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STUDIES OF KIDNEY PLASMA MEMBRANE ADENOSINE-3′,5′-MONOPHOSPHATE-DEPENDENT PROTEIN KINASE

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SUMMARY

Porcine kidney cortex was utilized for the preparation of plasma-membrane-enriched and soluble cytoplasmic (cytosol) fractions for the purpose of examining the relative properties of cyclic [^{3}H]AMP receptor and cyclic AMP-dependent protein kinase activities of these preparations. The affinity, specificity and reversibility of cyclic [^{3}H]AMP interaction with renal membrane and cytosol binding sites were indicative of physiological receptors.

Binding sites of cytosol and deoxycholate-solubilized membranes were half-saturated at approx. 50 nM and 100 nM cyclic [^{3}H]AMP. Native plasma membranes exhibited multiple binding sites which were not saturated up to 1 mM cyclic [^{3}H]-AMP. Modification of the cyclic phosphate configuration or 2′-hydroxyl of the ribose moiety of cyclic AMP produced a marked reduction in the effectiveness of the cyclic AMP analogue as a competitor with cyclic [^{3}H]AMP for renal receptors. The cyclic [^{3}H]AMP interaction with membrane and cytosol fractions was reversible and the rate and extent of dissociation of bound cyclic [^{3}H]AMP was temperature dependent. With the plasma-membrane preparation, dissociation of cyclic [^{3}H]AMP was enhanced by ATP or AMP.

Assay of both kidney subcellular fractions for protein kinase activity revealed that cyclic AMP enhanced the phosphorylation of protamine, lysine-rich and arginine-rich histones but not casein. The potency and efficacy of activation of renal membrane and cytosol protein kinase by cyclic AMP analogues such as N^6-butyryl-adenosine cyclic 3′,5′-monophosphate or N^6,O^2-dibutyryl-adenosine cyclic 3′,5′-monophosphate supported the observations on the effectiveness of cyclic AMP analogues as competitors with cyclic [^{3}H]AMP in competitive binding assays.

This study suggested that the membrane cyclic [^{3}H]AMP receptors may be closely associated with the membrane-bound catalytic moiety of the cyclic AMP-dependent protein kinase systems of porcine kidney.

INTRODUCTION

The existence and general properties of cyclic AMP-dependent protein kinase in many different species as well as in different cell types is well documented (for a

current review see ref. 1). Discovery of the involvement of protein phosphorylation as an underlying mechanism in the cellular mode of action of epinephrine [2] has ultimately led to the current level of understanding of the role of cyclic AMP-dependent protein kinase systems in hormone-mediated regulation mechanisms in a general sense. Soluble protein kinase enzymes are proposed to consist of a regulatory and catalytic subunit and the mechanism for cyclic AMP activation is considered to be mediated through binding of cyclic AMP to the regulatory (receptor) subunit producing dissociation of the regulatory subunit-cyclic AMP complex from the catalytic subunit [3, 4]. In preliminary reports [5, 6], we showed that porcine kidney plasma membranes exhibited both cyclic AMP-binding and cyclic AMP-dependent protein kinase properties. The existence of membrane-associated cyclic AMP-dependent protein kinase systems is supported by research with red cell ghosts [7] and membrane fractions of brain [8] and adrenal [9]. More recently, a report on the characteristics of porcine kidney kinase systems showed a soluble cyclic AMP-dependent protein kinase and a cyclic AMP-independent plasma-membrane protein kinase, but little attention was given in that study to the cyclic AMP-binding properties of the subcellular fractions [10].

The membrane-associated cyclic AMP-dependent protein kinase system was considered to be of very considerable interest since the plasma membrane of renal cells exhibits specific receptor properties for vasopressin [11] and thyrocalcitonin [12]; activation of renal membrane adenylate cyclase by parathyroid hormone [13, 14], vasopressin [13, 14], thyrocalcitonin [14] and β-adrenergic agents [14]; cyclic nucleotide phosphodiesterase activity [13]; and enzymatic properties which are apparently involved in the inactivation of parathyroid hormone (Forte, L. R., Chu, L. L. H., Hamilton, J. W., Anast, C. S. and Cohn, D. V., unpublished), vasopressin (Nardacc, N. I., Mukhopadhyay, S. and Campbell, B. J., personal communication) and thyrocalcitonin [12] at the level of the renal target cell.

The purpose of the present investigation was to compare the properties of soluble, cytoplasmic (cytosol) and plasma-membrane preparations, with respect to the cyclic AMP receptor and protein kinase activity of these subcellular fractions of porcine renal cotex.

EXPERIMENTAL PROCEDURES

Kidney subcellular preparations

Kidney plasma membranes were prepared from porcine kidney cortex and medulla by the method of Fitzpatrick et al., [15]. Kidneys were obtained from a local packing house within 60 min of slaughter and cooled on ice for transfer to the laboratory for use. Cortical tissue was dissected free from medulla prior to homogenization. The plasma-membrane-enriched preparation was suspended in 0.25 M sucrose/1 mM EDTA at a protein concentration of 5–10 mg/ml and then frozen and stored at -20 °C prior to use. Preparation of a soluble, cytoplasmic protein kinase from hog kidney was accomplished using the procedure described by Miyamoto et al. [16]. The purification of the soluble renal protein kinase (cytosol) preparation was carried through the $(NH_4)_2SO_4$ fractionation step which corresponds to Step 3 of this method [16]. This soluble protein kinase preparation was dissolved in 5 mM potassium phosphate buffer, pH 7.0, and then extensively dialyzed against the same buffer to remove resi-

dual $(NH_4)_2SO_4$. The preparation was then centrifuged to remove precipitated protein, frozen and stored at −20 °C. No loss of cyclic AMP-binding activity or protein kinase activity was observed in either the plasma membrane or the cytoplasmic protein kinase preparation when stored in this manner for a period up to 3 months.

Cyclic AMP-binding assay

Methodology employed for the determination of binding of cyclic AMP to the kidney receptor preparations was similar to that described by Gilman [17]. The incubation medium consisted of 40 mM Tris · HCl, pH 7.5, cyclic [^{3}H]AMP, specific activity 24.1 Ci/mmol (New England Nuclear) and approx. 50–100 μg "receptor" protein in a final volume of 50 μl. Most incubations were for 60 min at 0–2 °C in an ice bath, except for those studies designed to examine time and temperature parameters. At the end of the incubation, 2.0 ml 0.1 M Tris · HCl, pH 7.5, were added to the incubation tube, mixed and this solution was immediately filtered on 25-mm diameter nitrocellulose filters (Millipore Corporation, 0.45 μm pore size). The filters were washed with an additional 7.0 ml Tris · HCl buffer and filters were then transferred to counting vials. 10 ml of Brays [18] scintillation phosphor were added and tritium radioactivity measured in a Packard liquid scintillation spectrometer. Determination of cyclic [^{3}H]AMP binding to the filters was by incubation in the absence of binding protein and then filtering and washing as described above. The blank levels were approximately 0.05–0.1 % of the total cyclic [^{3}H]AMP in the incubation medium.

Protein kinase assay

Protein kinase activity of the plasma membrane and cytosol preparations was assayed by a method similar to that described by Rubin et al. [7]. The incubation medium consisted of 25 mM potassium phosphate buffer, pH 7.0, 5 mM Mg_2SO_4, 100 μg bovine serum albumin and 0.25 mM [γ^{32}P]ATP (50 000 cpm/nmol, New England Nuclear) in a final incubation volume of 100 μl. Incubation was for 5 min at 30 °C. At the end of the incubation, 0.5 ml trichloroacetic acid (10 %, w/v) was added and the denatured protein collected on nitrocellulose filters (Millipore Corporation, 0.45 μm pore size). The precipitate was then washed with 10 ml of 10 % trichloroacetic acid to remove acid-soluble ^{32}P. The filter was transferred to a counting vial and 10 ml Brays [18] scintillation phosphor added. ^{32}P radioactivity was measured by standard liquid scintillation methodology. Appropriate blanks with no protein kinase enzyme were employed and blank values were subtracted from the experimental values in all assays.

Adenylate cyclase assay

Adenylate cyclase activity of kidney preparations was assayed by the method of White and Zenser [19] as previously described [13] .This method employs [α-^{32}P]-ATP (ICN, Nuclear Division) as substrate. Protein was measured by the colorimetric method of Sutherland et al. [20] using bovine serum albumin as the reference standard.

Materials

All standard chemicals were of reagent grade and were used without further purification. Nucleotides were purchased from Sigma Chemical Company, St. Louis,

Mo., Calbiochem, La Jolla, Calif. and Plenum Scientific Research Inc., Hackensack, N. J. Sodium deoxycholate, Triton X-100, sodium dodecylsulfate, neutral alumina, Type WN-3, trypsin (bovine pancreas, Type III), protease (*Streptomyces griseus*, Type VI), trypsin inhibitor (soybean, Type 1-S), phospholipase C (*Clostridium welchii*, Type I), neuraminidase (*Clostridium perfringens*, Type V), *N*-ethylmaleimide, caffeine, theophylline, papaverine, protamine sulfate, Grade I, casein, lysine-rich histone, Type III, and arginine-rich histone, Type VIII were obtained from Sigma Chemical Company, St. Louis, Mo. Lubrol-WX was a gift from I.C.I. Organics, Inc., Providence, R.I.

RESULTS

In preliminary experiments, we verified that the tritium bound to both membrane and cytosol fractions was authentic cyclic [^{3}H]AMP by its elution characteristics using alumina chromatography [19]. The conditions of the cyclic [^{3}H]AMP-binding assays were examined with respect to time, temperature, protein concentration and pH. Maximum binding of cyclic [^{3}H]AMP was observed at 0 °C for 60 min over the pH range 6.0–8.5. Binding was proportional to protein concentration from about 20 to 200 μg protein in both the renal membrane and cytosol preparations. Therefore, these general conditions were employed for the subsequent experiments.

The renal membrane fraction was submitted to linear sucrose density-gradient centrifugation and it was found that the cyclic [^{3}H]AMP-binding activity cosedimented with the F^--activated adenylate cyclase (data not shown). Also the cyclic

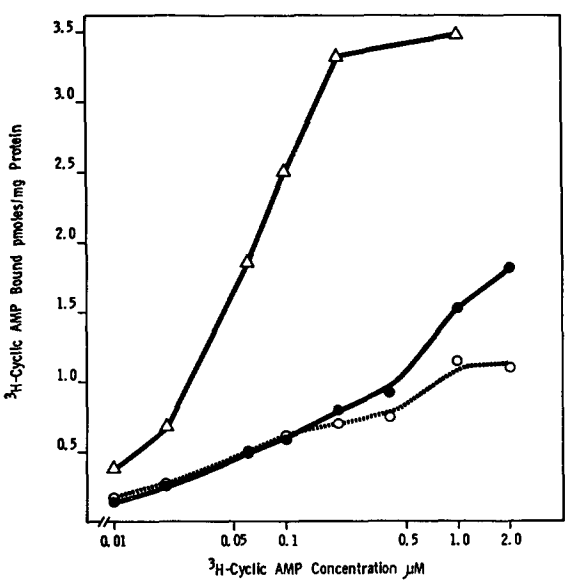

Fig. 1. Studies of the affinity of cyclic [^{3}H]AMP for renal membrane and cytosol receptors. Cyclic [^{3}H]AMP, 0.01–2.0 μM, was incubated with; ●—●, native plasma membranes, 95 μg; ○- - -○, deoxycholate-solubilized membranes, 130 μg; and △—△, cytosol, 100 μg for 60 min at 0 °C. Bound cyclic [^{3}H]AMP was determined by the standard filter procedure.

[^{3}H]AMP receptor of renal membranes could not be dissociated from the particulate membranes by various washing procedures but could be effectively solubilized with the detergents sodium deoxycholate, Triton X-100 and Lubrol-WX. Sodium dodecylsulfate solubilized membrane protein but destroyed the receptor activity.

The affinity of renal plasma membrane and cytosol fractions for cyclic [^{3}H]-AMP was examined by measuring the amount of cyclic [^{3}H]AMP bound at various concentrations of the cyclic nucleotide. Fig. 1 shows representative binding curves for cytosol, particulate membrane and deoxycholate-solubilized membranes. The cyclic AMP receptor sites of cytosol and deoxycholate-solubilized membranes were saturated at about 0.2 and 1.0 μM cyclic [^{3}H]AMP, respectively, whereas the particulate membrane preparation did not exhibit saturability over this concentration range. Half-maximal binding of cyclic [^{3}H]AMP to these receptors was approx. 50 nM for cytosol and 100 nM for deoxycholate solubilized membranes in these experiments. Additional experiments of this nature using concentrations of cyclic [^{3}H]AMP to 1.0 mM showed that the particulate plasma-membrane binding sites were not saturable. As previously reported [5], analysis of these binding curves by the procedure of Scatchard et al. [21] revealed that the cytosol fraction contained a single set of receptors whereas the curve for cyclic [^{3}H]AMP binding to native membranes was curvilinear suggesting multiple binding sites. Deoxycholate-solubilized membranes,

TABLE I

EXAMINATION OF THE SPECIFICITY OF THE CYCLIC [^{3}H]AMP RECEPTORS OF PORCINE KIDNEY CORTEX

Binding of cyclic [^{3}H]AMP, 0.2 μM, was measured at 0 °C for 60 min in the absence and presence of various potential competitors, over a range of competitor concentration from 0.1 μM to 10 mM.

Competitor	Competitor effectiveness ratio*	
	Plasma membrane	Cytosol
8-Bromo-adenosine cyclic 3′,5′-monophosphate	4	4
N^6-butyryl-adenosine cyclic 3′,5′-monophosphate	7	10
Inosine cyclic 3′,5′-monophosphate	20	2 500
O^2-butyryl-adenosine cyclic 3′,5′-monophosphate	500	300
N^6-O^2-dibutyryl-adenosine cyclic 3′,5′-monophosphate	500	3 000
2′-Deoxyadenosine cyclic 3′,5′-monophosphate	600	100
Guanosine cyclic 3′,5′-monophosphate	400	5 000
Adenosine cyclic 2′,3′-monophosphate	2 000	10 000
Uridine cyclic 2′,3′-monophosphate	10 000	50 000
Adenosine 5′-triphosphate	10 000	5 000
Adenosine 5′-monophosphate	10 000	5 000
Theophylline	No reduction	No reduction
Caffeine	No reduction	No reduction
Papaverine	No reduction	No reduction

* Competitor effectiveness ratio is the molar ratio of competitor to cyclic [^{3}H]AMP which reduced cyclic [^{3}H]AMP binding by 50 % divided by the molar ratio of cyclic AMP to cyclic [^{3}H]AMP which reduced cyclic [^{3}H]AMP binding by 50 %. The molar ratio of cyclic AMP to cyclic [^{3}H]AMP for 50 % reduction of bound cyclic [^{3}H]AMP in these experiments was about 1 for cytosol and 4 for membrane receptors.

however, showed a single set of binding sites which suggests that the low-affinity binding of cyclic [^{3}H]AMP to native membranes may be related to the vesicular nature of the plasma-membrane particles. It is possible that this binding is a reflection of uptake of cyclic [^{3}H]AMP into particulate membrane vesicles.

Specificity of the cyclic AMP-binding sites of renal plasma membrane and cytosol was examined using competitive binding experiments. A fixed concentration of cyclic [^{3}H]AMP (0.2 μM) and a range of concentrations of competing nucleotides and phosphodiesterase inhibitors were utilized in these experiments, which are summarized in Table I. The ratio of competitor to unlabeled cyclic AMP which reduced cyclic [^{3}H]AMP binding by one-half was used as an index of competitor effectiveness for displacement of cyclic [^{3}H]AMP. Cyclic AMP was the most effective competitor and a ratio of cyclic AMP:cyclic [^{3}H]AMP of 1 and 4 was found for renal cytosol and plasma membranes, respectively. Of the analogues of cyclic AMP examined, inosine cyclic 3',5'-monophosphate, guanosine cyclic 3',5'-monophosphate, and N^6,O^2-dibutyryl-adenosine cyclic 3',5'-monophospate were more effective in displacing cyclic [^{3}H]AMP from the renal membrane receptor than was observed in the experiments with cytosol receptor. Modification of the 2'-ribose hydroxyl moiety of cyclic AMP resulted in marked diminution of effectiveness as a competitor in this assay system. This was observed with the analogues of cyclic AMP such as O^2-2-butyryl-adenosine cyclic 3',5'-monophosphate, N^6,O^2-dibutyl-adenosine cyclic 3',5'-monophosphate and 2'-deoxyadenosine cyclic 3',5'-monophosphate, although the relative effectiveness of these compounds as competitors was different when comparing their action on renal membrane and cytosol receptors. Adenosine cyclic 2',3'-monophosphate, uridine cyclic 2',3'-monophosphate, ATP and AMP were essentially ineffective as competitors as were the cyclic nucleotide phosphodiesterase inhibitors, theophylline, caffeine, and papaverine. These cyclic nucleotide phosphodiesterase inhibitors actually enhanced cyclic [^{3}H]AMP binding by 20–30 % when used at concentrations which inhibit phosphodiesterase. The butyryl derivatives of cyclic AMP used in these experiments were shown to be, in fact, competitive with cyclic [^{3}H]AMP for renal receptors in additional experiments where the competitor concentration was kept constant and cyclic [^{3}H]AMP concentration varied. These assays showed that the effect of butyryl derivatives of cyclic AMP on reducing cyclic [^{3}H]-AMP binding was completely reversed by increasing the concentration of cyclic [^{3}H]AMP in the incubation medium.

Reversibility of the cyclic [^{3}H]AMP-binding reaction was studied by incubating renal membranes and cytosol with cyclic [^{3}H]AMP until equilibrium was reached, followed by addition of a large excess of unlabeled cyclic AMP. The rate of decline of bound cyclic [^{3}H]AMP was then estimated and the cyclic [^{3}H]AMP/cyclic AMP exchange rate was used as a reflection of the rate of dissociation of bound cyclic [^{3}H]AMP from the receptor sites. Since ATP has been shown by enhance the rate of dissociation of cyclic AMP from soluble cyclic AMP-receptor preparations [22, 23], we investigated the effect of this nucleotide on the rate of cyclic [^{3}H]AMP dissociation. Fig. 2 shows representative experiments of this type. The apparent rate and extent of dissociation over the 60-min period shown is dependent upon temperature. Incubation temperature of 0 °C markedly reduced the rate of cyclic [^{3}H]AMP dissociation. Inclusion of ATP in the incubation medium did not enhance cyclic [^{3}H]AMP dissociation from cytosol receptor, but did increase the rate in experiments

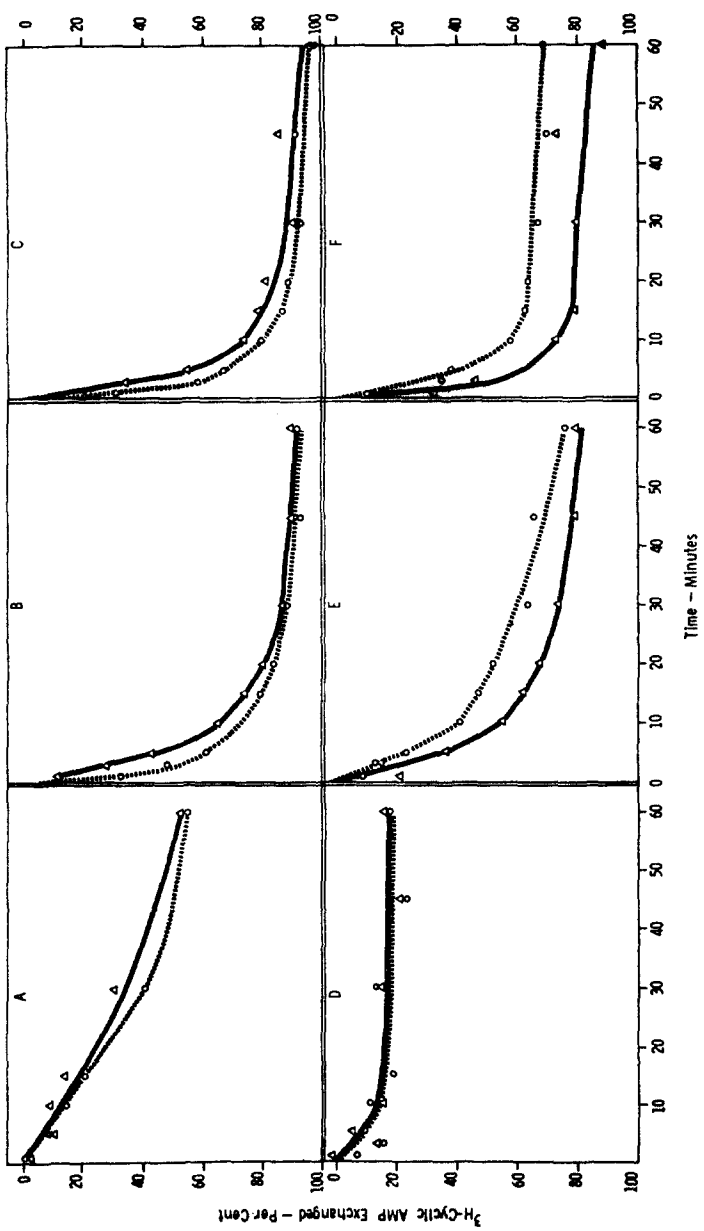

Fig. 2. Reversibility of cyclic [^{3}H]AMP binding to renal membrane and cytosol receptors. Cyclic [^{3}H]AMP, 0.2 μM, was preincubated with renal cytosol, 100 μg and plasma membrane, 95 μg for 15 min at the temperatures indicated and then cyclic AMP, 0.2 mM, plus 5 mM $MgCl_2$ (○- - -○), or cyclic AMP, 0.2 mM, plus 5 mM $MgCl_2$ and 1 mM ATP (△ — △), were added to the incubation medium. Samples were then removed from the incubation for determination of bound cyclic [^{3}H]AMP by the standard *filter procedure*. The data are presented as the percent of cyclic [^{3}H]AMP exchanged with cyclic AMP. Panels A–C, cytosol: A, 0 °C; B, 20 °C; C, 30 °C; Panels D–F plasma, membrane: A, 0 °C; B, 20 °C; C, 30 °C.

with renal membrane. However, this action of ATP on cyclic [^{3}H]AMP dissociation from the membrane receptor appeared to be temperature-dependent since ATP did not enhance this parameter at 0 °C. A more extensive examination of this action of ATP on cyclic [^{3}H]AMP dissociation was carried out at 20 °C (data not shown) and these experiments showed a repetitive and statistically significant action of ATP on enhancing the rate of cyclic [^{3}H]AMP dissociation from renal membrane receptors, whereas there was no effect of ATP on the rate of cyclic [^{3}H]AMP dissociation from the cytosol receptor. It was also shown that this action of ATP was absent at ATP concentrations less than 0.1 mM and that AMP also enhanced cyclic [^{3}H]-AMP dissociation from membrane receptors.

The nature of renal plasma membrane and cytosol cyclic AMP receptors was studied by treating membrane and cytosol fractions with various reagents and enzymes, followed by assay of cyclic [^{3}H]AMP binding to the treated fractions. These experiments are summarized in Table II. In some experiments, the F^--activated adenylate cyclase activity of renal membranes was assayed for the purpose of comparison of effects of the treatment procedure on binding of cyclic [^{3}H]AMP with effects on the renal membrane adenylate cyclase. Heating to 100 °C destroyed both receptor and enzymatic properties of renal membrane and cytosol and treatment of these preparations with the proteolytic enzymes, trypsin and protease, markedly reduced or abolished the cyclic [^{3}H]AMP binding property of both cytosol and plasma membranes. Soybean trypsin inhibitor effectively protected the cyclic AMP-receptor

TABLE II

EFFECT OF VARIOUS AGENTS ON CYCLIC [^{3}H]AMP BINDING AND ADENYLATE CYCLASE OF PORCINE KIDNEY CORTEX

Kidney cortex plasma membranes, 3.7 mg/ml, were incubated with the indicated reagents, enzymes, etc. in a medium consisting of sucrose, 0.25 mM, EDTA, 1 mM, Tris · HCl, 10 mM, pH 7.4. Cytosol, 8 mg/ml, was incubated in potassium phosphate buffer, 5 mM, pH 7.0. Incubation was for 60 min at 20 °C. Cyclic [^{3}H]AMP binding was then assayed at a concentration of 0.2 μM under standard conditions of 0 °C and 60 min for the binding incubation. Adenylate cyclase activated by 10 mM NaF, was assayed in those incubations indicated below. N.M. indicates not measured.

Treatment	Plasma membrane		Cytosol
	Cyclic [^{3}H]AMP bound (pmol/mg)	Adenylate cyclase (pmol/mg/10 min)	Cyclic [^{3}H]AMP bound (pmol/mg)
None	1.01	307	2.68
100 °C, 10 min	0	0	0
Trypsin, 250 μg/ml	0.16	N.M.	0.40
Soybean trypsin inhibitor, 500 μg/ml	1.20	N.M.	3.24
Trypsin+soybean trypsin inhibitor	0.92	N.M.	2.97
Protease (*Stm griseus*), 250 μg/ml	0	N.M.	0
Neuraminidase, 250 μg/ml	0.49	318	2.36
Phospholipase C, 100 μg/ml	1.04	242	2.70
Phospholipase C, 250 μg/ml	0.96	218	2.61
N-ethylmaleimide 0.01 mM	0.99	310	2.68
N-ethylmaleimide 0.1 mM	0.99	277	2.87
N-ethylmaleimide 1.0 mM	1.05	54	2.58

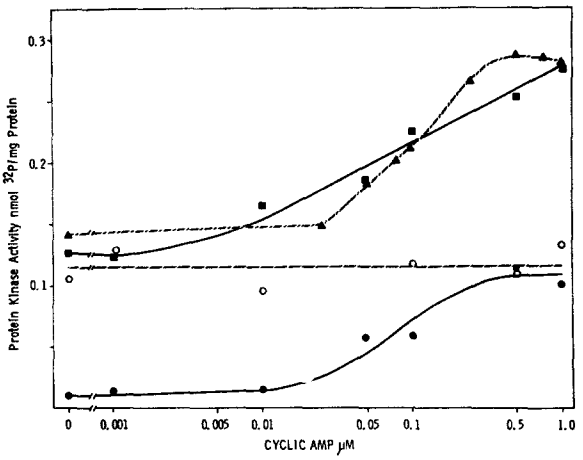

Fig. 3. Cyclic AMP-dependent protein kinase activity of renal membranes. Protein kinase activity of renal membranes, 125 μg, was determined using the exogenous acceptor proteins, 100 μg each; ▲- - -▲, protamine; ■—■, lysine-rich histone; ○- - -○, casein; and ●—●, arginine-rich histone as substrate. Assay conditions are described in Experimental Procedures. Incubation was for 10 min at 30 °C.

properties against trypsin degradation. Incubation of renal membrane and cytosol fractions with neuraminidase produced a 50 % reduction in cyclic [^{3}H]AMP-binding activity. The sulfhydryl-attacking reagent, *N*-ethylmaleimide, inhibited the renal membrane adenylate cyclase in a concentration-dependent manner but had essentially no effect on cyclic [^{3}H]AMP binding to either plasma membrane or cytosol preparations.

Figs 3 and 4 show representative experiments of cyclic AMP activation of renal plasma membrane and cytosol protein kinase. Four acceptor proteins were employed for the purpose of examining the relative specificity of the cyclic AMP-

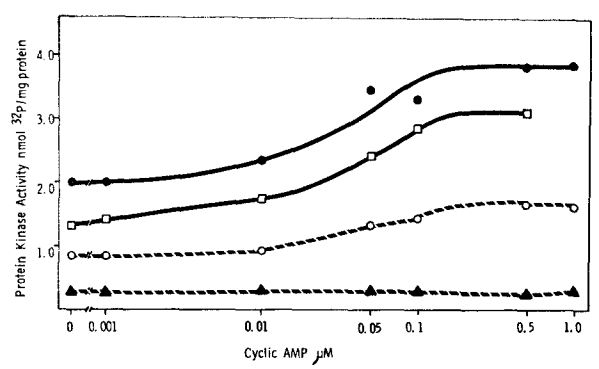

Fig. 4. Cyclic AMP-dependent protein kinase activity of renal cytosol. Protein kinase activity of renal cytosol, 100 μg, was determined using the exogenous acceptor proteins, 100 μg each; □—□, protamine; ○- - -○, lysine-rich histone; ▲- - -▲, casein; and ●—●, arginine-rich histone as substrate. Assay conditions are described in Experimental Procedures. Incubation was for 5 min at 30 °C.

dependent and -independent protein kinase systems. Renal membranes catalyzed the phosphorylation of protamine, lysine-rich histone, and casein to a greater extent than arginine-rich histone when cyclic AMP was not added to the assay system. Cyclic AMP activated this membrane-associated protein kinase over the cyclic nucleotide concentration range of 10 nm to 1 μM, with all the acceptor proteins except casein. A significant level of phosphorylation of casein was observed, but cyclic AMP did not enhance the phosphorylation of this acceptor protein. Fig. 4 depicts parallel experiments with the partially purified cytosol protein kinase. Phosphorylation of these acceptor proteins did occur in the absence of exogenous cyclic AMP but casein was a relatively poor phosphate acceptor protein. Cyclic AMP activation of the cytosol protein kinase was found over the same range of concentration of cyclic AMP (10 nM–1 μM) using protamine or histones as acceptor protein as was observed with the membrane protein kinase. The phosphorylation of casein was not stimulated by cyclic AMP. In consideration of these data, it was apparent that protamine would serve as a suitable acceptor protein for both the cytosol and membrane protein kinase systems in further studies.

The efficacy of several cyclic nucleotides as activators of the renal membrane and cytosol protein kinase was examined. Fig. 5 shows the phosphorylation of protamine catalyzed by renal plasma membrane protein kinase. Cyclic AMP and N^6-monobutyryl-adenosine cyclic 3′,5′-monophosphate were similar in potency and in maximal level (efficacy) of activation. Cyclic AMP produced a biphasic pattern of activation-inhibition. Activation of the kinase was observed over the concentration range from 10 nM to 10 μM with subsequent inhibition of phosphorylation of protamine at cyclic AMP concentration of 0.1 to 1 mM. This phenomenon was not observed with N^6-monobutyryl-adenosine cyclic 3′,5′-monophosphate. N^6,O^2-dibutyryl-adenosine cyclic 3′,5′-monophosphate was approx. 1 % as potent as cyclic AMP and also did not stimulate the phosphorylation of protamine to the same maximal level as cyclic AMP. The cyclic nucleotides, O^2-monobutyryl-adenosine 3′,5′-monophosphate and

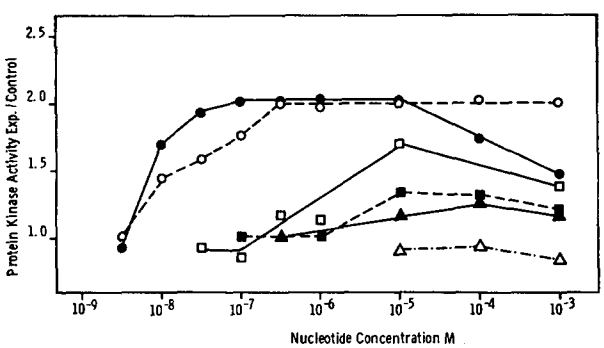

Fig. 5. Activation of renal membrane protein kinase by various cyclic nucleotides. Plasma membrane protein kinase activity was assayed as described in Experimental Procedures using 100 μg membrane protein and 100 μg protamine. Dose-response curves for activation of protein kinase for the cyclic nucleotides; ●—●, cyclic AMP; ○- - -○, N^6-butyryl-adenosine cyclic 3′,5′-monophosphate; □—□, N^6,O^2-dibutyryl-adenosine cyclic 3′,5′-monophosphate; ■—■, O^2-butyryl-adenosine cyclic 3′,5′-monophosphate; ▲—▲, cyclic 3′,5′-GMP; and △- . -△, cyclic 2′,3′-AMP, are depicted as the ratio of protein kinase activity in the presence of cyclic nucleotide (Exp) to the activity with no added cyclic nucleotide (control).

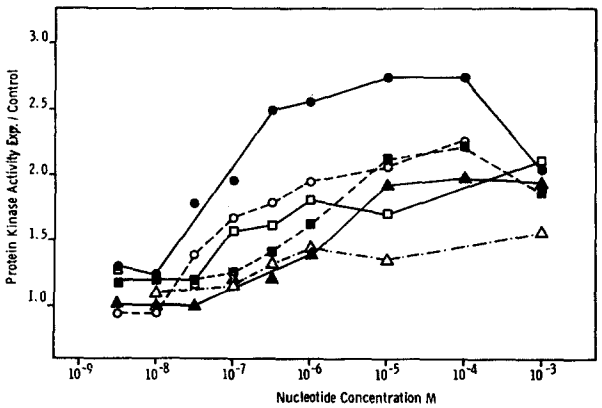

Fig. 6. Activation of renal cytosol protein kinase by various cyclic nucleotides. Cytosol protein kinase was assayed as described in Experimental Procedures using 100 μg cytosol protein and 100 μg protamine. The legend for this figure and the method of expression of data are the same as for Fig. 5 with respect to dose-response curves of the various cyclic nucleotides.

guanosine cyclic 3′,5′-monophosphate demonstrated even less potency and efficacy in this assay system, and adenosine cyclic 2′,3′-monophosphate was inactive.

Parallel experiments were performed with the renal cytosol protein kinase using protamine as acceptor protein. Fig. 6 shows representative concentration-response curves for a number of cyclic nucleotides. Cyclic AMP and N^6-monobutyryl-adenosine 3′,5′-monophosphate were similar in potency but the butyryl derivative stimulated to a lower maximum than did cyclic AMP. Cyclic AMP activation was biphasic as seen in experiments with renal membrane protein kinase. Inhibition of phosphorylation of protamine was found at 1 mM cyclic AMP. The activation curves for N^6,O^2-dibutyryl-adenosine cyclic 3′,5′-monophosphate, O^2-monobutyryl-adenosine cyclic 3′,5′-monophosphate and guanosine cyclic 3′,5′-monophosphate showed only minor differences in regard to potency and efficacy of activation of the cytosol protein kinase. As with the plasma-membrane system, adenosine cyclic 2′,3′-monophosphate was inactive in regard to enhancement of cytosol-catalyzed phosphorylation of protamine.

During the course of these studies with renal membrane protein kinase, we observed a small but notable stimulation by cyclic AMP of the phosphorylation of endogenous membrane protein when acceptor proteins such as protamine were omitted from the reaction mixture. In 16 replicate protein kinase assays, half of which contained 1 μM cyclic AMP, it was shown that cyclic AMP-independent phosphorylation of membrane protein (mean $\pm$S.E.) was 113 ± 15 pmol ^{32}P transferred per 5 min per mg protein whereas cyclic AMP increased this value to 165 ± 32, or a 46 % increase. This effect, however, was not statistically significant ($P < 0.2$), but stimulation by cyclic AMP of phosphorylation of endogenous membrane protein in particulate membrane preparations from bovine brain has previously been described [24].

DISCUSSION

This study suggests that porcine renal cortex plasma membrane cyclic AMP receptor and cyclic AMP-dependent protein kinase activities resemble those of the

more widely studied soluble protein kinase systems. The characteristics of the receptor moiety with regard to the affinity, specificity and reversibility of cyclic AMP interaction with the membrane receptor are indicative of a physiological receptor. Moreover, the affinity constants for binding of cyclic AMP to renal membranes and cytosol are similar to the activation constants observed for cyclic AMP stimulation of the respective protein kinase systems when the differences in incubation conditions are taken into consideration. The relative differences in apparent affinity and competitor effectiveness with regard to cyclic AMP binding to renal membranes and cytosol may be explained by the physiochemical differences between membrane-bound proteins and soluble proteins. The accessibility of the ligand to the receptor is likely to be different with membrane receptors than with soluble receptors. It is unlikely that these observed differences are due to the presence of cyclic nucleotide phosphodiesterase activities since the velocity of this reaction is severely limited by the reduced temperature of the binding incubation such that the concentration of nucleotide does not change significantly during the reaction period. It is not possible, at present, to suggest that the membrane-bound protein kinase system is a different enzyme system than the cytosol enzyme. The cyclic AMP receptor and kinase catalytic moieties must be isolated from the membrane for more direct comparison of chemical properties before this question can be answered.

Other studies have implicated membrane protein phosphorylation as an intermediary step in hormonal regulation of renal cell [25] and toad bladder epithelial cell transport processes [26]. Dousa et al. [25] showed that renal membranes served as phosphate acceptor for a soluble, bovine kidney cyclic AMP-dependent protein kinase. The physiological significance of this observation is questionable since the membranes used for substrated (acceptor) purposes were essentially denatured prior to use and the specificity of kidney cyclic AMP-dependent protein kinase, as shown in the present study, is quite low. The report by DeLorenzo et al. [26] that vasopressin and butyryl derivatives of cyclic AMP produced a decrease in the phosphorylation of a specific protein which appeared to be associated with membranes is of considerable interest since the examination of phosphorylation mechanisms in the intact cell is of primary importance in the determination of cell proteins which may serve as phosphate acceptors in vivo. This dephosphorylation mechanism is unique but is supported by a report that toad bladder membranes contain an endogenous protein kinase and cyclic AMP-activated phosphoprotein phosphatase [27]. The extrapolation of these reports from toad bladder to mammalian kidney, however, should be viewed with caution regarding the cellular mode of action of vasopressin in mammalian species.

In conclusion, this study suggests that renal cortical cells contain membrane-bound as well as soluble cyclic AMP-dependent protein kinase systems which conceivably may function in the sequence of metabolic events which are initiated by hormone-receptor interaction and culminate in alteration of cell function. The localization with respect to cell type of these protein kinase systems in a heterogenous mixture of renal cortical cells is impossible at present. Implication of the enzymes in expression of parathyroid hormone, vasopressin, thyrocalcitonin or catecholamine action via enhanced plasma membrane adenylate cyclase activity [13, 14] is of considerable importance as is identification of the physiological substrate(s) for these phosphorylating enzyme systems. Experimental attention to these points may further clarify the

role that cyclic AMP-dependent protein kinase plays in the cellular mode of action of hormones which regulate renal cell function.

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