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EFFECT OF VASOPRESSIN ON CYCLIC AMP-DEPENDENT PROTEIN KINASE IN TOAD URINARY BLADDER

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Summary

The effect of vasopressin on the toad urinary bladder has been shown to be mediated by cyclic AMP. It has been assumed that, as demonstrated for other systems, this involves activation of cyclic AMP-dependent protein kinase. In order to test this hypothesis we investigated the effect of vasopressin on cyclic AMP-dependent protein kinases in epithelial cells of toad bladders. About 80% of protein kinase activity and cyclic AMP-binding capacity was found to be in the cytosol. DEAE-cellulose chromatography showed a pattern of 15--20% type I and 80—85% type II cyclic AMP-dependent protein kinase. Cytosolic kinase was activated 3—4-fold by cyclic AMP with half-maximal activation at $5 \cdot 10^{-8}$ M. Similarly, half-maximal binding of cyclic AMP occurred at $7 \cdot 10^{-8}$ M. Incubation of toad bladders in Ringer's solution containing 0.1 mM 3-isobutyl-1-methylxanthine, prior to homogenization and assay, showed stable cyclic AMP-binding capacity and protein kinase ratio —cyclic AMP/+cyclic AMP. Exposure of bladders to 10 mU/ml of vasopressin for 10 min caused intracellular activation of protein kinase and decrease in cyclic AMP-binding capacity that were maintained for at least 30 min. Incubation of bladders with increasing concentrations of vasopressin (0.5 -100 mU/ml) resulted in a discrepancy between a progressive increase in cyclic AMP levels and a levelling off at 10 mU/ml of vasopressin for the changes in protein kinase ratio and cyclic AMP-binding capacity. The increase in kinase ratio was due to higher activity in the absence of exogenous cyclic AMP and was fully inhibitable by a specific protein kinase inhibitor. Using Sephadex G-25-CM50 column chromatography for separation of holoenzyme and free catalytic subunit we demonstrated that the activation of protein kinase in the vasopressin-treated bladders is due to intracellular dissociation of the kinase. These results show that the effect of vasopressin on the toad bladder involves activation of a cytosolic cyclic AMP-dependent protein kinase. The time course and the dose-

response curve of the kinase activation closely parallel vasopressin's effect on osmotic water flow.

Introduction

The action of vasopressin on toad urinary bladder and mammalian collecting duct was one of the first hormonal systems shown to be mediated via cyclic AMP as second messenger [1,2]. Vasopressin increases cyclic AMP generation in the mammalian renal medulla and isolated collecting tubule, as well as in the toad urinary bladder (for recent review see Ref. 3). Furthermore, exogenous cyclic AMP (or its analogues) simulates the action of vasopressin. From these studies a discrepancy between the effect of vasopressin on the generation of cyclic AMP and the effect of vasopressin on osmotic water flow has become apparent [3]. For example, cyclic AMP generation in the toad urinary bladder progressively increases with vasopressin concentrations of 10—200 mU/ml, while the effect on water flow is maximal with 10 mU/ml of vasopressin [4]. These findings are consistent with the receptor reserve hypothesis for vasopressin [5] and the concept of specific intracellular cyclic AMP pools [6]. Little is known about the mechanisms by which intracellularly generated cyclic AMP increases luminal membrane permeability. Amphibian urinary bladder has been described to contain cyclic AMP-stimulatable protein kinase [7,8]. As in other tissues (for recent review see Ref. 9), cyclic AMP generated in response to hormonal stimulation may activate a protein kinase in toad bladder and mammalian renal medulla [10]. Changes in the activity of cyclic AMP-dependent protein kinase would eventually (through a number of unknown steps) lead to alterations in the permeability characteristics of the luminal membrane.

The present study was designed to examine which types of cyclic AMP-dependent protein kinase are present in the toad urinary bladder and whether treatment of bladders with vasopressin does indeed result in activation of cyclic AMP-dependent protein kinase in intact tissue. Furthermore, measurement of the degree of cyclic AMP-dependent protein kinase activation in intact tissue in response to vasopressin treatment could provide further insight into the concepts of receptor reserve or separate intracellular cyclic AMP pools.

Some of these results have been published in abstract form in the programs of the 7th International Congress of Nephrology, Montreal, 1978, and the 11th Meeting of the American Society of Nephrology, New Orleans, 1978.

Materials and Methods

^{3}H-labeled cyclic AMP (30 Ci/mmol) and [γ-^{32}P]ATP (8 Ci/mmol) were purchased from ICN Pharmaceuticals Inc. (Irvine, CA). Protamine, calf thymus mixed histone and histone F_{2b}, and arginine vasopressin (9 U/ml) were obtained from Sigma Chemical Company (St. Louis, MO). Reagents for competition radioimmunoassay of cyclic AMP were purchased from Collaborative Research Inc. (Boston, MA). Sephadex G-25 fine and GM-Sephadex C50 were from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ). 1-Methyl-3-isobutylxanthine was from Aldrich Co. All other reagents and chemicals were obtained

from various suppliers and were of the purest grade available.

Female toads (National Reagents, Bridgeport, CT) were doubly pithed, each hemibladder was cut into two pieces and washed three times with 20 ml Ringer's solution (120 mM Na^+, 4.0 mM K^+, 0.5 mM Ca^{2+}, 116 mM Cl^-, 5 mM phosphate, pH 7.4). Each of the four bladder strips from one toad was then transferred into 36 ml of Ringer's gassed with room air and containing 0.1 mM 1-methyl-3-isobutylxanthine and different concentrations of vasopressin. Incubations were carried out for 30 min at room temperature (20—22°C). Incubations were terminated by scraping the epithelial cells from each bladder piece and immediate homogenization (20 s sonication) in 2–4 ml of ice-cold homogenization buffer (10 mM potassium phosphate, pH 6.8, 2 mM sodium EDTA, 100 mM KCl). The homogenates were centrifuged for 20 min at 30 000 × *g*, the supernatant was removed, and the pellet was washed with 0.5–1 ml of the buffer and centrifugation was repeated. The washing was added to the initial supernatant while the pellet was resuspended in 0.5–2 ml homogenization buffer. Protein kinase or cyclic AMP-binding assays were then performed on the same day. This method of preparation resulted in a 77 ± 2% recovery of protein in the supernatant ($n = 6$).

Assay for protein kinase. Protein kinase was determined by the method of DeLange et al. [11] as modified by Ehrlichman et al. [12]. The kinase reaction contained 20 mM potassium phosphate (pH 6.8), 10 mM magnesium acetate, 0.05 mM [^{32}P]ATP (containing $1–2 \cdot 10^6$ cpm), 80 μg histone F_{2b} and, when used, 2 μM cyclic AMP in a final volume of 0.1 ml. The mixture was preincubated at 30°C for 2 min and the reactions started by the addition of 3—30 μg protein of the supernatant preparation and carried out for 2 min at 30°C in a shaking water bath (100 cycles/min). The optimal conditions for the assay (e.g. type of histone, ATP concentration, protein concentration, time of incubation) were validated in preliminary experiments. We routinely included 100 mM KCl in the homogenization buffer, as we found that this ionic concentration prevented binding of free catalytic subunit to the pellet (80% of total kinase activity appearing in the supernatant), maintained the kinase ratio stable and only minimally dissociated the kinase (results not shown). Because of high phosphatase activity kinase could not be assayed in the pellet under our conditions.

Samples incubated without enzyme and with heat-inactivated enzyme served as blanks and accounted for less than 20% of the lowest experimental counts. Assays were carried out in triplicate, corrected for blanks and results expressed as means of the triplicate determination.

Types I and II cyclic AMP-dependent protein kinases were separated by DEAE-cellulose chromatography [13]. Epithelial cells from six hemibladders were sonicated in 2 ml of 10 mM potassium phosphate buffer, pH 6.8, 1 mM EDTA, 4 mM mercaptoethanol and spun at 30 000 × *g* for 20 min. A 1.5-ml aliquot of the supernatant was applied to a 0.9 cm inner diameter column containing 8 cm of Whatman DEAE-DE52 equilibrated with the same buffer. The column was eluted with 50 ml of the buffer and then a linear gradient (0—400 mM) of NaCl was applied. Fractions of 5 ml were collected and 20-μl aliquots were assayed for protein kinase activity.

When protein kinase holoenzyme and free catalytic subunit were to be

separated by column chromatography cells from each hemibladder were first sonicated in 1.5 ml of homogenization buffer without KCl. A 1-ml aliquot of the sonicated sample was adjusted with buffer to approximate 2 mg/ml protein by measuring ultraviolet absorbance at 280 nm and 260 nm by the method of Warburg and Christian [14]. A 50-μl aliquot of this was applied to a 0.5 cm inner diameter column containing 3 cm of Sephadex G-25 (fine) and 1 cm of CM-Sephadex C50 preequilibrated with a 40 mM potassium phosphate, 4 mM mercaptoethanol buffer, pH 6.1, according to Rangel-Aldao and Rosen (Rangel-Aldo, R. and Rosen, O.M., personal communication). The column was eluted with 1.8 ml of the same buffer and then with 1.2 ml of a 4 mM mercaptoethanol/0.3 M potassium phosphate buffer, pH 7.2. Fractions of 0.2 ml were collected and duplicate aliquots assayed for protein kinase activity (20 μl) and cyclic AMP-binding activity (80 μl).

Cyclic AMP levels were determined by transferring 0.5 ml of the sonicate into tubes containing 0.5 ml of boiling 50 mM sodium acetate, pH 6.2, and left in a boiling water bath for 3 min. These samples were then cooled on ice, centrifuged for 10 min at 2000 rev./min and aliquots of the supernatant were used at two different dilutions for determination of cyclic AMP by radioimmunoassay of Steiner et al. [15] as previously reported by us [16].

Assay for cyclic AMP-binding protein. Cyclic AMP binding was assayed by a modification of the method of Gilman [17]. Incubations were carried out for 2 h at 4°C or for 30 min at 30°C in 0.2 ml of 25 mM potassium phosphate buffer, pH 7.0, 0.8 mM EDTA, 0.2 mg/ml bovine serum albumin, 0.2 μM cyclic [^{3}H]AMP (400–800 000 cpm) and appropriate amounts of preparation. The protein bound cyclic [^{3}H]AMP was recovered by filtration over Millipore filters (0.45 μm pore size, type HA) after addition of 2 ml of ice-cold 25 mM potassium phosphate, pH 7.0. Each tube was rinsed three more times with 2 ml buffer and the filter then washed with an additional 10 ml of buffer. After drying, the filters were counted in 10 ml of Triton/toluene/omnifluor in a liquid scintillation counter. Samples incubated without or with heat-inactivated enzyme served as blanks and usually amounted to less than 15% of the lowest experimental counts. Assays were carried out in duplicate, corrected for respective blanks and expressed as means of the corrected duplicate determination. Binding of cyclic [^{3}H]AMP increased proportionately to the protein concentration at 20—200 μg per assay for the crude sonicate, 10—100 μg for the 30 000 × *g* supernatant and 20—200 μg for the pellet.

Protein kinase inhibitor was prepared from rabbit skeletal muscle through the steps of boiling and acid precipitation by the method of Walsh et al. [18].

Proteins were determined by the method of Lowry et al. [19]. Statistical evaluation was by Student's *t*-test using paired and unpaired analysis.

Results

Under our conditions, protein kinase activity in the 30 000 × *g* supernatant is activated about 3-fold by cyclic AMP with half-maximal activation at about 50 nM cyclic AMP (Fig. 1). The cyclic AMP protein kinase inhibitor prepared by the method of Walsh et al. [18] inhibited this activation in a dose-dependent manner; 10 μg totally abolished the increment produced by cyclic AMP,

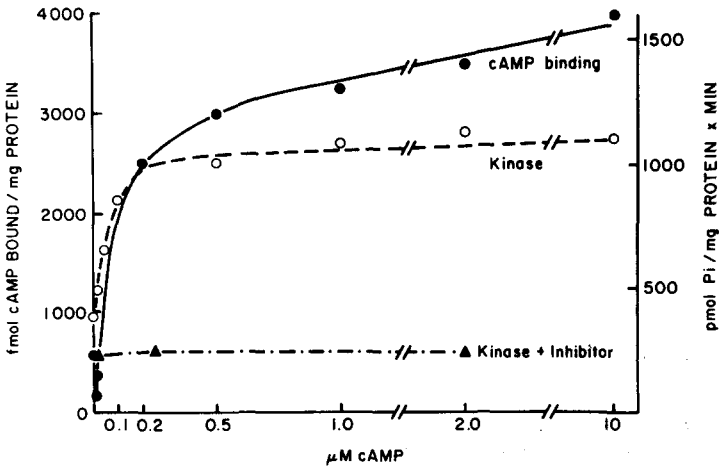

Fig. 1. Effect of different concentrations of cyclic AMP on binding capacity and protein kinase activation in 30 000 × *g* supernatants of sonicated toad bladder epithelial cells. Inhibitor, when present, was used at 25 μg protein per assay. Each point represents the mean of duplicate determinations from four experiments for cyclic AMP binding and of triplicate determinations from two experiments for protein kinase activation.

but also decreased basal protein kinase activity by about 30% (Fig. 1). No further inhibition was observed with up to 50 μg of inhibitor per assay. The 30% inhibition of basal kinase activity probably results from partial dissociation of cyclic AMP-dependent protein kinase under our conditions and is consistent with similar results obtained in other systems [9]. Non-inhibitable kinase activity represents cyclic AMP-independent protein kinase.

About 90% of the cyclic AMP binding was observed in the supernatant (Table I) with half-maximal binding at about 75 nM cyclic AMP (Fig. 1). Bound cyclic [^{3}H]AMP could be progressively displaced by increasing amounts of non-labeled cyclic AMP. 10 μM cyclic AMP displaced 95% of bound cyclic [^{3}H]AMP while either 10 μM adenosine or 5′-AMP only displaced 23%. Together with the cyclic AMP-binding curve this indicates predominantly specific binding sites, i.e. the regulatory subunit or protein kinase.

DEAE-cellulose chromatography showed that toad bladder contains 80% type II and 20% type I kinase. The type II kinase is stimulated about 10-fold by cyclic AMP (Fig. 2).

Fig. 3 shows the time course of the effect of vasopressin on activation of

TABLE I

BINDING CAPACITY FOR CYCLIC AMP IN CRUDE SONICATE, 30 000 × *g* SUPERNATANT AND PELLET FROM CONTROL TOAD BLADDER EPITHELIAL CELLS

Fractions of control toad bladder epithelial cells were prepared and assayed for cyclic AMP binding. Results represent mean ± S.E. of four experiments.

	Sonicate	Supernatant	Pellet
fmol cyclic AMP bound/mg protein	3498 ± 499	3692 ± 162	954 ± 120
% of total binding	100	87 ± 4	8.2 ± 2

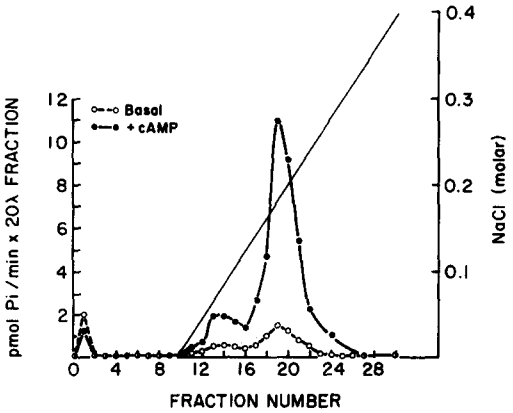

Fig. 2. DEAE-cellulose chromatography of protein kinase in the 30 000 × *g* supernatant of sonicated epithelial cells from toad bladder. Cells from four toads were prepared in a 10 mM potassium phosphate, pH 6.8, 1 mM EDTA, 4 mM mercaptoethanol buffer, and charged on a DEAE-cellulose column (0.9 × 8 cm) equilibrated with the same buffer as described in Materials and Methods. After washing the column with 40 ml of buffer, a linear gradient (0—0.4 M) of NaCl was started. Aliquots (20 μl) of the fractions (5 ml) were assayed for protein kinase activity in the absence or presence of cyclic AMP. The recovery of enzyme activity was approximately 90 and 80%, respectively. Results shown are from one of two similar experiments.

protein kinase and cyclic AMP-binding capacity in toad urinary bladders. Basal and cyclic AMP-stimulated kinase activity from control bladders remained stable for 30 min, while in the bladders exposed to 10 mU/ml vasopressin (a dose that elicits maximal osmotic water flow [3]), kinase activity in the absence of exogenous cyclic AMP was increased at 10 min, the earliest time point examined, and remained elevated for at least 30 min, while total kinase

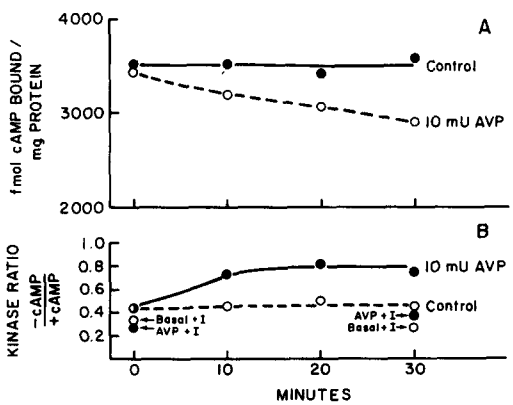

Fig. 3. Time course of cyclic AMP-binding capacity and protein kinase ratio in the absence or presence of cyclic AMP in toad urinary bladder under control conditions and after exposure to vasopressin (AVP). Pieces of bladder were incubated in Ringer's containing 0.1 mM 1-methyl-3-isobutylxanthine in the absence or presence of 10 mU/ml vasopressin for the time periods indicated. After scraping and sonication in homogenization buffer containing 100 mM KCl a 30 000 × *g* supernatant was prepared and assayed for cyclic AMP binding and protein kinase activity in the absence or presence of cyclic AMP. Kinase inhibitor (I), when present, was used at 25 μg protein per assay. Each point represents the mean of duplicate determinations for cyclic AMP binding and triplicate determination for protein kinase from two experiments each.

activity was unaffected. Thus, the kinase ratio increased after 10 min exposure to 10 mU vasopressin and remained stable up to 0.5 h (Fig. 3B). This time course is consistent with the effect of vasopressin on water permeability [3]. The increase in kinase activity and in kinase ratio after vasopressin treatment represents activation of cyclic AMP-dependent kinase, as it was totally inhibitable by 25 μg of the protein kinase inhibitor (Fig. 3B). This was confirmed by measurements of cyclic AMP-binding capacity (Fig. 3A). Under control conditions cyclic AMP-binding capacity remained stable while exposure of the bladders to vasopressin caused a progressive decrease in cyclic AMP-binding capacity. This is in good agreement with the time course of the kinase activity (Fig. 3B). No change of cyclic AMP-binding capacity was observed in the pellet fraction.

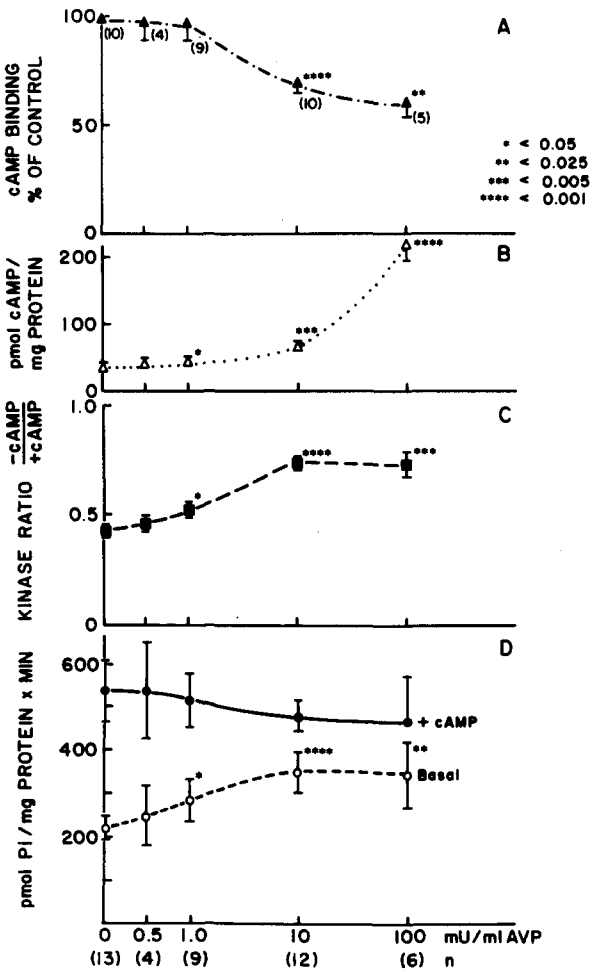

Fig. 4. Effect of increasing doses of vasopressin (AVP) on cyclic AMP concentrations, cyclic AMP-binding capacity, basal and cyclic AMP-stimulated protein kinase activity and kinase ratio in toad urinary bladders. Binding of cyclic AMP is expressed as percentage of binding observed in control bladders (i.e. without vasopressin). Numbers in brackets indicate number of experiments. * $P < 0.05$, ** $P < 0.025$, *** $P < 0.005$, **** $P < 0.001$, indicate P values compared to controls by paired analysis.

In all further experiments, strips of toad bladder were therefore exposed to vasopressin for 30 min prior to sonication. Increasing concentrations of vasopressin resulted in a progressive increase in cyclic AMP content (Fig. 4). Of note is that the major increase in cyclic AMP content occurs at vasopressin concentrations above 10 mU/ml, confirming similar results by Omachi et al. [4]. The protein kinase ratio also increased progressively with vasopressin treatment from 0.5 to 10 mU/ml, but showed no further increase at 100 mU/ml vasopressin (Fig. 4). This change is due to an increase in kinase activity measured without exogenous cyclic AMP. Of note, however, is a slight, though not significant, decrease in total protein kinase in bladders treated with 100 mU vasopressin. The reason for this decrease in total activity in the 30 000 $\times g$ supernatant of vasopressin-treated bladders is unlikely to be due to unspecific binding of catalytic subunit to particulate matter, as only 1.3 ± 0.4% (n = 4) of the total soluble kinase could be washed off the pellet with 300 mM KCl, accounting for only 3—4% of the kinase activity lost.

When cyclic AMP-binding capacity was assayed 30 min after exposure to varying concentrations of vasopressin, a dose-dependent decrease was observed (top line Fig. 4), which is a mirror image of the kinase ratio. In order to test whether this decrease in cyclic AMP-binding capacity of the bladders exposed to vasopressin represents occupation of binding sites by intracellularly generated cyclic AMP or unspecific loss of binding sites, cyclic AMP-binding assays were carried out at different temperatures. Binding for cyclic AMP was compared by incubating aliquots at 4°C for 2 h (the conditions used in Fig. 4), or at 30°C for 30 min. Toad bladder epithelial cells contain mostly type II kinase (see above) which would reassociate under the latter conditions and exchange bound cyclic AMP with free cyclic [^{3}H]AMP, while at 4°C only negligible exchange would occur [20]. That this is indeed the case is shown in Table II. In the absence of vasopressin, binding capacity for cyclic AMP was 40% higher when samples were incubated at 30°C instead of 4°C, indicating some occupancy of cyclic AMP-binding sites by endogenous cyclic AMP. Increasing doses of vasopressin again led to a progressive fall in the cyclic AMP-binding capacity (Table II) that was temperature reversible.

TABLE II

TEMPERATURE-DEPENDENT REVERSIBILITY OF CHANGES IN CYCLIC AMP-BINDING CAPACITY IN THE 30 000 $\times g$ SUPERNATANT OF SONICATED EPITHELIAL CELLS FROM CONTROL AND VASOPRESSIN-TREATED TOAD BLADDERS

Toad bladders were incubated, prepared and then assayed for cyclic AMP. Results represent means ± S.E. of four experiments. P value refers to paired analysis of binding assayed at 4°C for 2 h or at 30°C for 30 min. Data are fmol cyclic AMP bound/mg protein.

	Control	Vasopressin (mU/ml)		
		0.5	1.0	10
Binding for 2 h at 4°C	4346 ± 420	4242 ± 482	4009 ± 335	3137 ± 438 *
Binding for 30 min at 30°C	6116 ± 636	6399 ± 568	7085 ± 897	5974 ± 638
P value	<0.01	<0.005	<0.05	<0.005

* $P < 0.001$ as compared to respective control value.

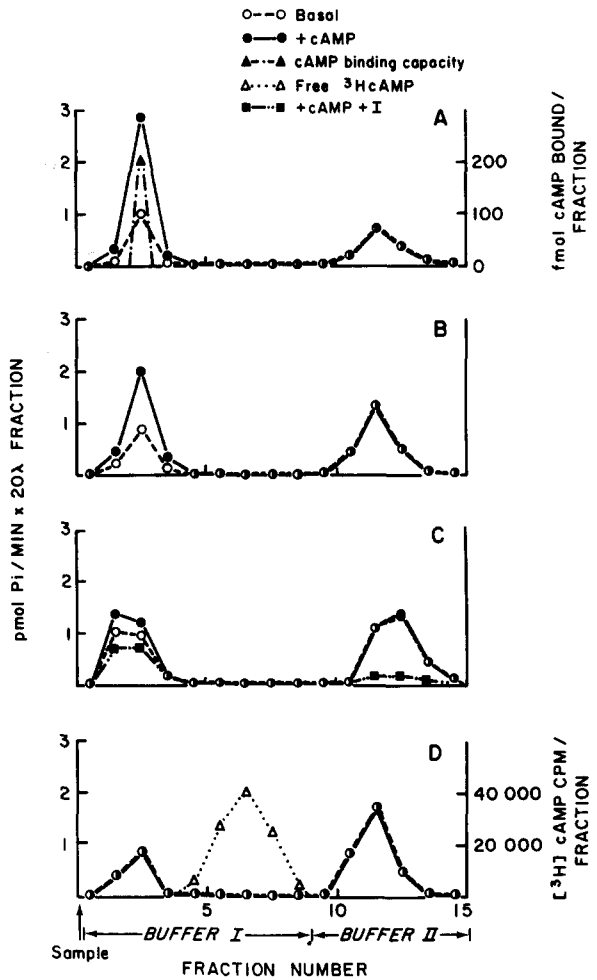

Fig. 5. Elution pattern of protein kinase activity in the absence or presence of cyclic AMP, cyclic AMP-binding capacity and free cyclic [^{3}H]AMP from Sephadex G-25 and CM-C50 column. 50-μl aliquots of sonicated epithelial cells, adjusted to the same protein concentrations, were applied to the column and eluted with 1.8 ml of 4 mM mercaptoethanol, 40 mM potassium phosphate, pH 6.0 (buffer I) followed by 1.2 ml of 4 mM mercaptoethanol, 0.3 M potassium phosphate, pH 7.2 (buffer II). 200-μl fractions were collected and assayed for cyclic AMP binding (80-μl aliquots) and protein kinase in the absence or presence of cyclic AMP (20-μl aliquots). Kinase inhibitor (I), when present was used at 25 μg protein per assay. Results are from one of two series of experiments. (A) Control bladder. (B) Bladder exposed to 10 mU/ml vasopressin. (C) Bladder exposed to 100 mU/ml vasopressin. (D) Sonicated epithelial cells from a control bladder were exposed for 30 min to 10 μM cyclic [^{3}H]AMP (100 000 cpm) prior to application and elution from column. In addition to protein kinase determinations 20-μl aliquots of the fractions were counted for ^{3}H and are shown as counts per total fraction.

In order to further examine whether the increase in protein kinase activity in vasopressin-treated bladders does indeed represent dissociation of protein kinase, kinase of unstimulated and vasopressin-stimulated bladders was separated into holoenzyme and free catalytic subunit by use of a column of Sephadex G-25 and GM-Sephadex C50. As shown in Fig. 5A, the cyclic AMP-dependent protein kinase (holoenzyme) eluted at an early peak, while the free

catalytic subunit was eluted with 0.3 M potassium phosphate (buffer II) as a second peak. This was verified by dissociating the protein kinase with 10 μM cyclic [^{3}H]AMP (100 000 cpm) prior to column chromatography. As shown in Fig. 5D the first peak no longer contains any cyclic AMP-stimulatable protein kinase (the remaining activity represents cyclic AMP-independent protein kinases), while the second peak representing the free catalytic subunit is increased. The activity, however, was not quantitatively recovered in the second peak probably due to instability of the free catalytic subunit. As also shown in Fig. 5A, cyclic AMP binding occurred only in the holoenzyme peak, while the free cyclic [^{3}H]AMP appeared between the peaks (Fig. 5D).

In unstimulated bladders the holoenzyme (peak 1) could be stimulated about 3-fold by cyclic AMP, while some free catalytic subunit (peak 2) was also apparent (Fig. 5A). This is consistent with the 30% dissociation of the kinase under our control conditions, as determined by use of the protein kinase inhibitor (see above). When the bladder had been exposed to increasing doses of vasopressin (10–100 mU/ml), the amount of holoenzyme decreased progressively (as evidenced by the decrease in activation achieved with cyclic AMP in peak 1), while more activity appeared as free catalytic subunit (Fig. 5B and C). As shown in Fig. 5C, the free catalytic subunit activity can be completely suppressed with specific protein kinase inhibitor [21]. The activation of protein kinase and the increase in kinase ratio of bladders exposed to vasopressin therefore represent true dissociation of the protein kinase into regulatory and catalytic subunits.

Discussion

The existence of two major types of cyclic AMP-dependent protein kinases in mammalian tissues is well established (see Ref. 9 for recent review). In the present study we show that cyclic AMP-dependent protein kinases in epithelial cells of toad urinary bladder are 80—90% cytosolic and consist of predominantly type II (80%) protein kinase, as determined by DEAE-cellulose chromatography. Whether this represents their distribution in all epithelial cell types of the toad bladder (e.g. granular versus mitochondria-rich cells) or whether it is due to different distribution in different cell types cannot be determined from these results. Our studies also show that exposure of toad urinary bladders to vasopressin results in activation of cytosolic cyclic AMP-dependent protein kinase that parallels the effect of vasopressin on osmotic water flow. As demonstrated by the results with Sephadex G-25-CM50 chromatography, vasopressin treatment of the toad bladder leads to activation through dissociation into the subunits of cyclic AMP-dependent protein kinase in the cells.

Under our control conditions (i.e. exposure of toad bladder pieces to 0.1 mM 1-methyl-3-isobutylxanthine and homogenization in buffer containing 100 mM KCl) cyclic AMP-dependent protein kinase is partially dissociated: kinase activity without added cyclic AMP is suppressed by 30% with kinase inhibitor, cyclic AMP-binding capacity increases at 30° C, and some free catalytic subunit elutes from Sephadex G-25-CM50. Similar findings have been obtained in other systems [9]. It has been argued that intracellular cyclic AMP-dependent protein kinase is always dissociated to some degree and that the physiological role of

the protein kinase inhibitor would be to suppress this basal activity and thus make the system more sensitive to small changes in cyclic AMP concentration [21]. In fact it is surprising that the degree of kinase activation is not higher under our control conditions.

Basal cyclic AMP levels were 20–40 pmol/mg protein, rising to 100–200 after vasopressin, while the concentration of cyclic AMP-binding sites was only 4–10 pmol/mg protein, so that the concentration of cyclic AMP was considerably in excess of the concentration of binding sites. Unless in vivo binding characteristics are remarkably different from those observed in vitro (as discussed by Terasaki and Brooker [20]) the discrepancy between the cyclic AMP concentrations and degree of kinase activation may indicate intracellular compartmentalization of cyclic AMP.

In this context it is of interest that the protein kinase ratio and the cyclic AMP-binding capacity show no further change when vasopressin concentrations are raised from 10 to 100 mU/ml. Maximal effects of vasopressin on osmotic water flow in the toad bladder are usually obtained with 10 mU vasopressin and higher concentrations, e.g. 100 mU, do not elicit an additional effect [3]. Stimulation of adenylate cyclase and intracellular concentrations of cyclic AMP on the other hand still show considerable increases from 10 to 100 mU/ml of vasopressin [3,4]. The latter findings have been interpreted as supporting the concepts of receptor reserve for vasopressin [5] and of specific intracellular cyclic AMP pools [6]. Our findings of no further increment in kinase ratio and no further decrease in cyclic AMP-binding capacity in spite of considerable further increases of cyclic AMP content when bladders were exposed to 100 instead of 10 mU/ml vasopressin would be consistent with these concepts.

In summary, our studies demonstrate that treatment of toad urinary bladder with vasopressin activates a cytosolic cyclic AMP-dependent protein kinase (mostly type II) in a manner that parallels the time course and dose-response of the vasopressin effect. Using Sephadex G-25-CM50 chromatography, we were also able to show that in intact toad bladder tissue (as in vitro) this activation is achieved by dissociation of the cyclic AMP-dependent protein kinase into its subunit. The endogenous substrate(s) for phosphorylation by the cyclic AMP-dependent protein kinase and the effect of their phosphorylation on membrane permeability remain to be determined.

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