Hormonal Modulation of Cyclic Adenosine 3', 5'Monophosphate-Dependent Protein Kinase Activity in Rat Renal Cortex

SPECIFICITY OF ENZYME TRANSLOCATION

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ABSTRACT Many of the intracellular actions of cyclic adenosine 3'.5'-monophosphate are expressed through phosphorylation reactions mediated by cAMP-dependent protein kinases, but little is known about hormonal control of endogenous protein kinase activity (PK) in kidney. In the present study, we examined the effects of parathyroid hormone, glucagon, and isoproterenol on cAMP and PK in slices of rat renal cortex. In the presence of 0.5 mM 1-methyl, 3-isobutyl xanthine, all three hormones activated PK in slices, as reflected by an increase in the ratio of enzyme activity assayable in homogenates of the slices without addition of cAMP to the kinase reaction mixture (cAMP-independent activity) over total enzyme activity (+2 \(\mu \)M cAMP in the reaction mixture). When enzyme activity was assayed in whole homogenates prepared from slices, the increase in the enzyme activity ratio (-cAMP/ +cAMP) which followed hormonal stimulation was due entirely to an increase in cAMP-independent activity, with no change in total activity. In general, a good correlation existed between the alterations in tissue cAMP levels mediated by the hormones and/or 1-methyl, 3-isobutyl xanthine and concomitant alterations in PK. All three hormones increased PK activity ratios to near unity, suggesting complete enzyme activation. However, the concentrations of parathyroid hormone and glucagon which produced maximal activation of PK were much lower than those required for maximal cAMP responses. Studies with charcoal indicated that these hormonal actions on PK reflected intracellular events rather than representing activation of the

enzyme during tissue homogenization, due to release of sequestered cAMP. Thus, homogenization of tissue in charcoal prevented activation of PK by subsequent addition of exogenous cAMP, but did not lower enzyme activity ratios in homogenates of hormone-stimulated cortical slices.

When PK was determined in the 20,000 g supernatant fraction of renal cortical slices incubated with the hormones, enzyme activity ratios also increased, but total enzyme activity declined. Lost activity was recovered by extraction of particulate fractions with 500 mM KCl or NaCl, results which implied particulate binding of activated PK. Activated soluble PK from renal cortex was bound equally well by intact, heat- and trypsin-treated renal cortical pellets and by intact and heated hepatic pellets. Accordingly, the apparent translocation of enzyme in hormone stimulated cortex does not necessarily represent binding of the activated PK to specific acceptor sites in the particulate cell fractions or constitute a physiologic hormonal action. Activation of renal cortical PK by increasing concentrations of salts suggests that the enzyme in this tissue resembles the predominant type found in heart.

INTRODUCTION

The regulation of renal cyclic adenosine 3',5'-monophosphate (cAMP)¹ by hormones and other agents has been extensively examined. However, only limited data are

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Received for publication 9 December 1975 and in revised form 9 February 1976.

¹ Abbreviations used in this paper: C, catalytic subunit of protein kinase; cAMP, cyclic adenosine 3',5'-monophosphate; KRBG, Krebs Ringer bicarbonate buffer containing 1 mg/ml of glucose and albumin with 95% O₂ and 5% CO₂ serving as the gas phase. MIX, 1-methyl 3-isobutyl xanthine; PTH, parathyroid hormone; R, regulatory subunit of protein kinase.

available concerning hormonal modulation of cAMPdependent protein kinase activity in kidney (1, 2). Observations in other tissues suggest that many of the intracellular actions of cAMP may be expressed through phosphorylation reactions mediated by this enzyme system (3-7). In studies of purified preparations of protein kinase from several sources it has been shown that the enzyme consists of a cAMP-binding, regulatory (R) subunit and a cAMP-independent, catalytic (C) subunit (8-10). Dissociation of R from R-C in the presence of cAMP results in enhanced catalytic activity (10). Studies on a number of intact cell systems (7, 11-14) including human renal medulla (1) support this general mechanism of kinase activation. Exposure of some tissues to hormonal agonists of cAMP has been shown to increase the proportion of enzyme activity in the cAMP-independent form (1, 7, 11–14), when the latter is subsequently determined in tissue homogenates from the ratio of kinase activity found in the absence of exogenously added cAMP (cAMP-independent activity) to that observed in the presence of added cAMP (total activity). There has also been considerable interest in the fact that total soluble protein kinase activity declines significantly in tissue, including renal medulla (1), after exposure to hormonal agonists of cAMP (15-17). This enzyme activity can be recovered from particulate fractions of the hormone-stimulated tissues (15-17). It has been suggested that this change in subcellular distribution of kinase activity might represent specific intracellular translocation of the catalytic subunit to acceptor sites in the particulate fraction, and possibly to specific protein substrates for phosphorylation on the plasma membrane. However, recent observations in heart by Keely and co-workers (18) have raised questions concerning the biologic significance of the reductions in soluble kinase activity which follows hormonal stimulation.

In the present study, we examined the effects of hormonal agonists of cAMP on cAMP-dependent protein kinase activity in slices of rat renal cortex. The specificity of the apparent intracellular translocation of kinase activity after hormonal stimulation of this tissue was evaluated. The effects of parathyroid hormone (PTH), glucagon, and isoproterenol on cAMP-dependent kinase activity were compared, since these three agents appear to stimulate the adenylate cyclase-cAMP system of renal cortex through interactions with separate tissue receptor sites.

METHODS

Preparation of tissue. Male Sprague-Dawley rats (Zivic-Miller, Pittsburgh, Pa.) weighing 300-350 g were anesthetized with pentobarbital, 5 mg/g body weight i.p., after an 18 h fast. The kidneys were excised and immediately placed in 0.85% saline at 4°C. The renal capsule was removed

manually and slices of cortex, 40-70 mg, prepared with a Stadie-Riggs microtome. A single slice was employed in each incubation vessel.

To assess tissue cAMP content and protein kinase activity, slices were initially incubated in a metabolic shaker in 25-ml flasks for 15 min at 37°C in 2 ml of Krebs Ringer bicarbonate buffer (pH 7.4) containing 1 mg/ml of glucose and bovine serum albumin (KRBG), with 95% O₂ and 5% CO₂ serving as the gas phase. Slices were then transferred to fresh buffer with or without 0.5 or 2 mM 1-methyl 3isobutyl xanthine (MIX) for a second 15 min incubation. Hormones were then added to appropriate flasks and the incubation routinely continued for 5 min, since preliminary data indicated peak cAMP responses occurred by this time (not shown). We found the initial incubation helpful in stabilizing basal protein kinase activity ratios at a low value. At the conclusion of the second incubation some slices were extracted for cAMP content by homogenization of slices in 0.5 ml of hot sodium acetate buffer (50 mM, pH 4.0), containing 2,000 cpm of [3H]cAMP to assess recovery. These homogenates were then heated for 10 min. The cAMP content of duplicate aliquots of appropriate dilutions of the supernatant fractions of the homogenates was then determined directly by the protein-binding method, as previously described (19). Authenticity of the cAMP determinations was confirmed by the linearity of sample dilutions, destruction of assayed cAMP by prior treatment of the extract with excess cyclic phosphodiesterase, and quantitative recovery of exogenously added cAMP (not shown). Other slices were homogenized at 4°C in 5 mM KH2PO4 buffer (pH 6.8) containing 2 mM EDTA and 0.5 mM MIX (50 mg tissue/ml buffer) for subsequent assay of protein kinase. After appropriate dilution and/or centrifugation of these homogenates at 20,000 g, enzyme activity was determined in the whole homogenates and the supernatant fractions as described below. Tissue was kept at 0-4°C until assay.

To assess the specificity of binding of activated soluble protein kinase activity from renal cortex to particulate cell fractions (see Table IV), the 20,000 g supernatant and particulate fractions were prepared from three separate rat renal cortical and hepatic homogenates (100 mg of tissue/ 6 ml 5 mM KH₂PO₄ buffer containing 2 mM EDTA and 0.5 mM MIX). Particulate material was resuspended in the original volume of the phosphate buffer. A 1.5 ml-aliquot was maintained at 4°C (intact pellets), while other 1.5 ml aliquots were heated for 5 min at 95°C or incubated for 15 min at 37°C with 150 µg of trypsin. At the conclusion of the latter incubations, trypsin was inactivated by addition of 300 µg of soybean antitrypsin. The intact or treated particulate preparations were then centrifuged and resuspended in 1.5 ml of the original renal cortical supernatant fractions, to which 2 µM cAMP was added. Control supernates received 2 µM cAMP alone without particulate material. Preparations were again centrifuged at 20,000 g and the kinase activity remaining in the supernatant fraction assayed in the presence of cAMP. The pellets obtained from the second centrifugation were resuspended in phosphate buffer containing 500 mM KCl and centrifuged at 20,-000 q for extraction and assay of bound kinase activity. Enzyme activity bound to the original 20,000 g renal cortical pellet was also extracted by resuspension in KCl.

Assay of cAMP-dependent protein kinase activity. The reaction mixture for the assay of protein kinase was as described by Corbin and Reimann (20). It contained at final concentrations 12 mM KH₂PO₁ (pH 6.8) 0.05 M NaF, 4 mM MgCl₂, 0.23 mM [α -³²P]ATP (2 × 10⁶ cpm), 0.5 mg of histone and, when present, 2 μ M cAMP. The reaction was

initiated by the addition of 20 μ l of an appropriate dilution of a renal cortical homogenate (16 µg of protein) or the 20,000 g supernatant fraction, (8 μ g of protein) to 50 μ l of the reaction mixture. After a 5-min incubation at 30°C, a 50-µl aliquot of the mixture was precipitated on a filter paper square (Whatman 31, 2×2 cm, Whatman, Inc., Clifton, N. J.) by immersion in 10% trichloroacetic acid kept at 0-4°C. The filter papers were then washed with four additional changes of 10% trichloroacetic acid for 15 min each with agitation at room temperature, and finally with 95% ethanol and ether for 5 min each. Filter papers were air dried before counting. The blank counting rate, obtained by substituting buffer for tissue, was less than 10 pmol of ³²P. Under the conditions of the assay, phosphorylation of endogenous substrate present in renal cortical homogenates was negligible when histone was omitted from the reaction mixture.

In the absence of NaF, the reaction was not linear with respect to time when either whole homogenates or 20.000 a supernatant fractions were used. This was shown to be due to the breakdown of ATP by endogenous phosphatases. The ATP content of standard reaction mixtures was measured after a 5-min incubation at 30°C. The reaction was stopped by the addition of HClO4 to a final concentration of 5% and ATP determined in neutralized extracts by the luciferin-luciferase procedure as previously described (21). Residual ATP was zero and 60% of control (no tissue) when whole homogenates or 20,000 g supernatant fractions were assayed in the absence of NaF. The inclusion of NaF (0.02-0.2 M) in the reaction mixtures resulted in significant ATP sparing. In the presence of 0.05 M NaF, the ATP content increased to 85% of control when either whole renal cortical homogenates or 20,000 g supernates were used. It should be emphasized that concentrations of NaF from 0.02 to 0.05 M gave similar protein kinase activities, although the ATP sparing effect was greatest with the latter concentration. However, at NaF concentrations of 0.1 M or above, protein kinase activity was inhibited by 90%. Under the standard assay conditions and in the presence of 0.05 M NaF, the reaction was linear with respect to time for 7 min and added protein from 10 to 40 μ g for whole homogenates and 5-20 μ g for 20,000 g supernatant fractions.

The specificity of the assay for cAMP-dependent protein kinase in whole homogenates of renal cortex was tested with a crude preparation of protein kinase inhibitor, isolated from beef cardiac muscle by the procedure described by Gilman (22). Under the standard assay conditions, approximately 80% of the total activity measured in the presence of cAMP was blocked by the addition of 50 μ g of the protein kinase inhibitor preparation to the kinase reaction mixture. Kinase activity is expressed as pmol ³²P incorporated into histone/min per mg protein. Activity ratios were calculated from the rates obtained in the absence to those obtained in the presence of 2 μ M cAMP in the reaction mixture.

Protein was determined by the Lowry et al. method (23). The significance of statistical differences between mean values of various parameters were analyzed using Student's t test for unpaired values. Results from a single representative experiment, repeated at least twice, are shown.

Materials. PTH (sp act 218 U/mg), isoproterenol, propranolol, activated neutral charcoal, calf thymus histone (Type II-A), trypsin, and soybean anti-trypsin were purchased from Sigma Chemical Co., St. Louis, Mo. Crystalline porcine glucagon (<0.008% insulin) was generously provided by Eli Lilly and Co., Indianapolis, Ind. MIX was obtained from Aldrich Chem. Co., Milwaukee, Wis.

[γ-32P]ATP (sp act, 3-10 Ci/mmol) and [3H]cAMP (sp act, 38.4 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. Whatman 31 filter paper was obtained from Fisher Scientific Co., Pittsburgh, Pa. Sources of all other materials have been previously described (19, 21).

RESULTS

Table I compares the effects of several agonists of cAMP in renal cortex on tissue protein kinase activity. In the absence of MIX in the incubation media, significant increases over control in tissue cAMP content (2.6fold), cAMP-independent kinase activity (1.8-fold), and kinase activity ratio (2-fold) were observed in response to 5 U/ml PTH, but not 0.01 mM glucagon or 0.1 mM isoproterenol. Incubation of slices with 2 mM MIX alone for 15 min increased cAMP 3.8-fold over control levels to 2.83±SE 0.2 pmol/mg wet weight. With 2 mM MIX, maximal stimulatory concentrations of PTH (5 U/ml), glucagon (0.01 mM), or isoproterenol (0.1 mM) further increased cAMP to 89.1±7.7, 27.5±2.3, and 21.0±1.9, respectively, and raised the protein kinase activity ratio to near unity in each instance (not shown). However, since 2 mM MIX alone increased the ratio from 0.29 ± 0.03 to 0.83 ± 0.07 , the measurable effects of the hormones on kinase activity were small under these conditions. By contrast, incubation of slices for 15 min with 0.5 mM MIX produced much smaller, though significant, increases in basal tissue cAMP content and the protein kinase activity ratio (0.46 ± 0.04) . Further, this lower concentration of MIX was effective in potentiating hormonal effects on both tissue cAMP accumulation and kinase activity. Thus, addition of 5 U/ml of PTH, 0.01 mM glucagon, or 0.1 mM isoproterenol to the incubates for the final 5 min of the 15 min incubations with 0.5 mM MIX, all increased tissue cAMP at least fivefold over levels seen with 0.5 mM MIX alone, and raised the kinase activity ratio to approximately unity in each instance. The latter change was due entirely to an increase in the proportion of kinase activity in the cAMP-independent form, when examined in whole homogenates prepared from slices which had been exposed to the hormones. Total (+ cAMP) kinase activity was similar in whole homogenates of slices incubated in either the presence or absence of hormones. An enzyme activity ratio of approximately one, due exclusively to an increase in cAMP-independent activity, would suggest maximal endogenous activation of protein kinase by the hormones. As also shown in Table I, addition of 0.5 mM propranolol to the slice incubates 30 s before the addition of isoproterenol blocked increases in both renal cortical cAMP accumulation and cAMPindependent protein kinase activity mediated by the latter agent. Propranolol did not alter cAMP or protein kinase responses to PTH or glucagon (not shown).

Table I

Effects of Agonists of cAMP on the Protein Kinase Activity of Renal Cortical Slices

	cAMP	Protein kinase activity		
Test agent		-cAMP	+cAMP	-cAMP +cAMP
	pmol/mg wet weight	pmol ¹² P inc/min per mg protein		mg protein
None	0.72 ± 0.06	106 ± 10	354 ± 22	0.29 ± 0.03
PTH, 5 U/ml	$1.89 \pm 0.13*$	$200 \pm 17*$	326 ± 24	$0.61 \pm 0.05*$
Glucagon, 0.01 mM	0.86 ± 0.06	138 ± 12	340 ± 25	0.40 ± 0.04
Isoproterenol, 0.1 mM	0.91 ± 0.08	127 ± 11	343 ± 21	0.37 ± 0.04
MIX, 2 mM	$2.83 \pm 0.21*$	$303 \pm 22*$	361 ± 29	$0.83 \pm 0.07*$
MIX, 0.5 mM	$1.35\pm0.10*$	$164 \pm 14*$	357 ± 27	$0.46\pm0.04*$
+PTH	$15.7 \pm 1.12 \ddagger$	$315 \pm 26 \ddagger$	332 ± 25	$0.95 \pm 0.04 \ddagger$
+Glucagon	$7.94 \pm 0.68 \ddagger$	$322 \pm 29 \ddagger$	322 ± 30	$0.99 \pm 0.08 \ddagger$
+Isoproterenol	$6.62 \pm 0.55 \ddagger$	$312 \pm 27 \ddagger$	339 ± 26	$0.92 \pm 0.06 \ddagger$
+Isoproterenol and propranolol	1.34 ± 0.12 §	163 ± 16 §	376 ± 33	0.49 ± 0.05 §

All slices were initially incubated for 15 min at 37°C in KRBG without MIX and then transferred for a second 15 min incubation to the same buffer, or to buffer containing 0.5 or 2 mM MIX, as indicated. Hormones and propranolol (0.5 mM) were present for only the final 5 min of the second incubation. At the conclusion of this incubation, the slices were either extracted for cAMP content or homogenized at 4°C in the standard 5 mM KH₂PO₄ buffer (pH 6.8) for subsequent assay of protein kinase activity. The latter was determined both in the absence and the presence of 2 μ M cAMP in the kinase reaction mixtures, using whole homogenates of the tissue. Values represent means±SE of determinations from four slices for each condition from the same experiment.

Table II summarizes alterations in renal cortical slice cAMP content and protein kinase activity ratios in response to various concentrations of PTH and glucagon, examined in the presence of 0.5 mM MIX. Concentrations of the hormones which mediated small but significant increases in tissue cAMP content under these incubation conditions concomitantly increased the kinase activity ratio. However, concentrations of PTH (5 U/ml) or glucagon (0.01 mM) which mediated maximal increases in tissue cAMP content were not required to produce maximal activation of endogenous protein kinase, as reflected by activity ratios of approximately one. The latter occurred in response to 50 mU/ml of PTH and 50 nM glucagon, concentrations of hormones which were submaximal relative to effects on tissue cAMP. A similar relationship was observed with isoproterenol + 0.5 mM MIX, where 1 μ M of the hormone produced a submaximal increase in cAMP (1.25±0.10 to 4.58±0.51 pmol/mg wet weight; maximal increase, 6.91 ± 0.74 in response to 0.1 mM isoproterenol), but raised the enzyme activity ratio to near unity. A temporal correlation between the actions of PTH on cAMP and protein kinase activity was also evident. Significant increases in both cAMP (2.5-fold over the 0.5 mM MIX

control) and the kinase activity ratio (0.41 ± 0.04) to (0.68 ± 0.07) were detectable with PTH (5 U/ml) at 30 s, the earliest time point examined. Peak increases in the enzyme activity ratio (0.96 ± 0.11) occurred at 2 min and preceded peak increases in cAMP (12-fold rise over 0.5 mM MIX alone at 5 min). However, with 5 U/ml PTH, cAMP had increased 6.7-fold over control to 8.94 ± 0.96 pmol/mg wet weight by 2 min, a change in cAMP of sufficient magnitude to activate protein kinase completely (Tables I and II).

Protein kinase activity and enzyme activity ratios were also determined in the 20,000~g supernatant fractions of slices which had been incubated with or without PTH, glucagon (Table III) or isoproterenol (not shown), under conditions identical to those described in Table I. Kinase activity ratios approached unity when assayed in the 20,000~g supernatant fractions prepared from slices which had been incubated with the hormones. However, the latter change reflected both a substantial decrease in total (+ cAMP) kinase activity in the supernatant tissue fraction, and some increase in cAMP-independent activity after hormone stimulation. As also shown in Table III, homogenization of hormone-stimulated slices in 5 mM phosphate buffer (pH 6.8) con-

^{*} P < 0.01 vs. slices incubated without MIX.

 $[\]ddagger P < 0.005 \text{ vs. } 0.5 \text{ mM MIX alone.}$

[§] P < 0.001 vs. isoproterenol alone.

TABLE II

Effects of Various Concentrations of PTH and Glucagon on the cAMP Content and Protein Kinase Activity

Ratio of Renal Cortical Slices

Test agent	Concentration	cAMP	Kinase activity ratio -cAMP/ +cAMP
	mU/ml	pmol/mg wet weight	
Experiment I			
None	_	1.56 ± 0.12	0.46 ± 0.04
PTH	1	1.64 ± 0.11	0.55 ± 0.05
	5	$2.18 \pm 0.17*$	0.71 ± 0.07
	10	$3.40\pm0.31*$	0.86 ± 0.09
	50	$6.19 \pm 0.56*$	$0.98\pm0.11^{\circ}$
	500	$12.53 \pm 1.14*$	$0.95\pm0.10^{\circ}$
	5,000	19.72 ±2.27*	$0.97\pm0.12^{\circ}$
	M		
Experiment II			
None		1.39 ± 0.10	0.38 ± 0.04
Glucagon	1 n M	1.60 ± 0.14	0.49 ± 0.05
	5 n M	$2.27 \pm 0.18*$	0.65 ± 0.06
	10 n M	$3.25 \pm 0.26*$	0.82 ± 0.08
	50 n M	$5.64 \pm 0.42*$	0.99 ± 0.12
	0.1 μ M	$6.73 \pm 0.59*$	0.96 ± 0.10
	10 μ M	$9.21 \pm 0.85*$	0.93 ± 0.11

Slices were initially incubated for 15 min at 37 °C in KRBG without MIX and then transferred for a final 15 min incubation to buffer containing 0.5 mM MIX. Hormones were present for only the final 5 min of the second incubation. At the conclusion of the second incubation, slices were either extracted for cAMP content or homogenized at 4°C in the standard 5 mM KH₂PO₄ buffer for assay of protein kinase activity in the absence and presence of cAMP in the enzyme reaction mixture. Enzyme activity was assayed using whole homogenates of the tissue. Values represent means ±SE of determinations from five slices for each condition from the two experiments.

taining 125 mM KCl was effective in preventing much of the loss of total kinase activity from the supernatant fraction. Addition of higher concentrations of KCl (150–500 mM) to the homogenizing buffer resulted in even greater recovery of soluble kinase activity from slices which had been incubated with PTH (Fig. 1). However, homogenization of tissue in buffer containing KCl concentrations above 125 mM also increased the kinase activity ratio of control slices, and thus prevented evaluation of hormonal effects on this parameter. The changes in total protein kinase activity and enzyme activity ratios in the supernatant fractions from PTH-stimulated and control slices were identical when various concentrations of NaCl were substituted for KCl in the homogenizing buffer (not shown).

As indicated in Table IV, loss of protein kinase activity from the supernatant fraction of tissue homogenates was the result of its binding to the particulate fraction. Thus, when the 20,000~g pellet from a control renal cortical homogenate was resuspended in the supernatant fraction from the same tissue preparation in

the presence of 2 µM cAMP and the particulate material again sedimented at 20,000 q total (+ cAMP) protein kinase activity in the supernatant fraction fell by 49%. The enzyme activity lost from the supernatant fraction could subsequently be recovered from the resultant 20,000 g pellet by resuspension of the latter in phosphate buffer containing 500 mM KCl (Table IV). Reduction in kinase activity in the supernatant fraction of renal cortex required the combined addition of both a particulate cell fraction and exogenous cAMP, since readdition of either the 20,000 g pellet alone (not shown) or cAMP alone (Table IV) did not detectably diminish total protein kinase activity in the supernate. The latter results implied that the moiety binding to the particulate material was the cAMP-independent form of the enzyme (catalytic subunit). As also shown in Table IV, approximately 10% of the total protein kinase activity assayable in whole homogenates of control renal cortex (i.e. no prior hormone exposure or addition of exogenous cAMP) could be extracted with 500 mM KCl from the original 20,-000 g pellet prepared from this tissue. Thus, in unstimulated renal cortex, a small proportion of total assayable kinase activity is found reversibly bound to the particulate cell fraction.

Reduction in the soluble protein kinase activity of renal cortical homogenates was clearly not dependent upon the specific readdition of intact particulate material from this same tissue (Table IV). In the presence of

TABLE III

Effects of Hormonal Stimulation of Renal Cortical Slices on
Soluble Protein Kinase Activity and Activity Ratios

	Protein kinase activity			
Test agent	-cAMP	+cAMP	-cAMP +cAMP	
	pmol 32P inc/min per mg protein			
	I	Phosphate buffer		
None	266 ± 18	701 ± 30	0.38 ± 0.03	
PTH, 5 U/ml	$393 \pm 35*$	$402 \pm 34*$	$0.98 \pm 0.10^{\circ}$	
Glucagon, 0.01 mM	395 ±32*	419±37*	0.94 ± 0.08	
	Phosphat	Phosphate buffer + 125 mM KCl		
None	341 ±29‡	773±52	0.44 ± 0.04	
PTH	588 ±42*‡	596 ±46*‡	$0.97 \pm 0.10^{\circ}$	
Glucagon	$602 \pm 39*1$	608 ±49*‡	$0.99\pm0.09^{\circ}$	

Slices were incubated in the presence or absence of hormones as described in Table II, and homogenized at 4°C in the standard 5 mM KH₂PO₄ buffer or in buffer containing 125 mM KCl. The 20,000 g supernatant fractions of these tissue homogenates were than assayed for protein kinase activity in the presence and absence of cAMP in the kinase reaction mixture. Values represent the means±SE of five slices for each condition from the same experiment.

^{*} P at least <0.02 vs. control slices from the same experiment.

^{*}P at least <0.05 compared to values from slices incubated without test agents and homogenized in corresponding buffer.

 $[\] P<0.01$ compared to corresponding values from slices incubated under identical conditions but homogenized in 5 mM KH_2PO_4 without added KCl.

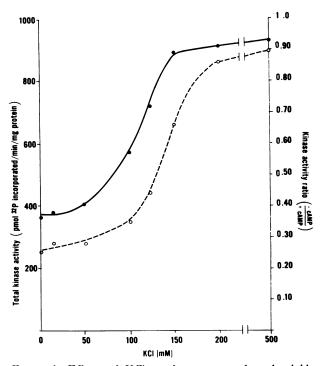


FIGURE 1 Effects of KCl on the recovery of total soluble protein kinase activity from PTH-stimulated renal cortical slices (•—••) and the enzyme activity ratios of control slices (O---•O). Slices were incubated with or without PTH as described in Table II and then homogenized in 5 mM KH₂PO₄ buffer (pH 6.8) containing the increasing concentrations of KCl shown. Kinase activity of the 20,000 g supernatant fractions of the homogenates was then determined in the presence and absence of 2 μ M cAMP in the kinase reaction mixture. For PTH-stimulated tissue, total assayable enzyme activities (i.e., +cAMP in the kinase reaction mixture) are shown for the supernatant fraction, while for control tissue kinase activity ratios are shown. Values represent means of determinations from three slices for each condition from the same experiment.

cAMP, addition of (a) intact 20,000 g pellets prepared from hepatic homogenates, (b) heat-denatured hepatic or renal cortical pellets, or (c) trypsin-treated pellets from renal cortex all produced a reduction in soluble protein kinase activity which was comparable to that observed upon readdition of the intact pellet from renal cortex. Further, the enzyme activity lost from the supernatant fraction was extracted from each of these particulate preparations by resuspension in 500 mM KCl (Table IV). Extraction of particulate fractions with 125 mM KCl resulted in recovery of most of the activity lost from the supernatant, but was less effective than 500 mM KCl (not shown).

Activation of protein kinase in renal cortical slices incubated with hormonal agonists of cAMP could reflect an intracellular event, or alternatively could have taken place after homogenization of the tissue due to

release of the excess cAMP which had been generated in response to the hormones. To separate these two possibilities, some control and hormone-stimulated slices were homogenized in phosphate buffer containing 10 mg/ml of neutralized, activated charcoal and 125 mM KCl (final pH 6.8), in accordance with procedures previously outlined by Corbin et al., (24). Exogenous [3 H] cAMP (0.1 μ M) was then added to a portion of some of the tissue homogenates and the protein kinase activity of all samples assayed in the 20,000 g supernate in both the presence and absence of cAMP in the reaction mixture. Charcoal completely prevented the activation of protein kinase mediated by the addition of exogenous cAMP to the tissue after homogenization (Table V). As reflected by the total disappearance of [*H] cAMP from the supernate (not shown), the added cAMP was probably bound to the charcoal. By contrast, the presence of charcoal in the tissue homogenizing buffer did not alter the increase in kinase activity ratios seen in homogenates prepared from renal cortical slices which had been incubated with PTH, glucagon (Table V), or isoproterenol (not shown). Thus, the effects of the hormones on protein kinase could not be attributed to activation of the enzyme by cAMP released after tissue homogenization, since the latter would have been abolished by the presence of charcoal. The

TABLE IV

Specificity of the Binding of Soluble Protein Kinase Activity
from Renal Cortex to Particulate Cell Fractions
in the Presence of cAMP

	Total protein kinase activity in fractions	
Cell fractions combined	Residual activity in the supernate	Activity extracted from the pellets with KCl
	pmol 32P	inc/min
Control supernate	$1,038 \pm 77$	
Original pellet	_	123 ± 10
Supernatant + intact pellet	534 ±45*	$726 \pm 63 \ddagger$
Supernatant + heated pellet	459 ±41*	654 ± 59 ‡
Supernatant + trypsin-treated pellet	$450 \pm 54*$	$638 \pm 60 \ddagger$
Supernatant + intact liver pellet	519±57*	732 ± 78 ‡
Supernatant + heated liver pellet	$496 \pm 50*$	579 ± 581

Total kinase activity (+2 μ M cAMP) remaining in the supernatant fractions of renal cortex or extracted from tissue pellets with 500 mM KCl is shown. The tissue fractions were treated as described in the text. Values represent means \pm SE of determinations from three separate supernates or pellet extracts for each condition from the same experiment. Mean total kinase activity present in the original whole homogenates of renal cortex was 1,365 \pm 117 pmol ³²P/min. Mean total enzyme activity of the original supernates before addition of cAMP and/or the pellets was 1,071 \pm 93. All enzyme activities were finally assayed in the presence of 2 μ M cAMP in the kinase reaction mixture.

^{*} P < 0.01 compared to control supernate.

 $[\]ddagger P < 0.01$ compared to original pellet.

TABLE V

Effects of Charcoal on Alterations in the Protein Kinase Activity Ratios Mediated by Incubation of Slices with PTH or Addition of cAMP to Tissue Homogenates

Test agent in slice incubate	Tissue homogenate		Kinase activity ratio
	Charcoal	Exogenous cAMP	(-cAMP/ +cAMP)
None	_	_	0.41±0.04
	_	+	$0.88 \pm 0.08*$
None	+	_	0.32 ± 0.04
	+	+	0.36 ± 0.041
PTH, $5 U/ml$	-	_	0.94 ± 0.10
PTH	+	_	0.86 ± 0.09
Glucagon, 0.01 mM	_	_	0.97 ± 0.11
Glucagon	+	_	0.90 ± 0.09

Slices were incubated with or without hormone as described in Table II and homogenized in 5 mM KH₂PO₄ buffer containing 125 mM KCl, in the presence or absence of 10 mg charcoal/ml of buffer as indicated. After this homogenization, [*H] cAMP was added to an aliquot of the homogenate (final concentration 0.1 μ M) prepared from each slice which had not been exposed to hormone. All homogenates were centrifuged at 20,000 g and protein kinase activity of the supernatant fraction assayed in the absence and presence of 2 μ m cAMP in the kinase reaction mixture. Each value represents the mean \pm SE of the kinase activity ratio (-cAMP/+cAMP in the reaction mixture) determined from five slices in the same experiment. *P < 0.001 compared to corresponding values without added exogenous cAMP.

 $\ \ P < 0.001$ compared to corresponding slices not homogenized in charcoal, but with added exogenous cAMP.

ability of charcoal to prevent activation of kinase by exogenous cAMP which was added to renal cortical homogenates after homogenization, and its inability to reverse hormone-induced increases in the activity ratio in cortex are analogous to its effects in epinephrine-stimulated cardiac muscle (24). Prior addition of excess charcoal presumably prevents the effects of exogenous cAMP by binding the nucleotide more rapidly than the R subunit of the kinase. Conversely, the failure of charcoal to reverse hormone-induced activation of the enzyme in renal cortex and heart implies that the affinity of the R subunit for cAMP is much greater than that of charcoal.

DISCUSSION

Kuo and co-workers (25) and Abou-Issa, et al. (26) have described the properties of partially purified cAMP-dependent protein kinases prepared from homogenates of whole bovine or swine kidneys. These properties, including the Km of the renal enzymes for ATP or cAMP, the optimal protein substrate or divalent cation cofactor and the effects of cAMP analogs, were quite similar to those of similarly purified kinases derived from several other tissues (25). However, specific hormonal modulation of cAMP-dependent protein kinase in intact renal cortex has not previously been reported.

The results of the present study demonstrate that changes in the cAMP content of cortex in response to PTH, glucagon, isoproterenol, and/or MIX correlate well with concomitant alterations in endogenous protein kinase activity. The concentrations of PTH, glucagon, or isoproterenol which produced maximal endogenous activation of protein kinase, as reflected by an enzyme activity ratio that approximated unity, were much lower than those required for maximum stimulation of cAMP. These observations imply that the marked increase in tissue cAMP that can be elicited in response to high concentrations of hormone in the presence of an inhibitor of phosphodiesterase activity are not required for complete activation of protein kinase. Analogous findings were reported in thyroid, where maximum kinase activity ratios were achieved with concentrations of thyroid-stimulating hormone below those which maximally increased cAMP (14). Glucagon and isoproterenol were much less potent agonists of cAMP in cortex than PTH and each hormone appears to stimulate the adenylate cyclase-cAMP system of kidney through an interaction with a separate tissue receptor site (27-29). However, the ability of all three agents to activate protein kinase maximally in cortex suggests that each hormone stimulates a large proportion of the cells in the slice preparation. A common subpopulation of target cells in cortex for these three hormones is also implied by the previous findings that each agent enhances cortical gluconeogenesis (30, 31) and inhibits proximal tubular reabsorption of sodium and phosphate (32-35) by cAMPmediated mechanisms. Release of excess cAMP from a small subpopulation of stimulated cortical cells during tissue homogenization might also account for complete activation of protein kinase by each hormone. However, studies with charcoal indicate that hormone-mediated increases in kinase activity in cortex reflect intracellular events and not changes that occur after cell disruption (Table V).

Since there is considerable evidence to suggest that many of the intracellular actions of cAMP in mammalian tissues are expressed through phosphorylation reactions mediated by protein kinases, the demonstration that several hormonal agonists of cAMP in renal cortex can also modulate endogenous protein kinase activity is of potential significance in further elucidating the mechanism of action of these hormones in kidney. In this regard, it is of interest that Ausiello and co-workers have recently reported that both PTH and cAMP can mediate the phosphorylation of a number of endogenous proteins in renal cortex (2). It seems likely that these effects are expressed through stimulation of cAMP-dependent protein kinase activity. However, the precise relationship between such hormonally induced phosphorylation reactions and the final expression of the physiologic action of the hormones in cortex remains unclear, since the specific endogenous substrate for the enzyme in this tissue is unknown. Both in kidney and in other tissues (1, 16-18) considerable significance has been attached to the apparent shift in subcellular distribution of protein kinase activity from the cytosol to the particulate fraction after hormonal stimulation of intact cells or the addition of exogenous cAMP to tissue homogenates. It has been suggested that this translocation of activity might reflect binding of the catalytic unit of the enzyme to specific substrates for phosphorylation on the plasma membranes, nucleus, or other insoluble cell components. Although membrane phosphorylation is a potentially attractive hypothesis to explain the physiologic action of hormones such as vasopressin (1) or PTH, our results in cortex would indicate that considerable caution is warranted in attributing biologic significance to the particulate binding of the cAMP-independent form of protein kinase. Not only was this binding largely reversible with salt solutions of physiologic ionic strength in cortex, but it also lacked specificity. Thus, soluble cortical protein kinase bound equally well to intact, heat- and trypsin-treated renal particulate material from cortex and to intact and heat-treated particulate material from liver. Similar observations with regard to the lack of specificity of the binding of the catalytic subunit of heart protein kinase to a variety of particulate materials have recently been reported by Keely and co-workers (19). The physiologic significance of the small fraction of protein kinase activity which was reversibly bound to the particulate fraction of unstimulated cortex is unclear, but could simply reflect binding of a portion of the cAMP-independent activity normally present in whole homogenates of control tissue. Forte and co-workers (36) described cAMP-dependent protein kinase activity associated with plasma membranes prepared from hog renal cortex, while Shlatz, et al. found a cAMP-dependent self-phosphorylating system in the luminal plasma membranes from bovine cortical epithelial cells (37). Our data do not exclude the possibility that this component of total kinase activity might be involved in the expression of hormone actions in cortex. However, specific hormonal control of membrane-bound activity has not been demonstrated, and some authors have found that protein kinases isolated from kidney plasma membranes and other particulate components are unresponsive to exogenous cAMP (26).

The particulate binding of cAMP-independent protein kinase led to considerable underestimation of total enzyme activity when the latter was assayed in the supernatant fractions of homogenates prepared from slices of renal cortex that had been stimulated with hormones. Thus, when assessed in the supernatant fraction, increases in enzyme activity ratios in response to

hormonal stimulation were largely due to a fall in total soluble enzyme activity. As reported in other tissues (11, 14, 19), increasing the ionic strength of the homogenizing buffer prevented binding of soluble activity to particulate fractions. However, the use of this technique was limited in renal cortex by the fact that concentrations of salt which completely inhibited the particulate binding of kinase activity also increased the kinase activity ratio of control homogenates. The latter effect may well reflect dissociation of the R-C subunits of the enzyme in the presence of increasing salt concentrations. With regard to its activation by salt, renal cortical protein kinase resembles the predominant enzyme system found in heart and differs from that of adipose tissue. The former but not the latter is readily activated by high concentrations of salt (24).

ACKNOWLEDGMENTS

We are greatly indebted to Mr. Charles Pfender, Ms. Nancy Oakman, and Ms. April Dworetz for expert technical assistance and to Mrs. Joan Klinger for secretarial support.

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