Modulation of Cyclic AMP-Dependent Protein Kinase by Vasopressin and Calcitonin in Cultured Porcine Renal LLC-PK₁ Cells

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We have previously demonstrated that a cultured porcine kidney cell, LLC-PK₁, maintains the characteristics of a polar renal epithelial cell in culture, and responds to salmon calcitonin and [arginine]vasopressin by increasing cyclic AMP content. To demonstrate the usefulness of this cell line as a model for the study of the biochemical events distal to cyclic AMP production, the activation of cyclic AMP-dependent protein kinase was examined. Intact cells in monolayer demonstrated progressive increases in cyclic AMP content and activation of protein kinase in response to [arginine]vasopressin (2–200 nM) and salmon calcitonin (0.03–30 nM) with both hormones fully activating the enzyme at a cell cyclic AMP content of 35 pmol/mg of protein. Of the total cyclic AMP-dependent protein kinase activity, 80% was found in the 27000g supernatant fraction of sonicated cell material, and this soluble protein kinase could be fully activated by hormone. Conversely, the 27000g pellet contained a significant proportion of cyclic AMP-independent protein kinase and only 20% of total cell cyclic AMP-dependent protein kinase; the latter showed little response to hormone. On the basis of DEAE-cellulose chromatography, type II protein kinase was the predominant isoenzyme in both soluble and particulate fractions of the LLC-PK₁ cells and the soluble fractions of rat and guinea-pig renal medulla. Thus, the LLC-PK₁ cell line can serve as a model for hormonal modulation of protein kinase and as a potential source for defining the endogenous substrates for these enzymes.

We have recently described a cell strain, LLC-PK₁, cultured from porcine kidney, which maintains morphological features characteristic of renal epithelia (Mills *et al.*, 1979), and retains hormonal responsiveness by increasing the content of cyclic AMP on exposure to salmon calcitonin or [arginine]vasopressin (Goldring *et al.*, 1978). In several systems, the step subsequent to activation of adenylate cyclase in the action of polypeptide hormones is believed to be activation of cAMP-PK (ATP-protein phosphotransferase, EC 2.7.1.37) (Krebs, 1972). These enzymes have been found in both renal cortex and medulla where they are modulated by several hormones, including calcitonin (Knox *et al.*, 1977) and antidiuretic hormone (Dousa & Barnes, 1977). The molecular form of these enzymes consists of regulatory subunits (R) and catalytic subunits (C) in the combination R₂C₂ (Erlichman *et al.*, 1973; Beavo *et al.*, 1975), and this holoenzyme is

Abbreviations used: cAMP-PK, cyclic AMP-dependent protein kinase; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

activated by dissociation in the presence of cyclic AMP. Recent studies have demonstrated that there are two types (I and II) of cAMP-PK isoenzymes in the mammalian cell, which differ solely in their regulatory subunits (Reimann *et al.*, 1971). Both protein kinases have been found in various proportions in soluble fractions of several tissues (Nimmo & Cohen, 1977), although the type II kinase seems to be unique to the particulate fraction in heart tissue (Corbin *et al.*, 1977). Little is known about the physiological role of type I or type II kinases. There are, however, studies that suggest that the proportion of kinase types in the renal cortex (DeRubertis & Craven, 1976) may be different from the renal medulla (Schlender & Reimann, 1975), although direct comparisons have not been made.

The present study was designed to determine if the LLC-PK₁ cells provide a model for the study of hormone-activated cAMP-PK. We have found that the salmon-calcitonin- and [arginine]vasopressin-stimulated increases in cyclic AMP content are accompanied by activation of cAMP-PK that is predomi-

nantly type II, similar to that identified in mammalian renal medulla.

A preliminary report of this study was presented at the VIIth International Congress of Nephrology, Montreal, Que., Canada, 1978.

Methods

Cell-culture procedures

The LLC-PK₁ strain was originally prepared from juvenile male Hampshire pig kidney as described by Hull *et al.* (1976). The cells that we used were provided by Dr. R. N. Hull and had been through more than 350 passages. Cells were carried in 10 cm-diameter plastic Petri dishes using Dulbecco's modification of Eagle's medium supplemented with 10% (v/v) foetal calf serum, plus 100 units of penicillin and 100 mg of streptomycin per ml (Goldring *et al.*, 1978). Cells were plated at 1×10^6 cells per dish and reached a density of $(1-2) \times 10^7$ cells per dish within 5-7 days at 37°C in air/CO₂ (19:1). All the studies described were conducted on cells at confluent levels within 5-7 days of transfer. Twenty passages were used over a 12 month period and no significant variation in protein kinase activity was observed.

Protein assay

Protein was assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Protein kinase assay

After removal of the culture medium, the cells were incubated with hormone in Dulbecco's phosphate-buffered saline supplemented with 0.2% (w/v) bovine serum albumin, 10 mM-glucose and 2.5 mM-Hepes, pH 7.4. After the appropriate incubation period at 37°C in air, the incubation medium was removed and cells were scraped at 4°C into a sonication buffer containing 5 mM-potassium phosphate, 2 mM-EDTA, 0.5 mM-3-isobutyl-1-methylxanthine and 150 mM-KCl, pH 6.8. The cells were sonicated at 4°C for 3 s. In preliminary experiments, 15 strokes in a tight-fitting Dounce homogenizer (1 mg of cell protein/ml) yielded similar results to those described below. Therefore, the more rapid and uniform method of cell sonication was utilized. Samples of sonicated cell material were taken for cyclic AMP determination. In some experiments, sonicated material was centrifuged at 4°C at 27 000 g in a Sorvall centrifuge (SS34 rotor; Ivan Sorvall, Norwalk, CT, U.S.A.) for 20 min to prepare supernatant and pellet fractions. Then 20 µl of cell sample was added to 50 µl of assay buffer containing (final concentrations): 12 mM-potassium phosphate, pH 6.8; 20 mM-NaF; 4 mM-MgCl₂; 500 mg of type II-A histone; 0.23 mM-[γ-³²P]ATP (sp. radioactivity 0.1 mCi/µmol); 2 µM-cyclic AMP (where added).

These solutions were incubated at 30°C for 4 min and the reaction stopped by the addition of 50 µl portions to Whatman 3MM discs, which were immediately dropped into 10% (w/v) trichloroacetic acid at 4°C. Discs were then treated as previously described (Corbin & Reimann, 1974) and the radioactivity of ³²P-labelled histone was determined in a liquid-scintillation counter (efficiency 95%). Each disc contained a minimum of 2000 c.p.m. above background.

Under these conditions the protein kinase assay was linear for 5 min for 7.5-75 µg of cell protein from whole sonicated material or the 27 000 g supernatant or the pellet fractions. There was no endogenous phosphorylation in the presence of histone as determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Separate studies were conducted by using charcoal in the sonication buffer as previously described (DeRubertis & Craven, 1976), which demonstrated that activation of protein kinase was a function of hormone stimulation in the intact cell and not an artefact of cyclic AMP release accompanying cell disruption. In some experiments, the protein kinase assay was conducted in the presence of a maximal concentration of catalytic inhibitor protein (40 µg) prepared from skeletal muscle by the method of Walsh *et al.* (1971).

The protein kinase activity is expressed as the kinase activity ratio, i.e. the ratio of activity in the absence of cyclic AMP to that in the presence of exogenous cyclic AMP (2 µM). Basal and hormone-stimulated activity ratios were unchanged in sonicated tissue kept at 4°C for 60 min.

Cyclic AMP determination

Cyclic AMP was determined by using a radioimmunoassay as previously described (Goldring *et al.*, 1978) on portions of sonicated material that had been boiled for 5 min in 5 mM-potassium phosphate/2 mM-EDTA/0.5 mM-3-isobutyl-1-methylxanthine/150 mM-KCl (pH 6.8), and centrifuged at 7000 g for 1 min. Preliminary experiments demonstrated that cyclic AMP measured in these supernatant solutions from boiled samples yielded results similar to those in samples prepared by trichloroacetic acid precipitation and ion-exchange chromatography. Therefore, the more rapid method was utilized in these studies. The validity of the cyclic AMP assay was confirmed as follows. (1) The recovery of exogenous cyclic AMP added to cells at time of scraping was complete. (2) There was linearity of the assay with sample dilution. (3) The hydrolysis of measured cyclic AMP was complete with cyclic nucleotide phosphodiesterase. (4) The addition of ATP, ADP or AMP at a 1000:1 ratio of cyclic AMP did not alter the amount of cyclic AMP measured.

DEAE-cellulose chromatography

DEAE-cellulose columns (0.9 cm $\times$ 3.5 cm) were prepared and equilibrated in cold 5 mM-Tris/HCl (pH 7.5) containing 1 mM-EDTA (column buffer). Approx. 50 mg of LLC-PK₁ cell protein was sonicated in this column buffer and centrifuged at 27000g for 20 min. The supernatant solution (10 ml) was chromatographed by using a linear gradient of NaCl (0–0.5 M). Fractions (2 ml) were collected and portions were assayed for protein kinase as described above. Recovery of enzyme activity was approx. 85%.

In a separate experiment, LLC-PK₁ cells were sonicated in column buffer containing 150 mM-KCl and a 27000g pellet prepared. The pellet was washed with the column buffer alone and centrifuged at 27000g. This procedure was repeated twice. The final pellet was suspended in a column buffer containing 0.2% (v/v) Triton X-100 and incubated for 1 h at 4°C with gentle mixing. The suspension was then centrifuged at 27000g. The supernatant was diluted 4-fold with column buffer and chromatographed on DEAE-cellulose as described. This fraction contained 90% of the total protein kinase activity of the original 27000g pellet.

Rat or guinea-pig renal medulla and papilla were dissected and homogenized at 4°C in column buffer with 10 strokes of a Dounce homogenizer. Homogenates were then sonicated and 27000g-supernatant solutions were prepared for chromatography as described.

Materials

Dulbecco's modified Eagle's medium was obtained from Grand Island Biological Co., Grand Island, NY, U.S.A., and foetal calf serum was from Microbiological Associates, Bethesda, MD, U.S.A. Histone (type II-A), [arginine]vasopressin (367 units/mg), 3-isobutyl-1-methylxanthine, Hepes and Triton X-100 were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. [γ -³²P]ATP (sp. radioactivity 2–10 Ci/mmol) and the cyclic AMP radioimmunoassay kit were purchased from New England Nuclear, Boston, MA, U.S.A. Synthetic salmon calcitonin was a gift from Dr. Henry Keutmann, Endocrine Unit, Massachusetts General Hospital. DEAE-cellulose (Whatman DE-52) was obtained from Metropolitan Supply Co., Cambridge, MA, U.S.A. Whatman 3MM filter paper was purchased from VWR Scientific Inc., Boston, MA, U.S.A. The catalytic-inhibitor protein of cAMP-PK, prepared by the method of Walsh *et al.* (1971), was a gift from Dr. Joseph Avruch, Diabetes Unit, Massachusetts General Hospital.

Results

Cyclic AMP response to hormone

Fig. 1 shows the time course of the increases in

Fig. 1. Effect of 200 nM-[arginine]vasopressin (O) and 300 nM-salmon calcitonin (Δ) on cyclic AMP content and protein kinase activity in LLC-PK₁ cells

Control (●) and hormone-stimulated cells were incubated at 37°C in air for 1–60 min in phosphate-buffered saline containing 2.5 mM-Hepes, 10 mM-glucose and 0.2% (w/v) bovine serum albumin, pH 7.4. At the end of each incubation, medium was removed and cells were sonicated at 4°C in 5 mM-potassium phosphate containing 2 mM-EDTA, 0.5 mM-3-isobutyl-1-methylxanthine and 150 mM-KCl, pH 6.8. Cyclic AMP content (a) and protein kinase activity (b) were measured in portions of whole sonicated material. Total protein kinase activity (+ 2 μM-cyclic AMP) was unchanged in control and hormone-stimulated cells (400 pmol/min per mg of protein). Each value represents the mean $\pm$ S.E.M. for duplicate assays from each of six culture dishes. The activity ratio is for protein kinase in the absence compared within the presence of cyclic AMP. * $P < 0.001$ for salmon calcitonin value versus control value or salmon calcitonin value versus [arginine]vasopressin value. These statistically significant differences were maintained at all later incubation times.

intracellular cyclic AMP content in the presence of maximal concentrations of either [arginine]vasopressin (200 nM) or salmon calcitonin (300 nM). Basal cyclic AMP content remained unchanged at 5 pmol/mg of protein. Cyclic AMP content increased during 10 min of incubation with salmon calcitonin and then declined, despite the continued presence of the hormone. However, the intracellular cyclic AMP content did not fall below 35 pmol/mg of protein. In contrast, [arginine]vasopressin maximally stimulated cyclic AMP production, within 1 min, to 35 pmol/mg of protein, which then declined over the next several minutes. These results could not be explained by differential increases in phosphodiesterase activity since qualitatively similar differences were obtained with [arginine]vasopressin

and salmon calcitonin in the presence of a maximal concentration of 3-isobutyl-1-methylxanthine (results not shown) despite cyclic AMP concentrations 10-fold higher than those in the presence of either hormone alone. When media containing submaximal (10nM) or maximal (200nM) concentrations of [arginine]vasopressin or salmon calcitonin were removed from cells after a 60min incubation and added to fresh cells, similar increases in cyclic AMP content were achieved when compared with control hormone solutions. There was, therefore, no significant degradation of hormones. Preliminary experiments have demonstrated that the decrease in hormonal responsiveness is secondary to an uncoupling of the hormone-receptor complex from adenylate cyclase (D. A. Ausiello & C. Roy, unpublished work).

Extrapolating from previous data on the relationship between tissue protein and intracellular water (Mills *et al.*, 1979), in the absence of phosphodiesterase inhibition, the basal cyclic AMP concentration per cell was $2\mu\text{M}$ ($0.75\mu\text{mol/kg}$ wet wt.) and the maximal concentration of cyclic AMP achieved was approx. $20\mu\text{M}$ with [arginine]vasopressin and $25\mu\text{M}$ with salmon calcitonin. This estimate ignores possible compartmentalization of cyclic AMP.

Protein kinase activity

In control sonicated cell material, exogenous $1\mu\text{M}$ -cyclic AMP fully activated cAMP-PK. Data from mammalian tissues indicate the cell content of cyclic AMP to be in the range $0.1\text{--}1.0\mu\text{mol/kg}$ wet wt. (Nimmo & Cohen, 1977), similar to LLC-PK₁ cells. The basal endogenous concentration of cyclic AMP ($2\mu\text{M}$) in LLC-PK₁ cells is twice the concentration of exogenous cyclic AMP necessary to fully activate cAMP-PK in sonicated cell material. This would suggest that cAMP-PK would always be fully activated in intact cells. This paradox is explained, however, by the conditions used in measuring cAMP-PK activity. If the standard assay for protein kinase *in vitro* is altered by utilizing physiological concentrations of cAMP-PK and catalytic-inhibitor protein, the concentrations of cyclic AMP necessary to activate cAMP-PK are similar to those observed with hormone stimulation in intact cells (Nimmo & Cohen, 1977). In the present study, total kinase activity was determined in the presence of $2\mu\text{M}$ -cyclic AMP and found to be similar with or without hormone stimulation at 400pmol of ^{32}P incorporated/min per mg of protein. Thus increases in the protein kinase activity ratio reflect changes only in the numerator, or free catalytic unit.

Fig. 1 also shows the activation of protein kinase by [arginine]vasopressin and salmon calcitonin. Control kinase activity ratios remained constant at 0.47 ± 0.02 (mean $\pm$ S.E.M.). At 1min both hormones fully activated protein kinase as indicated by

Fig. 2. Relationship between salmon calcitonin concentration, cell cyclic AMP content (○), and protein kinase activity (●)

LLC-PK₁ cells were incubated for 10min with increasing concentrations of salmon calcitonin and cyclic AMP content and protein kinase activity were measured as described in Fig. 1. Each value represents mean $\pm$ S.E.M. for two determinations from each of six culture dishes. For definition of activity ratio, see the legend to Fig. 1.

Fig. 3. Relationship between [arginine]vasopressin concentration, cell cyclic AMP content (○) and protein kinase activity (●)

LLC-PK₁ cells were incubated for 1min with increasing concentrations of [arginine]vasopressin, and cyclic AMP content and protein kinase activity were measured as described in Fig. 1. Each value represents mean $\pm$ S.E.M. for two determinations from each of six culture dishes. For definition of activity ratio, see the legend to Fig. 1.

the activity ratio of 1.0. Whereas salmon calcitonin maintained this activation for 60min, there was a progressive decrease in the activity of protein kinase in the presence of [arginine]vasopressin mimicking the similar decrease in intracellular cyclic AMP content described above. This would suggest that

minimum cyclic AMP content necessary to fully activate protein kinase is approx. 35 pmol/mg of tissue protein. Protein kinase activation and cell cyclic AMP responses to increasing concentrations of hormones were evaluated to document this observation. The effect of 10 min incubations with various concentrations of salmon calcitonin on cell cyclic AMP content and protein kinase activity ratios is shown in Fig. 2. Salmon calcitonin (0.03 nM) stimulated cyclic AMP production and protein kinase activity, and both cyclic AMP content and protein kinase activity increased up to a concentration of 30 nM salmon calcitonin. At the latter concentration cyclic AMP content reached 32 pmol/mg of protein, at which point protein kinase was fully active. Increasing concentrations of salmon calcitonin, however, continue to increase intracellular cyclic AMP content. With [arginine]vasopressin (Fig. 3) increases in cyclic AMP activation of protein kinase began at 2 nM and continued up to 200 nM, when cyclic AMP content again reached 35 pmol/mg of protein. Protein kinase activity was then maximal and was not altered by further increases in cyclic AMP.

Distribution of protein kinase

The distribution of protein kinase in the 27000g supernatant and pellet fractions in the presence or absence of hormone are shown in Table 1. This centrifugation condition was chosen so comparisons could be made with similar studies in kidney (DeRubertis & Craven, 1976) and other mammalian tissues (Corbin *et al.*, 1975). Approximately three-quarters of total kinase activity was found in the supernatant fraction of control cells; this distribution was not altered by the presence of maximal concentrations of either [arginine]vasopressin or salmon calcitonin. Of the total kinase activity 25% was localized in the pellet fraction. Most of the activation of protein kinase observed in the whole soni-

cated material in the presence of hormone is reflected in the activity ratios in the supernatant fraction, i.e. the control activity ratio of 0.34 was increased to 0.79 in the presence of [arginine]vasopressin, and 0.96 in the presence of salmon calcitonin. The control ratio in the pellet of 0.74 indicates that three-quarters of the protein kinases were in their active form even in the absence of hormone, and there was no significant increase in activation of the enzyme in the pellet fraction in the presence of [arginine]vasopressin or salmon calcitonin. The high activity ratio of the pellet could reflect one of two circumstances; either the cAMP-PK exists predominantly in the active dissociated form or there is a significant proportion of cyclic AMP-independent kinase activity. To evaluate these possibilities, the protein kinase studies were repeated in the presence of a specific inhibitor of the catalytic unit of cAMP-PK (Walsh *et al.*, 1971). Results were quantitatively similar when protein kinase activity was determined in the presence or absence of exogenous cyclic AMP. The amount of total kinase that was cyclic AMP-dependent was found to be 70% in the whole sonicated material, 70% in supernatants and only 50% in pellets. These values were not different in control or hormone-stimulated cell fractions. This supports the possibility that the high activity ratio of the pellet fraction is at least in part due to a large percentage of cyclic AMP-independent kinase. Therefore, the corrected distribution of cAMP-PK is 80% in supernatant fractions and 20% in the particulate fractions. Correcting the basal activity ratio to reflect only cAMP-PK yields values of 0.24 in sonicated material, 0.06 in supernatants and 0.48 in pellets.

Chromatographic separation of protein kinase isoenzymes

The elution pattern of the 27000g supernatant fraction chromatographed on DEAE-cellulose in the presence of a linear NaCl gradient (0–0.5 M) is

Table 1. *Distribution of protein kinase in LLC-PK₁ cells*

Cells were incubated for 10 min at 37°C with maximal concentrations of [arginine]vasopressin and salmon calcitonin. Cells were sonicated at 4°C as in Fig. 1. Whole sonicated material and 27000g supernatant and pellet fractions were assayed for protein kinase activity. Values are means $\pm$ S.E.M. for duplicate determinations in each of six culture dishes. Protein kinase activity is expressed in arbitrary units with the protein kinase activity ratio (mean $\pm$ S.E.M.) in parentheses. * $P < 0.001$ versus control. All other values are not significantly different from control values.

	Protein kinase activity		
	Sonicated material	Supernatant	Pellet
Control	100 (0.47 $\pm$ 0.02)	78 $\pm$ 4 (0.34 $\pm$ 0.02)	28 $\pm$ 2 (0.74 $\pm$ 0.08)
[Arginine]vasopressin (200 nM)	100 (0.84 $\pm$ 0.01)*	69 $\pm$ 2 (0.79 $\pm$ 0.03)*	33 $\pm$ 3 (0.81 $\pm$ 0.01)
Salmon calcitonin (300 nM)	100 (0.96 $\pm$ 0.01)*	77 $\pm$ 4 (0.96 $\pm$ 0.02)*	29 $\pm$ 2 (0.86 $\pm$ 0.02)

shown in Fig. 4. Two peaks of protein kinase activity were observed, both of which were stimulated in the presence of cyclic AMP. These results are con-

Fig. 4. DEAE-cellulose chromatography of protein kinases from LLC-PK₁ cells

Cells were sonicated at 4°C in 5 mM-Tris/1 mM-EDTA (pH 7.5) (column buffer), centrifuged for 20 min at 27000g and 50 mg of supernatant protein was applied to a column previously equilibrated in column buffer at 4°C. The column was eluted with a linear NaCl gradient (----). Portions of fractions were assayed for protein kinase activity with (O) or without 2 μM-cyclic AMP (●). Data are representative of four separate cell passages. One unit of protein kinase activity equals the transfer of 1 pmol of phosphate per min to histone.

sistent with the chromatographic pattern of protein kinase isoenzymes observed in mammalian tissue in general (Corbin *et al.*, 1975). Types I and II protein kinases were eluted at approx. 50 mM- and 150 mM-NaCl respectively.

Table 2 shows the protein kinase activities in the 27000g supernatant and pellet fractions of LLC-PK₁ cells and the 27000g supernatant fractions from rat and guinea-pig renal medulla. Quantitatively, over 85% of the protein kinase in the supernatant fraction of the LLC-PK₁ cells eluted as type II cAMP-PK at 150 mM-NaCl. To have an independent method of verifying the cyclic AMP dependency of the two protein kinase peaks observed, chromatography on DEAE-cellulose was repeated with a supernatant fraction that had previously been exposed to maximal concentrations of cyclic AMP (7 μM). Under these conditions, a large cyclic AMP-independent peak appeared in the void volume. A small cyclic AMP-independent peak corresponding to the fractions containing type I protein kinase (50 mM-NaCl) was identified; there was no protein kinase activity detected in the fractions previously found to contain type II protein kinase. The large peak of activity in the void volume is consistent with the elution of the free catalytic unit that had been dissociated by cyclic AMP from the regulatory units of type I and II enzymes. The presence of a small cyclic AMP-independent peak in the area of 50 mM-NaCl may indicate the co-elution of a cyclic AMP-independent protein kinase with type I enzyme.

Three peaks were observed in the chromatographic pattern of protein kinase activity extracted from the 27000g pellet fraction of LLC-PK₁ cells. The first eluted with the void volume and was cyclic AMP-independent. This probably represents free

Table 2. cAMP-PK activity in LLC-PK₁ cells and in rat and guinea-pig renal medulla

The 27000g supernatant fractions of LLC-PK₁ cells, rat, and guinea-pig renal medulla were prepared as described in the Methods section and chromatographed on DEAE-cellulose as described in the legend to Fig. 4. In Expt. 1(b), an LLC-PK₁ supernatant fraction was first exposed to 7 μM-cyclic AMP for 30 min at 4°C and then chromatographed. In Expt. 1(c), an LLC-PK₁ pellet fraction was washed and incubated at 4°C for 1 h in column buffer (see Fig. 4) containing 0.2% (v/v) Triton X-100. The sample was centrifuged at 27000g and the supernatant diluted 4-fold with column buffer before proceeding with chromatography. In each experiment 50 mg of cell protein was applied to the column. The protein kinase activity was quantified in column fractions preceding NaCl elution (void volume) and in those fractions containing type I (50 mM-NaCl) and type II (150 mM-NaCl) cAMP-PK as seen in Fig. 4. Data are expressed as the ratio of the units of protein kinase activity in the absence and presence of exogenous cyclic AMP (2 μM), and are representative of four separate cell passages or two renal medullae.

Experiment	Protein kinase activity		
	Void volume	50 mM-NaCl	150 mM-NaCl
1. LLC-PK ₁ cells			
(a) Supernatant	0	135/253	435/1696
(b) Supernatant plus cyclic AMP	1822/1912	344/348	0
(c) Pellet	192/198	54/42	54/159
2. Rat renal medulla	0	417/1182	675/3879
3. Guinea-pig renal medulla	0	441/570	597/4425

catalytic unit either dissociated from particulate-bound regulatory unit by treatment with Triton X-100 or bound non-specifically during cell fractionation. The second small peak eluted at 50mM-NaCl and was also cyclic AMP-independent. The third peak was a cAMP-PK consistent with the type II enzyme.

The chromatographic patterns of protein kinase activity in supernatant fractions of medullary tissue from guinea-pig and rat kidney demonstrated a predominance of the type II isoenzyme. It has previously been shown that the majority of the protein kinase activity in kidney medulla is located in the supernatant fractions (Barnes *et al.*, 1975; DeRubertis & Craven, 1976).

Discussion

We have demonstrated in these studies that [arginine]vasopressin and salmon calcitonin activate cAMP-PK in cultured kidney cells. The use of this cell line to study the biochemical action of these hormones has many advantages over renal tissue containing heterogeneous cell populations, only some of which may respond to a hormone. Our previous studies demonstrated that the apparent affinity constants (K_m) for the stimulation of cyclic AMP production by [arginine]vasopressin and salmon calcitonin were similar to those reported in rabbit and rat kidneys respectively (Goldring *et al.*, 1978). Although salmon calcitonin had been shown by others to stimulate cAMP-PK in hamster (Knox *et al.*, 1977) and rat (Sakai *et al.*, 1976) kidney, these studies utilized only supermaximal doses of hormone (0.9 μ M), and, therefore, there are no comparable data to those we have presented in the LLC-PK₁ cells. The regulation of protein kinase by vasopressin *in situ* in bovine renal medulla has been examined by Dousa & Barnes (1977). They found a variable basal kinase activity ratio in 40000g supernatant fractions between the ratios 0.17 and 0.27. LLC-PK₁ cells had a basal kinase activity ratio of 0.34 in the 27000g supernatant fractions, which decreased to 0.06 when the contribution of cyclic AMP-independent kinase was excluded.

Increasing the concentrations of both salmon calcitonin (0.03–30nM) and [arginine]vasopressin (2–200nM) quantitatively increased cAMP-PK activity in the cultured pig kidney cells until the activity ratio equaled 1.0, a 16-fold increase above basal values. In contrast, the maximum activity ratio achieved with 500nM-[arginine]vasopressin in bovine kidney was 0.03, only a 40% increase in activation (Dousa & Barnes, 1977). Salmon calcitonin increased the kinase activity ratio from 0.16 to 0.27 in hamster kidney, a 75% increase in activity (Knox *et al.*, 1977). These differences probably reflect the presence of cells in whole renal tissue that contain cAMP-PK, but are not sensitive to [arginine]vaso-

pressin or salmon calcitonin. Although the possibility exists that the LLC-PK₁ strain consists of more than one cell type, [arginine]vasopressin or salmon calcitonin are capable of fully activating cAMP-PK. These cells thus serve as a better model for hormonal modulation of these enzymes.

A question of potential physiological importance is the distribution of cAMP-PK and translocation with hormone stimulation. In both mammalian renal medulla (Barnes *et al.*, 1975) and cortex (DeRubertis & Craven, 1976), these enzymes are found predominantly in 40000g and 20000g supernatant fractions respectively. LLC-PK₁ cells similarly have 80% of cAMP-PK in the 27000g supernatant fraction. Almost half of the cAMP-PK found in the pellet was in the active catalytic state suggesting that some binding of soluble catalytic unit to particulate matter during separation of cell fractions occurred, similar to that reported for the rat kidney (DeRubertis & Craven, 1976). Therefore, the proportion of particulate-bound cAMP-PK may be overestimated. More definitive localization of these enzymes must await studies currently in progress with purified membranes.

In the presence of either [arginine]vasopressin or salmon calcitonin there was no translocation of kinase activity between the supernatant and pellet fractions with 150mM-KCl in the sonication buffer. When similar experiments were performed in the absence of KCl, over 30% of hormone-activated kinase was found in the pellet fraction. As has been noted in other studies, this 'translocation' can be avoided by the use of physiological salt concentrations and is non-specific i.e. supernatant kinase can bind to heat-inactivated protein from various tissues (DeRubertis & Craven, 1976). Therefore, its biological significance remains in question. Specific translocation of soluble protein kinase to nuclear fractions has been demonstrated in experiments with pig ovaries (Jungmann *et al.*, 1976) and adrenal medulla (Costa *et al.*, 1976a). The reverse may actually occur as has been suggested from studies of cardiac tissue, where translocation of particulate-bound catalytic unit to the supernatant with enzyme activation was found (Corbin *et al.*, 1977). Preliminary studies by Steiner *et al.* (1978) utilizing immunocytochemical techniques offer the best chance of clarifying translocation in the intact cell.

On the basis of DEAE-cellulose chromatography, the type II isoenzyme was found to comprise at least 85% of the total cAMP-PK in the supernatant fraction. This may be an underestimate, since cyclic AMP-independent protein kinase co-migrated with the type I enzyme. The only cAMP-PK in the particulate fraction was type II, with two peaks of activity consistent with catalytic unit and a cyclic AMP-independent kinase similar to that observed in supernatant fractions. Both type I and

type II kinases have been found in a variety of tissues in various proportions (Nimmo & Cohen, 1977). Data from rabbit kidney medulla have indicated a predominance of the type II kinase (Schlender & Reimann, 1975). Experiments in rat kidney cortex have suggested a large proportion of type I kinase (DeRubertis & Craven, 1976). In our studies, the type II isoenzyme predominated in LLC-PK₁ cells and rat and guinea-pig kidney medulla. The results of experiments in synchronous hamster ovary cells in culture (Costa *et al.*, 1976b) suggest that type I and type II kinase activity varies with the cell cycle. Although we do not know if this phenomenon is also true for the LLC-PK₁ cells, we utilized cultures that were confluent to minimize cell-cycle effects on protein kinase activity. Over 20 passages of the cell line have been studied with no variability in the patterns of protein kinase activation reported in the present paper.

The physiological significance of type I and type II kinases is unknown. These enzymes differ only in their regulatory units (Nimmo & Cohen, 1977). This accounts for differences in the activation of these enzymes by NaCl and cyclic AMP. Although these effects have only been determined *in vitro*, it is possible that they play a significant regulatory role *in vivo* as well. For instance, it is critical for a regulatory enzyme to have a low activity in the basal state. If kidney medulla contained type I instead of type II protein kinase as its predominant enzyme, the high osmolality and salt concentration in this segment of the nephron might result in full activation of the enzyme. Data for kidney medulla suggest that increases in extracellular osmolality actually lower basal protein kinase activity, but increase its responsivity to [arginine]vasopressin (DeRubertis & Craven, 1978). Current studies in our laboratory have demonstrated that increasing concentrations of NaCl and urea in incubation media alter both the binding of [³H]lysine]vasopressin to its receptor and the modulation of protein kinase by this hormone in LLC-PK₁ cells (D. A. Ausiello & C. Roy, unpublished work). Thus changes in extracellular fluid alter the intracellular environment, which may have a differential effect on the hormone activation of type I and type II protein kinases.

In conclusion, the LLC-PK₁ kidney cell line responds to [arginine]vasopressin and salmon calcitonin by stimulating cyclic AMP production and activating cAMP-PK, predominantly type II. This enzyme is also the major cAMP-PK found in kidney medulla. These cells can serve as a model for hormonal modulation of protein kinase and as a potential source for defining the endogenous substrates for these enzymes.

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