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SOLUBILIZATION AND PHOTOAFFINITY LABELING OF RENAL MEMBRANE CYCLIC AMP RECEPTORS *

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Summary

Renal cortical plasma membranes were solubilized with sodium deoxycholate. The membrane-bound cyclic AMP receptors retained biologic activity in the detergent-dispersed state exhibiting the properties of high affinity for cyclic AMP, saturability and specificity. Half-maximal binding of cyclic [^{3}H]-AMP to these receptors was found to occur at 0.06 μM and 1.5 pmol of cyclic [^{3}H]AMP was bound per mg membrane protein at saturation (0.5 μM cyclic [^{3}H]AMP). Sodium deoxycholate-solubilized membrane proteins were chromatographed on Biogel A-5m. Cyclic [^{3}H]AMP receptors eluted in the internal volume at positions equivalent to molecular sizes of 50 000 and 20 000 daltons and in the void volume at molecular size greater than 450 000. After photoaffinity labeling the renal membrane receptors with cyclic [^{3}H]AMP, we found peaks of tritium radioactivity which eluted at similar molecular size positions on this Biogel A-5m column. Further treatment of photoaffinity labeled membranes with sodium dodecyl sulfate, mercaptoethanol and urea, followed by polyacrylamide gel electrophoresis, showed bands of tritium-labeled receptor protein with relative mobilities corresponding to molecular sizes of 26 000 and 21 000 daltons. This study shows that porcine renal cortical membranes contain at least two molecular species of cyclic AMP receptors which may be associated with regulation of the membrane-bound cyclic AMP-dependent protein kinase.

Abbreviations: cyclic AMP, adenosine cyclic 3′,5′ monophosphate; cyclic GMP, guanosine cyclic 3′,5′ monophosphate.

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Introduction

Hormonal regulation of target cell function, which is mediated by the intracellular effector, adenosine 3′,5′ monophosphate (cyclic AMP), is thought to be via activation of specific protein kinase enzymes by the cyclic nucleotide resulting in the phosphorylation of cell protein (for review see ref. 1). These cyclic AMP-dependent protein kinase systems were first isolated from the cytoplasmic subcellular fraction of a number of tissues [2—5] and subsequently shown to be soluble enzymes consisting of two basic subunits [6—9]. These are a regulatory subunit, which serves as a receptor site for cyclic AMP and a catalytic subunit. Interaction of cyclic AMP with the regulatory subunit results in dissociation of this subunit as a cyclic AMP-regulatory subunit complex, thereby increasing the phosphotransferase activity of the catalytic subunit. More recently it has been shown that cyclic AMP-dependent protein kinases exhibit a more complex subcellular distribution than was originally considered. A number of mammalian tissues have been shown to contain both cyclic AMP receptors and cyclic AMP-dependent protein kinase activities which are components of the membrane systems of these cells [10—15]. We have described the characteristics of the cyclic AMP receptors and a cyclic AMP-dependent protein kinase activity associated with renal cortical plasma membranes [16,17]. The receptors show the necessary physiologic properties for the discrimination required of such a homeostatic control mechanism. Recently the cyclic AMP-dependent protein kinase of renal membranes was shown to be localized in the luminal membranes whereas the hormone-activated adenylate cyclase was found to be localized in the peritubular plasma membrane fraction [18—20]. These observations suggest a polarity of distribution of the renal enzymes which may be involved in the cellular transmission of information, beginning with the interaction of parathyroid hormone, vasopressin or calcitonin [21,22] with their respective receptors, followed by an increase in the intracellular levels of cyclic AMP, reacting in turn with specific receptors thereby controlling the level of phosphorylation of specific cell proteins involved in tubular reabsorption and/or secretion. The present study was designed to examine the biochemical properties of the cyclic AMP receptors of renal membranes after detergent solubilization of the membrane proteins. Both the number and molecular size of these receptor macromolecules were examined by gel filtration chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Materials and Methods

Kidney plasma membrane preparation. Porcine kidneys were obtained locally within 30 min after the animals were killed. The cortical portion of the tissue was dissected free of medulla and then homogenized in a solution containing 0.25 M sucrose, 10 mM Tris · HCl pH 7.4, and 1 mM EDTA (3 ml/g kidney). The plasma membrane-enriched subcellular fraction was prepared according to the method of Fitzpatrick et al. [23]. Membranes were suspended in the above buffer at a concentration of 5—10 mg/ml and stored at −20° C. Membrane protein was estimated using the method of Lowry [24].

Cyclic AMP-binding assay. Binding of cyclic [^{3}H]AMP to renal cortical mem-

brane preparations was assayed by the nitrocellulose filter (Millipore Corp.) assay previously described [17] which is a modification of the technique of Gilman [25]. Membranes were incubated in 40 mM Tris · HCl pH 7.5 at a protein concentration of 50-100 μg per 50 μl reaction volume. Incubation was generally for 60 min at 2° C. The binding of cyclic [^{3}H]AMP to membrane receptors was stable for several hours at that temperature.

Photoaffinity labeling of membrane cyclic AMP receptors. The method of Antonoff and Ferguson [26] was used for converting reversible cyclic [^{3}H]-AMP · receptor complexes to a trichloroacetic acid-resistant complex. Native or sodium deoxycholate-solubilized membranes were incubated with 1 μM cyclic [^{3}H]AMP under the conditions described above. After a 1 h preincubation at 2° C, a Mineralight UVS-11 lamp (peak wavelength 254.7 nm) was placed 2 cm from the quartz cuvettes containing the reaction media. The solution in the cuvettes was agitated continuously during the irradiation period. The reaction was stopped by making the reaction medium 10% with respect to trichloroacetic acid. This mixture was incubated for 2 h at 2° C and the precipitate was collected on a nitrocellulose filter (Millipore Corp., HAMK, 45 μm). The precipitate was then washed with 10 ml ice-cold 10% trichloroacetic acid and tritium radioactivity on the filter disc was measured by standard liquid scintillation spectrometry.

Protein kinase assay. The cyclic AMP-dependent and independent protein kinase activity of renal cortical membranes was assayed as previously described [17], which is a modification of the procedure of Rubin et al. [10]. Protamine (Sigma Chemical Co.) was used as exogenous phosphate acceptor protein.

Gel filtration chromatography of renal membrane proteins. A column of Biogel A-5m (6% agarose), 1.5 × 65 cm was equilibrated and eluted with a solution containing 0.3% sodium deoxycholate, 50 mM Tris · HCl pH 7.5, 50 mM NaCl and 0.02% NaN_3 at 2° C. The flow rate of this column was 25 ml/h at 80 cm H_2O. The gel was evaluated for its molecular sieving properties using macromolecules of known molecular size. These were; blue dextran 2—5 million, thyroglobulin 330 000, bovine albumin 65 000, ovalbumin 43 000 and cytochrome *c* 11 700 daltons. Elution volumes for these compounds were 50, 75, 95, 105 and 140 ml, respectively. Renal cortical membranes were solubilized by incubation of the membrane suspension with 1% sodium deoxycholate for 20 min at 2° C, followed by centrifugation at 39 000 × *g* for 10 min in a Sorvall RC-2B refrigerated centrifuge. 3 ml of the supernatant were applied to the above column and 3 ml fractions were collected.

Polyacrylamide gel electrophoresis of renal membrane proteins. Renal cortical membranes were treated with a solution containing 1% sodium dodecyl sulfate, 2% mercaptoethanol and 4 M urea for 5 minutes at 100° C [27]. Polyacrylamide gels (5%, 5 × 100 mm) were equilibrated with 0.1 M sodium phosphate, pH 7.0, containing 0.1% sodium dodecyl sulfate by electrophoresis using the same phosphate-sodium dodecyl sulfate buffer in the upper and lower reservoirs. Samples were loaded on the gel and run into the gel at 0.5 mA per tube for 0.5 h. The current was then increased to 5 mA per tube until the tracking dye (bromophenol blue) approached the end of the tube. Gels were then fixed and stained in a solution containing 0.025% Coomassie Brilliant Blue-R, 50% methanol and 10% acetic acid for 16 h. The gels were then destained in a Bio-

Rad Diffusion Destainer using 50% methanol/10% acetic acid solution. A chromatogram of each stained gel was obtained by scanning at 550 nm with a Gilford Model 2400-S spectrophotometer and linear transport attachment. Gels that were used for assay of tritium radioactivity were sliced in 1 mm thickness with no fixation or staining. This was done immediately after the electrophoresis was completed. The gels were sliced frozen at 1 mm thickness with a Mickle Gel Slicer. Each slice was then placed into a scintillation counting vial and digested in 0.5 ml of 30% H_2O_2 for 4 h at 60° C. Radioactivity was then measured by standard liquid scintillation methodology. The gels were calibrated using α-chymotrypsinogen (24 000 daltons) as a relative mobility of 1. Other standard proteins exhibit mobilities of 0.2 for thyroglobulin, 0.57 for bovine serum albumin and 0.75 for ovalbumin.

Materials. Radioactive compounds [γ^{32}P]ATP 7.3 Ci/mmol and cyclic [^{3}H]-AMP 37.7 Ci/mmol, were purchased from New England Nuclear, Boston, Mass. BioGel A-5M and the gel electrophoresis reagents were purchased from Bio-Rad Laboratories, Richmond, California. Detergent, standard proteins and other reagents were obtained from either Sigma Chemical Co., St. Louis, Mo. or Scientific Products Co., St. Louis, Mo.

Results

We previously reported that the detergent, sodium deoxycholate, effectively solubilized the cyclic AMP receptors of porcine kidney cortex plasma membranes [16]. The solubilized receptors were found to retain essentially complete cyclic [^{3}H]AMP binding activity such that the receptor's biochemical properties could be further examined. Fig. 1 shows the results of an experiment comparing the affinity of cyclic [^{3}H]AMP for the native, membrane-bound cyclic AMP receptors with the affinity of this cyclic nucleotide for sodium deoxycholate-solubilized membrane receptors. In these experiments, the detergent-solubilized membranes were assayed for cyclic [^{3}H]AMP binding in the presence of sodium deoxycholate. These data show that the binding of cyclic [^{3}H]AMP to receptors in the native membrane conformation exhibits the property of unsaturable binding. The binding curve shows a plateau for cyclic [^{3}H]-AMP binding at about 0.5 μM cyclic [^{3}H]AMP, but at concentrations of cyclic [^{3}H]AMP greater than 1 μM the binding increased sharply. Saturation of these binding sites was not achieved even at levels of cyclic [^{3}H]AMP up to 1 mM (data not shown). Sodium deoxycholate-solubilized membrane proteins, however, showed a finite number of cyclic AMP receptors since the binding of cyclic [^{3}H]AMP to this preparation was found to be saturated at approximately 0.5 μM cyclic [^{3}H]AMP. At saturation, the cyclic [^{3}H]AMP bound to sodium deoxycholate-solubilized membrane proteins was 1.5 pmol cyclic [^{3}H]AMP per mg of membrane protein. The Lineweaver-Burke analysis of cyclic [^{3}H]AMP binding to solubilized membranes (Fig. 1B, inset) shows an affinity constant for cyclic [^{3}H]AMP of 0.06 μM. These experiments demonstrate that the sodium deoxycholate-treatment of renal cortical membranes eliminates the low affinity binding of cyclic [^{3}H]AMP, which suggests that these binding sites are dependent upon the vesicular structure of the native membrane preparation. It is possible that the nonsaturable form of cyclic [^{3}H]AMP-membrane associa-

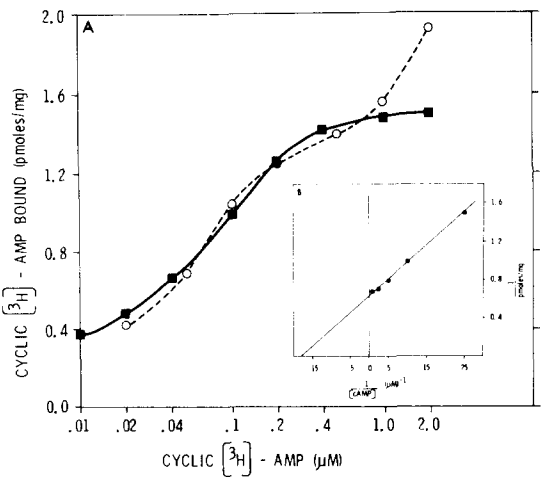

Fig. 1. Affinity of renal cortical membrane receptors for cyclic [^{3}H]AMP. Panel A: Both native plasma membranes (○- - - - -○) from procine renal cortex and membranes solubilized with 1% sodium deoxycholate (■- - - - -■) were assayed for cyclic [^{3}H]AMP binding activity over the range of cyclic [^{3}H]AMP concentration shown in the figure. Membranes solubilized with 1% sodium deoxycholate were assayed in the presence of sodium deoxycholate, but the final concentration of detergent was reduced to 0.2% in the binding assay. The data are representative of several experiments of this type and are the mean of triplicate assays at each experimental point. Panel B (inset): This plot is a Lineweaver-Burke analysis of the binding characteristics of the sodium deoxycholate-solubilized cyclic [^{3}H]AMP receptor shown in Panel A.

tion results from the uptake of cyclic [^{3}H]AMP into membranous vesicles, whereas the high affinity, saturable binding is with physiologic cyclic AMP receptors.

To further investigate the nature of the binding of cyclic [^{3}H]AMP to sodium deoxycholate-treated membranes we examined the effect of detergent solubilization on the specificity of these receptors. Competition experiments were employed as previously described [17] and reviewed in the figure legend. Fig. 2 shows a representative experiment of this type with both native membranes (Panel A) and sodium deoxycholate-solubilized membranes (panel B). These data show that the most effective competing cyclic nucleotides in this assay were cyclic AMP and 8-azido-cyclic AMP, and that a somewhat similar degree of displacement of cyclic [^{3}H]AMP was found with both native and sodium deoxycholate-solubilized membrane preparations. At the concentration of cyclic [^{3}H]AMP employed in this experiment (0.2 μM), 50 percent displacement of cyclic [^{3}H]-AMP bound to native membranes was achieved at a concentration of unlabeled cyclic AMP of 0.5 μM. This represents a molar ratio of unlabeled to labeled cyclic AMP of 2.5 to 1 in the binding assay. The same level of 50% displacement of cyclic [^{3}H]AMP from sodium deoxycholate-treated membranes by unlabeled cyclic AMP was found at 0.3 μM cyclic AMP which is a molar ratio of unlabeled to labeled cyclic AMP of 1.5 : 1. Dibutyryl cyclic AMP and cyclic GMP were found to displace cyclic [^{3}H]AMP only at high concentrations representing molar ratios of competitor to cyclic [^{3}H]AMP of 500 : 1. Cyclic 2′,3′-AMP did not compete with cyclic [^{3}H]AMP for the receptors of either native or sodium deoxycholate-treated membranes.

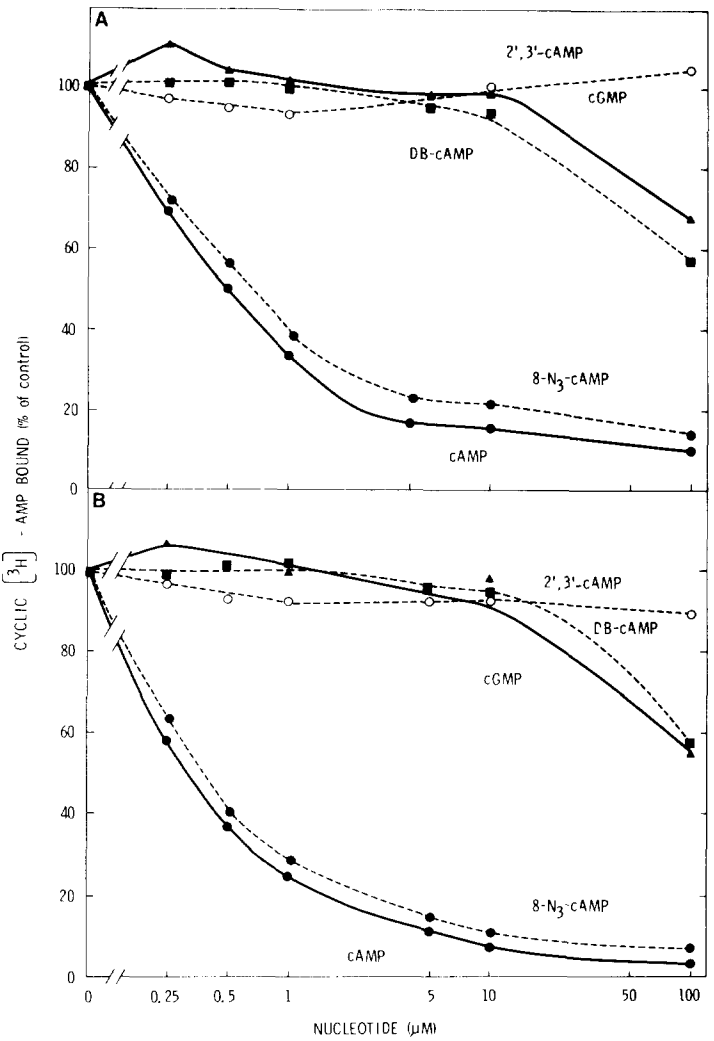

Fig. 2. Specificity of renal cortical membrane cyclic AMP receptors. Native plasma membranes (Panel A) from porcine renal cortex or membranes treated with 1% sodium deoxycholate (Panel B) were incubated with 0.2 μM cyclic [^{3}H]AMP under standard conditions in the absence or presence of the competing cyclic nucleotides at varying concentration. Cyclic nucleotides employed in this assay were: ●———●, cyclic 3′,5′-AMP (cAMP); ●- - - - -●, 8-azido-cyclic 3′,5′-AMP (8-N_3-cAMP); ▲———▲, cyclic 3′,5′-GMP (cGMP); ■- - - - -■, dibutyryl cyclic 3′,5′-AMP (DB-cAMP); ○- - - - -○, cyclic 2′,3′-AMP (2′,3′-cAMP). The data are the mean of duplicate assays and are presented as the percent of cyclic [^{3}H]AMP bound when no other nucleotide is present in the reaction medium.

The concentration vs. response relationship for cyclic AMP activation of the native membrane protein kinase was examined in order to compare this relationship with the binding curves for cyclic [^{3}H]AMP (Fig. 1). This assay was carried out in the absence and presence of theophylline, which inhibits membrane-associated cyclic nucleotide phosphodiesterase but does not impair the binding of cyclic [^{3}H]AMP to renal membranes [17]. Fig. 3 depicts a representative experiment. The cyclic-AMP dependent protein kinase activity was not altered by theophylline, whereas the methylxanthine did inhibit the cyclic

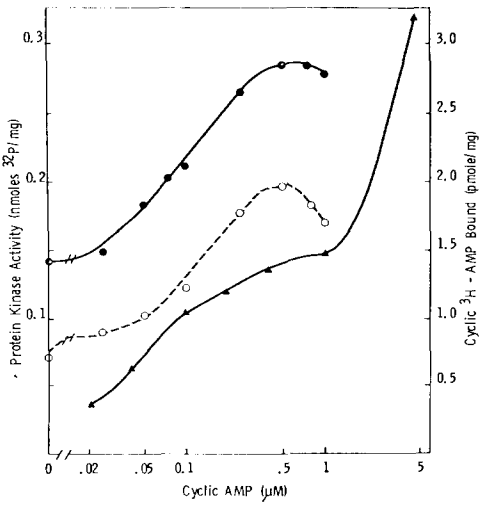

Fig. 3. Protein kinase activity of renal cortical membranes. Comparison of the activation by cyclic AMP with cyclic [^{3}H]AMP binding. Native plasma membranes (105 μg) from porcine renal cortex were assayed for protein kinase activity at 30°C for 10 min with 100 μg protamine as phosphate acceptor. Standard protein kinase conditions of assay were used (see Methods and Materials and ref. 17). Enzyme activity was determined in the absence of (●———●) and presence (○- - - - -○) of 5 mM theophylline. The levels of cyclic AMP employed are shown in the figure. Cyclic [^{3}H]AMP binding was assayed under standard conditions over the range of concentration shown above (▲———▲). The data are representative and are the mean of triplicate assays at each point.

AMP-independent protein kinase activity by about 50%. Activation by cyclic AMP occurred over the same range of cyclic AMP concentration observed for cyclic [^{3}H]AMP binding to high affinity membrane receptors. An activation constant (K_a) for cyclic AMP was found to be about 0.08 μM, which compares favorably to the affinity constant for cyclic AMP binding to membrane receptors (0.06 μM). Cyclic AMP-dependent protein kinase activity of the sodium deoxycholate-treated renal membrane is not presented since the detergent treatment produced marked inhibition of this enzyme activity.

Membrane proteins, solubilized with 1% sodium deoxycholate, were chromatographed on a column of Bio Gel A-5m (6% agarose) which was equilibrated and eluted with a solution containing sodium deoxycholate (0.3%). Each fraction was assayed for protein and for cyclic [^{3}H]AMP receptor activity. Fig. 4 shows the chromatographic profile for both the sodium deoxycholate-solubilized membrane proteins and the cyclic [^{3}H]AMP receptor components. Peaks of membrane protein were found at 50 ml (void volume) and about 110 ml with a shoulder occurring at 135—140 ml. Cyclic [^{3}H]AMP receptor activity eluted at 50 ml, 100—105 ml and about 135 ml, which corresponds to approximate molecular sizes of greater than 450 000, 50 000 and 20 000 daltons, respectively. Membrane cyclic AMP receptors eluting in the void volume of this column represent either exceptionally large macromolecules or the receptors are still associated with membranous components not completely solubilized by the sodium deoxycholate-treatment procedure. It is evident that the elution patterns of cyclic AMP receptor activity and membrane protein that elute in the internal volume of this gel are dissimilar. The 50 000 dalton receptor elutes

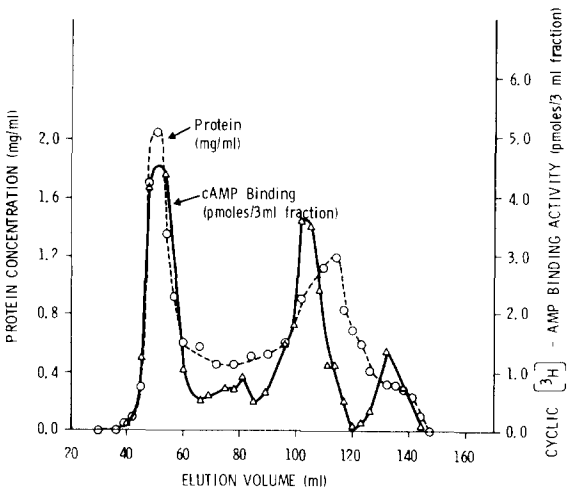

Fig. 4. Gel filtration chromatography of sodium deoxycholate-solubilized renal membrane cyclic AMP receptors. Plasma membranes (64 mg protein) from porcine renal cortex were solubilized with 1% sodium deoxycholate as described in Methods and Materials and chromatographed on a column of Biogel A5M (6% agarose, 1.5 X 65 cm), which was equilibrated and eluted with a solution of 10 mM Tris · HCl pH 7.5, 50 mM NaCl, 0.3% sodium deoxycholate and 0.02% NaN_3 at 2°C. Each 3 ml fraction was assayed for protein (○- - - - -○) and cyclic [^{3}H]AMP binding (△———△) using the methodology previously described. Elution volumes for the standard protein used to calibrate this column are given in the Methods and Materials.

before the larger protein peak seen in the internal volume suggesting that this receptor peak may represent a homogenous species of membrane cyclic AMP receptor protein.

In order to further study the number and molecular size of the renal membrane cyclic AMP receptors, we proposed to employ the method of Antonoff and Ferguson [26] for converting the reversible form of cyclic [^{3}H]AMP-receptor association to a trichloroacetic acid-stable form of cyclic [^{3}H]AMP-receptor bond. This method employs ultraviolet irradiation of the cyclic [^{3}H]AMP · receptor complex to convert the purine · receptor complex to a form that is stable to treatment with trichloroacetic acid, heat denaturation and sodium dodecyl sulfate. The experiment shown in Fig. 5 illustrates the time course for photoaffinity labeling of both native renal membranes and sodium deoxycholate-treated membranes. The reaction is relatively slow but after 10 h about one-fourth to one-third of the reversible form of cyclic [^{3}H]AMP receptor complex is converted to a form which is stable to treatment of the complex with 10% trichloroacetic acid. The reaction is dependent upon ultraviolet irradiation since the membrane · cyclic [^{3}H]AMP complex which was irradiated in a flint-glass cuvette was not converted to a trichloroacetate-stable form. In additional experiments, the conversion of reversible cyclic [^{3}H]AMP binding with both native and sodium deoxycholate-solubilized membranes to the trichloroacetic acid-resistant form of binding was examined in the presence and absence of an excess of unlabeled cyclic AMP (1000 fold). The membrane suspensions were irradiated for 0, 5 and 15 h under the conditions described in the legend to Fig. 5. In these experiments, specific cyclic [^{3}H]AMP binding to membranes was greater than 90% of the total binding. We found that after 15 h of photoaf-

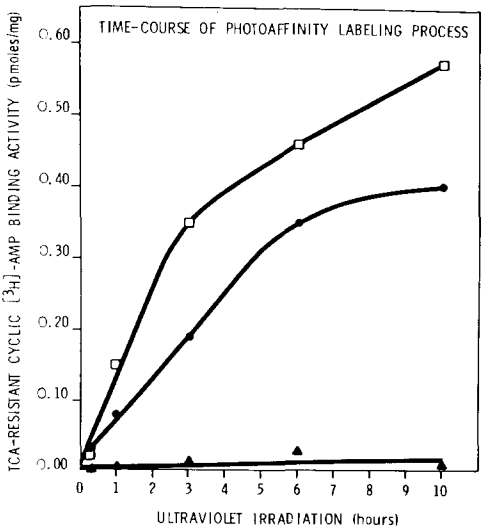

Fig. 5. Time-course for photoaffinity labeling of renal membranes with cyclic [^{3}H]AMP. Cortical membranes in the native state were preincubated with 1 μM cyclic [^{3}H]AMP for 60 min at 2°C and then irradiated in either quartz (●———●) or flint-glass (▲———▲) cuvettes for the period indicated above. Sodium deoxycholate-solubilized membranes (□———□) were irradiated in the presence of 1 μM cyclic [^{3}H]AMP under identical conditions. At the indicated intervals, trichloroacetic acid (TCA)-resistant cyclic [^{3}H]-AMP binding was measured. For complete details see Materials and Methods.

finity labeling, the conversion of specific, reversible cyclic [^{3}H]AMP binding to trichloroacetic acid-resistant binding was essentially complete in sodium deoxycholate-solubilized membranes, whereas with native membranes only about one-third of the specific binding of cyclic [^{3}H]AMP was converted from a reversible to an acid-resistant form. This may have been due to the enhanced clarity of the sodium deoxycholate-solubilized membrane preparation. The level of trichloroacetic acid-resistant specific cyclic [^{3}H]AMP binding after 15 h of irradiation was greater in the sodium deoxycholate-solubilized renal membranes (0.5 pmol/mg) than found in native membranes (0.4 pmol/mg). For these reasons, subsequent experiments were conducted using the sodium deoxycholate-solubilized membranes and a 15 h photoaffinity labeling period.

Renal cortical membranes, photoaffinity labeled with cyclic [^{3}H]AMP, were solubilized with 1% sodium deoxycholate and then chromatographed on a column of Biogel A-5m. Identical conditions were employed as were used in the experiment depicted in Fig. 4. Each eluted fraction was assayed for protein and trichloroacetic acid-resistant cyclic [^{3}H]AMP · protein complexes. A representative elution pattern for these parameters is shown in Fig. 6. The protein elution is similar to that of the previous gel filtration experiment (Fig. 4), with the exception that the shoulder of protein eluting at 135 ml is a more pronounced peak of membrane protein. The trichloroacetic acid-resistant tritium radioactivity eluted at positions which are similar to the elution of reversible cyclic [^{3}H]AMP · receptor activity (Fig. 4). These data suggest that the photochemical reaction involved in the photoaffinity labeling process is relatively specific for coupling the cyclic [^{3}H]AMP to the physiologically important receptor sites. It is conceivable that these cyclic AMP receptors are membrane proteins of 50 000

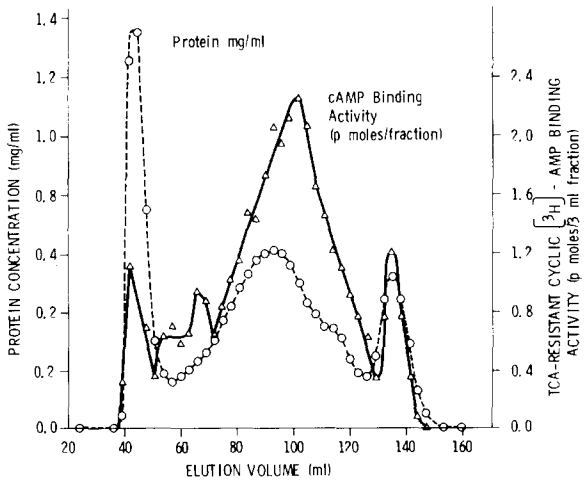

Fig. 6. Gel filtration chromatography of sodium deoxycholate-solubilized membranes after photoaffinity labeling with cyclic [^{3}H]AMP. Porcine renal cortical membrane suspensions were made 0.3% with respect to sodium deoxycholate and then photoaffinity labeled with 1 μM cyclic [^{3}H]AMP for 15 h as described in Methods and Materials. The suspension was then treated with a final concentration of 1% sodium deoxycholate, centrifuged at 39 000 $\times g$ for 10 min and the supernatant (64 mg protein) applied to the same Biogel column used for the experiment depicted in Fig. 4. Each 3 ml fraction was assayed for protein (○- - - - -○) and trichloroacetic acid (TCA)-resistant tritium radioactivity (△———△). The data are the mean of duplicate assays for both parameters.

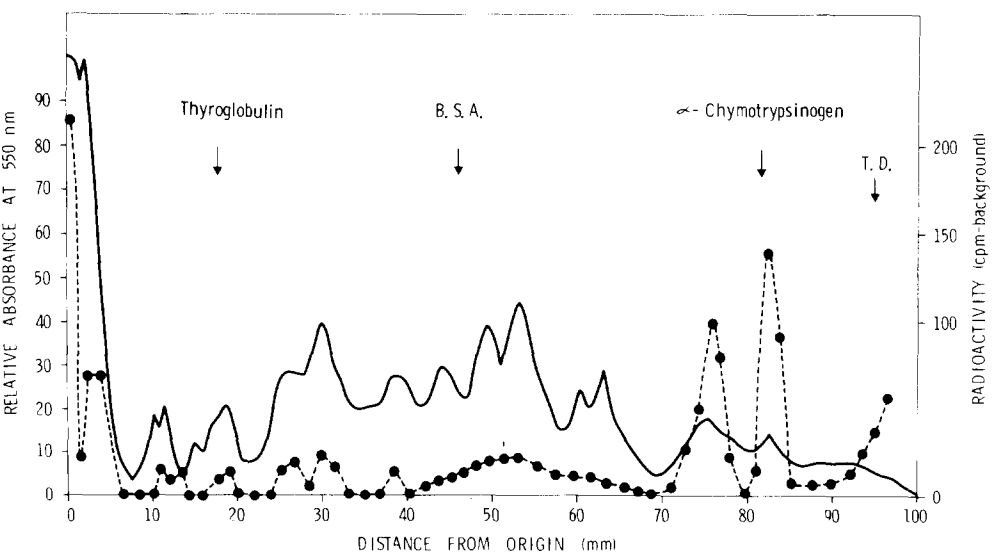

Fig. 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of renal membrane photoaffinity labeled with cyclic [^{3}H]AMP. Porcine renal cortical membranes (sodium deoxycholate-treated) were photoaffinity labeled with 1 μM cyclic [^{3}H]AMP for 15 h as described in Methods and Materials. The membranes were then treated with a solution containing 1% sodium dodecyl sulfate, 2% mercaptoethanol and 4 M urea for 5 min at 100°C [27]. After electrophoresis of the resulting protein mixture using 5% polyacrylamide gels (5 $\times$ 100 mm) one gel was fixed and stained in a solution of 0.025% Coomassie Brilliant Blue R, 50% methanol and 10% acetic acid. A duplicate gel was frozen and sliced at 1 mm thickness. Each slice was digested in 30% H_2O_2 and tritium radioactivity determined. The stained, duplicate gel was assayed for absorption at 550 nm using a Gilford 2400-S spectrophotometer with a linear transport attachment. The data are presented as tritium radioactivity in cpm per slice (○- - - - -○) and relative absorbance at 550 nm (———). B.S.A., bovine serum albumin. T.D., tracking dye, bromophenol blue.

and 20 000 daltons in molecular size in the presence of sodium deoxycholate.

This aspect was examined using a different method for estimation of molecular size of membrane proteins. Renal membranes, which were photo-affinity-labeled with cyclic [^{3}H]AMP in the presence of 0.3% sodium deoxycholate, were further treated with 1% sodium dodecyl sulfate, 2% mercaptoethanol and 4 M urea for 5 min at 100° C to provide complete dissociation and solubilization as well as denaturation of the membrane proteins [27]. The sample was then applied to 5% polyacrylamide gels and subjected to electrophoresis as described in Methods and Materials. Fig. 7 shows the results of such an experiment. The profile of radioactivity shows three areas of tritium which correlate with the protein remaining at the origin and two peaks of radioactivity at positions corresponding to apparent molecular sizes of 26 000 and 21 000 daltons. The protein banding patterns of the sodium dodecyl sulfate-treated membranes which had been exposed to ultraviolet light is essentially the same as the banding pattern for sodium dodecyl sulfate-treated membranes not exposed to irradiation (data not shown). A notable exception is the large quantity of protein which enters the gel only a few mm and probably represents the effect of ultraviolet irradiation resulting in the formation of membrane protein aggregates. Therefore, the smaller molecular size receptors found with sodium dodecyl sulfate-electrophoresis may be derived from receptors identified using sodium deoxycholate and Biogel A-5m chromatography.

Discussion

We previously reported the existence of a plasma membrane-bound, cyclic AMP-dependent protein kinase system and membrane receptors for the cyclic nucleotide [16,17]. The present study demonstrated that these membrane cyclic AMP receptors could be effectively dissociated from the membranous structure with retention of physiologically important receptor properties by treatment of the native, particulate membranes with sodium deoxycholate. The sodium deoxycholate-solubilized membrane proteins demonstrated high affinity binding of cyclic [^{3}H]AMP with an affinity constant of approximately 0.06 μM and 1.5 pmol cyclic [^{3}H]AMP bound per mg of membrane protein at a saturating concentration of 0.5 μM cyclic [^{3}H]AMP. The binding curve for cyclic [^{3}H]AMP was similar to the dose-response curve for cyclic AMP activation of the native membrane protein kinase, which showed half-maximal activation (K_a) at 0.08 μM cyclic AMP. This suggests that the cyclic AMP receptor sites may act in a regulatory manner with the protein kinase molecule in the membrane structure. It is possible that the receptor macromolecules are associated with the protein kinase macromolecule in a manner similar to the hormone-dependent adenylate cyclase, which exhibits specific receptors for the effector molecule associated with the adenylate cyclase catalytic component responsible for the intracellular synthesis of cyclic AMP [28,29]. It is unlikely that the cyclic AMP receptors are contaminants of the plasma membrane preparation resulting from adsorption of cytoplasmic, cyclic AMP-dependent protein kinase because we previously demonstrated that the receptor sites were not dissociated from the particulate membranes by extensive washing procedures [17]. The membrane

receptors are solubilized with detergents, suggesting that these receptors are indeed membrane components.

The renal tubular epithelial cell is differentiated such that the cell exhibits both morphologic and physiologic differences between the plasma membrane facing the filtrate (luminal membrane) and the membrane exposed to the extracellular fluid (peritubular membrane). This property of renal tubular cells is important for the establishment of vectorial pathways for transport of solutes in both reabsorptive and secretory modes of transport. Recently it has been shown that the cyclic AMP-dependent protein kinase system is preferentially localized in the luminal membrane, whereas the hormone-dependent adenylate cyclase system is localized in the peritubular membrane [18—20]. Our membranes prepared from porcine renal cortex exhibit both parathyroid hormone-dependent adenylate cyclase (data not shown) and cyclic AMP-dependent protein kinase activities, suggesting that these membranes are a mixture of luminal and peritubular membranes. The distribution of these two enzymes associated with the plasma membrane of renal target cells suggests that the hormone interacts with specific receptors at the peritubular side of the cell, thereby increasing intracellular cyclic AMP levels, which then react with specific receptors on the luminal membrane resulting in activation of the protein kinase. The phosphorylation of membrane proteins at the luminal membrane may regulate the transport of solutes and water across the luminal membrane.

The finding of multiple peaks of cyclic AMP receptor activity with both gel filtration and sodium dodecyl sulfate-electrophoresis may reflect alterations of the size of the receptor macromolecules as a result of the detergent solubilization procedures as well as effects of ultraviolet irradiation on membrane proteins. The high molecular weight binding sites revealed by both techniques may be related to incomplete solubilization of membrane protein by sodium deoxycholate resulting in elution of receptor material in the void volume of Biogel A-5m and/or protein-protein aggregates formed subsequent to ultraviolet irradiation of the membrane proteins followed by sodium dodecyl sulfate electrophoresis. These aggregates do not appear in membranes which are not treated with ultraviolet irradiation since all of the sodium dodecyl sulfate-treated membrane proteins enter the 5% polyacrylamide gels used in these experiments. However, two apparent cyclic AMP receptors solubilized by sodium deoxycholate were found with estimated molecular sizes of 20 000 and 50 000 daltons as measured by gel filtration on Biogel A-5m. These proteins showed reversible cyclic [^{3}H]AMP binding as well as photoaffinity labeling with cyclic [^{3}H]AMP after treatment of the reversible receptor · cyclic [^{3}H]AMP complex with ultraviolet light. In comparison, the molecular size of the two receptor proteins as determined by sodium dodecyl sulfate electrophoresis was approximately 21 000 and 26 000 daltons. One or both of these binding proteins may be a conversion product of the 50 000 dalton cyclic AMP receptor observed in the presence of sodium deoxycholate. It is therefore conceivable that these cyclic AMP receptors identified as different in molecular size by the two methods are derived from the same receptor proteins. Moreover, it is possible that the 50 000 dalton receptor is a dimer in the presence of sodium deoxycholate and is subsequently converted to a 26 000 dalton monomer by treatment with sodium dodecyl sulfate-urea. The 21 000 dalton cyclic AMP receptor protein

isolated by sodium dodecyl sulfate electrophoresis is similar in molecular size to the 20 000 dalton protein observed after sodium deoxycholate-solubilization of membranes and subsequent gel filtration chromatography of the membrane proteins. Another possibility is that the heterogeneity of cyclic AMP receptors may be produced through the proteolytic degradation of a single cyclic AMP receptor molecule. This would be more likely to occur in the photoaffinity labeling process due to the long incubation period.

Other investigators have reported the existence of cyclic AMP receptors associated with rat kidney membrane systems [30—32], but some have reported that the protein kinase system of renal membranes was not activated by cyclic AMP, whereas the cytoplasmic enzyme was a cyclic AMP-dependent protein kinase [31,32]. The present study and those previously reported by our group and others [16—20] clearly show the existence of a renal membrane cyclic AMP-dependent protein kinase as well as the appropriate cyclic AMP receptors. Whether the multiple cyclic AMP receptors seen in this study are associated with multiple forms of protein kinase due to the heterogeneity of renal cells or are involved in other cyclic AMP-regulated systems in the renal cortical cells is not presently known. Identification of the role of these membrane-bound cyclic AMP receptors is currently the subject of further investigation.

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References

1 Langan, T.A. (1973) in Advances in Cyclic Nucleotide Research (Greengard, P. and Robinson, G.A., eds.) Vol. 3, pp. 99—153, Raven Press, New York
2 Walsh, P.A., Perkins, J.P. and Krebs, E.G. (1968) J. Biol. Chem. 243, 3763—3765
3 Corbin, J.D. and Krebs, E.G. (1969) Biochem. Biophys. Res. Commun. 36, 328—336
4 Langan, T.A. (1969) Science 162, 579—580
5 Miyamoto, E., Kuo, J.F. and Greengard, P. (1969) Science 164, 63—65
6 Gill, G.N. and Garren, L.D. (1970) Biochem. Biophys. Res. Commun. 39, 335—343
7 Brostrom, M.A., Reimann, E.M., Walsh, D.A. and Krebs, E.G. (1970) Adv. Enzyme Regul. 8, 191—203
8 Kumon, A., Yamamura, H. and Nishizuka, Y. (1970) Biochem. Biophys. Res. Commun. 41, 1290—1297
9 Tao, M., Salas, M.L. and Lipmann, F. (1970) Proc. Natl. Acad. Sci. U.S. 67, 408—414
10 Rubin, C.S., Erlichman, J. and Rosen, O.M. (1972) J. Biol. Chem. 247, 6135—6139
11 Johnson, E.M., Maeno, H. and Greengard, P. (1971) J. Biol. Chem. 246, 7731—7739
12 Delorenzo, R.J. and Greengard, P. (1973) Proc. Acad. Sci. U.S. 70, 1831—1835
13 Chang, K., Marcus, J. and Cuatrecasas, P. (1974) J. Biol. Chem. 249, 6854—6865
14 Azhar, S. and Menon, K.M.J. (1975) Eur. J. Biochem. 58, 105—115
15 Roques, M., Rirard, A. and Lissitzky, S. (1975) Mol. Cell. Endocrinol. 2, 303—316
16 Forte, L.R., Chao, W.H., Walkenbach, R.J. and Byington, K.H. (1972) Biochem. Biophys. Res. Commun. 49, 1510—1517
17 Forte, L.R., Chao, W.H., Walkenbach, R.J. and Byington, K.H. (1975) Biochim. Biophys. Acta 389, 84—96
18 Schwartz, I.L., Shlatz, L.J., Kinne-Saffran, E. and Kinne, R. (1974) Proc. Natl. Acad. Sci. U.S. 71, 2595—2599

19 Shlatz, L.J., Schwartz, I.L., Kinne-Saffran, E. and Kinne, R. (1975) J. Memb. Biol. 24, 131—144
20 Kinne, R., Shlatz, L.J., Kinne-Saffran, E. and Kinne, R. (1975) J. Memb. Biol. 24, 145—159
21 Marx, S.J., Fedak, S.A. and Aurbach, G.D. (1972) J. Biol. Chem. 247, 6913—6918
22 Forte, L.R. (1972) Biochim. Biophys. Acta 266, 524—542
23 Fitzpatrick, D.F., Davenport, G.R., Forte, L. and Landon, E.J. (1969) J. Biol. Chem. 244, 3561—3569
24 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
25 Gilman, A.G. (1970) Proc. Natl. Acad. Sci. U.S. 67, 305—312
26 Antonoff, R.S. and Ferguson, J.J. (1974) J. Biol. Chem. 249, 3319—3321
27 Lenard, J. (1970) Biochemistry 9, 1129—1132
28 Davoren, P.R. and Sutherland, E.W. (1963) J. Biol. Chem. 238, 3016—3023
29 Birnbaumer, L. and Rodbell, M. (1969) J. Biol. Chem. 244, 3477—3482
30 Insel, P., Balakir, R. and Sacktor, B. (1975) J. Cyclic Nucleotide Res. 1, 107—122
31 Wombacher, H., Reuter-Smerdka, M. and Korber, F. (1973) FEBS Lett. 30, 313—316
32 Abou-Issa, H., Kratowich, N. and Mendicino, J. (1974) Eur. J. Biochem. 42, 461—473