.8, and 40% glycerol. After 2 min at 100°C, 90 µl of each mixture was applied to a 6% polyacrylamide gel with a 3% stacking gel (5). After electrophoresis (90 v, 4.5 hr) gels were stained with Coomassie blue, dried, and subjected to autoradiography (Kodak XR-2 film). Protein standards were: cardiac myosin heavy chains ($M_r$ 200,000), RNA polymerase ($\alpha$ subunit, $M_r$ 39,000, $\beta$ subunit, $M_r$ 155,000, $\beta'$ subunit, $M_r$ 165,000), $\beta$-galactosidase ($M_r$ 116,000), phosphorylase ($M_r$ 93,000) and bovine serum albumin ($M_r$ 67,000).

ATP and cyclic AMP were from Sigma, St. Louis, MO, [$^{32}$P]-ATP from ICN, Irvine CA, isobutylmethylxanthine from Aldrich, Milwaukee, WI.

## RESULTS AND DISCUSSION

Four groups of female mice were studied (Table 1): controls (strain VII +/+), mice bred to have a mild concentrating defect (strain DI +/+ nonsevere), and young and older mice bred to have a severe concentrating defect (strain DI +/+ severe). The latter two groups were differentiated because the concentrating ability of DI +/+ severe mice decreases with age (6). The characteristics of the groups of mice used are summarized in Table I, which shows that all mice responded to vasopressin; however, both DI +/+ nonsevere mice and young DI +/+ severe mice had a persistent mild concentrating defect, and older DI +/+ severe mice had a more pronounced defect.

Figure 1A shows that there are no appreciable differences in the protein staining patterns of renal medullary extracts from normal and DI mice.

Table I. Characteristics of control and DI mice

| Group | Age | Urine osmolality | | |
|---|---|---|---|---|
| | | Basal | | Post vasopressin |
| | months | mosmol | / | kg $H_2O$ |
| VII +/+ | 5 - 12 | 2447 ± 120* | (7)† | 2908 ± 208 |
| DI +/+ nonsevere | 5 - 12 | 806 ± 163 | (8) | 1094 ± 178 |
| DI +/+ severe (young) | 4 | 585 ± 171 | (4) | 1524 ± 86 |
| DI +/+ severe (old) | 5 - 12 | 273 ± 47 | (9) | 740 ± 18 |

*SEM

†(number studied)

The autoradiograms from typical experiments, shown in Figures 1B and 2, demonstrate that the general patterns of phosphorylation are also similar. Apparent differences in overall protein kinase activity were sometimes observed (Figure 2); in general protein kinase activity was similar in homogenates from different strains (Figures 1 & 3). However, the phosphorylation of a specific band (band 3) is reduced or absent in homogenates from DI mice. Significant reduction in phosphorylation of band 3 was observed in each of 16 separate experiments; in some of these a different gel electrophoresis system (8) which separated peptides with molecular weights down to 12,000 was employed. The phosphorylation of certain other bands (1,4,5 & 8) was sometimes reduced in extracts from DI mice, but the results were not consistent. The characteristics of band 3, in which consistent differences in phosphorylation were observed, were studied in more detail. In 6 of 12 experiments, band 3 could be resolved into two components (3a and 3b) whose molecular weights, after reduction in SDS, were 185,000 and 175,000; neither phosphorylated band corresponded in location to a stainable protein band (Fig. 1). The phosphorylation of band 3a was reduced more than that of band 3b in extracts

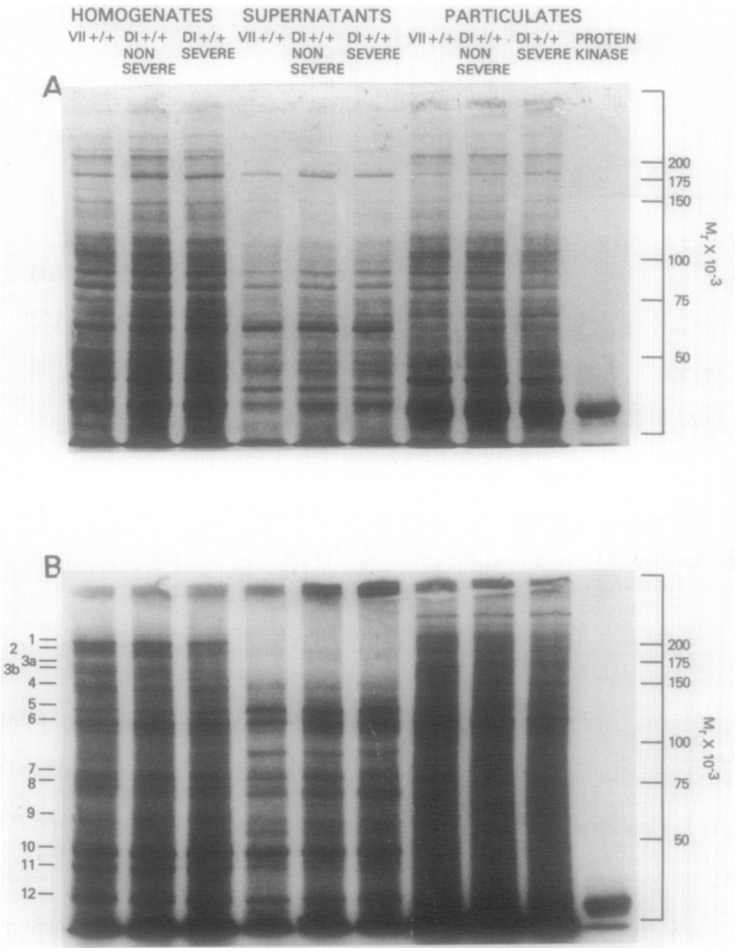


Figure 1. Gel electrophoresis of subcellular fractions. A. Protein staining. B. Autoradiography. Medullary extracts were centrifuged (40,000 x g, 20 min) and the pellets resuspended in homogenization buffer and recentrifuged. Fractions were phosphorylated for 2 min at 30°C; particulate fractions were phosphorylated in the presence of catalytic subunit of type II cyclic AMP-dependent protein kinase from bovine heart (75 U), which was purified by adsorption of the holoenzyme to DEAE cellulose, followed by elution of catalytic subunit with 10 mM cyclic AMP (7). Protein applied to the wells was 79 µg, wells 1-3; 32 µg, wells 4-6; 47 µg, wells 7-9.

from DI mice. In extracts from mice of strain VII +/+ and strain DI +/+ nonsevere, endogenous phosphorylation of band 3 was maximal after 15-20 seconds. Phosphorylation of band 3 was stimulated by cyclic AMP at early times (5-10 sec, not shown); at later times the effect of cyclic AMP was diminished (as in Fig. 2) or undetectable.

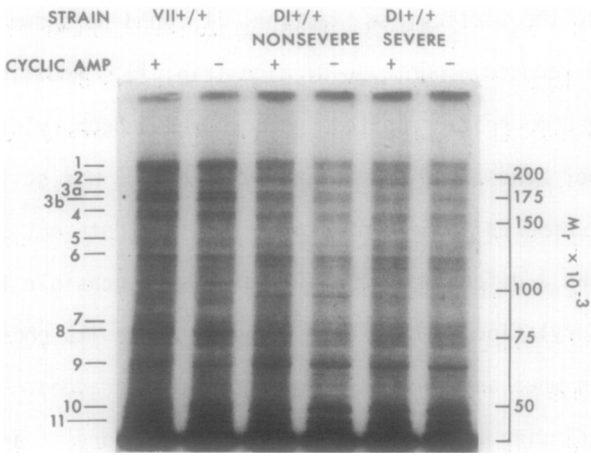


Figure 2. Autoradiography of phosphorylated medullary extracts. Extracts were phosphorylated for 30 seconds in the absence or presence of 10 μM cAMP; Gel electrophoresis and autoradiography were as in Figure 1, except that the separating gel concentration was 5.5%.

After fractionation of renal medullary extracts by centrifugation, band 3 was absent from the supernatant fraction (Fig. 1) and there was little endogenous phosphorylation of the particulate fraction, which appears to be poor in protein kinase activity (not shown). By adding purified catalytic subunit of cyclic AMP-dependent protein kinase from

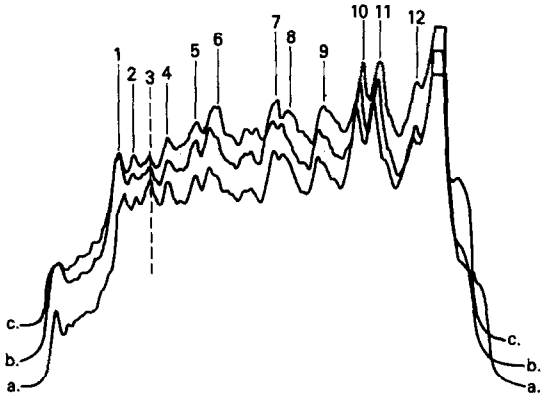


Figure 3. Densitometric scans of autoradiograms of phosphorylated medullary extracts. Extracts were phosphorylated for 30 seconds in the presence of 10 μM cAMP. Autoradiograms of 6% gels were scanned with an Ortec 4310 densitometer, with the baseline set on an unexposed area of the film and the same gain for all scans. a. strain VII +/+, b. strain DI +/+ severe (young), c. strain DI +/+ severe (old).

bovine heart to the particulate fraction, it could be shown that band 3 was present in reduced amounts in DI mice (Fig. 1). Addition of catalytic subunit to the homogenate resulted in increased phosphorylation of band 3 as well as other bands, but the intensity of band 3 was still diminished in DI mice as compared to mice of strain VII +/+ (data not shown). It is unlikely that a defective protein kinase is responsible for the reduced phosphorylation of band 3 in DI mice, since its phosphorylation is reduced even when an exogenous protein kinase is added. Several techniques, including two dimensional gel electrophoresis and sequential SDS gel electrophoresis and isoelectric focusing, were unsuccessfully employed in an attempt to purify band 3 sufficiently to allow quantitation of its phosphate content.

Additional studies were carried out to assess the specificity of the phosphorylation defect in DI mice and its relationship to urinary concentrating ability. First, several murine strains with normal concentrating ability (NFS, C57/BL6, $CAF_1$, C3H/HEN) were screened, and it was found that band 3 was phosphorylated in renal medullary extracts from all. Second, other tissues in mice of strain VII +/+ were examined. Band 3 was absent in renal cortex, liver and brain. The absence of band 3 from renal cortex could be viewed as inconsistent with a possible relationship of this protein to the vasopressin effect on water permeability, since the cortical collecting tubule is known to be a site of this effect (1). However, the cortical collecting tubule makes up so small a fraction of renal cortex that band 3 may be undetectable in extracts of unfractionated cortex, even though it is present in collecting tubule. Third, to assess the relationship of band 3 to concentrating ability, its phosphorylation was compared in DI mice with mild and severe concentrating defects. In renal medullary extracts from mice whose concentrating defect was not fully expressed (DI +/+ nonsevere, young DI +/+ severe), band 3 was phosphorylated more heavily than in extracts

from severely affected older mice of the DI +/+ severe strain (Figures 1-3). Thus phosphorylation of band 3 correlated with urinary concentrating ability.

The pathogenesis of diabetes insipidus in DI mice has previously been studied by Dousa and Valtin, who found that the maximal stimulation of renal medullary adenylate cyclase by saturating concentrations of vasopressin was reduced in DI mice, although the affinity of the receptor for vasopressin was unchanged (9). Studies of vasopressin responsiveness of the medullary collecting tubule, a segment of the nephron where concentration of the urine occurs, have recently been reported (10). Although vasopressin-responsive adenylate cyclase activity was present in medullary collecting tubules isolated from DI mice, vasopressin did not elicit an increase in cyclic AMP content. The failure to respond to vasopressin may have been related, at least in part, to increases in phosphodiesterase activity that were also observed, since the cyclic AMP response to vasopressin was partially restored by treatment with a phosphodiesterase inhibitor (10). The relationship of these findings to the defect in protein phosphorylation observed in the present study is uncertain. The proteins whose phosphorylation is defective in DI mice may be directly involved in the regulation of water permeability; alternatively, they may play a role in the control of phosphodiesterase activity. Studies of protein phosphorylation in isolated segments of collecting tubules may clarify these relationships, and further elucidate the role of protein phosphorylation in vasopressin action.

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