Proc. Nat. Acad. Sci. USA
Vol. 71, No. 10, pp. 3869–3873, October 1974

Aldosterone-Induced Increase in Protein Phosphatase Activity of Toad Bladder

(steroid action/protein phosphorylation/cAMP)

ALICE Y.-C. LIU AND PAUL GREENGARD

Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510

Communicated by Aaron B. Lerner, July 15, 1974

ABSTRACT **A study has been carried out of the effect of aldosterone on the endogenous phosphorylation and dephosphorylation of membrane-bound and of soluble proteins from toad bladder. Membrane-bound protein D (apparent molecular weight, 49,000), a protein which may possibly be involved in the regulation of sodium transport across the mucosal epithelium of toad bladder, contained a substantial fraction of the radioactive phosphate incorporated into membrane proteins; moreover, it was the only protein to appear consistently in autoradiographs of polyacrylamide gels of phosphorylated membrane proteins. Pretreatment of toad bladder slices with aldosterone caused an increase in the endogenous dephosphorylation of membrane-bound protein D. A half-maximal increase in this dephosphorylation occurred at an aldosterone concentration of 20–40 nM. The increase in protein D phosphatase activity induced by aldosterone was prevented by inhibitors of RNA and protein synthesis as well as by spironolactone, a specific antagonist of aldosterone. The mineralocorticoid, 9α-fluorohydrocortisone, also increased protein D phosphatase activity, but testosterone did not. Aldosterone also increased the removal of [^{32}P]phosphate from protein D in the cell sap. In contrast to the increase in protein D phosphatase activity, aldosterone had little effect on the phosphorylation of protein D by endogenous protein D kinase. In some experiments, effects of aldosterone and of cAMP, qualitatively similar to those found with protein D, were also observed on the phosphorylation and dephosphorylation of a protein with an apparent molecular weight of 37,000, in both the microsomal and cell sap fractions. No consistent effect of preincubation with aldosterone or of cAMP was observed on any membrane-bound or cell sap protein other than protein D and the 37,000 dalton protein.**

The studies of Orloff and Handler (1) have provided evidence that cAMP is involved in the mechanism by which antidiuretic hormone (ADH, vasopressin) regulates sodium and water permeability across the mucosal epithelium of the toad bladder. Recent studies (2–4) have raised the possibility that antidiuretic hormone and cAMP may control the permeability of the epithelial cells through regulation of the state of phosphorylation of a protein, designated protein D, found in membranous and cell sap subfractions. In those studies, evidence was obtained supporting the conclusion that cAMP might increase the rate of removal of phosphate from protein D by endogenous protein phosphatase (3). In view of the possibility that membrane-bound protein D may be involved in the regulation of sodium transport (4), we have investigated the possibility that aldosterone, which also increases active sodium transport across toad bladder epithelium (5), might regulate the phosphorylation or dephosphorylation of protein D.

METHODS

Toads, *Bufo marinus*, from Colombia, South America, were obtained through Tarpon Zoo, Tarpon Springs, Fla., and were kept on damp bedding at 25° without food for 1–4 weeks before use. Bladders were removed from 12 to 16 doubly pithed toads and rinsed in Ringer's solution (112 mM NaCl, 2.5 mM $KHCO_3$, 1 mM $CaCl_2$, 1 mM glucose; pH 7.8).

Preincubation of Sliced Bladder Preparations with Aldosterone and Antagonists. The rinsed hemibladder sacs were slit open and manually cut into slices of about 2 × 2 mm and the slices were rinsed four times with fresh Ringer's solution. The rinsed slices were divided into two equal portions, each of which was preincubated in 20 ml of Ringer's solution (aerated with 95% O_2–5% CO_2) at 25° for 10 hr, one portion in the absence and one portion in the presence of 1 μM aldosterone. In experiments in which various agents were tested for their possible ability to antagonize the effect of aldosterone, these agents were added to the preincubation medium 15 min prior to the addition of aldosterone and were present throughout the preincubation period.

Broken Cell Preparations. At the end of the preincubation period, bladder slices were washed twice with fresh Ringer's solution and were finely minced with a McIlwain tissue chopper (200-μm setting). The minced pieces were homogenized at 2000 rpm in Ringer's solution at 4° with a glass–Teflon homogenizer, and the homogenate was fractionated at 4°. The connective tissue and nuclear fraction were removed by centrifugation of the homogenate at 900 × *g* for 1 min. The supernatant was then centrifuged at 11,500 × *g* for 10 min and the mitochondrial pellet was removed. The microsomal fraction was obtained by centrifugation of the post-mitochondrial supernatant at 100,000 × *g* for 60 min. The high-speed microsomal pellet was washed once and resuspended in Ringer's solution, pH 7.8, to give a protein concentration of about 4–5 mg/ml. This microsomal preparation was used for the studies of the endogenous phosphorylation and dephosphorylation of membrane-bound protein D. The supernatant from the 100,000 × *g* centrifugation was used as the cell sap.

The standard reaction mixture used for studying phosphorylation of protein D by endogenous protein kinase contained 50 mM sodium acetate–60 mM Tris [tris(hydroxymethylaminomethane)] buffer (pH 6.4), 2.5 mM $ZnCl_2$, 3–5 μM [γ-^{32}P]ATP (1 to 3 × 10^7 cpm/nmol) and 2.5 mg/ml of membrane protein. [In the presence of Zn^{++}, the protein D kinase reaction, which requires a divalent cation, can proceed, but the protein D phosphatase reaction is completely inhibited (3).] Incubations were carried out at 30°. The phosphorylation reaction was initiated by the addition of radioactive ATP. At various time intervals thereafter, 100-μl aliquots, containing 250 μg of protein, were removed from the reaction flask and mixed with 50 μl of a solution containing 3% sodium dodecyl sulfate, 5% sucrose, 30 mM Tris·HCl (pH 7.4), 2 mM ethylenediaminetetraacetate (EDTA), 150 mM

Abbreviation: EDTA, ethylenediaminetetraacetate.

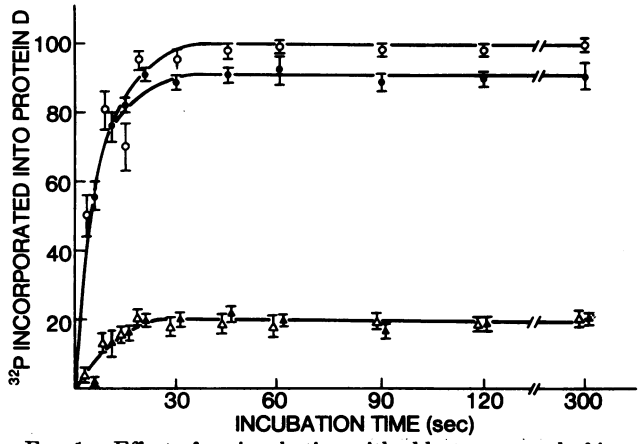

FIG. 1. Effect of preincubation with aldosterone, and of incubation with cAMP, on the rate of incorporation of radioactive phosphate into protein D, catalyzed by endogenous protein D kinase, in toad bladder membrane fraction. Flasks contained the standard reaction mixture for studying endogenous phosphorylation of protein D, with or without 10 μM cAMP. At the indicated times, 100-μl aliquots were removed and the amount of radioactive phosphate in protein D was analyzed as described in *Methods*. Results are expressed as percent of the maximum amount of [^{32}P]phosphate incorporated under the control conditions. The data represent mean ± SEM for five separate determinations. Explanation of symbols: membrane fraction, prepared from control slices, was incubated in the absence (○) or presence (△) of cAMP; membrane fraction, prepared from slices preincubated with 1 μM aldosterone, was incubated in the absence (●) or presence (▲) of cAMP.

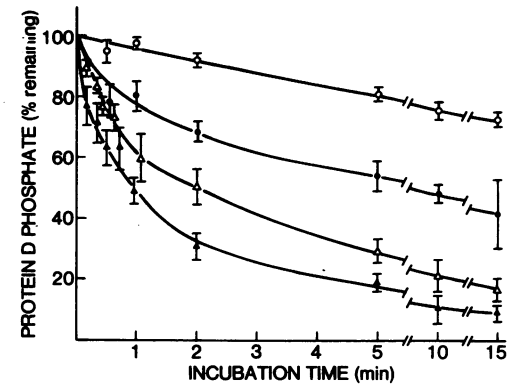

FIG. 2. Effect of preincubation with aldosterone, and of incubation with cAMP, on the rate of removal of [^{32}P]phosphate from protein D, catalyzed by endogenous protein D phosphatase, in toad bladder membrane fraction. Flasks containing the standard reaction mixture, including [γ-^{32}P]ATP, were preincubated for 1 min at 30° in the absence of cAMP in order to phosphorylate protein D. At zero time EDTA was added (50 mM final concentration), with or without cAMP (10 μM final concentration), to initiate the protein D phosphatase reaction. At the indicated times thereafter, 100-μl aliquots were removed and the amount of radioactive phosphate in protein D was analyzed as described in *Methods*. For each experimental condition, the amount of radioactive phosphate remaining in protein D is expressed as percent of the amount of [^{32}P]phosphate present in protein D at zero time. The data represent mean ± SEM for five separate determinations. Explanation of symbols: membrane fraction, prepared from control slices, was phosphorylated, and then dephosphorylation was measured in the absence (○) or presence (△) of cAMP; membrane fraction, prepared from slices preincubated with 1 μM aldosterone, was phosphorylated, and then dephosphorylation was measured in the absence (●) or presence (▲) of cAMP.

dithiothreitol, and 3 μM pyronin Y (sodium dodecyl sulfate–stop solution) to stop the reaction and solubilize the membrane proteins. Each sample was placed immediately in a boiling-water bath for 5 min to eliminate possible protease activity. The entire sample was then subjected to sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis (6, 7). In a typical experiment, after 1 min of phosphorylation in the presence of 3–5 μM [γ-^{32}P]ATP, the amount of ^{32}P incorporated into protein D was approximately 1000–2000 cpm/mg of total membrane protein or 250–500 cpm per gel slot. The gels were stained, destained, and dried, and autoradiographs were prepared, as described previously (7). Each autoradiograph was scanned with a Canalco model G-II microdensitometer and the areas under the peaks of the optical density tracings were used as a quantitative measure of the incorporation of ^{32}P into individual proteins (7).

For study of the effect of various agents on the removal of radioactive phosphate from protein D, by endogenous protein phosphatase, the membrane protein was first phosphorylated by incubation for 1 min under the standard reaction conditions used for studying endogenous protein kinase. EDTA (pH 6.4) was then added to a final concentration of 50 mM to initiate the protein phosphatase reaction. [The addition of EDTA, by removing free Zn^{++} from the reaction mixture, simultaneously (*a*) stops the protein D kinase reaction, and (*b*) relieves the inhibition of the protein D phosphatase reaction (3).] At various time intervals after the addition of EDTA, 100-μl aliquots were removed from the reaction mixture, immediately mixed with 50 μl of the sodium dodecyl sulfate-stop solution, heated, and subjected to polyacrylamide gel electrophoresis, autoradiography, and quantitative measurement of radioactive phosphate in individual protein bands, as described above.

[γ-^{32}P]ATP was prepared by the method of Post and Sen (8). Protein was determined by the method of Lowry *et al.* (9) with bovine serum albumin as standard. cAMP was obtained from Boehringer-Mannheim. Actinomycin D, spironolactone, puromycin, 9α-fluorohydrocortisone, testosterone, and aldosterone were obtained from Sigma. Cycloheximide was obtained from Calbiochem.

RESULTS

After incubation of the toad bladder microsomal fraction in the presence of [γ-^{32}P]ATP, the principle radioactive band seen on autoradiographs is protein D (apparent molecular weight, 49,000). The effect of preincubation with aldosterone, and of incubation with cAMP, on the incorporation of radioactive phosphate into protein D is shown in Fig. 1 as a function of incubation time. When assays were carried out in the absence of cAMP, the [^{32}P]phosphate content in protein D reached a maximum level within about 30 sec of incubation, and remained at that level for incubation periods up to 5 min, the longest interval studied. Pretreatment with aldosterone caused only a slight reduction in this maximal level of ^{32}P incorporation into protein D. In the presence of Zn^{++}, 10 μM cAMP caused a substantial reduction in the extent of phosphate incorporation into protein D (Fig. 1), as observed previously (3). Since protein D phosphatase activity is inhibited in the presence of Zn^{++} (3), this effect of cAMP is attributable

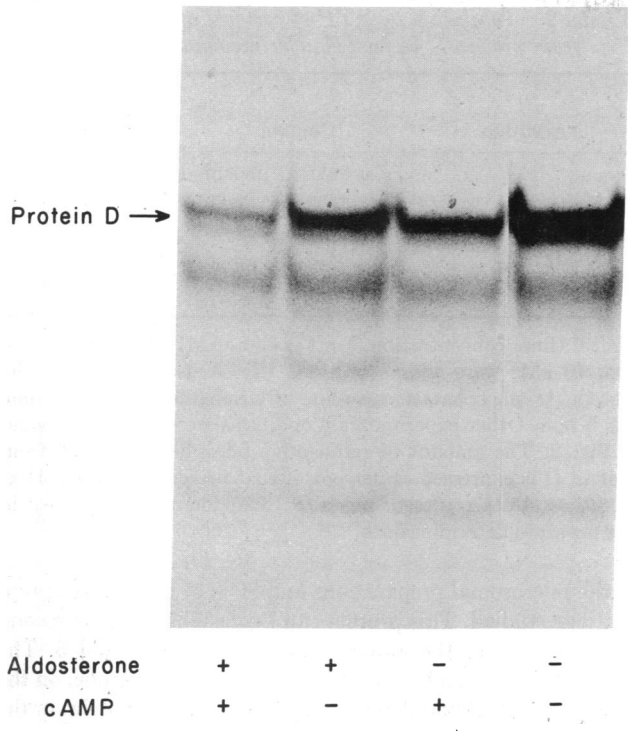

FIG. 3. Autoradiogram of phosphorylated proteins from toad bladder membrane. Membrane preparations prepared from control slices, and from slices preincubated with 1 μM aldosterone, were phosphorylated in the standard reaction mixture. The removal of [^{32}P]phosphate from protein D was carried out as described in the legend to Fig. 2. The reaction was stopped 2 min after initiation of the protein D phosphatase reaction and the solubilized proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

to an inhibition of protein D kinase activity. When assays were carried out in the presence of cAMP, the incorporation of ^{32}P into protein D in microsomal preparations from aldosterone-treated bladder slices was the same as that in microsomal preparations from control tissue.

The effect of preincubation with aldosterone on the removal of radioactive phosphate from protein D in the absence and presence of cAMP is shown in Fig. 2 as a function of incubation time. The dephosphorylation of protein D, by endogenous protein D phosphatase, was greater in microsomal preparations from aldosterone-treated bladder slices than from control bladder slices. cAMP stimulated the removal of ^{32}P from protein D in microsomal preparations from control tissue, as reported previously (3), as well as in microsomal preparations from aldosterone-treated tissue. The effects of aldosterone and of cAMP on removal of ^{32}P from protein D were approximately additive at time intervals up to 2 min.

A typical autoradiogram of the effect of aldosterone and of cAMP on the pattern of radioactivity in microsomal proteins, 2 min after initiating the protein D phosphatase reaction by addition of EDTA, is shown in Fig. 3. The ability of aldosterone to increase the removal of phosphate from protein D has been consistently observed in 40 separate experiments during the autumn, winter, and spring seasons.

The removal of radioactive phosphate from membrane-bound protein D, as a function of the concentration of aldosterone present in the preincubation mixture, is shown in Fig.

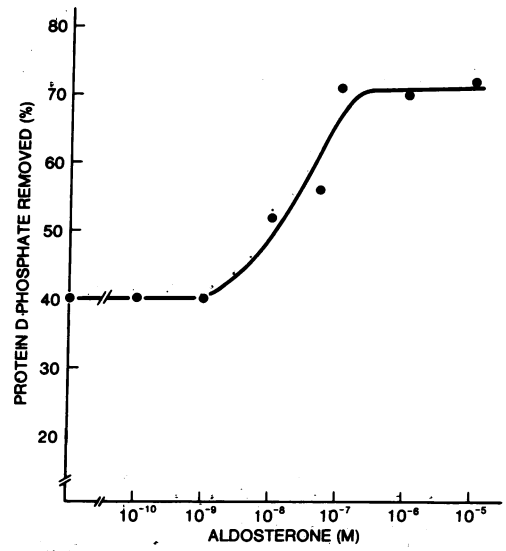

FIG. 4. Effect of preincubation with the indicated concentrations of aldosterone on the removal of [^{32}P]phosphate from protein D, catalyzed by endogenous protein D phosphatase, in toad bladder membrane fraction. The dephosphorylation of protein D was studied in the absence of cAMP, using the experimental conditions described in the legend to Fig. 2. Phosphatase reaction time was 10 min. The amount of radioactive phosphate removed from protein D is expressed as percent of the amount of [^{32}P]phosphate present in protein D at zero time. Each point represents a single determination. The results are representative of three separate experiments.

4. The data are presented for a 10-min interval after initiation of the protein D phosphatase reaction. A half-maximal increase in the removal of protein D phosphate was obtained at an aldosterone concentration of about 20–40 nM. [Similar values for the concentration of aldosterone causing a half-maximal effect were obtained with phosphatase reaction times of 1, 2, or 5 min (data not shown)]. This value is in the concentration range of aldosterone required to produce a half-maximal increase in sodium transport in intact toad bladder (10). A half-maximal binding of aldosterone to renal nuclei was also obtained at about this concentration of aldosterone (11).

The results of experiments in which various agents were tested for their possible ability to antagonize the effect of aldosterone on dephosphorylation of membrane-bound protein D are shown in Table 1. Spironolactone (0.5 mM), an aldosterone antagonist, abolished the aldosterone-induced increase in the rate of removal of radioactive phosphate from protein D. The stimulatory effect of aldosterone was also blocked by an inhibitor of RNA synthesis, actinomycin D (10 μg/ml), as well as by two inhibitors of protein synthesis, cycloheximide (2 μg/ml) and puromycin (100 μg/ml). Neither spironolactone, nor any of the inhibitors of RNA and protein synthesis; antagonized the cAMP-induced increase in protein D phosphatase activity, in contrast to the effectiveness of these substances as antagonists of the action of aldosterone.

Other steroids were tested, at 1 μM, for their possible ability to mimic the effect of aldosterone on the removal of radioactive phosphate from membrane-bound protein D, using phosphatase reaction times of 2 and 5 min. 9α-Fluorohydrocortisone, a mineralocorticoid, mimicked the effect of aldosterone, but testosterone, an androgen, had no effect.

ATP was found to inhibit the removal of [^{32}P]phosphate from protein D in microsomal fractions from control slices,

TABLE 1. *Effect of spironolactone, actinomycin D, cycloheximide, and puromycin on aldosterone-induced increase in rate of removal of [^{32}P]phosphate from protein D in toad bladder membrane fraction*

Test agent	−cAMP		+cAMP	
	normal	aldosterone	normal	aldosterone
	protein D phosphate removed (%)			
None	10	34	50	71
Spironolactone, 0.5 mM	6	10	60	50
Actinomycin D, 10 μg/ml	10	10	52	55
Cycloheximide, 2 μg/ml	10	11	49	56
Puromycin, 100 μg/ml	8	9	60	60

The experimental conditions for preincubation with aldosterone (1 μM) and the various test agents are described in *Methods*, and those for measuring the rate of removal of [^{32}P]phosphate from protein D, in the absence and presence of 10 μM cAMP, are described in the legend to Fig. 2. Phosphatase reaction time was 2 min. For each experimental condition, the amount of radioactive phosphate removed from protein D is expressed as percent of that present in protein D at zero time. The data are based on single determinations. Results qualitatively similar to those shown were observed in the same experiment using reaction times of 1 and 5 min. The results are representative of three separate experiments.

as well as from slices preincubated with aldosterone, when assays were carried out in the absence of cAMP (Table 2). This inhibitory effect of ATP was not observed in the presence of cAMP. Under other experimental conditions, ATP was able to accelerate the rate of removal of [^{32}P]phosphate from protein D both in the absence and presence of cAMP (3). Layne and Najjar have independently found an inhibition by ATP of liver membrane phosphoprotein phosphatase activity (12).

cAMP-dependent protein phosphatase activity has recently been found in cell sap from a variety of vertebrate tissues, including toad bladder (13). In the present study, aldosterone and cAMP were found to have effects on protein D phosphorylation and dephosphorylation in toad bladder cell sap which were qualitatively similar to the effects of these agents on the microsomal preparations. Under the standard conditions used for studying protein D kinase, in which Zn^{++} was present to inhibit protein D phosphatase, cAMP caused a marked inhibition of the phosphorylation of protein D in the cell sap. Aldosterone caused only a slight reduction in the phosphorylation of protein D in the absence of cAMP, and had no measurable effect in the presence of cAMP. Under the standard conditions used for studying protein D phosphatase activity, aldosterone and cAMP each increased the removal of radioactive phosphate from protein D in the cell sap. It was not possible to carry out a quantitative study of factors affecting the phosphorylation and dephosphorylation of protein D in the cell sap, because in most experiments other radioactive proteins appeared in juxtaposition to protein D on the polyacrylamide gels (which was not the case with the microsomal preparations). An autoradiogram of the effect of aldosterone and of cAMP on the radioactivity in cell sap proteins, 10 min after initiating the protein D phosphatase reaction by addition of EDTA, is shown in Fig. 5.

Incorporation of radioactive phosphate into a protein with an apparent molecular weight of 37,000 occurred in about 25% of the microsomal preparations and 80% of the cell sap preparations studied. This protein can be visualized as the second darkest band in the autoradiograms of Figs. 3 and 5. The effects of cAMP and of preincubation with aldosterone, on the phosphorylation and dephosphorylation of this protein, in the microsomal as well as in the cell sap fractions, were qualitatively similar to the effects of these agents on protein D. No consistent effect of cAMP or of preincubation with aldosterone was observed on any microsomal or cell sap proteins other than protein D and the 37,000 dalton protein.

TABLE 2. *Inhibition by ATP of removal of [^{32}P]phosphate from protein D in toad bladder membrane fraction*

Addition	Control	Aldosterone preincubation
	protein D phosphate removed (%)	
None	19.0 ± 2.7	45.8 ± 5.2
ATP	5.4 ± 1.9	4.1 ± 6.0
cAMP	70.6 ± 3.7	81.0 ± 2.8
ATP + cAMP	74.8 ± 2.5	86.0 ± 3.0

ATP (final concentration, 1 mM) and cAMP (final concentration, 10 μM) were added with the EDTA used to initiate the protein D phosphatase reaction. Phosphatase reaction time was 5 min. Other experimental conditions were as in the legend to Fig. 2. The amount of radioactive phosphate removed from protein D is expressed as percent of that present in protein D at zero time. Data represent mean ± SEM for results obtained in five separate determinations.

DISCUSSION

The results of the present study indicate that preincubation of toad bladder slices with aldosterone causes an increase in the rate of removal of [^{32}P]phosphate from membrane-bound (and cell sap) protein D by endogenous protein phosphatase. This effect of aldosterone was prevented by preincubation of the slices with inhibitors of RNA or protein synthesis. In contrast, the increase in protein D phosphatase activity resulting from cAMP was unaffected by preincubation with these inhibitors. One possible explanation for these results is that aldosterone induces the *de novo* synthesis of a protein phosphatase capable of removing phosphate from protein D. However, our results with aldosterone are also compatible with other possibilities; for instance aldosterone might induce the synthesis of a protein phosphatase activator. Interestingly, the phosphorylation of protein D, by endogenous protein D kinase, was only slightly affected by preincubation with aldosterone; these results suggest that in contrast to its action on protein D phosphatase, aldosterone does not markedly affect either the level of protein D kinase or the amount of protein D.

It was recently postulated, as a working hypothesis, that the state of phosphorylation of membrane-bound protein D might determine the permeability of the apical membrane of toad bladder epithelial cells to sodium (2–4). It is of particular interest, therefore, that both antidiuretic hormone (acting through cAMP) and aldosterone lead to an increase in the dephosphorylation of protein D, since both hormones also cause an increase in active sodium transport across the toad bladder epithelium. The effects of various other agents on protein D phosphatase activity, reported in the present study, also correlate well with their effects on sodium transport across toad bladder epithelium. Thus, spironolactone,

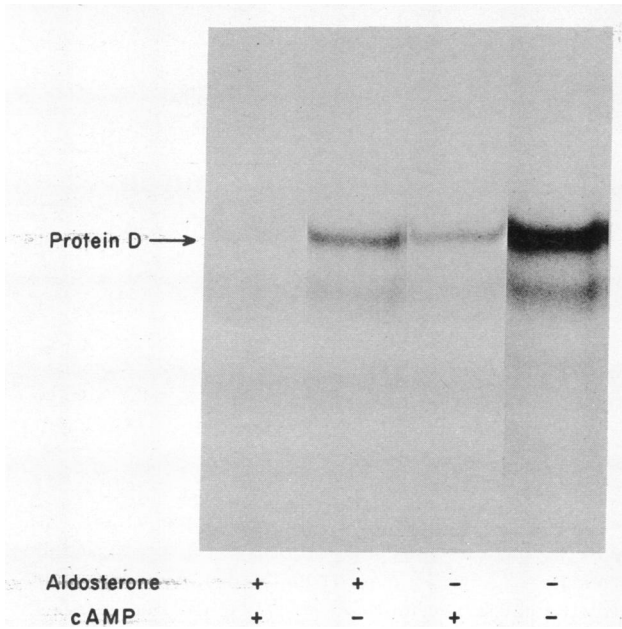

FIG. 5. Autoradiogram of phosphorylated proteins from toad bladder cell sap. Cell sap prepared from control slices, and from slices preincubated with 1 μM aldosterone, was phosphorylated in the standard reaction mixture. The removal of [^{32}P]phosphate from protein D was carried out as described in the legend to Fig. 2. The reaction was stopped 10 min after initiation of the protein D phosphatase reaction and the proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

actinomycin D, cycloheximide, and puromycin, which antagonize the effect of aldosterone on protein D phosphatase activity, also antagonize the effect of aldosterone in increasing sodium transport (10, 14). These inhibitors had no effect on the cAMP-induced increase in protein D phosphatase activity, and have little or no effect on antidiuretic hormone-induced stimulation of sodium transport (15). Moreover, 9α-fluorohydrocortisone, which was found to cause an increase in protein D phosphatase activity, also causes an increase in sodium transport across intact toad bladder (16). Conversely, testosterone, which had no effect on protein D phosphatase activity, is devoid of mineralocorticoid activity.

Several other important considerations are raised by the present study: (*a*) Is the regulation of sodium transport by aldosterone and by cAMP related to their regulation in membranes of the state of phosphorylation of protein D and/or the 37,000 dalton protein? (*b*) What is the biological significance of the presence in the *cell sap* of protein D and of the 37,000 dalton protein, the dephosphorylation of which are affected by aldosterone and by cAMP? (*c*) What is the substrate specificity of the protein phosphatase(s)? For example, are the dephosphorylation of protein D and the dephosphorylation of the 37,000 dalton protein catalyzed by the same enzyme system? (*d*) Is the protein phosphatase system that is affected by aldosterone the same as that which is affected by cAMP? (*e*) Do other steroid hormones acting on other target organs achieve some of their biological effects through actions on protein phosphatase (or protein kinase)?

Note Added in Proof. Vokaer, A., Iacobelli, S. & Kram, R. (*Proc. Nat. Acad. Sci. USA*, in press) have made the interesting observation that an estrogen-induced protein prepared from rat uterus contains phosphoprotein phosphatase activity.

This work was supported by US Public Health Service Grants NS-08440 and MH-17387.

1. Orloff, J. & Handler, J. (1967) *Amer. J. Med.* **42**, 757–768.
2. DeLorenzo, R. J., Walton, K. G., Curran, P. F. & Greengard, P. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 880–884.
3. DeLorenzo, R. J. & Greengard, P. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1831–1835.
4. Walton, K. G., DeLorenzo, R. J., Curran, P. F. & Greengard, P. (1974) *J. Gen. Physiol.*, in press.
5. Crabbé, J. (1961) *J. Clin. Invest.* **40**, 2103–2110.
6. Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617.
7. Ueda, T., Maeno, H. & Greengard, P. (1973) *J. Biol. Chem.* **248**, 8295–8305.
8. Post, R. L. & Sen, A. K. (1967) in *Methods in Enzymology*, eds. Colowick, S. P. & Kaplan, N. O. (Academic Press, New York), Vol. X, pp. 773–776.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
10. Sharp, G. & Leaf, A. (1964) *Nature* **202**, 1185–1188.
11. Fanestil, D. D. & Edelman, I. S. (1966) *Proc. Nat. Acad. Sci. USA* **56**, 872–879.
12. Layne, P. P. & Najjar, J. L. (1974) *Fed. Proc.* **33**, 1249.
13. Malkinson, A. M., Krueger, B. K., Rudolph, S. A., Casnellie, J. E., Haley, B. E. & Greengard, P. (1975) *Metabolism*, in press.
14. Fanestil, D. D. & Edelman, I. S. (1966) *Fed. Proc.* **25**, 912–916.
15. Edelman, I. S., Bogoroch, R. & Porter, G. A. (1963) *Proc. Nat. Acad. Sci. USA* **50**, 1169–1176.
16. Porter, G. A. & Edelman, I. S. (1964) *J. Clin. Invest.* **43**, 611–620.