Regulation of Phosphorylation of a Specific Protein in Toad-Bladder Membrane by Antidiuretic Hormone and Cyclic AMP, and Its Possible Relationship to Membrane Permeability Changes

(monobutyl cyclic AMP/vasopressin/phosphoprotein phosphatase)

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ABSTRACT Phosphorylation of a specific protein was decreased in intact toad bladders by exposure to either antidiuretic hormone or monobutyl cyclic AMP. The decrease in phosphorylation caused by these agents preceded the change in electrical potential difference (an indicator of the rate of sodium ion transport) observed in response to the same compounds. The addition of cyclic AMP to homogenates of toad bladder led to a decrease in phosphorylation of the same, or a similar, protein. In subcellular fractionation studies, the effect of cyclic AMP on the phosphorylation of this protein was observed in those fractions rich in membrane fragments, but not in the nuclear or cell-sap fractions. These and other results are compatible with the possibility that the regulation by vasopressin and cyclic AMP of sodium and/or water transport in toad bladder may be mediated through regulation of the phosphorylation of this specific protein.

Recently, the cyclic AMP-dependent endogenous phosphorylation of a specific protein was demonstrated in synaptic membrane fractions from rat cerebrum (1), and it was suggested that this protein might control the permeability of some synaptic membranes to inorganic ions. However, it would be difficult to compare the level of phosphorylation of specific proteins of synaptic membranes with the permeability characteristics of these membranes, either *in situ* or in isolated intact preparations of nervous tissue, because of the relatively fast rate constants associated with potential changes of the synaptic membranes (e.g., excitatory and inhibitory postsynaptic potentials). In view of the possible relationship between the level of phosphorylation and ion permeability of membranes, suggested by our studies with synaptic membranes, we have undertaken a study of isolated toad bladders.

There is good evidence in toad bladders (2) that the regulation of sodium and water transport by antidiuretic hormone is mediated through cyclic AMP (cAMP). Moreover, the bladder lends itself to simultaneous physiological and biochemical measurements, since the time constants involved are sufficiently slow to permit biochemical measurements at various times during the course of the physiological response to the regulatory hormone. Our results demonstrate that antidiuretic hormone (ADH) and cAMP cause a decrease in the phosphorylation of a specific protein in toad-bladder membrane, and thus raise the possibility that the effect of cAMP on sodium and/or water transport in this tissue might be mediated through the level of phosphorylation of this

specific protein. Experiments to be reported elsewhere (manuscript in preparation) indicate that the decrease in phosphorylation of this specific protein can be partially accounted for by the activation, in the presence of cAMP, of a membrane-bound phosphoprotein phosphatase.

MATERIALS AND METHODS

Toads, *Bufo marinus*, from Colombia, South America, were obtained through Tarpon Zoo, Tarpon Springs, Fla., and maintained on damp bedding at 25°. Bladders were removed (4) from doubly-pithed toads and rinsed in Ringer's solution [112 mM NaCl-2.5 mM KHCO₃-1 mM CaCl₂-2 mM glucose (pH 7.8)].

Intact-Bladder Preparations. Intact-bladder experiments used hemibladder sacs that were tied (mucosal surface inward) to a piece of plastic tubing. The bladder was filled with Ringer's solution and suspended in a beaker containing Ringer's solution. The electrical potential difference across the bladder was measured with calomel cells connected to the bathing solutions by Ringer-agar bridges. [Increases in potential difference caused by addition of antidiuretic hormone (ADH) or monobutyl cAMP were taken as an indication of an increase in the rate of active sodium transport from mucosa to serosa, since changes in potential difference caused by ADH are parallel, although not identical, to changes in short-circuit current, a direct quantitative measure of active sodium transport (5).] In order to generate the radioactive ATP needed for labeling of proteins, the bladder sacs were then prelabeled from the serosal side for 1 hr in Ringer's solution containing 100 μ Ci/ml of [³²P]orthophosphate (New England Nuclear) while the potential difference across the bladder was being monitored. After prelabeled, paired hemibladders from the same toad were washed twice with fresh Ringer's solution and then placed into fresh Ringer's solution minus (control) or plus (experimental) antidiuretic hormone (0.2 units/ml, Parke-Davis) or monobutyl-3',5'-cyclic AMP (2 mM), the potential difference being monitored simultaneously. At various times the bladders were removed from the tubing and cut open. The mucosal surface was rapidly scraped from the bladders with a microscope slide and homogenized in 3% sodium dodecyl sulfate (SDS) containing 10 mM sodium phosphate buffer (pH 7.2). In order to obtain homogeneous samples, it was necessary to use a slow up-and-down hand homogenization, with a volume sufficient to give a final protein concentration of 2-3 mg/ml. An aliquot

Abbreviations: ADH, antidiuretic hormone; SDS, sodium dodecyl sulfate.

of the homogenate was taken for protein determination by the method of Lowry *et al.* (6). 2-Mercaptoethanol was then added to the remainder of the sample, to a final concentration of 8%, and the sample was incubated for 4 hr at 30°. An aliquot (containing 200 μ g of protein) of each bladder preparation was then subjected to acrylamide gel electrophoresis for 5 hr at 120 mA on a vertical plate gel of 5 1/2% polyacrylamide with a running buffer of 40 mM Tris–20 mM sodium acetate–2 mM EDTA–1% SDS, pH adjusted to 7.4 with acetic acid (7). The dimensions of the gel were 11 cm $\times$ 16 cm $\times$ 4 mm, and the apparatus was similar to that described (8). The gel was stained for protein with 0.025% Coomassie blue in 25% isopropyl alcohol and 10% acetic acid, and then destained for 6 hr with 0.0025% Coomassie blue in 10% isopropyl alcohol and 10% acetic acid, and finally in 10% acetic acid (7). The stained gel was dried and placed in contact with Kodak Royal X-Omat film, and the film was developed 3–7 days later (1). Quantitative measurements of phosphate incorporation into individual protein bands were made by liquid scintillation counting of individual protein bands cut from the gel and/or by scanning the autoradiographs with a Joyce–Loebl double-beam recording microdensitometer. In experiments in which the two methods of quantitation were compared, they gave similar results; the data presented were obtained by liquid scintillation counting. Molecular weights were determined electrophoretically (9).

Broken-Cell Preparations. In some experiments, we obtained bladder homogenates by mincing each bladder with scissors and homogenizing in 5–7 volumes of 0.32 M sucrose in a 40-ml glass homogenizer with a Teflon pestle of 0.15-mm clearance at 1800 rpm (20–30 strokes). The connective tissue was removed by centrifugation at 400 $\times$ *g* for 1 min. In other experiments, homogenates were prepared from mucosal scrapings. In still other experiments, bladders were treated with collagenase (10), followed by gentle scraping of the mucosal surface to obtain epithelial cells. In the latter two types of preparation, tissue was homogenized as above, but at 900 rpm with 10–12 up-and-down strokes. The three types of homogenate preparation gave similar results.

For studies with cellular subfractions, homogenate preparations were separated into four fractions by differential centrifugation (11) and examined by electron microscopy. The nuclear fraction was composed primarily of unbroken nuclei, but also contained some unbroken cells and large cell fragments. The mitochondrial fraction contained the mitochondria, other cellular organelles, and large membrane fragments. The microsomal fraction consisted entirely of microsomes and membrane fragments. The cell sap represented the soluble fraction of the homogenate.

The standard reaction mixture used for studies of phosphorylation in homogenates and subcellular fractions contained 250 μ g of protein, 50 mM sodium acetate buffer (pH 6.4), 10 mM magnesium acetate, and 5 μ M [γ -³²P]ATP [prepared according to Post and Sen (12)], with or without 5 μ M cAMP, in a total volume of 0.2 ml, and was incubated for 1 min at 30°. The reaction was terminated by addition of 0.1 ml of a solution containing 3% SDS, 5% sucrose, 30 mM Tris·HCl (pH 8.0), 3 mM EDTA (pH 8.0), 120 mM dithiothreitol, and 3 μ M pyronin y, a marker dye (7). Equal protein aliquots (usually 200 μ g) were subjected to electrophoresis, autoradiography, and quantitative measurement of phosphate incorporation, as described above.

FIG. 1. Effects of ADH and monobutryl cAMP on protein phosphorylation in intact toad bladders and of cAMP on protein phosphorylation in toad-bladder homogenates. The concentrations of the test agents and the times of incubation were: ADH, 0.2 units/ml, 5 min; monobutryl cAMP, 2 mM, 5 min; cAMP, 5 μ M, 1 min. For the experiments with intact tissue, toad bladders were first incubated with radioactive inorganic phosphate. For the experiments with homogenates, [γ -³²P]ATP was added directly to the reaction mixture. Experimental details are given in *Methods*.

In order to obtain further evidence that phosphate was incorporated into protein and to identify the amino acid residues being phosphorylated, labeled protein bands were extracted from the gel (1), the lipids were removed (13), and the protein was hydrolyzed by acid hydrolysis (14). Hydrolysates were subjected to high-voltage paper electrophoresis, and the radioactivity in phosphoserine and phosphothreonine was determined by liquid scintillation counting. Lipids were extracted and analyzed, as described elsewhere (13, 15).

RESULTS

The effect of ADH and of monobutryl cAMP on the pattern of phosphorylation of proteins in intact toad bladders is shown in Fig. 1. When ADH was applied to the serosal side of intact half-bladders, there was a substantial decrease in the amount of [³²P]phosphate associated with the principal radioactive band seen in the autoradiographs. When either monobutryl cAMP (Fig. 1) or dibutryl cAMP was substituted for ADH, the results were virtually identical to those obtained with the hormone. The effect of cAMP on the pattern of phosphorylation of proteins in a homogenate of epithelial cells that had been scraped from the mucosal side of the toad bladder is also shown in Fig. 1. The principal radioactive band obtained with the homogenate was in the same position as that observed in the experiments with intact cells. Moreover, there was a substantial decrease in the radioactivity of this band when cAMP was added to the homogenate. This band was the only band whose phosphorylation was consistently observed to be affected by ADH or monobutryl cAMP in intact bladder experiments or by cAMP in broken-cell preparations. (In some of the experiments with broken-cell preparations, the phosphorylation of two other bands was slightly stimulated in the presence of cAMP.) Experiments to be described below indicate that the radioactivity in this band was in the form of phosphoprotein. Results qualitatively similar to those shown in Fig. 1 were observed in about 110

Fig. 2. Changes in the amount of phosphorylation of protein D, and in the potential difference across the bladder, as a function of time after application of ADH. The potential difference (○), measured at the time of removal of hemibladders for determination of phosphorylation, is expressed as the percent increase above the value measured on the same hemibladder immediately before addition of ADH. The amount of protein D phosphate (●), measured by liquid scintillation counting, is expressed as a percent of that in the control hemibladder. For the protein D phosphate curve, the time of incubation with ADH includes 1 min required to excise the bladder, scrape the mucosal surface, and transfer it to the SDS homogenizing medium. The results of the experiment illustrated are representative of five individual experiments.

experiments with intact bladders and in about 200 experiments with homogenates. It seems likely that the protein affected by the hormone in intact bladders is identical to that affected by cAMP in the homogenates. Interestingly, although this protein was very highly labeled, it appears from the protein-staining pattern of the gels to represent only a minor component of the total protein in these preparations. For convenience, this protein, which shows a decreased phosphorylation in response to ADH or cAMP, has been termed "protein D."

The apparent molecular weight of protein D was estimated by SDS-gel electrophoresis to be about 50,000 by the procedure of Weber and Osborn (9), and about 54,000 by the procedure of Fairbanks *et al.* (7). The same molecular weights were observed whether the protein samples were from intact-tissue experiments or from broken-cell experiments. When the protein bands, corresponding to protein D, from intact-tissue and broken-cell preparations, were eluted from the gels, mixed, and again subjected to electrophoresis, a single radioactive band was observed, indicating that the protein D formed in the intact-tissue preparation was probably the same as that formed in the broken-cell preparation.

Fig. 2 compares the time course of the ADH-induced change in the level of phosphorylation of protein D with the time course of the ADH-induced change in potential difference across the toad bladder. The potential difference provides a reasonable estimate of the rate of Na transport (see *Methods*), and was measured in the same tissues in which the amount of protein D phosphate was determined. The change in the level of phosphorylation of protein D was half-maximal within 2 min and maximal within 4 min after addition of ADH (Fig. 2). The change in potential difference was half-maximal at about 4 min and maximal at about 6 min after addition of ADH. In the studies of Singer *et al.* (16) and Lichtenstein and Leaf (17), the increase in short circuit current upon

exposure to ADH showed a time course similar to that observed in the present studies of potential difference measurements. Moreover, those authors observed that the time course of the change in water permeability was about the same as that of the change in the short circuit current. Therefore, the change in amount of protein D phosphate upon addition of ADH was sufficiently rapid that it could account for the various physiological responses to the hormone.

Fig. 3 presents quantitative data on the effects of various agents on the level of phosphorylation of protein D. ADH and monobutyl cAMP caused a 50–60% decrease in the level of phosphorylation of protein D in intact bladders, and cAMP produced the same effect in broken-cell preparations. When intact bladders or bladder homogenates were first incubated in the presence of ouabain, and then exposed to

Fig. 3. Effects of ADH, monobutyl cAMP, cAMP, and ouabain on phosphorylation of protein D in intact and broken-cell preparations of toad bladders. The effects of ADH and monobutyl cAMP on protein D phosphate were determined in 10 separate experiments with pairs (control compared to experimental) of intact hemibladders in which the potential difference increased by 60% or more during a 5-min exposure to these agents. For each pair of hemibladders, protein D phosphate was calculated as percent of control. The data give the mean values and ranges for the ten pairs of hemibladders. For examination of the effects of the test agents in the presence of ouabain in intact bladders, five pairs of hemibladders were first incubated for 60 min with ouabain in Ringer's solution, followed by 60-min incubation with radioactive inorganic phosphate, and then by a 5-min incubation with either ADH or monobutyl cAMP, or with Ringer's solution alone (controls). The data in the presence of ouabain give the mean values and ranges for the five pairs of hemibladders. In experiments with homogenates of scraped toad-bladder mucosa, the effect of a 1-min incubation with cAMP was measured either under standard conditions or in homogenates that had first been incubated for 30 min with ouabain. Data obtained with homogenates give the mean values and ranges for 10 experiments, each experiment having been done with a different homogenate preparation. Data on serine- PO_4 incorporation give mean values and ranges for four experiments in which protein D was hydrolyzed by HCl. The concentrations of the test agents were: ADH, 0.2 units/ml; monobutyl cAMP, 2 mM; cAMP, 5 μM ; ouabain, 1 mM. All data were obtained by liquid scintillation counting.

ADH, monobutyl cAMP, or cAMP, these three agents still caused a decrease in protein D phosphate, when compared to the ouabain-treated controls (Fig. 3). Since ouabain inhibits the active transport of sodium in toad bladders (18), its inability to block the effects of ADH or monobutyl cAMP on protein phosphorylation suggests that the reduced amount of protein D phosphate in intact bladders is not dependent on active sodium transport or on the operation of a Na-K ATPase.

We have confirmed earlier experiments (15) indicating that phospholipids, which are also phosphorylated during intact-tissue experiments, incorporated only a small amount of phosphate compared with that in the protein, and that this incorporation was not influenced by cAMP. More definitive evidence that the radioactivity in the band corresponding to protein D indeed represents incorporation into protein was provided by the following experiments. High-voltage electrophoresis of HCl hydrolysates of protein D, which had been isolated by extraction of the protein from gel slices with SDS, indicated that 89% of the label was present in the form of serine phosphate. In addition, the decrease in the amount of radioactive phosphate in protein D, brought about by cAMP, was attributable to changes in the amount of serine phosphate (Fig. 3).

The effect of various concentrations of cAMP on the level of phosphorylation of protein D in a homogenate of scraped toad-bladder epithelium is shown in Fig. 4. The concentration of cAMP required to produce a half-maximal decrease in the level of protein D phosphate was about 10 nM. The phosphorylation of protein D was reduced about 65% in the presence of optimal concentrations of cAMP.

The effect of 5 μ M cAMP on the amount of protein D phosphate was independent of ATP concentration over the range from 1 μ M to 1 mM. These results demonstrate that the decrease in protein D phosphorylation was not due to a competition between cAMP and ATP. The results further indicate that the effect of cAMP on protein D phosphorylation is not attributable to an indirect action of the cyclic nucleotide causing a decrease in the concentration of ATP.

Table 1 shows the results of incubation of various subcellular fractions of a homogenate of toad-bladder mucosa, in the absence and presence of cAMP, on the amount of radioactivity appearing in the gels in a position corresponding

TABLE 1. Effect of cAMP on phosphorylation of protein D in subfractions of toad-bladder epithelium

Subfraction	Protein μ g	Radioactivity in protein D nmol			% Decrease due to cAMP
		Minus cAMP	Plus cAMP	Difference	
Homogenate	250	10.8	5.70	5.10	47
Nuclear	250	7.76	7.13	0.63	8
Mitochondrial	250	2.05	1.35	0.70	34
Microsomal	250	3.08	1.48	1.60	52
	100	1.53	0.80	0.73	49
Cell sap	250	20.3	17.4	2.82	12
Cell sap + microsomal	250				
	+	13.1	8.20	4.92	38
	100				

cAMP concentration was 5 μ M. The data represent the averages of duplicate determinations with one homogenate preparation, pooled from the mucosal scrapings of five bladders, and subcellular fractions from the same homogenate. Experimental details are given in *Methods*. Identical experiments were performed on six additional preparations, four from mucosal scrapings and two from homogenates of chopped, whole bladders. Though absolute values showed some variation from preparation to preparation, the relative decreases due to cAMP were similar to those shown. Radioactivity was determined by liquid scintillation counting.

to protein D. The presence of cAMP in the incubation medium caused a substantial reduction in the amount of [32 P]protein D in those subcellular fractions (mitochondrial and microsomal) found by electron microscopy to contain membrane fragments. The cell-sap fraction showed a relatively large amount of [32 P]protein D, but this radioactive band was not significantly affected by the presence of cAMP in the incubation medium. However, when the cell sap was incubated with the microsomal subfraction, the amount of [32 P]protein D found was much lower than that observed with the cell sap alone, and cAMP further decreased the amount of radioactive protein D.

DISCUSSION

The functional significance of the effect of cAMP on protein phosphorylation in toad bladder reported in this paper remains to be elucidated. It is generally accepted that cAMP mediates the effects of ADH on Na transport as well as on H₂O transport in toad bladder (2). The following observations reported in this paper suggest the possibility of a connection between the cAMP-induced lowering of the level of phosphorylation of a specific protein in toad-bladder membrane and the physiological effects of ADH on Na and/or H₂O transport in this tissue: (1) ADH, monobutyl cAMP, and dibutyl cAMP each cause a decrease in the phosphorylation of this specific protein in intact toad bladders; (2) cAMP, when added to bladder homogenates, causes a decrease in phosphorylation of what appears to be the same specific protein; (3) this effect of cAMP was observed only in subcellular fractions containing membrane fragments; and (4) the change in phosphorylation of this protein appears sufficiently rapid to permit a role in the production of permeability changes leading to increased Na and/or H₂O transport.

FIG. 4. Effect of cAMP concentration on level of protein D phosphate in a homogenate of mucosal scrapings of toad bladder. For each concentration of cAMP tested, five aliquots of the homogenate were separately incubated for 1 min and subjected to gel electrophoresis. Radioactivity was determined by liquid scintillation counting. The data give mean values and ranges for each concentration, expressed as percentage of the mean phosphorylation observed in the absence of added cAMP.

Moreover, the membrane fragments present in the microsomal subfraction of toad-bladder homogenates contain an endogenous protein kinase capable of phosphorylating the specific protein and also an endogenous protein phosphatase capable of dephosphorylating this protein (3).

Existing evidence indicates that the effect of ADH on Na transport is mediated through an action of cAMP at the mucosal surface of toad bladders. cAMP apparently increases the effective permeability of the apical surface of the mucosal epithelium, permitting an increased entry of Na into the cells, from which it is extruded by the sodium pump at the serosal side of the mucosal epithelium (2, 3, 5). If the change in sodium transport is, in fact, the result of a changed amount of phosphorylation of protein D, then the data of Frazier *et al.* (19) on the time required for transport of sodium across the toad-bladder epithelium provide a possible explanation for the short time-lag observed in the present study between phosphorylation change and Na transport change. Further experiments are necessary to determine whether the observed change in phosphorylation of protein D is responsible for the action of antidiuretic hormone on Na transport or on H₂O transport or on both.

Other laboratories have speculated on the possibility of a relationship between cAMP stimulation of protein phosphorylation and the cellular effects of ADH. Jard and Bastide (10) suggested such a connection based on their observation, using histone as substrate, of protein kinase activity in soluble and particulate fractions of toad bladders. Dousa *et al.* (20), using a partially purified cAMP-dependent protein kinase from bovine kidney (21), were able to phosphorylate fractions tested as substrate. Unfortunately, such experimental data do not provide much support for a possible relationship between protein phosphorylation and membrane transport phenomena, since cAMP-dependent protein kinases are ubiquitous (22) and exhibit a relatively low specificity for substrates, phosphorylating a variety of particulate and soluble proteins (23).

Based upon the widespread distribution of cAMP-dependent protein kinases in nature, a general hypothesis was proposed (22, 24) for the mechanism of action of cAMP. According to this hypothesis, the diverse effects of cAMP in various tissues and species are mediated through regulation of the activity of protein kinases, the specificity of the action of cAMP residing in the nature of the protein kinases and, particularly, of the substrates for the protein kinases in the various tissues. According to this general mechanism, the altered level of phosphorylation of the substrate protein would result in an altered functional state of the substrate, thus producing the specific physiological effects of cAMP.

In most cases studied, the effect of cAMP on protein kinases has been to increase the activity of the enzyme. However, a few instances have been found (14, 21, 23, 25) in which cAMP appeared to cause a decrease in protein kinase activity. In these cases, the level of substrate phosphorylation was lower in the presence than in the absence of cAMP. Such a net decrease in the level of phosphorylation of a substrate protein, observed in earlier and in the present studies, could result

either from a cAMP-induced decrease in the activity of a protein kinase or from a cAMP-induced increase in the activity of a protein phosphatase, or both. Experiments to be reported in detail elsewhere (manuscript in preparation) indicate that cAMP can cause the activation of protein D phosphatase, and raise the possibility that the effect of cAMP on the phosphatase may be a direct one. If cAMP does indeed cause the direct activation of a protein phosphatase, this would represent the first example, in a eukaryotic organism, of an action of this nucleotide not mediated through a cAMP-dependent protein kinase.

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