Regulation of Protein Phosphorylation
and Sodium Transport in Toad Bladder

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ABSTRACT It is well established that active sodium-ion transport and water flow across isolated toad bladder are increased by antidiuretic hormone (ADH) and by cAMP. These agents were also observed in previous studies to cause changes in the amount of radioactive phosphate in a specific protein in the toad bladder. This protein, found by SDS-polyacrylamide gel electrophoresis of toad-bladder epithelial preparations, had an apparent molecular weight of 49,000 daltons. In the present study, a correlation was found between the ability of a variety of substances to affect the amount of radioactive phosphate in this 49,000-dalton protein and their ability to alter the rate of sodium transport. Thus, several agents (ADH, cAMP, theophylline, adenine, prostaglandin E₁, and MnCl₂) caused a decrease in the amount of radioactive phosphate in the 49,000-dalton protein and also stimulated active sodium transport across the bladder. Conversely, ZnCl₂ produced an increase in the amount of radioactive phosphate in this protein and an inhibition of sodium transport. With each of these agents, the time-course of change in phosphorylation of this protein was, in general, similar to that for sodium transport. A second phosphoprotein, with an apparent molecular weight of about 42,000 daltons, showed changes in parallel with, but less extensive than, those observed in the 49,000-dalton protein. There was no consistent relationship between changes in level of phosphorylation of either the 49,000- or 42,000-dalton protein and changes in osmotic water permeability. The results are compatible with the possibility that regulation by ADH and by cAMP of sodium transport in the toad bladder epithelium may be mediated through regulation of the amount of phosphate in a specific protein.

INTRODUCTION

Antidiuretic hormone (ADH)stimulates net active transport of sodium ion across the mucosal epithelium of toad bladder and increases the rate of net...
water flow across the tissue in response to an osmotic pressure difference between the solutions bathing the two sides of the bladder. It is now widely accepted that these two effects of ADH are mediated by cAMP (1, 2). Stimulation of sodium transport is accomplished primarily by a cAMP-induced increase in permeability of the apical membrane of the mucosal cells to sodium ion, rather than by a direct action of cAMP on the active transport mechanism that is assumed to be located at the basal border of the mucosal cells (3). Increased water flow in response to an osmotic gradient results from an increased permeability of the apical membrane to water (4). The effects of ADH on the permeability of this membrane to sodium and water can be dissociated, suggesting that they may be due to separate hormone-activated adenylate cyclases (5, 6). One of the major questions regarding this system concerns the nature of the mechanism(s) by which cAMP can produce alterations in membrane permeability to sodium and water.

Based upon the discovery of cAMP-dependent protein kinase activity in skeletal muscle (7), liver (8), and brain (9), a unifying hypothesis was formulated which stated that the mechanism of action of cAMP in producing its diverse effects in various tissues might involve regulation of the activity of protein kinases in these tissues (10), with a consequent alteration in the level of phosphorylation of tissue-specific substrate proteins. The primary impetus for undertaking a study of protein phosphorylation in toad bladder was provided by the observation that cAMP stimulates the rapid phosphorylation of two specific proteins in preparations of synaptic membranes (11, 12). Those results, together with other experimental evidence (13–15), led to the suggestion (16) that the state of phosphorylation of specific synaptic membrane proteins might regulate the ion permeability of postsynaptic membranes, and thereby mediate postsynaptic potential changes associated with certain types of synaptic transmission. The time-course of the postsynaptic potential changes which have been observed in various types of neural tissue is so rapid that it would be extremely difficult to attempt correlations between these electrophysiological changes and any biochemical parameters. In contrast, ADH and cAMP cause relatively slow changes in the membrane permeability of toad-bladder epithelium to sodium and water. Therefore, the toad bladder was chosen to investigate the possible relationship between membrane permeability and phosphorylation of membrane proteins.

It was found, in our initial investigation of the toad bladder, that the amount of radioactive phosphate present in a specific phosphoprotein was selectively decreased in intact bladders by the addition of either ADH or monobutyryl cAMP (17). This phosphoprotein, designated “protein D,” was found on SDS-polyacrylamide gels, and had an apparent molecular weight of about 50,000 daltons. The time-course of the ADH-induced decrease in protein D phosphate was similar to that of the ADH-induced increase in sodium
transport. Furthermore, the addition of cAMP to whole homogenates or membrane fractions of bladder led to a net decrease in incorporation of radioactive phosphate from ATP into the same, or a similar, protein. In subsequent studies, a protein kinase capable of phosphorylating protein D, and a phosphoprotein phosphatase capable of dephosphorylating protein D, were both demonstrated in membrane preparations of toad bladder (18). By the selective use of inhibitors of these two enzymes, evidence was obtained supporting the conclusion that the effect of cAMP on membrane-bound protein D phosphate is mediated, at least in part, through an increase in the activity of protein D phosphatase in the membrane fraction. The results of those earlier studies were consistent with the hypothesis that alterations in the amount of phosphate present in protein D might be involved in the changes of membrane permeability to sodium and/or water which occur in response to application of ADH. In the present study we have examined a variety of agents, known to affect the permeability of toad bladder epithelium to sodium or water, or both, for their effects on the amount of radioactive phosphate in the proteins of intact cells from toad bladder.

**EXPERIMENTAL PROCEDURE**

**Materials**

Toads, *Bufo marinus*, were obtained from South America through Tarpon Zoo (Tarpon Springs, Florida), and maintained at 25° on damp bedding for 3–15 days before use. In most cases, physiological and biochemical experiments were carried out on the same batch of toads. Bladders were excised (19) from doubly pithed toads and placed in Ringer's solution (112 mM NaCl, 2.5 mM KHCO₃, 2 mM glucose, 1 mM CaCl₂ [pH 7.8]).

[γ-³²P]ATP was prepared by the method of Post and Sen (20). Other substances were obtained from the following sources: cAMP and ATP from Schwarz Bio Research Inc., Orangeburg, N. Y.; cytochrome C, myoglobin, chymotrypsinogen, ovalbumin, and bovine serum albumin from Mann Research Laboratories Inc., New York; phosphorylase a (two-times crystallized), theophylline, adenine, and Coomassie blue (brilliant blue R), from Sigma Chemical Co., St. Louis, Mo.; PGE₁ from Upjohn (courtesy of Dr. John Pike) (Upjohn Co., Kalamazoo, Mich.); amilomide from Merck & Co., Rahway, N. J.; ADH (Pitressin, vasopressin injection) from Parke, Davis & Co., Detroit, Mich.; ³²P₁ ([³²P]-orthophosphate) from New England Nuclear, Boston, Mass.; acrylamide and pyronin y from Eastman Kodak Co., Rochester, N. Y.; SDS (sodium dodecyl sulfate) from Alcolac Chemical Corp., Baltimore, Md. Pronase (120 P. U. K./mg, B Grade, nuclease-free) was obtained from Calbiochem, La Jolla, Calif. and pancreatic ribonuclease (25 U/mg) was obtained from E. Merck. Other reagents were analytical grade.

**Sliced Bladder Preparation**

In a previous study of phosphorylation in intact bladders, single “experimental” hemibladders were compared with matched “control” hemibladders from the same
toad (17). Radioactive protein D phosphate and changes in electrical potential difference (a measure of changes in sodium transport) were both measured in each hemibladder. However, because of large individual differences among toads, such experiments had to be performed in excessive numbers. To overcome this problem, the following procedure was used.

Hemibladder sacs were cut open and, with a single-edged razor blade, manually sliced into pieces approximately 2 × 2 mm on a paraffin surface. Approximately 20 hemibladders were sliced for each experiment and washed for 30 min in 20–30 ml of aerated Ringer’s solution at 22 °. Prelabeling of bladder proteins was carried out as follows: 32P_i solutions were prepared by mixing 1 ml (10 mCi) of 32P_i with 4 ml of Ringer’s solution, 0.1 ml of 1 M Tris buffer, pH 7.4, 2 µl of 1 mM potassium phosphate buffer, pH 7.4, and adjusted to about pH 7.5 with 0.2 N NaOH using Pan-pH indicator paper; approximately 2 ml (4 mCi) of this solution were added to the 20–30 ml of aerated Ringer’s solution containing the tissue. After prelabeling for 2 h at 22 °, tissue slices were rinsed four times with fresh Ringer’s solution containing 1 mM sodium phosphate buffer, pH 7.4 (rapid settling of the slices made centrifugation unnecessary), and finally resuspended in 30–50 ml of a solution of the same composition. (In preliminary experiments a prelabeling time of only 30 min gave results comparable to those obtained using the 2-h prelabeling period. However, the longer prelabeling period permitted shorter exposure times for autoradiographs as well as use of lower radioactivity in the medium.)

Uniform 1-ml samples from the suspension of prelabeled bladder slices were obtained by stirring the suspension just before each sample was removed. Such 1-ml samples were placed successively into various control and experimental beakers, and the process repeated until each beaker contained 5–8 ml of suspension (30–50 mg of protein). Small volumes of Ringer’s solution (control) or Ringer’s solution containing test reagents (experimental) were then added to the appropriate beakers and the suspensions incubated at 22 °. The number of experimental conditions ranged from four to eight per experiment. At various time intervals, 0.5-ml aliquots of the incubating bladder slices were removed from the suspensions immediately after stirring and mixed with 1 ml of a boiling solution containing 6 % SDS and 10 mM sodium phosphate buffer, pH 7.1, in a glass test tube and allowed to cool slowly in air at 22 °. (A control experiment showed that this procedure for stopping the reaction gave results on the incorporation of 32P_i into protein which were virtually the same as when the samples were boiled for various periods from 10 s to 10 min.) Within 2 h after the incubations were completed, each sample was homogenized by hand (12-14 strokes in 2 min) in a glass homogenizer with a Teflon pestle of 0.15-mm clearance. Approximately 95 % of the bladder tissue was dissolved under these conditions. The small amount of undissolved material was then removed by centrifugation for 10 min at 1,200 g at room temperature. A sample of the supernatant was taken for protein determination by the method of Lowry et al. (21), using bovine serum albumin as the standard, and 2-mercaptoethanol was added to the remainder of the supernatant to a final concentration of 8 %. The samples, containing 3–5 mg of protein, were then covered and left at room temperature overnight. An aliquot of the sample, containing 200 µg of protein, was then mixed with 50 µl of a solution (pH 7.4) containing 3 % SDS, 30 mM Tris-HCl, 5 % sucrose, and 3 µM pyronin y as a marker dye (22), and
subjected to SDS-polyacrylamide gel electrophoresis, followed by estimation of the amount of radioactive phosphate in various proteins as described below. Results obtained on the 49,000-dalton protein, using this sliced bladder procedure, were similar to those obtained earlier using intact hemibladders. For instance, the time-course of the effect of ADH on the amount of radioactive phosphate present in the 49,000-dalton protein, observed in the present study (Fig. 3), is in good agreement with previous results obtained using intact hemibladders (17).

Electrophoresis and Autoradiography

Electrophoresis was carried out for 4–5 h at 100–150 mA on a vertical plate gel of 5.6% polyacrylamide in the presence of 1% SDS, with a running buffer of 0.04 M Tris-acetate, pH 7.4, containing 2 mM EDTA and 1% SDS (22). The gel dimensions were 11 cm × 16 cm × 4 mm, and the apparatus used was that described by Reid and Bieleski (23). The gel was stained for protein with 0.025% Coomassie blue in 25% isopropyl alcohol–10% acetic acid. It was destained with 0.0025% Coomassie blue in 10% isopropyl alcohol–10% acetic acid, and finally with several changes of 10% acetic acid (22). This procedure also washed the gel free of unbound 32Pi. The stained gel was dried (24) on Whatman no. 50 filter paper under vacuum and heat, and placed in close contact with Kodak Royal X-Omat Film. The film was exposed for 2–8 days, depending upon the level of radioactivity in the dried gel, and then developed. The resulting autoradiograph, of the type illustrated in Fig. 1, demonstrated those protein

![Figure 1](image_url)
bands containing radioactive phosphate. Approximate molecular weights of toad-bladder proteins were determined on SDS-polyacrylamide gels using protein standards of known molecular weights (cytochrome c, 11,700; myoglobin, 17,200; chymotrypsinogen, 25,700; ovalbumin, 43,000; bovine serum albumin, 68,000; phosphorylase a, 94,000) as gel markers.

Quantitative Evaluation of Extent of Phosphorylation of Protein Bands

Quantitative evaluation of the extent of phosphorylation of specific protein bands observed in the autoradiograph was carried out using a Joyce-Loebl microdensitometer (Joyce, Loebl & Co., Inc., Burlington, Mass.), as described elsewhere (12). Recordings of microdensitometer scans of autoradiographs of the gels were made on uniform, heavy-grade graph paper. To determine the area under individual peaks, base lines were constructed for each peak, as illustrated in Fig. 2 of reference 12, and the individual peaks cut out and weighed. Aliquots from each of two samples taken at the start of the incubation ("zero-time" samples) were electrophoresed on each gel and the weights of all peaks on each gel were standardized against the mean weight for these two zero-time samples. In each experiment, the value for the amount of radioactive phosphate incorporated into the individual protein bands at each time point, under both the experimental (presence of test substance) and control (absence of test substance) conditions, was determined on duplicate samples, except where a larger number of samples is indicated. In each experiment, every sample was analyzed on duplicate gels. Data for each experimental condition are presented as percent of the value in control tissue incubated for the same period. Unless otherwise noted, each data point represents the mean ± SEM for the number of experiments indicated in the figure legends; the significance of the difference of these values from 100% was evaluated by Student's t test. In some experiments, the method of quantitation by densitometry was compared to a method involving scintillation counting of gel slices. Similar results were obtained by the two methods, but the densitometric method was found to be more reproducible and less time consuming.

The measured level of radioactive phosphate in the individual proteins in bladder slices represents a balance between protein kinase activity and protein phosphatase activity. The term “dephosphorylation,” as used in the present study, is intended to indicate simply a decrease in the amount of [32P]phosphate present in the proteins without regard to the mechanism involved, i.e., it is not intended to imply specifically either a decrease in protein kinase activity or an increase in protein phosphatase activity.

In most experiments, protein D was the most intensely radioactive protein band observed on the autoradiographs. The apparent molecular weight of protein D was estimated to be between 47,000 and 51,000 in numerous experiments. The mean estimate of 49,000 was lower than the 50,000–54,000 estimated in our initial experiments (17).

Short-Circuit Current (SCC) Measurements

Short-circuit current was measured by established procedures (25, 26). Briefly, half bladders were mounted as flat sheets between Lucite chambers and bathed on both
sides by Ringer's solution containing 115 mM NaCl, 2.5 mM KHCO₃, and 1.0 mM CaCl₂. The solutions were oxygenated and stirred by bubbling with air. Electrical potential difference was measured with a pair of calomel electrodes connected to the bathing solutions via Ringer-agar bridges. Current was passed through the tissue from an external battery via Ag-AgCl electrodes that were connected to the bathing solutions by a second pair of agar bridges. After an initial equilibration period of 20–30 min, the bladders were short-circuited by passing sufficient current to reduce the open-circuit potential difference to zero. The magnitude of this current provided a direct measure of the rate of net active sodium transport across the bladder from mucosa to serosa (26).

Twenty minutes after short-circuiting, reagents to be tested were added to the solutions bathing both sides of the experimental hemibladder, and changes in SCC were followed. One hemibladder from a toad served as control and the other hemibladder was treated with a test agent. In the experiments of Figs. 2 and 6, the data on SCC at any time were expressed as percent of SCC at time zero in the same hemibladder. In all other cases, the results for SCC were calculated as follows:

$$\text{Percent of Control} = \left( \frac{SCC_t}{SCC_0} \right)_{\text{experimental}} \times 100,$$

where $SCC_0$ is SCC at zero time, taken just before addition of the test agent to the experimental hemibladder, and $SCC_t$ is SCC at any subsequent time, $t$. This procedure of expressing the data takes account of spontaneous variation of SCC with time. Each data point in the figures represents the mean value ± SEM for the number of experiments indicated in the figure legends. The significance of the difference of these values from 100% was evaluated by Student's $t$ test.

Osmotic Water Flow

Half-bladders were tied as sacs to small Lucite cannulas. They were then filled with Ringer's solution diluted with 4 vol of distilled water and suspended in beakers containing Ringer's solution. In this manner, an osmotic gradient of approximately 160 mosmol/liter was imposed across the tissue. Water flow in response to this osmotic gradient was determined by periodically weighing the sac and cannula on an analytical balance. A control period always preceded treatment of the bladder with the agent being tested. Results are expressed as microliters of fluid loss per unit time per hemibladder (mean value ± SEM); differences between control and test conditions were evaluated by Student's $t$ test.

RESULTS

Endogenous Phosphorylation of Tissue Slices

Typical autoradiographic and protein-staining patterns observed following SDS-polyacrylamide gel electrophoresis of solubilized proteins from intact slices of toad bladder are shown in Fig. 1. The principal radioactive band, corresponding to an apparent molecular weight of about 49,000 daltons, and the radioactive band corresponding to an apparent molecular weight of
42,000 daltons, were less radioactive in the presence than in the absence of ADH. The effect of ADH on radioactive phosphate in the 49,000-dalton protein in the slice preparation was similar to the effect, reported earlier (17), of ADH and of monobutyryl cAMP on radioactive phosphate in this protein in intact hemibladders.

About $10^8$ cpm/g wet weight of bladder (amounting altogether to about 2.5% of the total $^{32}$P in the medium) was taken up by the toad-bladder slices. When the SDS solution containing 200 µg of dissolved bladder-slice protein was subjected to SDS-polyacrylamide gel electrophoresis, as in the experiment illustrated in Fig. 1, the total amount of radioactivity in the protein bands on the gel was about 10,000 cpm. Of this radioactivity the 49,000-dalton protein band contained, in a typical experiment, about 1,300 cpm in the absence, and 900 cpm in the presence, of ADH. (The radioactivity in the protein was determined by subtracting a background of 150-300 cpm in the manner described in reference 12.) In some experiments (A. Liu and P. Greengard, unpublished observations), some samples were treated with Pronase (0.2 mg/ml, 15 min at room temperature) before performing the standard procedures of electrophoresis and autoradiography. When this was done, all the radioactivity in the gels appeared in a broad band in the low molecular weight region overlapping the marker dye, whereas ribonuclease (0.2 mg/ml, 15 min at room temperature) had no effect. These results indicate that the radioactive bands studied represent phosphoproteins on the gels.

While protein patterns were very similar throughout the entire series of experiments, autoradiographic patterns varied somewhat from experiment to experiment. For example, although the band corresponding to the 49,000-dalton protein was usually the most intensely radioactive, the phosphorylated bands of about 18,000 and 22,000 daltons were sometimes relatively low and at other times quite high in intensity. Another type of variation was noticed in the band corresponding to a molecular weight of about 42,000 daltons. In some experiments, this peak was partially resolved into two smaller bands. The resolution of these two small bands, however, was too poor to permit separate quantitative estimation of the extent of their phosphorylation. Other bands were sometimes visible and sometimes not, but in all experiments, they were too low in radioactivity to permit accurate quantitative measurement by the methods used.

The gels from the experiments in which bladder slices were incubated with ADH for various periods of time (see Fig. 3) were analyzed for the effects of ADH on radioactive phosphate in different protein bands. The effect of a 5-min period of incubation with ADH, expressed as mean ± SEM of the difference from control ($n = 10$ samples from three experiments), was as follows: the radioactivity in the 49,000-dalton protein was reduced by 40 ± 3% ($P < 0.001$); the radioactivity in the band of about 42,000 daltons (see
Fig. 1) was reduced by 13 ± 5% (P < 0.05). The effect of ADH on the amount of radioactive phosphate in the 49,000- and 42,000-dalton proteins was maximal at 5 min. Of the other protein bands with sufficient radioactivity to permit quantitative measurement, none was significantly affected by ADH at any incubation time. Similar comparisons were made for the effects of cAMP, theophylline, adenine, Zn²⁺, Mn²⁺, and PGE₂. With these other agents, the radioactivity of the 42,000-dalton band always changed in the same direction as that in the 49,000-dalton band, but the radioactivity of the other protein bands was not significantly affected by any of these other agents. The fact that most of the radioactive protein bands on the autoradiographs were not affected by ADH or other test agents, under conditions in which the bands corresponding to molecular weights of 49,000 and 42,000 daltons did show changes, suggests that the observed effects of those test agents were not due to alterations in the amount or specific activity of the labeled ATP.

The effect of various agents on the level of radioactive phosphate in individual proteins, on sodium transport (as measured by SCC) and on water flow, was studied in toad-bladder preparations as a function of time. The values for control tissue remained relatively constant throughout the duration of the experiment for all three types of measurement. The time-course and variability exhibited by controls, in the amount of radioactive phosphate present in the 49,000- and 42,000-dalton proteins, and in the rate of sodium

![Figure 2. Changes in the amount of radioactive phosphate in the proteins of 49,000 daltons (●) and 42,000 daltons (□) in toad-bladder slices (n = 18), and in the SCC across intact hemibladders (○, n = 20), as a function of time in Ringer's solution. Protein phosphate and SCC values were calculated as percent of the zero-time values.](image-url)
transport, is shown in Fig. 2. The two different types of controls used in experiments on water flow can be seen in Figs. 3 B and 5 B.

**Effects of ADH, cAMP, and Theophylline**

The effects of ADH on protein phosphate in the slice preparation and on SCC in the intact hemibladder are shown as a function of time in Fig. 3 A. A decrease in the level of phosphate in the 49,000-dalton protein preceded an increase in SCC. The times required for ADH (0.2 U/ml) to cause a half-

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**Figure 3.** (A) The effect of ADH (0.2 u/ml) on the amount of radioactive phosphate in the proteins of 49,000 daltons (○—○) and 42,000 daltons (■—■) in toad-bladder slices (n = 6), and on SCC (○—○, n = 17) across intact hemibladders. (B) The effect of ADH (0.2 u/ml) on water flow in hemibladders (n = 10). ADH was added at the time indicated by the arrow.
maximal decrease in radioactive phosphate in the 49,000-dalton protein and a half-maximal increase in sodium transport were approximately 2.5 and 5 min, respectively. These results are similar to those reported earlier (17) in which measurements of phosphate in the 49,000-dalton protein and of sodium transport (as indicated by changes in electrical potential difference across the bladder) were carried out on the same hemibladder. Phosphate levels in the 42,000-dalton protein were affected by ADH to a much smaller extent than in the 49,000-dalton protein. The ability to show any significant effect of ADH on the phosphorylation of the 42,000-dalton protein in the present studies, but not in our initial studies of toad bladder, may be attributable to the fact that the variability observed earlier was considerably reduced in the present studies by the use of pooled slices from many bladders.

The stimulation of water flow by ADH has been studied by numerous investigators. Fig. 3 B shows the effect of ADH on water flow determined under our experimental conditions. No attempt was made to obtain accurate measurements for intervals of less than 15-min duration. However, the fact that the integrated rate of water flow during the first 15 min after addition of ADH was approximately one-half of the maximum rate indicates that the time required for a half-maximal response of water flow to ADH is of the order of 5–10 min, a value similar to that for sodium (see also reference 27).

The addition either of cAMP itself, or of theophylline, an inhibitor of cyclic nucleotide phosphodiesterase, mimics the stimulation by added ADH of sodium and water movement across the toad bladder (1, 28). In the present study, incubation of toad bladder preparations with 2 mM cAMP for 15 min caused an increase in SCC of 58 ± 4% (mean ± SEM; three experiments; \( P < 0.01 \)) and decreases in radioactive phosphate in the 49,000- and 42,000-dalton proteins of 32 ± 12% and 29 ± 10%, respectively (\( n = 10 \) samples from three experiments; \( P < 0.01 \)). Similarly, incubation of toad bladder preparations with 10 mM theophylline for 12 min caused an increase in SCC of 35 ± 8% (five experiments; \( P < 0.01 \)) and a decrease in radioactive phosphate in the 49,000- and 42,000-dalton proteins of 44 ± 5% and 34 ± 13%, respectively (\( n = 10 \) samples from three experiments; \( P < 0.01 \)). The time-courses of change for both sodium transport and for radioactive phosphate in the 49,000-dalton protein, observed after addition of cAMP and of theophylline, were similar to those observed with ADH. No decrease in the level of radioactive phosphate in proteins was observed when 5'-adenosine monophosphate was used instead of cAMP (A. Liu and P. Greengard, unpublished observation).

**Effects of Adenine**

Adenine has been reported to cause a transient stimulation of Na\(^+\) transport in toad-bladder preparations (29). Fig. 4 shows the effect of adenine on SCC,
on radioactive phosphate present in the 49,000- and 42,000-dalton proteins, and on water flow. Adenine caused a transient increase in SCC and a transient decrease in radioactive phosphate in both the 49,000- and the 42,000-dalton proteins. The magnitude of the increase in SCC brought about by adenine was considerably smaller than the magnitude of the decrease in radioactive phosphate in these proteins. However, incubation with adenine for periods greater than 20 min caused a progressive reduction of SCC, in agreement with previous observations (29). It may be that the early part of

**Figure 4.** (A) The effect of adenine (1 mM) on the amount of radioactive phosphate in the proteins of 49,000 daltons (●—●) and 42,000 daltons (■—■) in toad-bladder slices (n = 6), and on SCC (○—○, n = 7) across intact hemibladders. SCC in the presence of adenine was significantly greater than control (P < 0.01) at 4, 6, 8, 10, and 12 min. (B) The effect of adenine (1 mM) on water flow in hemibladders (n = 8). Adenine was added at the time indicated by the arrow. Changes in water flow were not statistically significant.
the SCC curve represents a difference of two opposing effects of adenine, with the stimulatory effect being overcome after 20 min by the inhibitory effect. As shown in Fig. 4 B, no significant effect of adenine on water transport was seen under the conditions of these experiments (although the possibility of a transient effect on water flow, lasting for only a few minutes, cannot be excluded). Thus, 1 mM adenine caused an increase in SCC, and a reduction of radioactive phosphate present in the 49,000- and 42,000-dalton proteins, without causing a significant change in water flow.

Effects of Divalent Metal Ions

Some divalent metal ions have been shown to affect sodium transport in toad bladder. Thus, 10^{-4} M Zn^{2+} inhibits and 10^{-4} M Mn^{2+} stimulates sodium transport (30). Water flow in the absence of added ADH was unaffected by either of these ions at the concentrations used. In the present study, Zn^{2+} caused a marked inhibition of SCC (Fig. 5 A) and, with a similar time-course, caused a large increase in the level of radioactive phosphate in the 49,000-dalton protein and a smaller increase in the level of radioactive phosphate in the 42,000-dalton protein. The change in phosphorylation of these two proteins, caused by Zn^{2+}, was opposite to that induced by ADH, cAMP, theophylline, and adenine; likewise the effect of Zn^{2+} on sodium transport was opposite to that seen with those other agents. Zn^{2+}, rather than inhibiting water flow, had a delayed stimulatory effect (Fig. 5 B). (The concentration of Zn^{2+} used in these studies was 10 times the concentration used in the studies [30] reporting the absence of an effect of Zn^{2+} on water flow.) Thus, as in the case of adenine, Zn^{2+} caused differential effects on sodium and water transport, with sodium transport, but not water flow, again maintaining a consistent, reciprocal relationship to the level of phosphate in the 49,000- and 42,000-dalton proteins.

Zn^{2+} has also been shown to block ADH-induced stimulation of sodium transport across toad bladder (30). In the present study, too, Zn^{2+} blocked stimulation of sodium transport by ADH (Fig. 6), even though different concentrations of these agents and somewhat different experimental conditions were employed. In the presence of ADH plus 1 mM ZnCl₂, the increase in SCC, which occurred in response to ADH alone, was prevented. On the other hand, the decrease in SCC, which occurred in response to ZnCl₂ alone, was slightly but significantly delayed in the presence of these two agents; a statistically significant (P < 0.01) decrease in SCC occurred after 4 min in the presence of ZnCl₂ alone (Fig 5 A), but only after 14 min in the presence of ZnCl₂ plus ADH (Fig. 6). Under conditions similar to these, ADH plus ZnCl₂ caused an increase in the level of phosphate in the 49,000- and the 42,000-dalton proteins in bladder slices (Fig. 6), rather than the decrease seen in the presence of ADH alone. Thus, in the presence of ADH plus
Figure 5. (A) The effect of ZnCl₂ (1 mM) on the amount of radioactive phosphate in the proteins of 49,000 daltons (●●●) and 42,000 daltons (■■■) in toad-bladder slices (mean values ± range for two experiments), and on SCC (○○○, n = 5) across intact hemibladders. (B) The effect of ZnCl₂ (1 mM) on water flow in hemibladders (n = 7). For the purpose of comparison, water flow was also measured in paired hemibladders from the same toads in the absence of ZnCl₂ (control) during the same 3-h period. Water flow in the presence of zinc was significantly greater than in paired control bladders (P < 0.01) in the second, third, and fourth 30-min intervals after addition of the metal ion.
Protein Phosphorylation and Sodium Transport in Toad Bladder

ZnCl₂, reciprocal changes were again observed in SCC and protein phosphate.

Mn²⁺ (0.25 mM) caused a significant stimulation of sodium transport (Fig. 7 A), a result consistent with published observations (30). In addition, Mn²⁺ also caused a decrease in the level of radioactive phosphate in the 49,000- and 42,000-dalton proteins. Thus, as with other agents, Mn²⁺ caused SCC and the level of phosphate in these two proteins to change in opposite directions. No change in water flow was detected during the first hour, but a significant inhibition occurred during the second hour, after addition of MnCl₂ (Fig. 7 B).

Effects of Prostaglandin E₁

Prostaglandin E₁ (PGE₁) has been reported to increase sodium transport and cAMP level in toad bladder (6) without affecting water flow (6, 31). PGE₁
Figure 7. (A) The effect of MnCl₂ (0.25 mM) on the amount of radioactive phosphate in the proteins of 49,000 daltons (●—●) and 42,000 daltons (■—■) in toad-bladder slices (mean values ± range for two experiments), and on SCC (○—○, n = 8) across intact hemibladders. SCC in the presence of MnCl₂ was significantly greater than control (P < 0.01) at 6-25 min. (B) The effect of MnCl₂ (0.25 mM) on water flow in hemibladders (n = 8). Water flow in the third and fourth 30-min intervals after MnCl₂ addition was significantly different (P < 0.01) from water flow before addition of the metal ion.

was found, in the present study, to cause a gradual increase in sodium transport and a correspondingly gradual decrease in radioactive phosphate in the 49,000- and 42,000-dalton proteins (Fig. 8 A), but had no significant effect on water flow (Fig. 8 B), although ADH produced a large increase in water flow in hemibladders from the same group of toads.
Figure 8. (A) The effect of PGE₁ (2.5 × 10⁻⁸ M) on the amount of radioactive phosphate in the proteins of 49,000 daltons (■—■) and 42,000 daltons (□—□) in toad-bladder slices (mean values ± range for two experiments), and on SCC (○—○, n = 5) across intact hemibladders. SCC in the presence of PGE₁ was significantly different from control (P < 0.01) at all times greater than 5 min. (B) Water flow in paired hemibladders (n = 3) in the absence (left) or presence (right) of PGE₁ (2.5 × 10⁻⁸ M). No statistically significant effect of PGE₁ was observed at any time.

Effects of Amiloride

The results presented above indicate a correlation between the rate of sodium transport across the toad bladder and the level of radioactive phosphate in specific proteins. Such a correlation might conceivably arise if the level of protein phosphate were regulated by the rate of sodium transport. To test this possibility, experiments were carried out with amiloride, an agent that inhibits sodium transport across the toad bladder by preventing sodium entry into the cells across the apical membrane (32–34). As shown in Fig. 9, a con-
centration of amiloride sufficient to produce a 90% reduction in SCC had no significant effect on phosphorylation of the 49,000- or 42,000-dalton proteins.

**DISCUSSION**

The neurohypophyseal hormone, ADH, is known to stimulate active sodium transport across several types of epithelium (1) and also to increase the magnitude of water flow across them in the presence of an osmotic pressure difference. The toad bladder has frequently been used to study the effects of ADH and other hormones on transport of sodium and on water permeability. The structure of the bladder has been studied extensively with both the light and electron microscope and the left side of Fig. 10 shows a schematic representation of this structure. Active sodium transport across the cells of the mucosal epithelium involves a two-step process (3) as indicated in the right side of Fig. 10. Sodium is thought to cross the apical cell membrane by a nonenergy-requiring process, moving from a relatively high concentration in the mucosal solution to a relatively low concentration in the cells. It is then extruded actively at the serosal membrane, presumably via the action of a Na-K-ATPase similar to that present in a variety of other cells. Several lines of evidence indicate that the entry step at the apical membrane plays a major role in controlling the rate of overall active sodium transport (3, 4, 38-40).

A number of the factors involved in the mechanism of action of ADH on the permeability of the bladder to sodium have already been elucidated. Evidence has been obtained (1, 28) that ADH activates an adenylate cyclase located along the serosal side of the mucosal epithelial cell, producing cAMP
SEROSAL SUB-MUCOSAL

MUCOSAL

ADH

Adenylate Cyclase

Cyclic AMP

PROTEIN X - PO₄

PROTEIN X PROTEIN X PHOSPHATASE KINASE

PROTEIN X ATP

PO₄

MUCOSAL EPITHELIAL CELL

LOW PERMEABILITY STATE

Na⁺

K⁺

ATPase

NO3

NO3

HIGH PERMEABILITY STATE

Na⁺

Figure 10. Model of proposed mechanism by which ADH and cAMP might regulate the sodium permeability of the toad bladder by controlling the state of phosphorylation of a specific membrane protein. (Left) Schematic illustration of the three layers of the toad bladder (4, 35, 36): the serosal (a simple layer of squamous epithelium), the submucosal (containing an interlacing network of collagen and smooth muscle bundles embedded in a matrix containing capillaries and venules), and the mucosal (consisting of specialized epithelial transporting cells and several other cell types). ADH reaches the bladder through the blood vessels in the submucosal layer, and alters the permeability of the mucosal epithelial cells, allowing Na⁺ to enter the cells more rapidly. (Right) Proposed mechanism by which ADH regulates the sodium permeability of the luminal surface of the bladder. ADH activates an adenylate cyclase on the serosal surface of the mucosal epithelial cells, causing an increased cAMP level within the cells. The newly formed cAMP diffuses through the cell to the luminal surface, where it increases the activity of protein phosphatase in the apical membrane of the mucosal epithelial cells, resulting in an increased conversion of protein X from its phosphorylated to its dephosphorylated state. Protein X is converted from its dephosphorylated state back to its phosphorylated state by the action of a protein kinase. (cAMP may, along with its stimulation of protein phosphatase activity, simultaneously inhibit protein kinase activity [18, 37], but, for the sake of simplicity, this possibility is not illustrated in the diagram.) Na⁺ readily permeates the apical membrane at sites where protein X is in the dephosphorylated form, but cannot enter the cell, or can do so only slowly, in areas of the membrane where protein X remains phosphorylated. The overall effect of ADH, according to the model, is to shift the steady state between the phosphorylated and dephosphorylated forms of protein X toward the dephosphorylated form. Protein X could be the 49,000-dalton protein, or possibly the 42,000-dalton protein, studied in the present investigation. This model is based upon data reviewed by Orloff and Handler (1) and Ferguson and Price (2), on recent studies from this laboratory (17, 18) and other (3, 4) laboratories, and on the present investigation.
within the cell (Fig. 10). There appear to be two distinct adenylate cyclases which are stimulated by ADH (5, 6), one for controlling sodium permeability and the other for controlling water permeability. Exogenous cAMP mimics the effect of ADH on sodium and water transport (28). The permeability changes induced by ADH have been localized to the apical surface of the mucosal epithelium, which is in direct contact with the lumen of the bladder (3, 4, 39, 40). From these and other (1, 2) findings, the sequence of events involved in the actions of ADH in toad bladder appears to be: (a) ADH from the bloodstream interacts with a "receptor" on the serosal surface of the mucosal epithelium, and thereby (b) stimulates production of cAMP, which (c) diffuses across the cell to the inner surface of the apical membrane, and (d) causes a change in membrane permeability. Step d in this sequence, the mechanism by which cAMP appears to cause a change in permeability of the apical membrane to sodium and water, has been the focus of the present investigation. This problem was approached using the working hypothesis that regulation, by cAMP, of the state of phosphorylation of a specific membrane protein might be responsible for the observed changes in permeability of the membrane to sodium or water or both, a possibility considered previously by Jard and Bastide (41).

Table I summarizes qualitatively the effects of various agents on dephosphorylation of the 49,000- and 42,000-dalton proteins, transport of sodium, and water flow induced by an osmotic gradient. With the exception of amiloride, all agents affected sodium transport and dephosphorylation of the 49,000-dalton protein in the same direction. Moreover, with all agents tested, the

**Table I**

**SUMMARY OF THE PREDOMINANT EFFECT OF VARIOUS AGENTS ON THE DEPHOSPHORYLATION OF 49,000- AND 42,000-DALTON PROTEINS, ON TRANSPORT OF Na⁺ (SCC) AND ON WATER FLOW IN TOAD BLADDER**

| Agent          | Dephosphorylation of 49,000- and 42,000-dalton proteins* | Transport | |
|----------------|----------------------------------------------------------|-----------|-----------|
|                |                                                          | Na⁺       | H₂O       |
| ADH (0.2 u/ml) | Stimulates                                               | Stimulates| Stimulates|
| cAMP (2 mM)    | Stimulates                                               | Stimulates| Stimulates|
| Theophylline (10 mM) | Stimulates               | Stimulates| Stimulates (28)‡ |
| Adenine (1 mM) | Stimulates                                               | Stimulates| No effect |
| ZnCl₂ (1 mM)   | Inhibits                                                 | Inhibits  | Delayed stimulation |
| MnCl₂ (0.25 mM) | Stimulates                                               | Stimulates| Delayed inhibition |
| PGE₁ (0.025 mM) | Stimulates                                               | Stimulates| No effect |
| Amiloride (0.01 mM) | No effect           | Inhibits  | No effect (32)‡ |

* As indicated in Experimental Procedure, "dephosphorylation of proteins" refers strictly to a decrease in amount of radioactive phosphate present in the proteins, without implication concerning the molecular mechanism involved.

‡ Not studied in the present investigation. The numbers in parentheses refer to the references in which the indicated effect is described.
amount of radioactive phosphate in the 42,000-dalton protein changed in the same direction as that in the 49,000-dalton protein, although to a smaller extent. Thus, in general, the present studies indicate a correlation between changes in the state of phosphorylation of two specific proteins and changes in the permeability of toad bladder membranes to sodium. In contrast, the present studies failed to demonstrate a correlation between the effects of these agents on levels of protein phosphate and their effects on water flow.

The reciprocal relationship between sodium permeability and level of protein phosphate, observed under a variety of experimental conditions in the present study, is compatible with the possibility of a causal relationship between these parameters. If such a relationship exists, either protein phosphate level could determine sodium permeability or, conversely, changes in sodium permeability could regulate the level of protein phosphate. The results obtained with amiloride favor the former possibility. Amiloride is a very potent inhibitor of sodium transport in toad bladder and frog skin, apparently acting by decreasing the rate of sodium movement across the apical membrane in both types of epithelia (32, 34). In addition, the rapidity of amiloride action and its ready reversibility suggest that it acts very near the outer surface of the apical membrane, presumably at or near the site of sodium entry into that membrane. The observation that amiloride inhibits sodium transport without significantly affecting radioactive phosphate in either the 49,000- or 42,000-dalton proteins (Fig. 9) indicates that the state of phosphorylation of these proteins is not affected by large changes in the rate of sodium movement across the apical membrane. Thus, if there is a causal relationship between protein phosphate level and permeability, it would appear to be one in which the state of phosphorylation of a protein regulates membrane sodium permeability rather than the reverse.

In addition to the effect of ADH on sodium permeability at the apical border of the mucosal epithelium, there is evidence that there may also be some effect of ADH on the Na-K-ATPase at the opposite border (40). Interestingly, Na-K-ATPase, purified from widely different species, appears to contain a subunit of about 50,000 daltons (42, 43). The phosphorylation of the catalytic site of Na-K-ATPase involves the formation of a relatively labile acyl bond (44) in a 100,000-dalton subunit (45), whereas the phosphorylation of the 49,000-dalton protein in the toad bladder involves the formation of a more stable phosphoester bond (17). Ouabain did not alter the effects either of ADH or monobutyryl cAMP on radioactive phosphate in the 49,000-dalton protein in toad hemibladders, or of cAMP on radioactive phosphate in this protein in toad-bladder homogenates (17). The results with ouabain indicate that the effects of ADH and cAMP on the amount of radioactive phosphate in this protein do not depend on active sodium transport or on the activity of Na-K-ATPase.
The schematic model shown on the right in Fig. 10 illustrates one possible relationship between protein phosphate and sodium permeability in toad bladder. The model suggests a mechanism by which cAMP might mediate step d (above) by initiating the dephosphorylation of a specific membrane protein, through the stimulation of a membrane-bound protein phosphatase (18). Na⁺ could readily enter the membrane in those regions where the protein is in the dephosphorylated state, but could not penetrate the membrane, or could penetrate only slowly, at sites where the protein is in the phosphorylated state.

The model is compatible with the decrease in the level of phosphate in specific proteins which occurs in bladder slices in the presence of ADH, theophylline, or PGE₁. Each of these agents is known to increase cAMP in toad bladder. The extra cAMP generated may cause a stimulation of protein phosphatase activity in intact cells in a manner similar to that observed in isolated membrane preparations (17, 18). Although this seems the most plausible explanation for the selective decrease in radioactive phosphate in the 49,000- and 42,000-dalton proteins in bladder slices, we cannot exclude an alternative mechanism involving a decrease in the specific activity of the ATP used to phosphorylate these two proteins, but not of the ATP used to phosphorylate any other proteins.

The mechanism by which adenine, Zn²⁺, and Mn²⁺ each caused reciprocal changes in sodium transport and in the state of phosphorylation of proteins is not known. These substances have been found to affect endogenous protein kinase and protein phosphatase activity in isolated membrane fractions from mammalian brain (12) and from toad bladder (18). However, in view of the fact that each of these agents has several known biochemical actions, it would be premature to conclude that their effects on protein phosphate levels in intact toad bladder cells are due to a direct action on protein kinase or protein phosphatase.

Several radioactive bands in addition to the 49,000- and 42,000-dalton protein bands were observed on the autoradiographs. Conceivably, one of these other phosphoproteins may be involved in the regulation, by ADH, of sodium or water transport in the toad bladder. However, evidence to support this possibility was not obtained in the present study.

A paper has appeared by Ferguson and Twite (46) confirming that ADH, applied to intact hemibladders, produces dephosphorylation of a toad-bladder protein of about 50,000 mol wt. The authors reported that incubation with vasopressin, for 5 min, at a concentration of 50 mU/ml, produced dephosphorylation of this protein and maximal effects on sodium transport and water flow; incubation with vasopressin for 5 min at a concentration of 10 mU/ml produced a maximal effect on sodium transport, but no significant effect on protein phosphorylation or water flow. Based on these data, they
concluded that the dephosphorylation of this protein was likely to be associated with water flow rather than with sodium transport. We are unable to explain the results of Ferguson and Twite within the context of the present study.

A phosphoprotein with an apparent molecular weight of 49,000 has been found in the soluble and particulate fractions of a variety of tissues from several species (37). Moreover, the dephosphorylation of this protein by an endogenous protein phosphatase was markedly stimulated by cAMP in the cytosol and particulate fractions of all tissues studied. There is evidence that this protein may be the regulatory subunit of a protein kinase (37, 47). Several studies have indicated that the regulatory subunit of some protein kinases can be phosphorylated by the catalytic subunits of these kinases (47, 48). The 49,000-dalton protein studied in the present investigation may also be the regulatory subunit of a protein kinase. In any case, it will be important to elucidate the basis for the correlation between the state of phosphorylation of this protein and the state of sodium permeability of the toad bladder epithelium.

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Note Added in Proof Recently, evidence has been obtained (Liu, A., and P. Green-gard. 1974. Aldosterone-induced increase in protein phosphatase activity of toad bladder. Proc. Natl. Acad. Sci. U.S.A. 71:3869) that aldosterone, which increases active sodium transport across toad-bladder epithelium, also stimulates the dephosphorylation of the 49,000 dalton protein in this tissue.

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