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CHARACTERIZATION OF THE ADENYL CYCLASE OF RAT KIDNEY PLASMA MEMBRANES

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SUMMARY

1. This paper examines the characteristics of a renal plasma membrane adenyl cyclase with respect to NaF and hormonal activation, enzyme kinetic parameters, effects of solubilization procedures on adenyl cyclase activity, and gel-filtration chromatography of a "soluble" membrane adenyl cyclase.

2. Renal membrane adenyl cyclase was stimulated by NaF, vasopressin and parathyroid hormone in a concentration-dependent manner.

3. A K_m for ATP of 0.1 mM was obtained for both the renal cortex and medulla membrane adenyl cyclase whereas the divalent cation dependency was found to be: cortex, $Mg^{2+} > Mn^{2+} > Co^{2+}$; medulla, $Mg^{2+} = Mn^{2+} > Co^{2+}$, for sustaining adenyl cyclase activity in the presence of NaF.

4. The particulate plasma membrane adenyl cyclase was solubilized by use of the detergent, Lubrol WX or by intense shear forces with a French pressure cell. Addition of NaF to the membrane suspension prior to solubilization was necessary for protection of adenyl cyclase activity. Gel-filtration chromatography with 6% agarose gave two adenyl cyclase peaks of different specific activity and molecular size.

INTRODUCTION

The adenyl cyclase system has been implicated in the cellular mechanism of action of both polypeptide and catecholamine hormones[1]. This enzyme system which catalyzes the formation of cyclic 3',5'-AMP from ATP is considered to be specifically localized in the plasma membrane of cells[2,3]. A plasma membrane preparation from rat kidney has been described which exhibits high specific activity adenyl cyclase and (Na^+–K^+)-dependent ATPase[4]. Urinary excretion of cyclic AMP is enhanced by vasopressin in man[5] and by parathyroid hormone in rats[6]. *In vitro* activation of rat kidney adenyl cyclase of crude "nuclear" preparations by vasopressin and parathyroid hormone[7,8] has been reported but examination of the hormone-sensitive adenyl cyclase system of renal cortex and medulla plasma membrane preparations has not been extensively studied.

Abbreviations: ACTH, adrenocorticotropin hormone.

The particulate nature of membrane-bound adenyl cyclase systems has prevented investigators from characterizing this enzyme with respect to the usual criterion (*i.e.* molecular size, *etc.*). However, some progress with solubilization of this enzyme has been made. Sutherland *et al.*[9] reported the partial solubilization of adenyl cyclase from heart, brain, liver and skeletal muscle with the detergent, Triton X-100. A soluble adenyl cyclase from mouse adrenal tumor tissue has been prepared by the use of physical methods (sonication, French pressure cell) which was activated *in vitro* by adrenocorticotropin hormone (ACTH) and by NaF[10]. Addition of exogenous phospholipids and NaF to the preparation prior to solubilization was necessary to maintain hormone sensitivity. Lubrol-PX, a nonionic detergent, has been employed to solubilize an adenyl cyclase from cat myocardium[11]. This preparation exhibited the property of activation by NaF but was rendered insensitive to hormonal activation. Subsequent reports indicated that removal of the detergent from the solubilized myocardial adenyl cyclase *plus* the addition of exogenous phosphatidylserine restored the glucagon responsiveness to the solubilized adenyl cyclase preparation[12]. The rat kidney plasma membrane preparation employed in this study has been solubilized through the use of both detergent (Lubrol-WX) and physical methods (sonication) for the purpose of characterizing the membrane (Na^+–K^+)-dependent ATPase and of the nature of *in vivo* binding of organic mercurial compounds to plasma membranes[4,13].

The present investigation is primarily concerned with an examination of the general characteristics of the rat kidney plasma membrane adenyl cyclase and the effects of solubilization on the hormone-sensitive adenyl cyclase system.

METHODS

The preparative procedure and characterization of the rat kidney plasma membrane preparation employed in this study has been previously reported[4].

Adenyl cyclase activity of kidney plasma membranes was determined by the method of White and Zenser[14]. This assay employs [α-^{32}P]ATP as substrate and cyclic 3′,5′-[^{3}H]AMP to calculate the recovery of cyclic 3′,5′-[^{32}P]AMP formed during the incubation. The incubation medium consists of: 20 μg bovine serum albumin, 90 nmoles ATP, $1 \cdot 10^5$–$2 \cdot 10^5$ cpm [α-^{32}P]ATP, 3 μmoles Tris–HCl, pH 7.5, 0.5 μmole $MgCl_2$, 1.2 μmoles caffeine, 80 nmoles cyclic AMP, 0.9 μmole creatine phosphate, 0.4 μg creatine phosphokinase and approx. 100 μg membrane protein. The final volume was 75 μl. Cyclic AMP was added as a trap to prevent degradation of the cyclic [^{32}P]AMP formed during the incubation and creatine phosphate–creatine phosphokinase was added for regeneration of ATP from ADP produced by the ATPase in plasma membrane preparations. Incubation was carried out for 20 min at 30 °C in a Dubnoff metabolic shaking incubator and the reaction was terminated by the addition of 20 μl EDTA, 0.1 M, which contained 5000 cpm of cyclic 3′,5′-[^{3}H]-AMP. The tubes were then heated for 2 min in a heating block maintained at 100 °C. 1 ml of 0.05 M Tris–HCl, pH 7.6, was added and the denatured protein precipitated by centrifugation in a clinical centrifuge for 5 min. Neutral alumina columns were employed to separate the cyclic 3′,5′-[^{32}P]AMP from other radioactive compounds according to the method of White and Zenser[14]. ^{3}H and ^{32}P radioactivity was determined in a Packard liquid scintillation counter. The recovery of cyclic 3′,5′-AMP

from the incubation mixture utilizing alumina columns was approx. 70–80 %. Protein was estimated by the method of Sutherland *et al.*[15] with bovine serum albumin as the reference standard.

Renal phosphodiesterase activity was estimated by measuring the disappearance of cyclic 3',5'-[^{3}H]AMP in an incubation medium consisting of: 20 μg bovine serum albumin, 3 μmoles Tris–HCl, pH 7.6, 0.5 μmole $MgCl_2$, 80 nmoles cyclic 3',5'-[^{3}H]-AMP and 100–200 μg enzyme protein. The final incubation volume was 75 μl. Incubation was carried out for 30 min at 30 °C in a Dubnoff metabolic shaking incubator and the reaction was terminated by the addition of 20 μl caffeine, 60 mM, and heating for 2 min in a heating block maintained at 100 °C. 1 ml of 0.05 M Tris–HCl, pH 7.6, was then added and the denatured protein precipitated by centrifugation. Chromatographic separation of cyclic 3',5'-[^{3}H]AMP from other ^{3}H-labeled compounds was the same as described for the adenyl cyclase assay[14]. Triplicate blanks without enzyme, were carried through the procedure to determine the per cent recovery of cyclic 3',5'-[^{3}H]AMP. The variability of recovery within one experiment was less than 5 % which did not significantly alter the data obtained by this method.

Methods employed for the determination of membrane (Na^+–K^+)-dependent ATPase and Mg^{2+}-ATPase activities have been previously described[4]. Measurement of inorganic phosphate formed during the incubation was by the colorimetric procedure of Lowry and Lopez[16].

Methods for solubilization of rat kidney plasma membrane preparations were:

French pressure cell method. Rat kidney plasma membranes suspended in 5.0 ml 0.25 M sucrose–1 mM EDTA, 3–5 mg protein per ml, were passed one time through an Aminco pressure cell (No. 4-3398A) with pressure maintained constant at 20000 lb/inch^2 with a Carver 12 ton laboratory press according to the procedure of Cauldwell and Schumaker[17]. Flow rate through the pressure cell was approximately 2–3 ml/min. After passage of the membrane preparation through the pressure cell it was centrifuged at 37000 × *g* for 20 min in a Sorvall RC-2B refrigerated centrifuge. Sedimentation of the particulate plasma membranes prior to this solubilization procedure could be accomplished by centrifugation at 12000 × *g* for 10 min.

Lubrol-WX method. Rat kidney plasma membranes suspended in sucrose–EDTA as above were incubated with various concentrations of Lubrol-WX for 15 min at 0–2 °C as previously described[4]. Centrifugation was as described above. All solubilization and subsequent chromatographic procedures were carried out at 0–2 °C.

Gel filtration chromatographic methods were as previously described[4]. Gels employed in the present study were 6 % agarose (Gelarose, Aldrich Chemical Co.) bead size 50–200 μm and Sephadex G-25 obtained from Sigma Chemical Co., bead size 50–150 μm. Column dimensions for both gels were 2.5 × 40 cm. Flow rates for the above columns were approximately 1 ml/min for the 6 % agarose column and 1.5 ml/min for the Sephadex G-25 column. These columns were calibrated with blue dextran, bovine thyroglobulin, bovine gamma globulin, bovine hemoglobin and bovine heart cytochrome *c* all obtained from the Sigma Chemical Co., St. Louis, Mo. Fractions eluted from the agarose column were concentrated by ultrafiltration to assure adequate protein concentration for adenyl cyclase assays. An Amicon Model 10PA cell and type PM-10 membranes were used in this procedure.

Cyclic 3′,5′-[^{3}H]AMP and [α-^{32}P]ATP were purchased from International Chemical and Nuclear Corp., Irvine, Calif. Synthetic lysine vasopressin-type I-S, arginine vasopressin, grade V, ATP, brain phospholipid (Folch Fraction V), creatine phosphate, creatine phosphokinase, sodium deoxycholate and neutral alumina (chromatographic type WN-3) were purchased from Sigma Chemical Co., St. Louis, Mo. Parathyroid hormone (parathyroid, U.S.P., Eli Lilly Co.) was obtained from the hospital pharmacy. Bray's[18] scintillation phosphor was used for measurement of radioactivity. Other standard reagents were purchased from Fisher Scientific Co., St. Louis, Mo.

RESULTS

Experiments with the rat kidney plasma membrane adenyl cyclase indicated that the formation of cyclic AMP was linear over a range of 20–225 μg membrane protein in the assay. Linearity with respect to time was found over a range from 5–30 min of incubation at 30 °C. These experiments were performed with 10 mM NaF included in the incubation medium. These preliminary experiments indicated that use of approximately 100 μg membrane protein with an incubation of 20 min would yield optimal ratios of cyclic [^{32}P]AMP to blank ^{32}P radioactivity. The cyclic AMP phosphodiesterase activity of the renal membrane preparation was examined and compared with the activity of a 100000 $\times$ g supernatant fraction from rat kidney. Phosphodiesterase activity of the plasma membranes was 59 nmoles/mg per

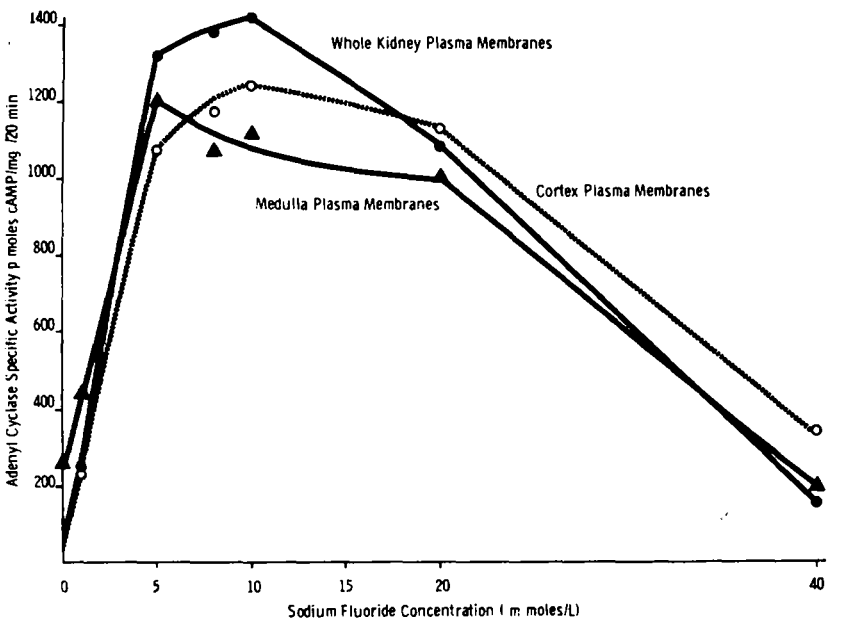

Fig. 1. Effect of increasing concentration of NaF on the formation of cyclic 3′,5′-AMP by rat kidney plasma membranes. Membranes were incubated for 20 min at 30 °C in the medium described in Methods, *plus* NaF from a concentration of 1–40 mM. The data is the result of one experiment with duplicate assays at each point and is expressed as pmoles cyclic 3′,5′-AMP formed per 20 min per mg protein at each point.

30 min at 30 °C whereas the activity of the soluble cytoplasmic fraction was 171 nmoles/mg per 30 min. The highest specific activity of adenyl cyclase from rat kidney was consistently observed in the plasma membrane preparation whereas no adenyl cyclase activity was detected in the soluble cytoplasmic fraction.

Stimulation of adenyl cyclase activity by NaF in broken cell preparations is a characteristic property of this enzyme system. The kidney plasma membrane adenyl cyclase is markedly stimulated by NaF. Fig. 1 depicts the increase in adenyl cyclase activity observed with various concentrations of NaF. NaF stimulated the kidney adenyl cyclase over a relatively narrow range of concentration from 1–10 mM for whole kidney and cortex membrane preparations and 1–5 mM for medulla membranes. Increasing the concentration of NaF above that necessary for maximal activation of the adenyl cyclase subsequently inhibited the adenyl cyclase activity.

Renal adenyl cyclase preparations have been shown to be activated *in vitro* by vasopressin and parathyroid hormone[7,8]. Using crude "nuclear" preparations Chase and Aurbach[7] found that renal cortex and medulla adenyl cyclase demonstrated different characteristics in that parathyroid hormone activated the cortical adenyl cyclase whereas vasopressin stimulated the medullary adenyl cyclase. For characterization of the nature of hormone activation properties of the plasma membrane adenyl cyclase preparation from renal cortex and medulla, dose response experiments were performed with parathyroid hormone and vasopressin. Fig. 2 shows a representative curve obtained with concentrations of vasopressin from $2.5 \cdot 10^{-8}$–$1.5 \cdot 10^{-5}$ M. The dose response curves for arginine and lysine vasopressin derivatives were found to be markedly different. Arginine vasopressin stimulated

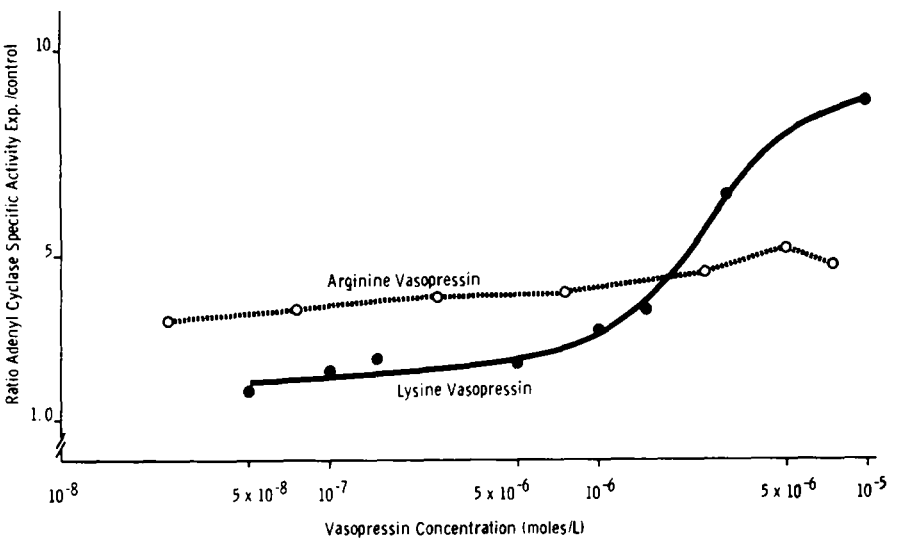

Fig. 2. Dose response curve for *in vitro* activation of renal medulla membrane adenyl cyclase by arginine and lysine vasopressin. Adenyl cyclase activity of rat kidney medulla plasma membranes was determined in the absence and presence of various concentrations of lysine and arginine derivatives of vasopressin as described in Methods. This is one representative experiment with the results expressed as the ratio; vasopressin-stimulated adenyl cyclase activity/basal adenyl cyclase activity. Each point represents the mean of duplicate assays in control and vasopressin-activated adenyl cyclase assays.

the medulla adenyl cyclase 3.5-fold at a concentration of $2.5 \cdot 10^{-8}$ M and produced a maximal stimulation of 5-fold at $5 \cdot 10^{-6}$ M. Arginine vasopressin enhanced the medullary adenyl cyclase activity quantitatively more at the lower concentrations than did the lysine derivative. In contrast, lysine vasopressin demonstrated a quantitatively larger stimulation with a 9-fold increase in adenyl cyclase activity at a concentration of $1 \cdot 10^{-5}$ M. Arginine vasopressin, however, produced very significant (3.5 fold) stimulation of the medullary adenyl cyclase at the lowest concentrations employed. Arginine vasopressin is the naturally occurring hormone in the rat. The dose-response relationship with parathyroid hormone and the cortex membrane adenyl cyclase exhibited a characteristic sigmoidal shaped curve illustrated in Fig. 3. A 14-fold increase in adenyl cyclase activity was obtained with the highest concentration of parathyroid hormone employed in these experiments.

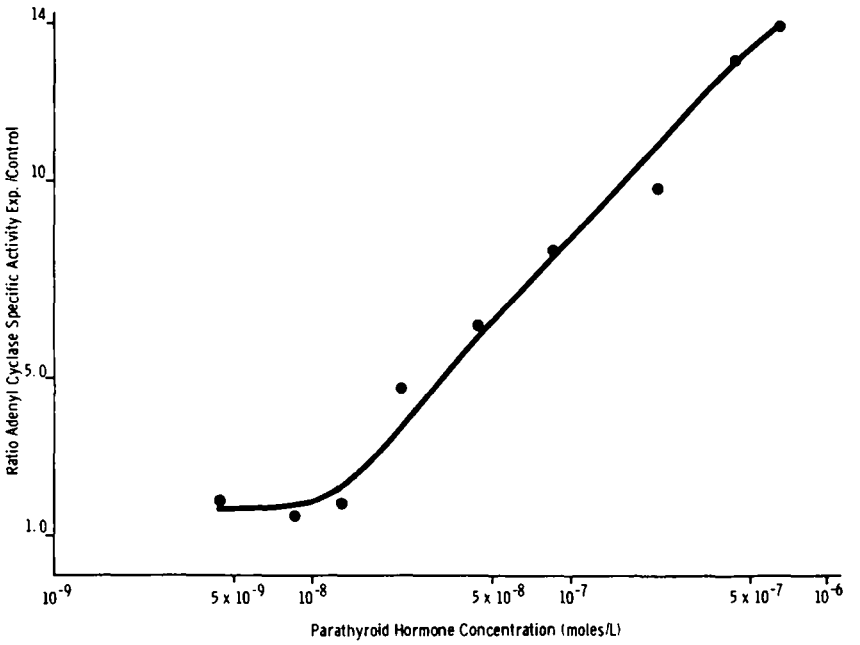

Fig. 3. Dose–response curve for *in vitro* activation of renal cortex membrane adenyl cyclase by parathyroid hormone. Adenyl cyclase activity of rat kidney cortex plasma membranes was determined in the absence and presence of various concentrations of parathyroid hormone as described in Methods. This is one representative experiment with the results expressed as the ratio; parathyroid-stimulated adenyl cyclase activity/basal adenyl cyclase activity. Each point represents the mean of duplicate assays in control and parathyroid hormone-activated adenyl cyclase assays.

Experiments were designed to further compare and characterize the properties of the renal medulla and cortex adenyl cyclase systems with respect to substrate kinetics and divalent cation dependence (Mg^{2+}, Mn^{2+} and Co^{2+}). Fig. 4 shows the effect of varying the concentration of ATP on the renal cortex and medulla plasma membrane adenyl cyclase activity. It is evident that the two enzyme systems are essentially identical with respect to this parameter. A K_m for ATP can be derived from these data of approximately $1 \cdot 10^{-4}$ M utilizing the reciprocal plot of Lineweaver and Burk[19].

The most striking difference observed between the cortex adenyl cyclase and the medullary adenyl cyclase was obtained upon comparison of the divalent cation dependency of the membrane adenyl cyclase. Figs 5A and 5B compare the cortical and medullary adenyl cyclase systems, respectively, with respect to the effect of various Mg^{2+} concentrations on the basal activity and on the vasopressin, parathyroid hormone, or NaF-activated adenyl cyclase activity. It can be seen that maximal activity of the NaF-activated, cortical adenyl cyclase is obtained with a concentration of 3 mM Mg^{2+} but the maximal medullary adenyl cyclase activity with NaF is not achieved with Mg^{2+} concentrations as high as 50 mM. The basal activities of these two systems are different throughout the entire range of Mg^{2+} concentrations employed, with the medullary adenyl cyclase specific activity being greater than the cortical activity. Hormonal stimulation of these membrane adenyl cyclase systems produced a parallel shift in the curve to a level of activity greater than the basal activity at all Mg^{2+} concentrations. An additional interesting observa-

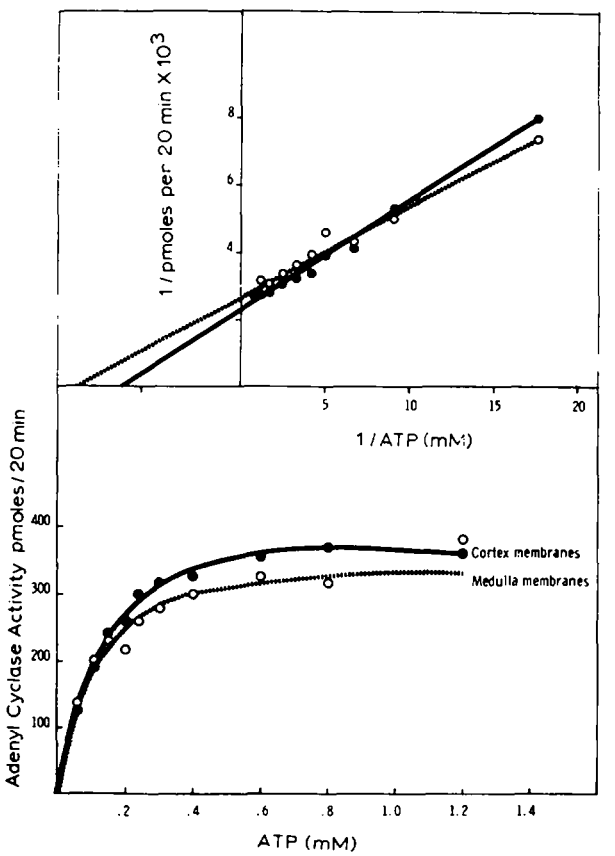

Fig. 4. Kinetic effects of ATP on rat kidney cortex and medulla plasma membrane adenyl cyclase. Renal cortex and medulla plasma membranes (165 μg protein each) were incubated with various concentrations of ATP and fixed concentration of both 6.7 mM $MgCl_2$ and 10 mM NaF for 20 min at 30 °C for determination of adenyl cyclase as described in Methods. The upper curve is a Lineweaver–Burk[19] plot of the reciprocals of ATP concentration and velocity (pmoles cyclic AMP per 20 min).

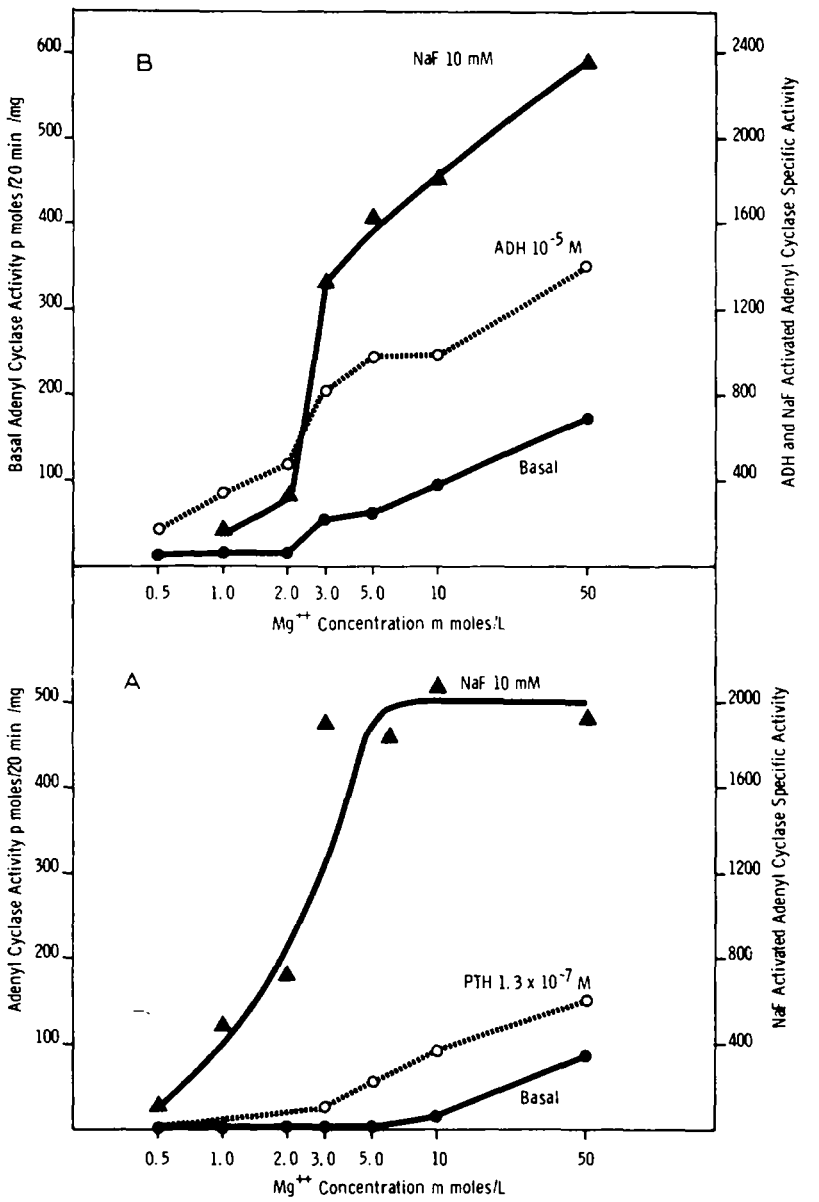

Fig. 5. Kinetic effects of Mg^{2+} on rat kidney plasma membrane adenyl cyclase. (A) Renal cortex plasma membranes were assayed for adenyl cyclase activity as described in Methods. This is one representative experiment with a single membrane preparation. ATP concentration was 1.2 mM. (B) Renal medulla membranes were assayed for adenyl cyclase activity as above. This is one representative experiment with a single membrane preparation. ATP concentration was 1.2 mM. In both A and B the incubations contained the hormones depicted or NaF or no activator (basal activity). Different scales for adenyl cyclase activity were necessary and are appropriately labeled on the figure. ADH, vasopressin; PTH, parathyroid hormone.

tion derived from these experiments is that valid comparisons of cortical and medullary adenyl cyclase activity in the presence of NaF is not possible unless a wide range of Mg^{2+} concentrations are employed. NaF-activated adenyl cyclase activity of medulla plasma membrane preparations is greater than the cortical membrane activity at high Mg^{2+} concentrations (50 mM) but the reverse was found with low

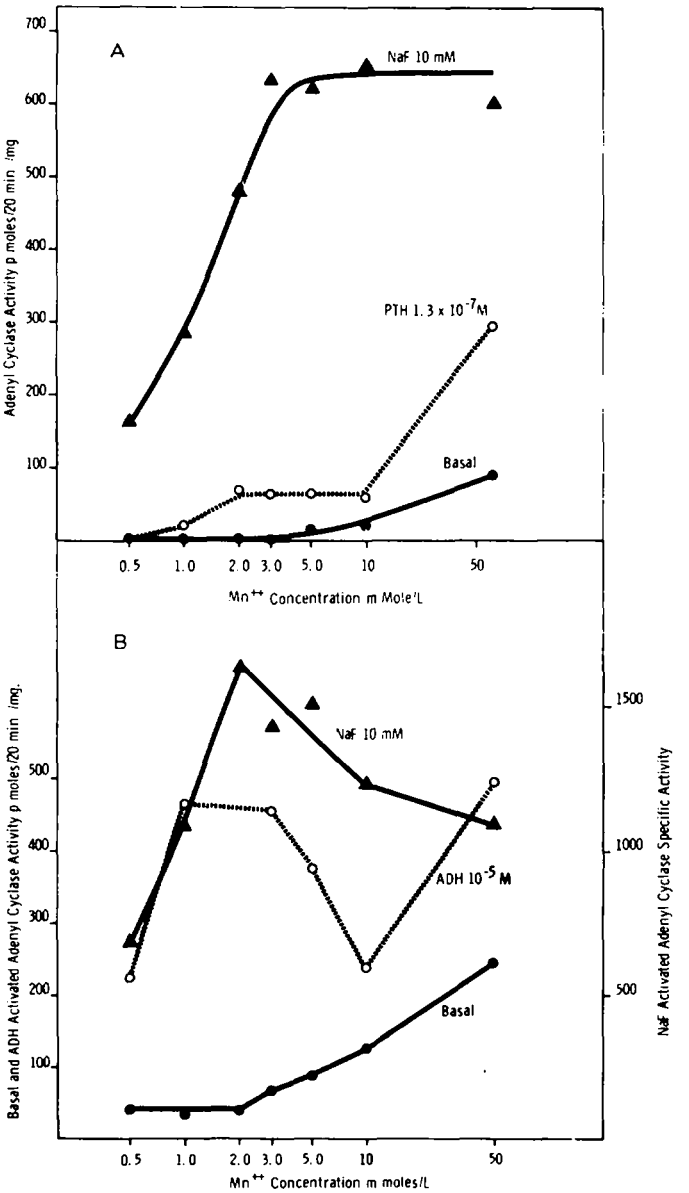

Fig. 6. Kinetic effects of Mn^{2+} on rat kidney plasma membrane adenyl cyclase. Renal plasma membranes were assayed for adenyl cyclase activity as described in Fig. 5 except that various concentrations of $MnCl_2$ were used instead of $MgCl_2$. (A) Kidney cortex plasma membranes. (B) Kidney medulla plasma membranes.

Mg^{2+} (1–5 mM) concentrations. A striking difference in cofactor specificity was observed when Mn^{2+} was substituted for Mg^{2+} in the adenyl cyclase incubation medium. Fig. 6A and 6B show the results of experiments employing various concentrations of Mn^{2+} on the renal cortex membrane adenyl cyclase and the renal medulla membrane adenyl cyclase activities. The shape of the curves obtained with Mg^{2+} and Mn^{2+} as cofactors for the adenyl cyclase (NaF-activated) of cortex membranes was similar but the activity of the cortical adenyl cyclase was reduced with saturating concentrations of Mn^{2+} to approximately one-third the level of activity achieved with saturating concentrations of Mg^{2+}. In contrast, the medullary membrane adenyl cyclase (NaF activated) more effectively utilized Mn^{2+} as a replacement for Mg^{2+} with a maximal activity produced at 2 mM Mn^{2+}. The ratio of activities with this level of Mn^{2+} (Mg^{2+}/Mn^{2+}) is approximately 1. Concentrations of Mn^{2+} higher than 2 mM subsequently inhibited the medullary adenyl cyclase preparation when NaF of vasopressin was present. However, the basal medullary enzyme activity continued to increase without plateauing with concentrations of Mn^{2+} as high as 50 mM. The basal and parathyroid hormone activated cortical adenyl cyclase also did not show a saturation phenomenon with concentrations of Mn^{2+} of 50 mM. Cobalt was found to support both cortical and medullary membrane adenyl cyclase activity to approximately the same extent when NaF was included in the incubation medium (Fig. 7). Approx. 3–5 mM Co^{2+} produced maximal activity and higher concentrations inhibited the adenyl cyclase of both preparations.

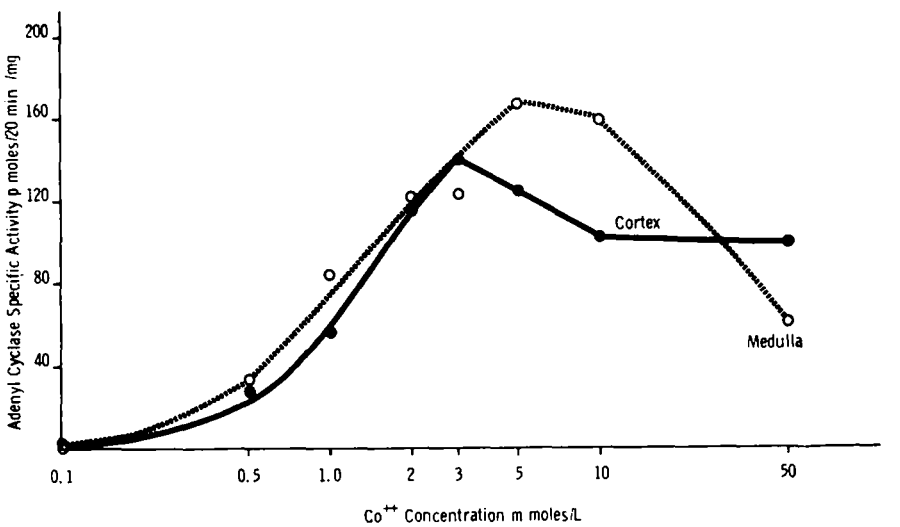

Fig. 7. Kinetic effect of Co^{2+} on rat kidney plasma membrane adenyl cyclase. Rat kidney cortex and medulla plasma membranes were incubated with various concentrations of $CoCl_2$ and fixed concentrations of 1.2 mM ATP and 10 mM NaF for 20 min at 30 °C. Adenyl cyclase assay was performed in duplicate as described in Methods.

The particulate nature of adenyl cyclase systems has prevented investigators from applying standard types of procedures for characterization of the enzyme with respect to molecular size, *etc.* However, some progress has been made in the solubilization of membrane-bound adenyl cyclase with maintenance of enzymatic

activity[9-12]. The rat kidney membrane preparation used in this study has been solubilized with detergents or by sonication for the purpose of studying membrane subunits[4]. The criterion utilized for definition of a "soluble" plasma membrane preparation is that centrifugation of the solubilized membranes at 37000 × *g* for 20 min does not sediment these membranes. In addition the "soluble" plasma membranes prepared by detergent or by French pressure cell methods were checked for sedimentation characteristics at 105000 × *g* for 60 min and found that insignificant quantities (less than 5 %) of the soluble plasma membranes sedimented with this additional centrifugation. The experiments reported in this study utilized the lower centrifugation (37000 × *g*).

TABLE I

FRENCH PRESSURE CELL SOLUBILIZATION OF RAT KIDNEY PLASMA MEMBRANES

Rat kidney plasma membranes suspended in 5.0 ml 0.25 M sucrose–1 mM EDTA were passed one time through an Aminco, French pressure cell at 20000 lb/inch². Details of solubilization are described in Methods. Results are expressed as the mean ± S.E. or are single experiments and are the membrane suspension protein concentration before and after centrifugation at 37000 × *g* for 20 min. The numbers in parentheses refer to the number of experiments.

Addition	*Protein concentration (mg/ml)*	
	Before centrifugation	*After centrifugation*
None	4.86 ± 0.37 (8)	4.16 ± 0.35 (8)
NaF, 25 mM	3.61 ± 0.22 (5)	2.75 ± 0.07 (5)
NaF, 25 mM + Folch Fraction V, 2.5 mg/ml	4.96 (1)	3.20 (1)

Table I shows the quantitative degree of solubilization of rat kidney plasma membrane protein utilizing the French pressure cell method. One passage of the particulate membrane preparation through the pressure cell "solubilized" approx. 85 % of the membrane protein. Addition of 25 mM NaF to the particulate membrane suspension prior to passage through the pressure cell, for protection of adenyl cyclase activity, reduced the efficiency of solubilization as did the combination of NaF and exogenous phospholipid (Folch Fraction V). The effect of pressure cell solubilization on the membrane adenyl cyclase and total ATPase ((Na^+-K^+)-dependent *plus* Mg^{2+}-dependent ATPase) activities are shown in Table II. Solubilization of renal plasma membranes suspended in 0.25 M sucrose–1 mM EDTA resulted in marked loss of adenyl cyclase activity (81 %) when assayed in the presence of 10 mM NaF. ATPase activity declined somewhat (35 %)but not as drastically as did the adenyl cyclase activity. Addition of 25 mM NaF to the particulate membrane suspension prior to passage through the pressure cell resulted in the maintenance of 55 % of the adenyl cyclase activity as compared to 19 % in the absence of NaF. It was anticipated that the further addition of exogenous phospholipid emulsion to the membrane suspensions before solubilization, according to the procedure of Pastan[10], *et al.* would provide additional protection of the soluble adenyl cyclase activity. However, the addition of 2.5 mg/ml of a crude brain phospholipid emulsion (Folch Fraction V) reversed the protection achieved by NaF alone.

TABLE II

EFFECT OF FRENCH PRESSURE CELL SOLUBILIZATION ON KIDNEY PLASMA MEMBRANE ADENYL CYCLASE AND ATPase ACTIVITIES

Rat kidney plasma membranes were solubilized by use of an Aminco, French pressure cell as described in Table I and Methods. Results are expressed as the mean ± S.E. or are single experiments. Numbers in parentheses refer to the number of experiments. Adenyl cyclase activity is expressed as pmoles cyclic 3′,5′-AMP formed per mg protein per 20 min at 30 °C. ATPase activity is the activity measured in the presence of Na^+, K^+ and Mg^{2+} and represents the basal Mg^{2+}-dependent ATPase *plus* the (Na^+–K^+)-dependent ATPase activities as μmoles P_i liberated per mg protein per 20 min at 37 °C.

Addition	*Adenyl cyclase activity (pmoles/20 min/mg)*		*ATPase activity (μmoles P_i/20 min/mg)*	
	Native	*"Soluble"*	*Native*	*"Soluble"*
None	1646 ± 24 (5)	342 ± 96 (5)	14.0 ± 0.9 (6)	9.4 ± 0.9 (6)
NaF, 25 mM	1694 ± 99 (4)	930 ± 116 (4)	—	—
NaF, 25 mM + Folch Fraction V, 2.5 mg/ml	1159 (1)	201 (1)	—	—

TABLE III

EFFECT OF NaF ON THE SOLUBILIZATION OF RAT KIDNEY PLASMA MEMBRANE PROTEIN BY LUBROL-WX

Rat kidney plasma membranes suspended in 0.25 M sucrose–1 mM EDTA were incubated with Lubrol-WX for 15 min at 0–2 °C and then centrifuged at 37000 × *g* for 20 min. The data are expressed as the mean ± S.E. of 4 experiments without NaF and 3 experiments with 25 mM NaF added to the membrane suspension. Percentage of native protein concentration was calculated by: protein concentration (mg/ml) after centrifugation/protein concentration before centrifugation × 100.

Lubrol concn (%, w/v)	*% of native protein concn*	
	No NaF	*25 mM NaF*
0.05	80.7 ± 2.6	37.3 ± 2.1
0.1	79.1 ± 1.6	44.4 ± 4.3
0.4	82.5 ± 4.3	56.2 ± 4.6
0.8	95.6 ± 2.8	55.6 ± 3.2
1.2	96.4 ± 2.3	63.8 ± 3.8

The non-ionic detergent, Lubrol-WX was also employed for solubilizing renal plasma membranes for comparison with the French pressure cell method. Table III shows the quantitative solubilization of the particulate membrane protein with a range of Lubrol-WX concentrations from 0.05 to 1.2 % w/v, in the presence and absence of 25 mM NaF. NaF produced a more marked decrease in the amount of membrane protein solubilized with Lubrol-WX than with the French pressure cell technique. The addition of NaF was necessary for maintenance of adenyl cyclase activity of the soluble membrane preparation. Only 4–17 % of the particulate adenyl cyclase activity was maintained after solubilization with Lubrol-WX in the absence of NaF (Table IV). Addition of 25 mM NaF prior to detergent solubilization yielded soluble membrane adenyl cyclase activities of up to 45 % of the native particulate membrane adenyl cyclase activity. These data indicate that Lubrol-WX concentra-

TABLE IV

PROTECTION AGAINST LUBROL INHIBITION OF RAT KIDNEY PLASMA MEMBRANE ADENYL CYCLASE ACTIVITY BY NaF

Rat kidney plasma membrane suspensions were treated with Lubrol-WX as described in Table III. The adenyl cyclase specific activity of the native membrane suspension and soluble membranes is expressed as the mean of 3 experiments with NaF and the results of one experiment without NaF. The Lubrol-WX concentration in the enzyme assay was one-third the above concentration (25 μl membrane preparation used with final incubation volume of 75 μl). The final concentration of NaF in all the adenyl cyclase assays was 10 mM.

Lubrol (%, *w/v*)	*Adenyl cyclase activity (pmoles cyclic AMP/20 min/mg)*			
	Native	*"Soluble"*	*Native + NaF (25 mM)*	*"Soluble" + NaF (25 mM)*
0	1409	—	2042	—
0.05	309	237	2339	344
0.1	271	161	2294	369
0.4	218	174	1990	828
0.8	175	59	1661	902
1.2	115	83	1206	666

tions of 0.4–0.8 % yield optimal solubilization of membrane protein and maintenance of adenyl cyclase activity in the presence of NaF. Renal plasma membrane ATPase activity is also inhibited by Lubrol-WX solubilization procedures as shown in Fig. 8. Both the native particulate and soluble ATPase appears to be inhibited to the same extent with the concentration of detergent employed.

Particulate rat kidney plasma membrane preparations exhibit a hormone-sensitive adenyl cyclase so that the nature of hormone activation with respect to solubilization was investigated. Renal membranes were solubilized without the addition of NaF, for protection of the adenyl cyclase, because hormonal activation

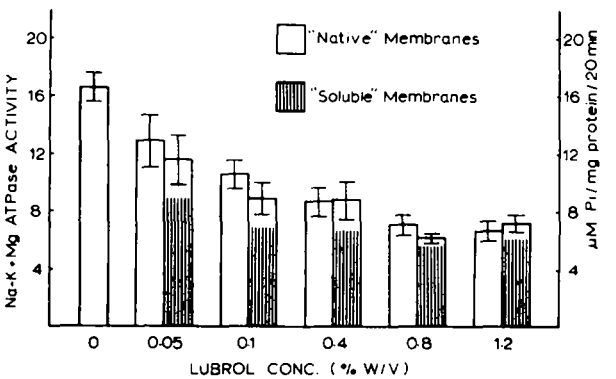

Fig. 8. Effect of addition of Lubrol-WX to rat kidney plasma membrane suspensions on the native and soluble (Na^+–K^+)- and Mg^{2+}-ATPase activity. Membranes were incubated with the depicted concentrations of Lubrol-WX for 15 min at 0–2 °C prior to centrifugation at 37000 × *g* for 20 min. Native membranes refers to the membrane suspension prior to centrifugation. Soluble membranes refers to the membranes not sedimented by this centrifugation. The results are the mean ± S.E. of 4 experiments. (Na^+–K^+)- and Mg^{2+}-ATPase activity is that measured in the presence of Na^+, K^+ and Mg^{2+} and represents the sum of the Mg^{2+}-dependent ATPase and the (Na^+–K^+)-dependent ATPase activities. Data are expressed as μmoles P_i formed per 20 min per mg protein at 37 °C.

TABLE V

EFFECT OF SOLUBILIZATION ON RENAL PLASMA MEMBRANE ADENYL CYCLASE ACTIVITY

Rat kidney plasma membranes suspended in 5.0 ml 0.25 M sucrose–1 mM EDTA were solubilized by passage through a French pressure cell under 20000 lb/inch^2 pressure as described in Methods. Adenyl cyclase activity of the membrane preparation was determined before and after solubilization. Results are expressed as the mean ± S.E. of 4 experiments employing membrane fractions prepared on different days. NaF or the hormones indicated were added to the adenyl cyclase assay medium at the concentrations shown.

Addition	*Adenyl cyclase (pmoles cyclic AMP/20 min/mg)*	
	Native membranes	*"Soluble" membranes*
None	52 ± 6	46 ± 19
NaF, 10 mM	2054 ± 340	739 ± 211
Lysine vasopressin, $1 \cdot 10^{-5}$ M	239 ± 18	73 ± 30
Parathyroid hormone, $1 \cdot 10^{-8}$ M	354 ± 42	68 ± 41

of the adenyl cyclase is prevented by addition of NaF. The French pressure cell method was employed in these and subsequent experiments. Table V shows the results of 4 separate experiments utilizing membrane preparations prepared on different days. The particulate adenyl cyclase activity was enhanced by NaF, vasopressin and parathyroid hormone but after solubilization of the renal plasma membranes only NaF activation could be achieved. In these experiments the activation achieved by NaF, 10 mM, was reduced from a 40-fold increase in adenyl cyclase activity in particulate membranes to about a 16-fold increase in the activity of the soluble adenyl cyclase. The basal adenyl cyclase activity was not significantly reduced by solubilization of the particulate membrane preparations. Additional experiments were designed to further investigate the effect of solubilization on the hormone-sensitive adenyl cyclase. Addition of NaF to the particulate plasma membrane suspension prior to passage through the French pressure cell afforded partial protection of the adenyl cyclase activity as shown in Table II. It was conceivable that NaF may protect the hormone-sensitive component of the renal membrane adenyl cyclase system and that removal of the NaF after solubilization would yield a soluble hormone-sensitive adenyl cyclase. Dialysis of the solubilized plasma membrane preparation, which contained 25 mM NaF, against 250 vol. sucrose–EDTA solution, for 16 h at 2 °C resulted in complete loss of enzymatic activity. Control experiments where the particulate membrane suspension (25 mM NaF added) was dialyzed under the same conditions also resulted in complete loss of activity indicating that the rat kidney membrane adenyl cyclase is not sufficiently stable to withstand this experimental procedure. Therefore an alternative method of removal of NaF after solubilization was necessary and the use of a Sephadex G-25 column was subsequently employed. Figs. 9 and 10 depict the elution profile of solubilized plasma-membrane protein and adenyl cyclase activity when NaF, 25 mM, is included or excluded from the column. Exclusion of NaF from the column resulted in complete loss of adenyl cyclase activity (Fig. 9) presumably due to removal of NaF from the eluted soluble membrane proteins. The soluble membrane proteins are excluded from Sephadex G-25 with all the protein peak contained in the external volume of the

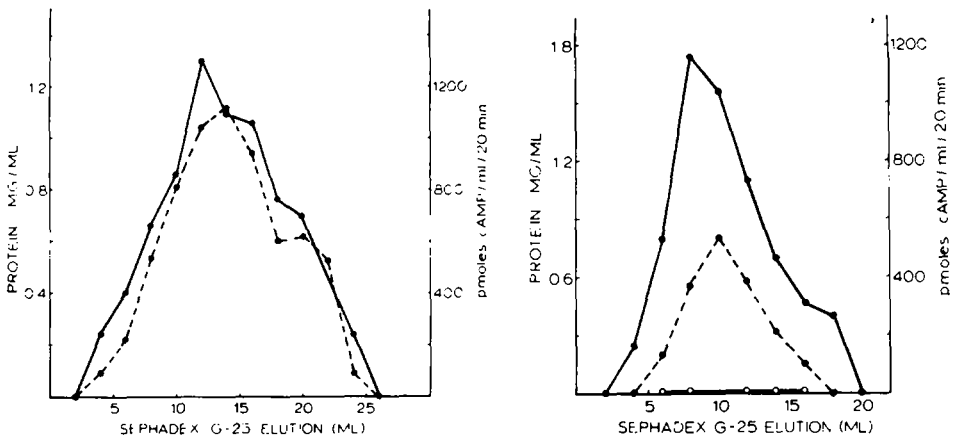

Fig. 9. Gel-filtration chromatography of soluble renal plasma membranes with Sephadex G-25. Membranes solubilized by passage through a French pressure cell were chromatographed with Sephadex G-25 column. Column dimensions were 2.5 cm × 40 cm and flow rate was approx. 1.5 ml/min. The column was equilibrated and eluted with 0.1 M NaCl, 10 mM Tris–HCl, pH 7.5, and with (●---●) or without (○—○) 10 mM NaF. Adenyl cyclase activity was determined with 10 mM NaF in the incubation medium in both cases. ●—●, protein elution profile.

Fig. 10. Effect of inclusion of EDTA and NaF in Sephadex G-25 columns on rat kidney adenyl cyclase activity of eluted fractions. Conditions same as in Fig. 9 except that the column was equilibrated and eluted with 0.1 M NaCl, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 10 mM NaF. ●—●, protein elution profile; ●---●, adenyl cyclase activity with 10 mM NaF in assay.

column. Addition of 25 mM NaF to the eluting solution resulted in a loss of adenyl cyclase specific activity of about 50 % after passage of the soluble membranes through the Sephadex G-25 column. The further addition of 1 mM EDTA *plus* NaF (Fig. 10) resulted in no loss of adenyl cyclase activity after Sephadex chromatography. This indicated that the experimental procedure of Sephadex chromatography was not altering the soluble membrane adenyl cyclase by removal of some small molecular weight component necessary for activity as long as NaF and EDTA remained present in the eluting medium. Since the NaF could not be removed without loss of adenyl cyclase activity, this method was deemed unfeasible for further examination of a potential soluble, hormone sensitive renal membrane adenyl cyclase.

The above experiments did, however, define the conditions which were necessary for maintenance of adenyl cyclase activity which precluded further examination of the soluble renal adenyl cyclase by gel-filtration chromatography. Since this soluble membrane preparation had been previously examined for its chromatographic properties with respect to the (Na^+–K^+)-dependent ATPase and Mg^{2+}-ATPase utilizing 6 % agarose-gel columns, investigation of the chromatographic properties of the soluble membrane adenyl cyclase was undertaken. Fig. 11 shows the elution profile of soluble membrane protein and adenyl cyclase activity. This column was calibrated with blue dextran (M_r approx. $2 \cdot 10^6$), bovine thyroglobulin (M_r approx. $6 \cdot 10^5$), bovine gamma globulin (M_r approx. $1.6 \cdot 10^5$) and bovine hemoglobin (M_r approx. $6.8 \cdot 10^4$) and the elution peak of these compounds are noted on the chromatogram by arrows. Two protein peaks are eluted with the first eluting in the external volume (void volume) of the column and the second broad protein peak eluting within

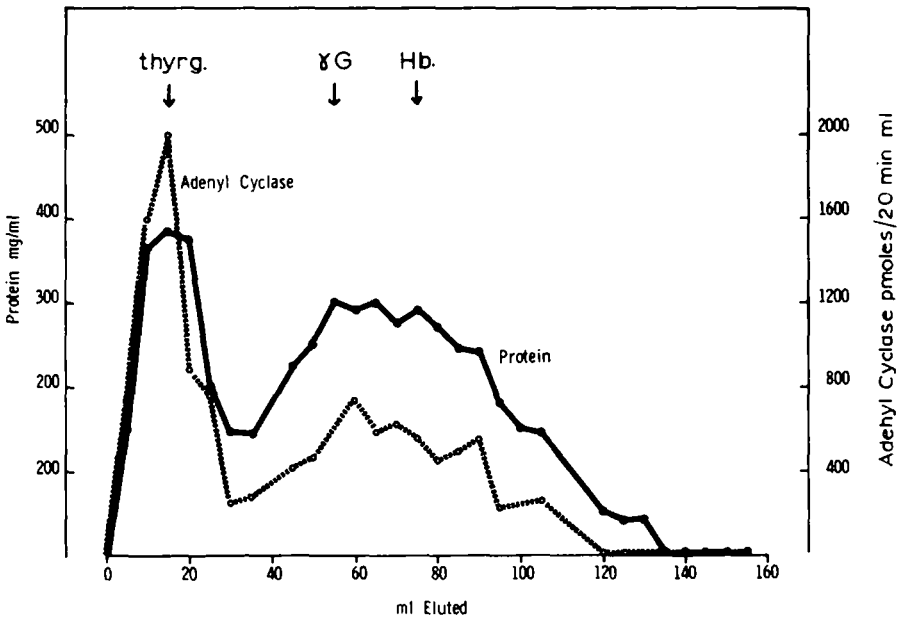

Fig. 11. Gel-filtration chromatography of soluble renal plasma membranes with 6% agarose. Membranes solubilized by passage through a French pressure cell were chromatographed with a 6% agarose (Gelarose) column. Column dimensions were 2.5 cm × 40 cm and the flow rate was approx. 1.0 ml/min. The column was equilibrated and eluted with 0.1 M NaCl, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 10 mM NaF. Adenyl cyclase activity was determined in the eluted fractions with 10 mM NaF in the incubation medium. ●—●, protein elution; ○---○, adenyl cyclase activity.

TABLE VI

AGAROSE CHROMATOGRAPHY OF SOLUBLE RAT KIDNEY PLASMA MEMBRANE ADENYL CYCLASE

Rat kidney plasma membranes suspended in 0.25 M sucrose, 1 mM EDTA, 25 mM NaF were solubilized by passage through a French pressure cell as described in detail in Methods. The soluble membrane preparation was chromatographed on 6% agarose column (2.5 cm × 40 cm) which was equilibrated and eluted with a solution of 0.1 M NaCl, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 25 mM NaF. Flow rate was approx. 1 ml/min. Adenyl cyclase activity of the soluble membrane preparation applied to the column and the first chromatographic peak were obtained without concentration whereas the fractions retarded by the gel (second peak) were concentrated by ultrafiltration employing Amicon type PM-10 membranes. Final concentration of NaF in the adenyl cyclase assay was 10 mM. The mean ± S.E. of several assays on different fractions in each peak is reported.

Fraction	*Adenyl cyclase specific activity (pmoles cyclic AMP/ 20 min/mg)*
Soluble membranes	537
First peak	1702 ± 150
Second peak (concentrated 3 times)	849 ± 72

the exclusion limit of this gel. Estimation of the molecular weight of these two peaks would place the first peak at $>6 \cdot 10^5$ and the second protein peak consisting of a wide range of different size membrane subunits with a mean molecular weight of about $1 \cdot 10^5$. Adenyl cyclase activity was found to be associated with both protein peaks but it is obvious that the specific activities of the two adenyl cyclase systems are quite different. The specific activity of the first peak adenyl cyclase is approx. 3-fold increased over the specific activity of the soluble membrane adenyl cyclase applied to this column, whereas the specific activity of the second peak adenyl cyclase is somewhat lower than the specific activity of the soluble preparation applied to the column. Due to relatively low membrane protein concentration and adenyl cyclase activity of the second chromatographic peak, it was felt that additional analysis of the second peak adenyl cyclase was necessary to substantiate this observation. In subsequent experiments the second peak fractions were concentrated approximately 3-fold by ultrafiltration with Amicon Diaflo membranes. The second-peak proteins did not pass through this membrane. Table VI shows the results of such an experiment. The specific activity of the first peak and second peak adenyl cyclase is presented as the mean of several assays in the respective elution peak. The adenyl cyclase specific activity of the soluble membrane preparation applied to the agarose column was 537 pmoles cyclic AMP per mg per 20 min whereas the activity of the first peak was enhanced to 1702 and the activity of the second peak adenyl cyclase was 849. With concentration of the second peak fractions there was no difficulty in obtaining accurate measurements of enzymatic activity in the second peak fractions.

DISCUSSION

The mammalian kidney is a very interesting tissue for studying the mechanism of hormone specificity with regard to hormone-receptor interactions in that this tissue demonstrates morphological differentiation with respect to the localization of hormone-sensitive adenyl cyclase systems. Chase and Aurbach[7] have demonstrated that parathyroid hormone activates the adenyl cyclase of crude renal cortex fractions whereas vasopressin stimulates the adenyl cyclase located in renal medulla. The present study utilizing a plasma membrane preparation prepared from cortex and medulla of rat kidney supports those observations. Parathyroid hormone activated the cortical membrane adenyl cyclase considerably more than did vasopressin and vasopressin activated the medullary membrane adenyl cyclase more than parathyroid hormone. Comparison of the plasma membrane preparation of renal cortex with renal medulla indicated that the activity of the cortical adenyl cyclase was approximately one-third the basal specific activity of the medulla adenyl cyclase. It is conceivable that the adenyl cyclase of mammalian kidney is regionally distributed along the nephron in a functional manner such as has been observed for the (Na^+-K^+)-dependent ATPase, which also demonstrates a higher specific activity in the renal medulla of the rat kidney as demonstrated by Hendler *et al.*[20]. These investigators also compared the adenyl cyclase activity of rat renal cortex and medulla ($2200 \times g$ sediment) and concluded that little difference in activity between cortex and medulla was present. However, the adenyl cyclase assay included 10 mM NaF to produce maximal activation of the adenyl cyclase and under these conditions

little difference in the cortex and medulla plasma membrane adenyl cyclase activity was found in the present study unless the Mg^{2+} concentration is varied.

A K_m for ATP was determined for the renal cortex and medulla adenyl cyclase which was approximately 0.1 mM for both preparations. This is similar to values derived from experiments with a cardiac adenyl cyclase[21]. The most marked difference observed between the cortex and medulla adenyl cyclase preparations was the nature of divalent cation dependence. The renal cortex enzyme activity with different divalent cations in the assay showed the order of activity of $Mg^{2+} > Mn^{2+} > Co^{2+}$ whereas the medulla adenyl cyclase was $Mg^{2+} = Mn^{2+} > Co^{2+}$. It is difficult to interpret these differences in divalent cation dependency observed in these studies since the enzyme preparation employed is very crude and other Mg^{2+} dependent enzymes are present in this membrane preparation.

Experiments with the solubilization methods reported in this study indicate that the rat kidney plasma membrane adenyl cyclase can be solubilized with the detergent, Lubrol-WX or by intense shear force with a French pressure cell. Significant protection of the adenyl cyclase activity was achieved by the addition of 25 mM NaF to the membranes prior to solubilization. Removal of the NaF after solubilization resulted in complete loss of activity. This interesting observation may be of significant potential in regard to clarification of the mechanism of activation of adenyl cyclase by NaF. Studies are presently being done in this area.

In conclusion, it is felt that the renal plasma membrane preparation employed in this study is an excellent preparation for studying hormone–adenyl cyclase interactions. This plasma membrane adenyl cyclase preparation should also serve as a convenient preparation for solubilization of the particulate adenyl cyclase so that more sophisticated methods of fractionation (electrophoresis, gel-filtration chromatography) of the adenyl cyclase from other plasma membrane subunits can be achieved.

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