Proc. Nat. Acad. Sci. USA
Vol. 70, No. 6, pp. 1831–1835, June 1973

Activation by Adenosine 3′:5′-Monophosphate of a Membrane-Bound Phosphoprotein Phosphatase from Toad Bladder

(cyclic AMP/protein D/antidiuretic hormone)

ROBERT J. DELORENZO AND PAUL GREENGARD

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

Communicated by Edward A. Adelberg, April 4, 1973

ABSTRACT **Adenosine 3′:5′-monophosphate (cyclic AMP) caused a decrease in the net rate of incorporation of radioactive phosphate into a specific protein (protein D) in a membrane fraction from toad bladder. Moreover, when the membrane protein was prelabeled with radioactive phosphate, cyclic AMP caused an increase in the net rate of removal of radioactive phosphate from this specific protein. Certain agents were shown to be selective inhibitors of membrane-bound protein D kinase or protein D phosphatase. With the help of these agents, it was concluded that cyclic AMP caused the activation of membrane-bound protein D phosphatase. The present data, together with earlier studies, are compatible with the possibility that the cyclic AMP-induced activation of a membrane-bound phosphoprotein phosphatase in toad bladder, with the consequent dephosphorylation of protein D, may be responsible for the physiological effects of antidiuretic hormone on sodium and/or water transport in this tissue.**

Addition of antidiuretic hormone or monobutyryl cyclic AMP to preparations of intact toad bladder which had been preincubated with radioactive inorganic phosphate caused a decrease in the amount of radioactive phosphate present in a specific protein (protein D) in the toad bladder (1). The decrease in the amount of phosphate present in protein D brought about by these agents in intact bladders slightly preceded the change in electrical potential difference (an indicator of the rate of sodium ion transport) observed in response to the same compounds. Addition of cyclic AMP to toad-bladder homogenates caused a decrease in the net phosphorylation of the same, or a similar, protein. This effect of cyclic AMP was most pronounced in those subcellular fractions of toad bladder rich in membrane fragments. The data were compatible with the possibility that regulation by antidiuretic hormone of sodium and/or of water permeability of the epithelial membrane of toad bladder might be mediated through regulation of the level of phosphorylation of protein D. It, therefore, seemed of importance to elucidate the mechanism by which cyclic AMP causes a decrease in the level of phosphorylation of protein D, and for that reason the present investigation was undertaken.

The ability of cyclic AMP to cause a decrease in net incorporation of radioactive phosphate into protein D could result either from a cyclic AMP-induced decrease in the activity of a protein D kinase, or from a cyclic AMP-induced increase in the activity of a protein D phosphatase, or both. In an effort to distinguish among these possibilities, we have searched for and found selective inhibitors of protein D kinase (adenosine; EDTA) and of protein D phosphatase ($ZnCl_2$), and have used these inhibitors to determine whether the effects of cyclic AMP on the amount of protein D phosphate would be reduced or abolished by either class of inhibitor. Our results indicate that cyclic AMP causes an increase in the activity of membrane-bound protein D phosphatase, and can account, at least partially, for the effect of cyclic AMP and of antidiuretic hormone on the amount of protein D phosphate in preparations of intact toad bladder.

METHODS

Toads, *Bufo marinus*, were obtained from South America through Tarpon Zoo (Tarpon Springs, Fla.), and maintained at 25° on damp bedding. Bladders were excised (2) from doubly-pithed toads and placed in Ringers solution [112 mM NaCl–2.5 mM $KHCO_3$–1 mM $CaCl_2$–2 mM glucose (pH 7.8)].

Scrapings of toad-bladder mucosal epithelium (1) were homogenized in 6–8 volumes of 0.32 M sucrose in a 40-ml glass homogenizer with a Teflon pestle of 0.15-mm clearance at 900 rpm (10–15 strokes). The connective tissue was removed by centrifugation at 400 *g* for 1 min, and the supernatant was used directly in experiments involving toad-bladder epithelial homogenates. The microsomal subfraction, rich in membrane fragments, was prepared from homogenates by differential centrifugation as follows. The nuclear fraction, obtained by centrifugation of the homogenate at 900 × *g* for 10 min, was washed twice with 0.32 M sucrose. From the combined supernatants, a crude mitochondrial fraction was obtained by centrifugation at 11,500 × *g* for 10 min, and the mitochondrial pellet was washed twice with 0.32 M sucrose. The microsomal subfraction was then obtained by centrifugation of the postmitochondrial fraction (combined supernatants) at 100,000 × *g* for 60 min. The microsomal pellet was washed once with 0.32 M sucrose and suspended in 0.32 M sucrose. This preparation, which appears to consist of microsomes and membrane fragments (1), is the preparation referred to as the toad-bladder membrane fraction in the experiments described.

The standard reaction mixture used for studying phosphorylation and dephosphorylation of protein D contained 50 mM sodium acetate buffer (pH 6.2), 10 mM magnesium acetate, 5 μM [γ-^{32}P]ATP [prepared according to Post and Sen (3)], and 1.25 mg of membrane protein per ml, with other additions as indicated. The reaction was initiated by addition of radioactive ATP. Preincubations and incubations were done at 30°.

To study the effect of various agents on incorporation of radioactive phosphate into protein D (Fig. 1), reaction tubes were incubated under standard conditions for various periods

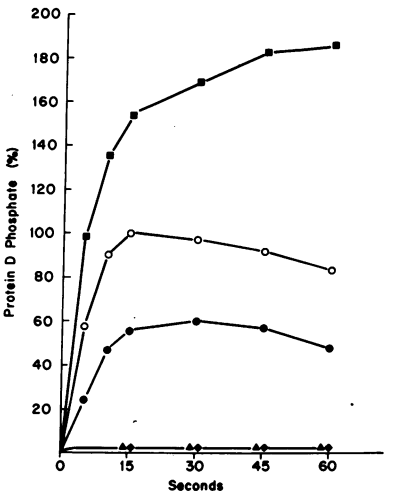

FIG. 1. Effect of cyclic AMP, of protein kinase inhibitors (adenosine, EDTA), and of a phosphoprotein phosphatase inhibitor ($ZnCl_2$) on the net rate of incorporation of radioactive phosphate into protein D of the toad-bladder membrane fraction. Reaction tubes contained the standard incubation mixture in a volume of 0.2 ml (containing 250 μg of membrane protein), including 5 μM [γ-^{32}P]ATP, and the following additions: none, (○——○); 50 mM EDTA, (◆——◆); 1 mM adenosine, (▲——▲); 5 μM cyclic AMP, (●——●); and 10 mM $ZnCl_2$, (■——■). The reaction was terminated at the indicated times by the addition of 0.1 ml of Na dodecyl sulfate-stop solution. The solubilized protein solution was then analyzed for protein D phosphate, as described in *Methods*. The data are expressed as percent of the maximum amount of [^{32}P]phosphate incorporated under the control conditions (which occurred at 15 sec of incubation). Each point represents a single determination. The results of the experiment illustrated are representative of six separate experiments.

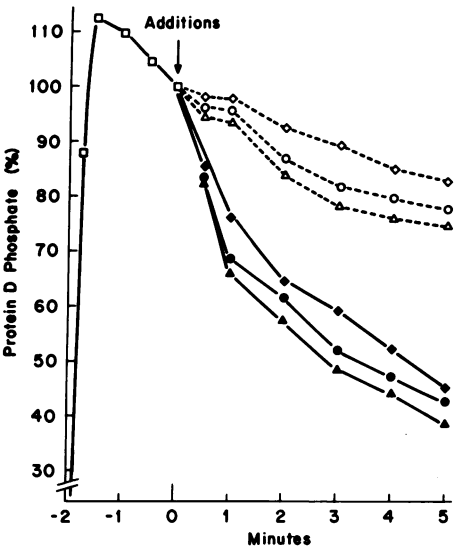

FIG. 2. Effect of cyclic AMP on the rate of removal of [^{32}P]phosphate from protein D in toad-bladder homogenates in the absence or presence of inhibitors (adenosine, EDTA) of protein D kinase. Homogenate, containing 3 mg of protein, was preincubated for 2 min in a volume of 2.4 ml, with 5 μM [γ-^{32}P]-ATP, under standard assay conditions (□——□). At zero time, various test agents were added in a volume of 0.05 ml to yield the following concentrations: 1 mM adenosine (△- - -△; ▲——▲), 50 mM EDTA (◇- - -◇; ◆——◆), or neither (○- - -○; ●——●) of these inhibitors, without (*open symbols*) or with (*solid symbols*) 5 μM cyclic AMP. (Data have not been corrected for the 2% dilution caused by addition of the test agents.) At the indicated times, before and after the addition of the test agents, 200-μl aliquots (containing approximately 250 μg of protein) were removed from the incubation flasks, mixed with 0.1 ml of the Na dodecyl sulfate-stop solution to terminate the reaction, and protein D phosphate was analyzed as described in *Methods*. Data are expressed as percent of the value of protein D phosphate present at zero time. Each point represents a single determination. The results of the experiment illustrated above are representative of four separate experiments.

of time in a volume of 0.2 ml. The reaction was terminated by addition of 0.1 ml of a "Na dodecyl sulfate-stop" solution containing 3% sodium dodecyl sulfate, 5% sucrose, 30 mM Tris·HCl (pH 8.0), 3 mM EDTA (pH 8.0), 120 mM dithiothreitol, and 3 μM pyronin Y, a marker dye. The Na dodecyl sulfate-stop solution abolished enzymatic activity and solubilized the membrane components. The solubilized preparation was then analyzed for incorporation of [^{32}P]phosphate into protein D (1). For this purpose, a 200-μl sample of the solubilized protein was subjected to acrylamide gel electrophoresis, as described previously (4), for 5 hr at 120 mA on a vertical plate gel of 5.6% polyacrylamide, with a running buffer of 40 mM Tris–20 mM sodium acetate–2 mM EDTA–1% Na dodecyl sulfate, pH adjusted to 7.4 with acetic acid (5). The gel was stained for protein with 0.025% Coomassie blue in 25% isopropyl alcohol–10% acetic acid. It was destained for 6 hr in 0.0025% Coomassie blue in 10% isopropyl alcohol–10% acetic acid, and finally in 10% acetic acid with several changes (1). The stained gel was dried and placed in contact with Kodak Royal X-Omat Film, and the film was developed 3–7 days later (4). The band corresponding to protein D was located on the dried gel by superimposing the autoradiograph on the gel. The band was then cut from the gel and the [^{32}P]phosphate incorporated into protein D was determined by liquid scintillation counting.

To study the effect of various agents on the rate of removal of radioactive phosphate from protein D, the protein was first labeled by preincubating the homogenate or membrane protein fraction for 2 min, either under standard conditions (Figs. 2–4) or by substitution of zinc chloride (10 mM) for magnesium acetate (Fig. 5). Various agents were then added to determine their effect on dephosphorylation of protein D. Equal protein aliquots (usually 0.2 ml) were removed from the reaction mixture at various time intervals during preincubation and after the addition of test agents, and immediately mixed with 0.10 ml of the Na dodecyl sulfate-stop solution. The [^{32}P]phosphate present in protein D at each time interval was determined by subjecting equal aliquots to acrylamide gel electrophoresis, autoradiography, and quantitative measurement, as described above.

[γ-^{32}P]ATP remaining in the standard reaction mixture (0.2 ml) at various time intervals was determined by terminating the reaction with 4 ml of cold 20% trichloracetic acid, followed by centrifugation of the sample for 10 min at 900 × g. The supernatant was removed and neutralized with 0.1 N NaOH. [γ-^{32}P]ATP was isolated by thin-layer chromatography (6), and the radioactivity was determined by liquid scintillation counting.

RESULTS

The effect of cyclic AMP on the net rate of incorporation of radioactive phosphate into protein D is shown as a function of incubation time in Fig. 1. In the absence of any additions to the standard incubation mixture, the [^{32}P]phosphate in protein D reached a peak at about 15 sec of incubation, and slowly decreased thereafter (Fig. 1). In the presence of 5 μM cyclic AMP, a decreased amount of protein D phosphate was observed throughout the reaction.

For measurement of the rate of removal of [^{32}P]phosphate from protein D under various conditions, homogenate or membrane protein was preincubated for 2 min with [γ-^{32}P]-ATP in the standard incubation mixture, by which time the phosphorylation of protein D had passed its peak and was decreasing (Figs. 2–4). Thus, within 2 min the rate of incorporation of phosphate, catalyzed by protein D kinase, had fallen below the rate of removal of phosphate, catalyzed by protein D phosphatase. A decrease in the rate of incorporation of phosphate into protein D can be explained by a depletion of ATP: we observed that, after 1 and 2 min of incubation of the membrane fraction under standard conditions, the concentration of radioactive ATP remaining in the incubation solution had fallen from 5 μM to about 0.7 μM and 0.2 μM, respectively; when incubations were done for 1 min with 0.2 μM radioactive ATP in place of the 5 μM ATP present under standard conditions, the net incorporation of [^{32}P]phosphate into protein D was about 5% of that observed with 5 μM ATP. Therefore, we studied the effect of various agents on protein D phosphatase activity by adding them to the reaction mixture after 2 min of preincubation to minimize any contribution of protein D kinase activity to the effects of the test agents. With this experimental procedure, we found that cyclic AMP (5 μM) markedly stimulated the rate of removal of [^{32}P]phosphate from protein D in homogenates (Fig. 2) and in microsomal fractions (Figs. 3 and 4). Therefore, this effect of cyclic AMP is attributable to a stimulation of protein D phosphatase activity rather than to an inhibition of protein D kinase activity. This conclusion was supported by studies in which selective inhibitors of protein D kinase or protein D phosphatase were used.

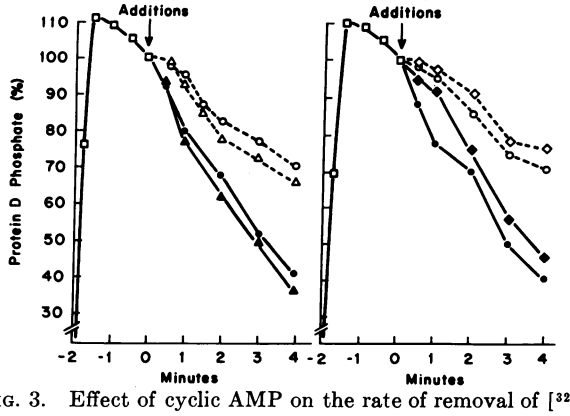

FIG. 3. Effect of cyclic AMP on the rate of removal of [^{32}P]-phosphate from protein D in toad-bladder membrane fraction, in the absence or presence of inhibitors (adenosine, *left panel;* EDTA, *right panel*) of protein D kinase. The toad-bladder membrane fraction was prepared as described in *Methods*. Other experimental details were the same as described in the legend to Fig. 2, except for the use of the membrane fraction (containing 3 mg of protein) rather than of the whole homogenate in the incubation mixture. The symbols for adenosine, EDTA, and cyclic AMP are identical to those used in Fig. 2. The results of the experiment illustrated are representative of four separate experiments.

Our search for selective inhibitors of protein D kinase and protein D phosphatase activity of toad-bladder membranes was facilitated by our earlier studies of mammalian protein kinases and phosphoprotein phosphatases. In studies of partially purified protein kinases from mammalian brain (7) and several other mammalian tissues (8), protein kinase activity required the presence of a divalent cation such as Mg^{++} or Zn^{++}, and was inhibited by adenosine. In studies (9) of partially purified phosphoprotein phosphatases from mammalian brain, phosphoprotein phosphatase activity was not stimulated by addition of any divalent cation and, in fact, was strongly inhibited by Zn^{++}. Therefore, in searching for selective inhibitors, we examined the effects of adenosine, of EDTA, and of Zn^{++} on the protein D kinase and protein D phosphatase activities of toad-bladder homogenates and membrane fractions. The effects of these agents on the membrane-bound protein kinase and phosphoprotein phosphatase activity of toad bladder, observed in the present study, indicated that adenosine and EDTA are selective inhibitors of protein D kinase activity and that Zn^{++} is a selective inhibitor of protein D phosphatase activity.

Adenosine (1 mM) and EDTA (50 mM) caused an almost complete inhibition of the net rate of incorporation of phosphate into protein D (Fig. 1), while having little or no effect on the removal of phosphate from protein D (Figs. 2–4). Considered together, the results indicate that the effect of adenosine and EDTA on the net phosphorylation of pro-

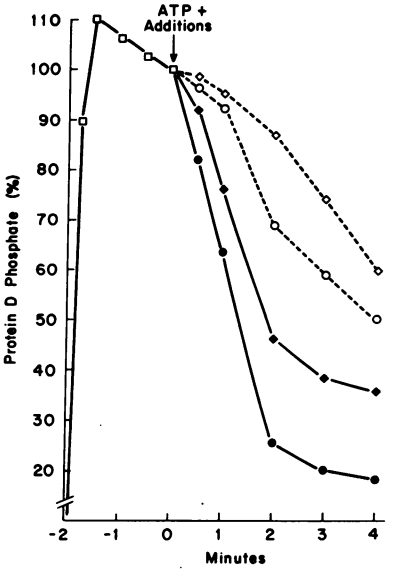

FIG. 4. Effect of cyclic AMP on the rate of removal of [^{32}P]-phosphate from protein D in toad-bladder membrane fraction in the presence of a large amount of nonradioactive ATP, in the absence or presence of an inhibitor (EDTA) of protein D kinase. Membrane fraction, containing 3 mg of protein, was preincubated for 2 min in a volume of 2.4 ml, with 5 μM [γ-^{32}P]ATP, under standard assay conditions (□——□). At zero time, nonradioactive ATP was added, alone (○- - -○), with EDTA (◇- - -◇), with cyclic AMP (●——●), or with EDTA plus cyclic AMP (◆——◆), in a volume of 0.05 ml. The final concentrations of these additions were: ATP, 1 mM; EDTA, 50 mM; cyclic AMP, 5 μM. Other experimental details were as described in the legends to Figs. 2 and 3. The results of the experiment illustrated are representative of five separate experiments.

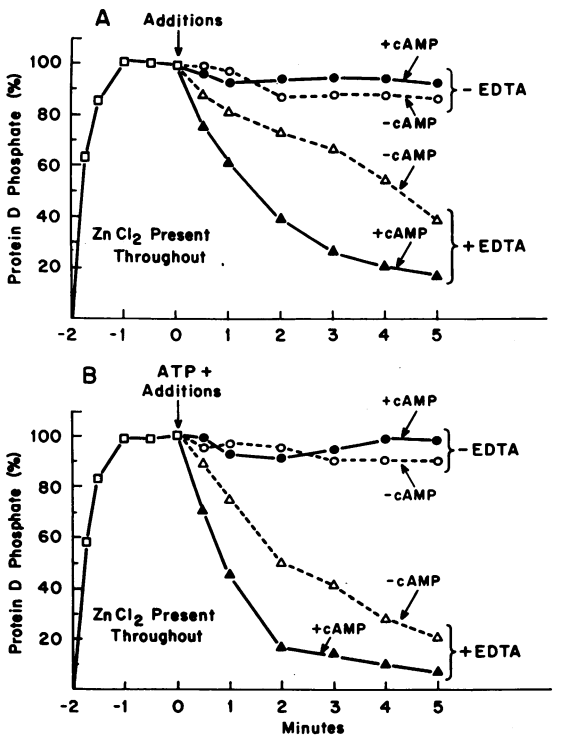

FIG. 5. Effect of cyclic AMP on the rate of removal of [^{32}P]-phosphate from protein D in toad-bladder membrane fraction in the presence of an inhibitor ($ZnCl_2$) of protein D phosphatase, and after the removal of the phosphatase inhibitor by chelation with EDTA. Membrane fraction, containing 3 mg of protein, was preincubated for 2 min in a volume of 2.4 ml, with 5 μM [γ-^{32}P]-ATP, under the standard assay conditions, except for the substitution of 10 mM $ZnCl_2$ for 10 mM magnesium acetate (□——□) At zero time, test agents were added without (*A*) or with (*B*) nonradioactive ATP (final concentration 1 mM), in a volume of 0.05 ml. The test agents added, and their final concentrations, were: none (○- - -○); 50 mM EDTA (△- - -△); 5 μM cyclic AMP (●——●); 50 mM EDTA plus 5 μM cyclic AMP (▲——▲). Other experimental details were as described in the legends to Figs. 2 and 3. The results of the experiment illustrated are representative of four separate experiments.

tein D (Fig. 1) is attributable to a selective inhibition by these substances of protein D kinase activity.

The protein kinase inhibitors, adenosine (1 mM) and EDTA (50 mM), each abolished the incorporation of radioactive phosphate into protein D not only in the absence (Fig. 1), but also in the presence of cyclic AMP. In contrast to the strong inhibition of protein D kinase activity caused by adenosine and EDTA, these agents had only slight effects on protein D phosphatase activity, either in the absence or presence of cyclic AMP. Adenosine (1 mM) caused a slight increase in the net rate of removal of [^{32}P]phosphate from protein D, in homogenates (Fig. 2) and in membrane fractions (Fig. 3), both in the absence and in the presence of cyclic AMP. This small effect of adenosine may be attributable to inhibition of the slight amount of phosphate incorporation still occurring at that time. The increase in the rate of removal of radioactive phosphate from protein D, due to cyclic AMP, was virtually unaffected by the presence of adenosine. EDTA (50 mM) caused a slight decrease in the net rate of removal of [^{32}P]phosphate from protein D, in homogenates (Fig. 2) and in membrane fractions (Fig. 3), both in the absence and in the presence of cyclic AMP. This small effect of EDTA is probably attributable to a slight inhibition of protein D phosphatase activity, since we have found in other experiments that Mg^{++} causes a slight stimulation of casein phosphatase activity of toad-bladder membrane.

When a large amount of nonradioactive ATP was added after 2 min of preincubation, to stop any slight incorporation of [^{32}P]phosphate into protein D from trace amounts of [γ-^{32}P]ATP still remaining, cyclic AMP still increased the rate of removal of [^{32}P]phosphate from protein D (Fig. 4). Moreover, the combination of ATP plus EDTA (each of which should have abolished any residual incorporation of [^{32}P]-phosphate into protein D still occurring after 2 min of preincubation) did not significantly reduce the stimulatory effect of cyclic AMP on the dephosphorylation of protein D in membrane fractions (Fig. 4).

Preincubation of toad-bladder membrane fraction, in the absence of cyclic AMP, under standard conditions except for the substitution of 10 mM $ZnCl_2$ for 10 mM magnesium acetate, led to a net rate of incorporation of radioactive phosphate into protein D greater than that observed under standard incubation conditions (Fig. 1). In contrast, in the presence of $ZnCl_2$, the subsequent removal of [^{32}P]phosphate from protein D in the absence of cyclic AMP was almost completely inhibited (compare Figs. 3–5). These results indicate that $ZnCl_2$ acts as a selective inhibitor of protein D phosphatase. The stimulation by $ZnCl_2$ of the net rate of incorporation of phosphate into protein D may be attributable to inhibition by $ZnCl_2$ of protein D phosphatase activity.

The stimulatory effect of cyclic AMP on the rate of dephosphorylation of protein D was abolished by $ZnCl_2$, both in the absence (Fig. 5*A*) and presence (Fig. 5*B*) of nonradioactive ATP (1 mM). If EDTA was added after 2 min of preincubation to remove free Zn^{++} from the incubation mixture, removal of [^{32}P]phosphate from protein D was again observed and the cyclic AMP stimulation of this dephosphorylation was restored, both in the absence (Fig. 5*A*) and presence (Fig. 5*B*) of 1 mM ATP.

We observed in experiments with membrane fractions (compare Figs. 3 and 4; also Fig. 5*A* and *B*) that the rate of removal of [γ-^{32}P]phosphate from protein D was greatly accelerated by ATP. This effect of ATP was greater than could be accounted for by prevention of further incorporation of radioactive phosphate into protein D from the trace amount of [γ-^{32}P]ATP remaining.

DISCUSSION

The results of the present study indicate that the mechanism by which cyclic AMP causes a decrease in the net incorporation of [^{32}P]phosphate into protein D in toad-bladder membrane probably involves activation of a protein D phosphatase. The main basis for this conclusion is that cyclic AMP stimulated the removal of [^{32}P]phosphate from protein D under conditions in which, for several reasons, there was little or no ongoing incorporation of [^{32}P]phosphate into protein D from [γ-^{32}P]ATP: (*i*) too little [γ-^{32}P]ATP remained in the reaction mixture after the preincubation period used to phosphorylate protein D to a significant extent; (*ii*) addition of a large amount of nonradioactive ATP after the preincubation period prevented the incorporation into protein D of [^{32}P]phosphate from any remaining traces of

[γ-^{32}P]ATP; and (*iii*) the presence of either EDTA or adenosine inhibited protein D kinase activity. Stimulation by cyclic AMP of the removal of [^{32}P]phosphate from protein D was still clearly demonstrable when all three of these conditions existed (Fig. 4).

Other results compatible with the conclusion that cyclic AMP causes activation of protein D phosphatase were obtained in experiments in which an inhibitor of protein D phosphatase was used. Thus, cyclic AMP did not stimulate the removal of [^{32}P]phosphate from protein D when protein D phosphatase activity was inhibited by $ZnCl_2$. Moreover, the removal of the Zn^{++}-induced inhibition of protein D phosphatase by the addition of EDTA restored the ability of cyclic AMP to stimulate the dephosphorylation of protein D.

The present results do not exclude the possibility that the decreased net phosphorylation of protein D observed in the presence of cyclic AMP may also be due in part to an inhibition of protein D kinase activity. Miyamoto *et al.* (7) reported that the stimulatory effect of cyclic AMP on phosphorylation of histone by a purified cyclic AMP-dependent protein kinase from bovine brain was converted, in the presence of Zn^{++} or Ca^{++}, into an inhibitory effect. The inhibitory effect of cyclic AMP on this purified brain enzyme, observed in the presence of Zn^{++}, is attributable to an inhibition of the protein kinase activity of the preparation, and not due to stimulation of a phosphoprotein phosphatase contaminant.* Similarly, in the presence of Zn^{++}, cyclic AMP inhibits the endogenous activity of membrane-bound protein kinase present in a synaptic membrane fraction from rat cerebrum,* as well as inhibits the endogenous protein D kinase activity present in the toad-bladder membrane (R. J. DeLorenzo and P. Greengard, unpublished experiments). It therefore seems possible that, under physiological conditions, cyclic AMP might, in addition to its stimulatory action on protein D phosphatase, also inhibit the activity of protein D kinase.

Further investigation will be necessary to determine the molecular mechanism by which cyclic AMP causes activation of protein D phosphatase. The effect of cyclic AMP might be a direct one on phosphoprotein phosphatase itself. Another possibility is that cyclic AMP activates a protein D phosphatase kinase, leading to phosphorylation and activation of protein D phosphatase. Such a mechanism would be consistent with the general theory (10) that the diverse effects of cyclic AMP are mediated through regulation of the activity of protein kinases. Still a third possibility is that cyclic AMP causes an increased availability of protein D to protein D phosphatase. The use of model substrates, such as [^{32}P]-casein and [^{32}P]histone, may help to elucidate the detailed mechanism by which cyclic AMP can stimulate membrane-bound phosphoprotein phosphatase activity.

Evidence has been presented suggesting that cyclic AMP may cause the inactivation of soluble phosphorylase phosphatase in bovine-adrenal cortex (11) and pigeon-breast muscle (12): ATP, in the absence of Mg^{++}, caused inactivation of the enzyme; ATP plus Mg^{++} caused activation of the enzyme; cyclic AMP plus ATP plus Mg^{++} caused inactivation of the enzyme. It is not at all clear whether there is any relationship between inactivation of soluble phosphorylase phosphatase and activation of membrane-bound phosphoprotein phosphatase.

The evidence obtained in the present study, indicating an activation by cyclic AMP of a membrane-bound phosphoprotein phosphatase in toad bladder, has led us to examine other biological membranes for the possible occurrence of a similar phenomenon. Using a subcellular fraction of rat cerebrum rich in synaptic membranes (13), it has been found* that cyclic AMP can, under certain experimental conditions, increase the rate of removal of radioactive phosphate from a membrane-bound substrate protein (designated protein II) through an increase in the activity of a membrane-bound phosphoprotein phosphatase. The stimulation by cyclic AMP of protein D phosphatase activity in toad-bladder membrane and of protein II phosphatase activity in synaptic membrane suggests that phosphoprotein phosphatase activation by cyclic AMP may be a fairly widespread phenomenon.

This work was supported by Grants NS-08440 and MH-17387 from the United States Public Health Service. R. J. D. was supported by Training Grant GM 02044 from the United States Public Health Service.

1. DeLorenzo, R. J., Walton, K., Curran, P. & Greengard, P. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 880–884.
2. Bentley, P. J. (1960) *J. Endocrinol* **21**, 161–170.
3. Post, R. L. & Sen, A. K. (1967) in *Methods in Enzymology*, eds. Estabrook, R. W. & Pullman, M. E. (Academic Press, New York), Vol. X, pp. 773–776.
4. Johnson, E. M., Ueda, T., Maeno, H. & Greengard, P. (1972) *J. Biol. Chem.* **247**, 5650–5652.
5. Fairbanks, G., Steck, T. L. & Wallach D. F. H. (1971) *Biochemistry* **10**, 2606–2617.
6. Hochman, H. I., Agrawal, K. C. & Sartorelli, A. C. (1972) *Biochem. Pharmacol.* **21**, 3223–3233.
7. Miyamoto, E., Kuo, J. F. & Greengard, P. (1969) *J. Biol. Chem.* **244**, 6395–6402.
8. Kuo, J. F., Krueger, B. K., Sanes, J. R. & Greengard, P. (1970) *Biochim. Biophys. Acta* **212**, 79–91.
9. Maeno, H. & Greengard, P. (1972) *J. Biol. Chem.* **247**, 3269–3277.
10. Kuo, J. F. & Greengard, P. (1969) *Proc. Nat. Acad. Sci. USA* **64**, 1349–1355.
11. Merlevede, W. & Riley, G. A. (1966) *J. Biol. Chem.* **241**, 3517–3524.
12. Chelala, C. A. & Torres, H. N. (1970) *Biochim. Biophys. Acta* **198**, 504–513.
13. Maeno, H., Johnson, E. M. & Greengard, P. (1971) *J. Biol. Chem.* **246**, 134–142.

* Maeno, H., Ueda, T., Rudolph, S. A. & Greengard, P., manuscript in preparation.