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RADIOLIGAND RECEPTOR ASSAY FOR PROLACTIN USING CHICKEN AND TURKEY KIDNEY MEMBRANES*

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Abstract—1. A sensitive and specific radioreceptor assay for measuring prolactin in tissue extracts, culture media and serum has been developed utilizing membrane fractions prepared from either chicken or turkey kidney, and an ovine prolactin standard and tracer.

2. Assay sensitivity was 1.0 ± 0.1 ng per tube, 50% inhibition of binding occurred with 12.8 ± 1.2 ng of unlabeled ovine prolactin standard, and intra- and interassay coefficients of variation were 3.9% and 8.8%, respectively.

3. The 47,800 *g* kidney membrane preparation, which yielded maximum specific binding of 20–40%, offered advantages over current methods including use of an easily available and inexpensive tissue, no need for pretreatment of the donor animal, a high yield of receptor protein, and a simplified method of membrane preparation.

INTRODUCTION

It is generally accepted that, in mammals, one of the principal target tissues for prolactin is the mammary gland. Specific binding sites for prolactin in mammals have been localized and characterized in a variety of organs, including liver, kidney, mammary gland, adrenal cortex, brain, gonads, seminal vesicle, and prostate (Shiu and Friesen, 1974; Parke and Forsyth, 1975; Barkey *et al.*, 1977, 1979; Posner *et al.*, 1974; Elberg *et al.*, 1979; Hayden *et al.*, 1979; Nicoll *et al.*, 1980; Katikineni *et al.*, 1981). Cell receptors have been used for the development of tissue receptor assays which are sensitive, specific and biologically relevant. The diverse physiological actions of prolactin in mammalian species have been well documented (Nicoll, 1974; deVlaming, 1979; Clarke and Bern, 1980). In recent years, the role of prolactin in avian reproduction has been studied using homologous and heterologous radioimmunoassays for prolactin (for a review, see Goldsmith, 1985). In birds, prolactin binding sites have been localized and characterized by biochemical and autoradiographic techniques in pigeon crop sac mucosa (Shani *et al.*, 1972; Kledzik *et al.*, 1975; Forsyth *et al.*, 1978; Shani *et al.*, 1982). The existence of specific binding sites for prolactin in the brain and liver of the ring dove has been shown by Buntin *et al.* (1984) and Buntin and Ruzycki (1987). Posner *et al.* (1974) obtained preliminary evidence for the existence of prolactin binding sites in frog kidney. Later, prolactin binding sites in the kidney were reported in duck and rabbit (Nicoll *et al.*, 1980). In this study, we demonstrate the properties of prolactin binding sites in chicken and turkey kidneys and validate a sensitive radioreceptor assay for measuring avian and mammalian prolactin.

MATERIALS AND METHODS

Reagents

Ovine prolactin (NIADDK-0-PRL-17 and NIH-PRL-I-1), ovine growth hormone (NIADDK-oGH-14), bovine growth hormone (NIH-GH-B-18), and ovine luteinizing hormone (NIH-LH S-20) were gifts of the National Hormone and Pituitary Program and the National Institute of Diabetes, Digestive and Kidney Diseases. Porcine growth hormone (USDA-pGH-B-1), bovine prolactin (USDA-bPRL-B-1), bovine thyroid stimulating hormone (USDA-bTSH-I-1) and bovine follicle stimulating hormone (USDA-bFSH-B-1) were gifts from the USDA Animal Hormone Program. Recombinant-derived chicken growth hormone was provided by AMGEN Biologicals (Thousand Oaks, CA). Turkey prolactin was prepared as described by Proudman and Corcoran (1981). Bovine pancreatic trypsin and phospholipase C were purchased from Sigma Chemical Co. (St Louis, MO). The protease inhibitor 4-amino benzamidine dihydrochloride was obtained from Aldrich Chemical Co. (Milwaukee, WI).

Hormone iodination

Iodination grade ovine prolactin (NIH-PRL-I-1) was iodinated by a lactoperoxidase method (Thorell and Johansson, 1971) with modifications described previously (Krishnan *et al.*, 1989). The spec. act. of ^{125}I-ovine prolactin was 32–43 μCi/μg as determined by the self displacement method (Catt *et al.*, 1974).

Membrane preparation

The kidneys used for binding studies were obtained from adult male chickens and turkeys. Kidneys were removed, immediately frozen, and maintained at −70°C until use. To prepare the membranes, kidneys were thawed, minced, and homogenized in HEPES–sucrose buffer, pH 9.0, as described earlier (Krishnan *et al.*, 1989). Briefly, the homogenate was filtered through cheese cloth and centrifuged at 1900 *g* for 30 min, the supernatant was again filtered and

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centrifuged at 47,800 *g* for 90 min. The pellet from the high speed centrifugation was resuspended in 25 mM HEPES containing 0.01% thimerosal, pH 7.6, at a concentration of 1.0 g of pellet per 2 ml of buffer and then filtered through cheese cloth, aliquoted and stored at −70°C. A 100,000 *g* membrane preparation was also prepared, using the method of Shiu *et al.* (1973). This membrane pellet was resuspended and stored as described above. The protein content of the final pellet was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard.

Prolactin receptor assay

Receptor assays were carried out in 50 mM HEPES buffer, pH 7.6, containing 50 mM $CaCl_2$, 0.2% (w/v) BSA and 0.01% thimerosal. The membrane aliquots were thawed, homogenized with a glass–Teflon homogenizer and 50 μl of membrane preparation (3.8–4.0 mg of protein) were added to 12 × 75 mm polypropylene tubes containing 50 μl of ^{125}I-ovine prolactin (50,000–90,000 cpm) and 400 μl of assay buffer or buffer containing various concentrations of unlabeled hormones. With the exception of the incubation time and temperature studies, the assay tubes were incubated at 4°C for 16–20 hr. Assays were terminated by the addition of 1 ml of cold assay buffer without BSA followed by centrifugation at 3500 *g* for 30 min at 4°C. The supernatant was decanted and the cpm of ^{125}I-labeled ovine prolactin bound to the membrane pellet were determined. For measurement of ovine prolactin added to sera from hypophysectomized chickens and turkeys or to tissue culture medium, the assay conditions were as noted above except that the $CaCl_2$ concentration of the assay buffer was increased to 80 mM. Specific binding was determined by subtracting the counts bound in the presence of excess (5 μg) unlabeled ovine prolactin (non-specific binding) from the counts bound in the absence of unlabeled hormone (total binding). Duplicate or triplicate tubes were run for each total binding and non-specific binding determination. Estimates of standards or unknowns were calculated using the "log-logit" program of the Micromedic Gamma Counter (ICN Micromedic Systems, Huntsville, AL).

Statistical analysis

Analysis of variance was used to determine the effects of changes in assay conditions or treatments on receptor binding, and to assess differences in assay parameters such as sensitivity and ED_{50}. All tests were performed at the 5% level of significance unless otherwise stated. Values for per cent binding were subjected to arc sine transformation prior to analysis. Treatment means were compared using either a least square means procedure or Tukey's range test, as appropriate.

RESULTS

Specific binding of ^{125}I-labeled ovine prolactin to chicken and turkey kidney membranes (47,800 *g* pellet) increased as a function of the amount of membrane protein used. Normally, we used 3.8–4.0 mg protein per tube of chicken or turkey membranes in our receptor assays. Under these conditions 14.5 ± 0.35% and 7.8 ± 0.20% (mean ± SEM, $n = 3$ experiments) of labeled hormone was specifically bound to chicken and turkey kidney membranes, respectively. Non-specific binding ranged from 5 to 5.4% of the total counts added (Fig. 1).

In experiments performed to compare the two methods for preparation of the kidney membranes, we observed that the specific binding of ^{125}I-labeled ovine prolactin was approximately the same as shown above using either the 47,800 *g* pellet or the 100,000 *g* pellet, respectively. However, the total amount of membrane protein obtained was greater in the 47,800 *g* pellet than in the 100,000 *g* pellet. Therefore in subsequent studies we have used the 47,800 *g* membrane pellet.

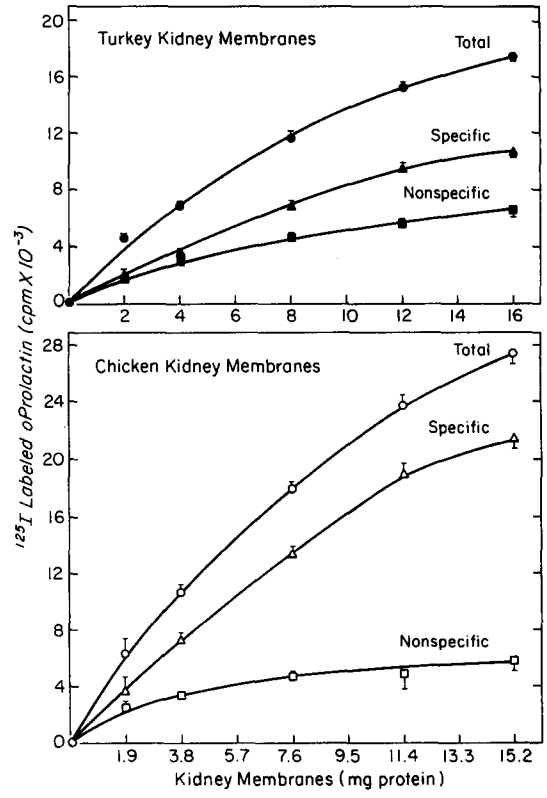

Fig. 1. Effect of increasing concentrations of chicken (lower panel) and turkey (upper panel) kidney membranes on binding of ^{125}I-labeled ovine prolactin. Values are the mean ± SEM from three membrane preparations.

The specific binding of ^{125}I-ovine prolactin to chicken kidney membranes was time and temperature dependent. Specific binding at 4°C increased from 0.7% at 1 hr to 12.6% at 20 hr, whereas at 37°C binding reached a peak of 3.8% during the first 8 hr, and then gradually declined to 0.5% by 24 hr, probably due to degradation of either the hormone and/or the receptor at elevated temperatures. In subsequent assays, we used a 16–20 hr incubation time at 4°C.

Binding of labeled ovine prolactin was enhanced by the presence of both monovalent and divalent cations. Increased binding was observed with addition of 10–100 mM calcium or magnesium (Fig. 2). Both divalent cations significantly increased ($P < 0.05$) the binding of labeled ovine prolactin to kidney membranes with increasing concentrations up to 50 mM. Further increase in the divalent cation concentration to 100 mM did not increase binding further. Monovalent cations (sodium and potassium) were less effective than divalent cations in enhancing prolactin binding, although binding was significantly increased by 10 to 100 mM monovalent cation ($P < 0.05$).

The binding of labeled ovine prolactin to chicken kidney membranes was pH dependent. A peak in specific binding occurred between pH 6.0 and pH 7.6; binding was dramatically decreased ($P < 0.05$) below pH 6.0 and above pH 7.6.

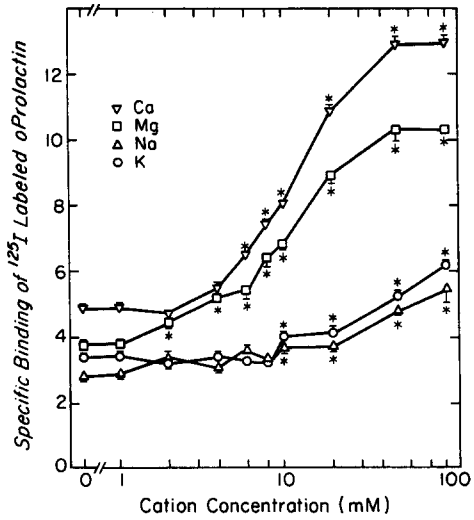

Fig. 2. The effect of cations on specific binding of ^{125}I-labeled ovine prolactin to chicken kidney membranes. The incubation medium contained 50 mM HEPES (pH 7.6), 0.2% BSA, 0.01% thimerosal and various concentrations of cations. Values are the mean ± SEM of two experiments performed in duplicate. *Denotes means significantly different from the 0 value.

We tested the effect of enzyme treatments on specific binding of labeled ovine prolactin to kidney membranes (Table 1). Brief (30 min) exposure of chicken or turkey kidney membranes to trypsin (5 or 50 μg) resulted in a 44% and 73% decrease, respectively, in specific binding, suggesting that protein is a functionally important part of the receptor binding site. Similarly, incubation of the receptor with 5 or 50 μg of phospholipase C also significantly reduced binding (by 27% and 68%, respectively), suggesting that phospholipids may also play a significant role in the binding of prolactin to its receptor.

The number and affinity of prolactin receptors on chicken and turkey kidney membranes were determined by Scatchard analysis (Scatchard, 1949). Analysis of the saturation data (Fig. 3A and B) suggested the existence of only one class of high affinity binding sites in kidney membranes from both species. The affinity constant (K_a) (Fig. 3C) for the binding of ^{125}I-labeled ovine prolactin to chicken kidney membranes was 2.13×10^9 l/M with an apparent binding capacity of 12.3 fmol/mg protein. For turkey kidney membranes, the K_a was 1.91×10^9 l/M and the apparent binding capacity was 11.5 fmol/mg protein.

Table 1. Effect of enzyme treatment on binding of ^{125}I-labeled ovine prolactin by chicken kidney membranes

Enzyme	n	Concentration (μg/ml)	Specific binding of ^{125}I-oPRL (% of control ± SEM)
Control	2	0	100%[a]
Trypsin	2	5	56.6[b] ± 0.18
	2	50	27.6[c] ± 0.61
Phospholipase C	2	5	73.5[d] ± 2.89
	2	50	32.8[e] ± 1.01

[a–e]Values with different superscripts differ significantly ($P < 0.01$). n = Mean of two experiments each performed in triplicate.

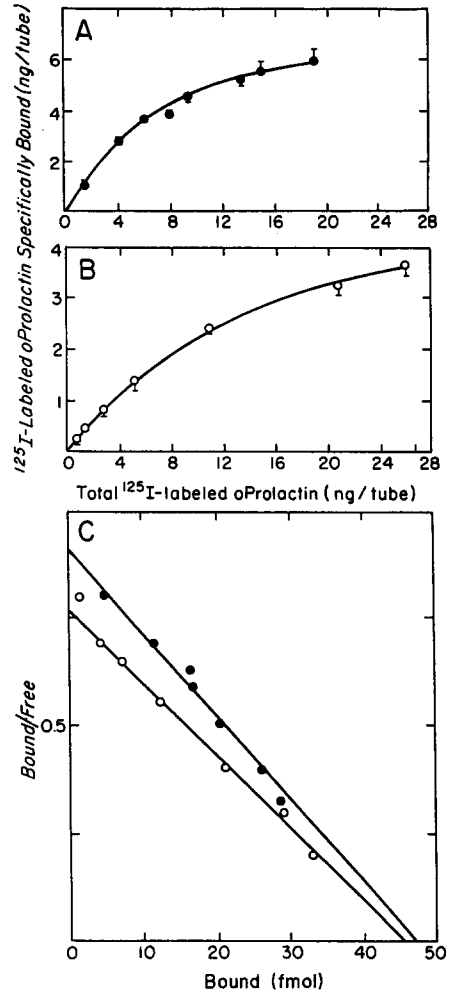

Fig. 3. Effect of ^{125}I-labeled ovine prolactin concentration on specific binding of (A) chicken (●), and (B) turkey (○), kidney membranes. Values represent mean ± SEM of four total binding and four non-specific binding assay tubes for each specific binding determination. (C) Scatchard plot derived from specific binding data obtained from chicken (●) (binding affinity $K_a = 2.13 \times 10^9$ l/M; binding capacity = 12.3 fmol/mg protein; $r = 0.991$), and turkey (○) ($K_a = 1.91 \times 10^9$ l/M; binding capacity = 11.5 fmol/mg protein; $r = 0.998$) kidney membranes.

The specificity of the chicken kidney prolactin receptors for prolactin was evaluated by determining the competition of unlabeled prolactin and growth hormone from various species, as well as bovine follicle stimulating hormone, ovine luteinizing hormone, and bovine thyroid stimulating hormone, for prolactin binding sites on chicken kidney membranes (Fig. 4). Similar displacement data were obtained for turkey kidney membranes. But these data did not differ significantly from those for chicken membranes and are not shown. Bovine, porcine, and turkey prolactin competed 92%, 42% and 28%, respectively, with chicken kidney membranes and 82%, 42% and 20% with turkey kidney membranes. Bovine growth hormone cross-reacted 0.7% with chicken kidney membranes and 0.2% with turkey kidney membranes, whereas ovine and chicken growth hormones showed lower cross-reaction (<0.05%) with both

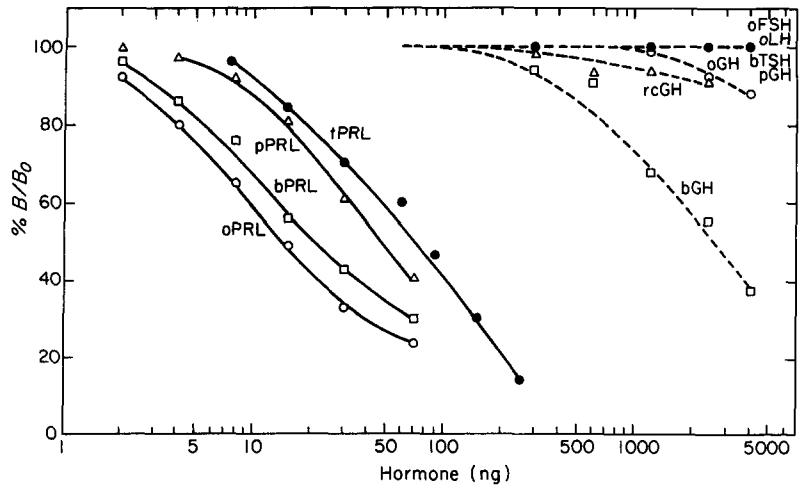

Fig. 4. Displacement of ^{125}I-labeled ovine prolactin from chicken kidney membranes by pituitary hormones from various species. Each point represents the mean of three experiments.

membrane preparations. There was no significant cross-reactivity with porcine growth hormone, ovine luteinizing hormone, bovine follicle stimulating hormone or thyroid stimulating hormone (<0.01%).

The displacement curve for an homogenate of fresh chicken pituitary glands is shown in Fig. 5. The chicken pituitary preparation produced a dose–response curve parallel to that observed for ovine prolactin.

The sensitivity of the assay, defined as the amount of hormone measured which was different from zero by two SDs, was 1.0 ± 0.1 ng (mean $\pm$ SEM; $n = 6$) and the midrange of the standard curve (dose of ovine prolactin required to produce 50% inhibition of binding) was 12.8 ± 1.2 ng ($n = 8$). The intra- and interassay coefficients of variations were 3.9% and 8.8%, respectively, ($n = 10$). Preincubation of the receptor with standard for 5 hr prior to adding labeled hormone shifted the midrange of the standard curve to 7.9 ± 0.32 ng ($n = 2$, $P < 0.05$), but the sensitivity of the assay was unchanged.

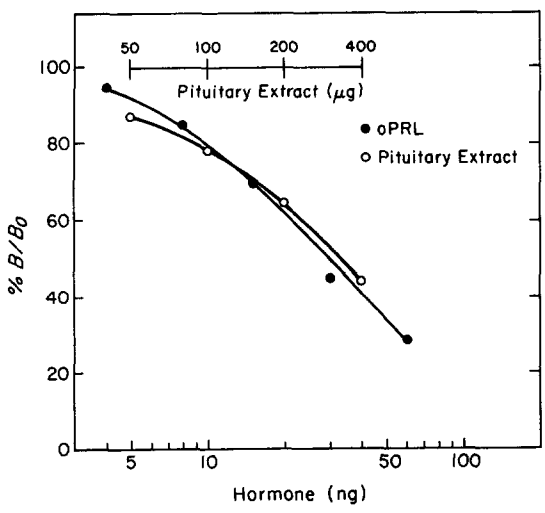

Fig. 5. Displacement of ^{125}I-labeled ovine prolactin from chicken kidney membranes by increasing doses of ovine prolactin and by a homogenate of fresh chicken pituitaries. Each point represents the mean of triplicate determinations.

The ability of the system to accurately measure prolactin in physiological samples was assessed by adding known amounts of ovine prolactin to sera from hypophysectomized chickens and turkeys (hypox sera), and to Dulbecco's modified Eagles tissue culture medium containing 5% fetal calf serum. The hypox sera (5–40 μl) and culture medium (10–100 μl) did not interfere with the binding of ^{125}I-labeled ovine prolactin to the receptor. Binding in the presence of hypox sera was 100.9 ± 0.4% (range 100–103%) of the binding observed without sera ($n = 6$). Similarly, the binding in the presence of culture medium was 100 ± 0.5% (range 98–101%) of the binding observed without medium ($n = 6$). There was no significant effect of the dose of sera or medium used. The recovery of 2–60 ng of ovine prolactin added to 15 μl of hypox chicken or turkey sera or to 100 μl of medium was quantitative (Fig. 6). Recovery of ovine prolactin added to hypox chicken serum was 103 ± 3.1% (range 91–115%), while recovery from hypox turkey serum was 99.8 ± 2.0% (range 95–107%), and from tissue culture medium was 99 ± 1.9% (range 92–106%).

DISCUSSION

Binding of ovine prolactin to a number of different tissues in a variety of species has been previously described (Posner *et al.*, 1974; Forsyth, 1978; Nicoll *et al.*, 1980; Shani *et al.*, 1982), but the characteristics of binding to chicken or turkey kidneys, in particular the hormone-binding site interaction, has not been shown. The studies reported here describe some of the basic characteristics of the binding of ovine prolactin to subcellular membranes from chicken and turkey kidneys.

The use of a 47,800 *g* membrane pellet rather than a 100,000 *g* membrane pellet simplified membrane preparation and produced a much higher yield of receptor protein. Furthermore, we have found that the membrane homogenate can be stored at 4°C for

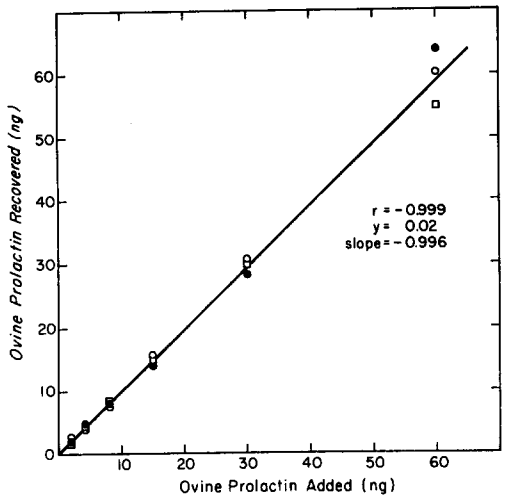

Fig. 6. Measurement of ovine prolactin (2–60 ng) added to 15 μl of serum from hypophysectomized chickens (●) or turkeys (○) and to 100 μl of Dulbecco's modified Eagles medium (□). Each point represents the mean of three experiments with duplicate determinations.

2–4 days and at −70°C for at least 5 months without loss of prolactin binding activity.

The per cent specific binding of prolactin to the subcellular material used in this study is higher than that reported in pigeon crop sac by Kledzik *et al.* (1975) and Forsyth *et al.* (1978). The membrane protein concentration used here was higher than in earlier studies due to low receptor concentration in the kidneys. The binding of ovine prolactin depended on several parameters including time, temperature, pH and membrane concentration, as has been described for other protein hormones and their target-tissue binding sites (Kahn *et al.*, 1974; Bhalla and Reichert, 1974). We observed high specific binding at 4°C but not at 37°C. We observed relatively low levels of non-specific binding using our chicken kidney preparation in contrast to the higher non-specific binding observed using pigeon crop sac (Forsyth *et al.*, 1978) and drove dove liver membranes (Buntin *et al.*, 1984). The slow increase in the specific binding at 4°C with respect to time and temperature could be due to the unmasking of the binding sites or to their reorganization during the incubation (Barkey *et al.*, 1979).

The prolactin receptor membrane preparation showed no significant cross-reaction ($P < 0.05$) with ovine, porcine, or chicken growth hormones. Bovine growth hormone had weak affinity for the prolactin binding site, but cross-reaction (<0.7%) was much less than that of any mammalian or avian prolactin studied. This binding of bovine growth hormone to prolactin binding sites may be due to contamination of this preparation with prolactin (reported to be as high as 1.4%). Ovine and bovine prolactin were clearly the most effective of the hormones tested in their ability to compete with labeled ovine prolactin. These findings are consistent with ovine prolactin binding data in pigeon crop sac (Forsyth *et al.*, 1978), and dove liver and brain (Buntin *et al.*, 1984), Buntin and Ruzycki, 1987), and a variety of other vertebrate tissues (Nicoll *et al.*, 1980), which indicate that ovine prolactin is widely recognized by prolactin binding sites. The ovine prolactin was two times more potent than porcine prolactin, and about four times more potent than turkey prolactin, in both chicken and turkey membrane assays. We did not test the cross-reactivity of chicken prolactin since this hormone was unavailable at the time of these studies. The level of activity of turkey prolactin which we observed in our assay system is similar to that reported in a dove liver radioreceptor assay of this preparation (Buntin *et al.*, 1984), but substantially higher than that obtained in a dove brain prolactin radioreceptor assay (Buntin and Ruzycki, 1987). The lower binding of turkey prolactin to turkey and chicken prolactin receptors, compared to the greater binding of ovine or bovine prolactin, is consistent with numerous reports of growth hormone, prolactin and insulin radioreceptor assays which show that the binding affinity of receptors for hormones from foreign species is often greater than for those from homologous or closely related species. Ovine prolactin binds similarly to receptors from the major vertebrate groups, and thus is a widely used standard for prolactin radioreceptor assays (for a review, see Nicoll *et al.*, 1986).

The K_a values of ovine prolactin in preparations of diverse organs in varied species are generally about the same (about $10^9\,M^{-1}$). Thus, with respect to binding affinity for ovine prolactin, prolactin receptors seem to be very similar among the major vertebrate groups (Nicoll, 1986). The binding affinity (K_a) obtained with our chicken and turkey kidney membranes was very similar to that obtained using duck kidney (Nicoll, 1980). However, the binding capacity (11–13 fmol/mg protein) of avian kidneys was 5–6-fold less than that of pigeon crop sac (Forsyth *et al.*, 1978) and rabbit mammary gland (Shiu and Friesen, 1974). The differences in binding capacity could be a reflection of pronounced up-regulation of prolactin receptors during the priming with prolactin (Kledzik *et al.*, 1975; Shani *et al.*, 1982) or placental lactogen and cortisone (Friesen, 1966) which is required in these eariler procedures. A major advantage of using avian kidney receptor for the estimation of prolactin activity is the easy availability of tissue without any priming or manipulation of the animal.

A substantial increase in binding was found with increasing divalent cation concentrations of up to 50 mM. Moore *et al.* (1980) have shown a specific effect of cations on the tertiary conformation of human growth hormone that may relate to this effect on binding, suggesting that the cationic effect resides with the hormone rather than the receptor.

To our knowledge, our observations represent the first characterization of prolactin receptors in chicken and turkey kidney membranes. Receptors from kidneys of both species have a single class of binding sites, and similar affinity constants and binding capacities. Destruction of receptor activity by treatments with trypsin and phospholipase suggests that protein and phospholipids are structural components essential for formation of the hormone–receptor complex. The radioreceptor assay method described here is suitable to estimate potencies of prolactin preparations and to monitor pituitary prolactin at various stages of purification in both avian and

mammalian species. Furthermore, the assay system will also facilitate the measurement of bioactive prolactin in serum and in tissue culture media.

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