Goldstein, David L., Vishala Reddy, and Kimberly Plaga. Second messenger production in avian medullary nephron segments in response to peptide hormones. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R847–R854, 1999.—We examined the sites of peptide hormone activation within medullary nephron segments of the house sparrow (Passer domesticus) kidney by measuring rates of hormone-induced generation of cyclic nucleotide second messenger. Thin descending limbs, thick ascending limbs, and collecting ducts had baseline activity of adenyl cyclase that resulted in cAMP accumulation of 207 ± 56, 147 ± 31, and 151 ± 41 fmol·mm-1·30min-1, respectively. In all segments, this activity increased 10- to 20-fold in response to forskolin. Activity of adenyl cyclase in the thin descending limb was stimulated approximately twofold by parathyroid hormone (PTH) but not by any of the other hormones tested [arginine vasotocin (AVT), glucagon, atrial natriuretic peptide (ANP), or isoproterenol, each at 10-6 M]. Thick ascending limb was stimulated two- to threefold by both AVT and PTH; however, glucagon and isoproterenol had no effect, and ANP stimulated neither cAMP nor cGMP accumulation.

METHODS

All studies were performed on kidneys taken from adult house sparrows (Passer domesticus). Male and female birds were captured throughout the year in Greene County, OH, and were maintained in captivity for 1–2 wk before experimentation. Captive birds received a mixed seed diet with unlimited access to water.

Isolation of Tubule Segments

Birds were killed by anesthetic overdose (a mix of diallyl barbituric acid and urethan), and their kidneys were quickly removed and placed in ice-cold modified Hanks’ solution (composition in mM: 137 NaCl, 5 KCl, 0.8 MgSO4, 0.33 Na2HPO4, 0.44 KH2PO4, 1 MgCl2, 10 Tris·HCl, pH 7.4; Ref. 26) containing 1 mM CaCl2 and 1 mg/ml BSA. Medullary cones (individual subdivisions of the avian renal medulla) were dissected from the kidney in this same solution and then were transferred to Hanks’ solution containing 1 mg/ml collagenase (Worthington Scientific CLS2) and incubated at 30°C for 35–40 min. Cones were returned to ice-cold Hanks’ solution after incubation. To isolate nephron segments, cones were transferred to low-Ca2+ Hanks’ solution (same composition as previously described but 0.25 mM CaCl2), and individual tubule segments were separated from the medullary cones under a stereomicroscope, using insect pins mounted in glass holders. Typically, thick ascending limbs were easily dissectible in segments 0.75–1.5 mm in length. Isolation of collecting ducts and thin descending limbs was more difficult, and we usually obtained shorter pieces of these segments, 0.25–0.5 mm in length; we often combined two pieces of these segments onto a single slide for analysis so that the total length of tubule was at least 0.5 mm. Individual nephron segments were transferred in a 2-µl droplet onto a small spot of bovine serum albumin that was dried onto the center of an organosilane-coated depression slide. The droplet was then sealed into this chamber by placing a second Petrolatum-coated depression slide over the first, and the sample in its chamber was kept in a pan on ice until further experimentation. Within each experiment (i.e., from any single bird), three
to six tubules were used in each of the conditions examined (control or agonist; see below).

Assay for Activity of Adenylyl Cyclase

Our procedure for assessing activity of adenylyl cyclase in the tubule segments was based on the protocols described by Imbert et al. (16) and Mored (25) for measuring accumulation of cAMP. In brief, we replaced the droplet of Hanks’ solution with 0.5 µl of a hyposmotic incubation medium (composition in mM: 0.25 EDTA, 1 MgCl₂, 1 Tris-HCl, 5 EGTA, 5 × 10⁻³ GTP, and 0.1% BSA, pH 7.4; Ref. 3) with or without various potential agonists (forskolin, hormone). In some experiments we also added 1 mM IBMX to test for the requirement of including a phosphodiesterase inhibitor in the incubation medium. The hyposmotic solution helped to permeabilize the tubules to provide access to the various reactant chemicals. Both baseline control tubules (no agonist) and positive controls (forskolin) were included in all experiments. Agonists that we tested (all from Sigma) were forskolin (10⁻⁵ M, in DMSO), AVT (10⁻⁶ – 10⁻⁷, and 10⁻⁹ M), PTH (bovine fragment 1–34, 10⁻⁷ – 10⁻¹¹ M), glucagon (porcine, 10⁻⁶ M), ANP (chicken ANP, 10⁻⁶ M), and isoproterenol (β-adrenergic agonist, 10⁻⁶ M). Preliminary tests demonstrated that DMSO, the vehicle for dissolving forskolin, had no significant effect on accumulation of cAMP, and we did not include this compound in any incubation solutions other than forskolin. Moreover, as described in Statistical Analyses, responsiveness to hormones was evaluated by comparing hormone-treated tubules with control tubules, all of which were free of DMSO. Tubules were further permeabilized by brief freezing (application of the slide to a block of dry ice), and incubation medium (1 mM cAMP, 0.25 mM EDTA, 4 mM MgCl₂, 0.24 mM ATP, 16 U creatine phosphokinase, 2.5 × 10⁻² M phosphocreatine, 0.1 M Tris-HCl, pH 7.4; Ref. 3) containing 37 kBq (1 µCi) [α³²P]ATP was added in a 2-µl droplet. The tubules were then incubated in their slide chambers at 30°C or in some experiments at 40°C. After 30 min, the reaction was terminated by addition of ice-cold stop solution containing excess ATP (3.3 × 10⁻³ M) and cAMP (5 × 10⁻³ M) in Tris-HCl (5 × 10⁻² M) with pH 7.6. The stop solution additionally contained ~330 Bq (20,000 dpm) of [³²P]AMP, which was used to estimate recovery of cAMP during the following procedures. All steps from removal of the kidney to application of the stop solution were completed in 1 day. The stopped solution was then frozen overnight pending assay for [³²P]cAMP.

Radioactively labeled cAMP was separated from other [³²P]-containing compounds with a two-step column chromatography procedure consisting of columns first of Dowex resin and then of aluminum oxide (40). Recovery of cAMP through the chromatography was 35–60%, and background counts of [³²P], measured from slides incubated without any tubule, were ~0.5 Bq (25–30 dpm). We converted counts of radioactive cAMP into measures of cAMP accumulation rate (fmol/30 min) on the basis of the specific activity of the precursor [³²P]ATP (see Ref. 25) and then corrected these for recovery and standardized the measures per millimeter of tubule length.

Assay for cGMP and Its Activation by ANP

In mammals, ANP exerts its action with cGMP, not cAMP, as a second messenger. We therefore conducted an additional set of experiments in which we evaluated the ability of ANP to stimulate guanylyl cyclase in thick ascending limb and collecting ducts with a modification of the protocol described by Chabardès et al. (5). To do this, we isolated tubule segments and sealed them in a 2-µl droplet between depres-
tion with the thick limb, had a diameter notably thinner than the terminal proximal tubule, and were associated together in a central core of the cone.

Basic Findings: Baseline Activity of Adenylyl Cyclase, Individual Variation, Effects of Incubation Temperature, and Phosphodiesterase Inhibition

Adenylyl cyclase activity under control conditions resulted in cAMP accumulation at rates ranging from 150 to 200 fmol·mm⁻¹·30 min⁻¹ in three nephron segments. These rates did not differ between segments (Table 1). Because of differences in tubule diameter, however, activities expressed per unit tubule surface area were highest in thin descending limb (Table 1).

We found no significant variation among individuals in adenylyl cyclase activity in collecting ducts [F(20,70) = 1.23, P < 0.26] or in thick ascending limb [F(17,82) = 1.66, P < 0.07]. In thin descending limb, however, baseline adenylyl cyclase activity did vary significantly between individuals [F(8,27) = 29, P < 0.02].

Control levels of adenylyl cyclase activity in tubules incubated at 40°C were not significantly different than in tubules incubated simultaneously at 30°C for either collecting ducts or thick ascending limbs. Similarly, adding the phosphodiesterase inhibitor IBMX did not influence the amount of cAMP accumulating in control incubations. Moreover, the stimulatory effects of AVT and forskolin at 40°C or with IBMX were not different from those at 30°C or in the absence of IBMX (Table 2).

Actions of Hormones on Medullary Nephron Segments

AVT. AVT at 10⁻⁶ M elicited an increase in adenylyl cyclase activity in collecting ducts and, to a lesser extent, in thick ascending limbs (Fig. 1). In the collecting ducts, AVT-induced increases in adenylyl cyclase activity were greater at 10⁻⁶ and 10⁻⁷ M than at 10⁻⁵ M or in control tubules (Fig. 2). In the thick ascending limbs, only the highest dose tested (10⁻⁶ M) significantly enhanced rates of cAMP accumulation; the intermediate rates measured at the two lower doses were not significantly different than at either the higher dose or the control. Adenylyl cyclase activity in thin descending limb was not significantly affected by AVT.

PTH. PTH exerted its strongest action in the thick ascending limb, where adenylyl cyclase activity overall was 2.6 times the basal rate in the presence of 10⁻⁶ M hormone (Fig. 1). In one of the six birds tested, however, there was no effect of PTH. The effect of PTH on thick ascending limb was significantly greater than control only at the highest dose tested, 10⁻⁶ M (Fig. 3). PTH had a lesser but significant effect also on thin descending limb; it had no effect on collecting ducts.

Glucagon, isoproterenol, and ANP. Adenylyl cyclase activity in the presence of glucagon was not significantly different from baseline in any of the three tubule segments (Fig. 1). Similarly, in no case was there evidence of significant stimulation by isoproterenol or ANP (Fig. 1). We also measured rates of cGMP production in thick ascending limb and collecting ducts. Again, the hormone was without stimulatory effect in these segments (Table 3).

Summary by tubule segment. In the thin descending limb, we were able to demonstrate a small but significant enhancement of adenylyl cyclase activity only by PTH. In thick ascending limb, adenylyl cyclase activity was increased in response to both PTH and AVT at 10⁻⁶ M but not in response to glucagon or ANP. In collecting ducts, AVT at 10⁻⁶ and 10⁻⁷ M stimulated adenylyl cyclase activity, but the other hormones were without effect.

Table 1. Control and forskolin-stimulated rates of cAMP production in three tubule segments

<table>
<thead>
<tr>
<th>Segment</th>
<th>Control fmol·mm⁻¹·30 min⁻¹</th>
<th>Forskolin (10⁻⁶ M) fmol·mm⁻¹·30 min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thin descending limb</td>
<td>207.5 ± 55.6 (10,38)</td>
<td>1,014.0 ± 156.6 (7,19)</td>
</tr>
<tr>
<td>Thick ascending limb</td>
<td>147.3 ± 31.5 (20,89)</td>
<td>1,500.2 ± 218.8 (19,75)</td>
</tr>
<tr>
<td>Collecting duct</td>
<td>151.4 ± 40.8 (21,86)</td>
<td>2,051.7 ± 300.5 (18,59)</td>
</tr>
</tbody>
</table>

Values are means ± SE of cAMP production rates; nos. in parens, no. of animals and no. of tubules, respectively. Rates of cAMP production per unit tubule surface area are expressed in fmol·mm⁻¹·30 min⁻¹ and were calculated on the basis of measured tubule diameters (thin descending limb = 15.0 ± 3.2 µm, n = 20; thick ascending limb = 25.9 ± 4.1 µm, n = 35; collecting duct = 40.9 ± 2.6 µm, n = 30), assuming a simple cylindrical shape to tubules.

Table 2. Effects of elevated incubation temperature and of the phosphodiesterase inhibitor IBMX on activity of adenylyl cyclase activity in avian medullary tubule segments

<table>
<thead>
<tr>
<th>Segment</th>
<th>40°C Control</th>
<th>40°C IBMX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thin descending limb</td>
<td>186.5 ± 14.7 (2,6)</td>
<td>159.5 ± 32.2 (4,18)</td>
</tr>
<tr>
<td>Thick ascending limb</td>
<td>80.8 ± 22.8 (6,16)</td>
<td>180.2 ± 147.4 (5,27)</td>
</tr>
<tr>
<td>Collecting duct</td>
<td>147.2 ± 12.5 (2,5)</td>
<td>132.1 ± 43.2 (3,9)</td>
</tr>
</tbody>
</table>

Data are means ± SE and are expressed as fmol cAMP·mm⁻¹·30 min⁻¹; nos. in parens, no. of animals and no. of tubules, respectively. In no case, for either 40°C or IBMX incubations, did values measured under those conditions differ from values obtained for tubules incubated simultaneously at 30°C and without IBMX (all P > 0.05). Similarly, when adenylyl cyclase activity in presence of arginine vasotocin (AVT) or forskolin is expressed as a multiple of activity in control incubations, ratios at 40°C or with IBMX do not differ from those at 30°C and without IBMX (all P > 0.05).
DISCUSSION

Measures of second messenger production in defined segments of individual renal tubules have contributed substantially to delineating the heterogeneity of tubular function within the mammalian nephron. However, such measures have been only recently applied to nonmammalian vertebrates, including an amphibian (46) and a lizard (3), and the present study represents the first application to the avian kidney.

We found no significant effect of phosphodiesterase inhibition on levels of cAMP accumulation in the avian tubules. This result is similar to previous findings with this same method on mammalian renal tubules (26), suggesting that phosphodiesterase activity is relatively low and that, in the presence of the relatively high concentration of cold cAMP (1 mM) in the incubation medium, it was not necessary to include the phosphodiesterase inhibitor.
Activity of adenyl cyclase was also not significantly different at the two temperatures that we tested, 30 and 40°C. Avian body temperature is closer to the latter of these temperatures. However, in the absence of any significant effect of temperature on our experimental results, we chose to conduct the remainder of the studies at 30°C to facilitate comparison with other studies of both mammals (e.g., 4, 17) and reptiles (3) that have been conducted with this technique at this same temperature.

Baseline rates of adenyl cyclase activity in the avian tubules were similar to those measured for a reptile (3), although both of these were higher than baseline rates measured in rat or rabbit tubules (see Ref. 28). Maximal rates of adenyl cyclase activity were similar in all of these taxa.

AVT

AVT is well characterized as the avian ADH. Circulating concentrations of AVT rise when birds are dehydrated (e.g., 15, 43), and infusion of the hormone in birds, including house sparrows, induces a diminution of urine flow (e.g., 8, 42). In mammals, the primary mechanism of action of ADH is to enhance the permeability to water of the medullary collecting ducts. However, in birds, the mechanism of AVT has remained controversial. One consistent action of AVT on the avian kidney is to reduce the rate of glomerular filtration (GFR; Refs. 8, 42). The consequent reduction in fluid flow through the renal tubules could lead to enhanced solute and water reabsorption from the renal tubules, processes that are sensitive to rates of solute delivery (34). Hence, an AVT-induced increase in fractional water reabsorption could be a secondary effect to the reduced GFR, rather than representing a direct action of the hormone on the renal tubules. On the other hand, several lines of evidence do suggest a direct action of AVT on the avian renal tubule. First, in some studies, the AVT-induced antidiuresis occurs, at least at low doses of hormone, with little or no change in GFR (1, 42). Second, in house sparrows, a chemical analog of ADH was able to enhance the antiuretic actions of AVT without any effect on the GFR (8). Finally, Nishimura et al. (32) have recently reported that AVT at a concentration of 10^{-8} M can induce an increased water flux across isolated, perfused collecting ducts from water-restricted J apanese quail. Thus the present findings are consistent with this growing body of evidence for a tubular action of AVT in birds.

Table 3. Rates of cGMP production in thick ascending limbs and collecting ducts in response to ANP in presence of phosphodiesterase inhibitor IBMX

<table>
<thead>
<tr>
<th>Segment</th>
<th>Control</th>
<th>ANP (10^{-6} M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thick ascending limb</td>
<td>0.04 ± 0.01 (6,26)</td>
<td>0.06 ± 0.02 (6,29)</td>
</tr>
<tr>
<td>Collecting duct</td>
<td>0.05 ± 0.03 (2,5)</td>
<td>0.02 ± 0.01 (2,5)</td>
</tr>
</tbody>
</table>

Values are means ± SE and are expressed in fmol/mm. Nos. in parenth. no. of animals and no. of tubules, respectively. ANP, atrial natriuretic peptide.

Previous studies of slices from medullary cones have measured either small (32) or insignificant (7, 9) enhancement of cAMP production by AVT. This difficulty in detecting a response to AVT in medullary slices may occur because the most responsive elements to AVT, the collecting ducts, are relatively few in number and are surrounded within the medullary cone by other tubule segments (11). The thick ascending limbs are less responsive to AVT than the collecting ducts and, although located more peripherally, are bound together by connective tissue. Thus AVT may gain insufficient access to receptors on the renal tubules in these slices.

In the present study, we were able to demonstrate a lesser but significant activation of adenyl cyclase activity in thick ascending limb in addition to collecting ducts. This finding contrasts with that of Miwa and Nishimura (24), who could not detect any stimulation of Cl^- transport or volume flux in perfused thick ascending limbs from J apanese quail. However, our finding is consistent with the action of ADH in the mammalian nephron, where the hormone stimulates CAMP production in (17) and enhances electrolyte absorption from the thick ascending limb. Moreover, AVT stimulates adenyl cyclase activity in both collecting ducts and in intermediate segments from the lizard *Ctenophorus ornatus* (3); the avian thick ascending limb may be homologous to the intermediate segment of avian reptilian-type nephrons (48) and thus may be evolutionarily related to the reptilian intermediate segment. Thus the dual site of action of ADH within the nephron appears to be quite general among the amniote vertebrates that have been studied.

The present experiments do not define the physiological action of AVT on the spiral renal tubules. However, our current understanding of the avian urea-concentrating mechanism (32, 34) suggests that the actions of AVT are likely to differ in thick ascending limb and collecting duct, analogously to the action of ADH in the mammalian nephron. Thus AVT in birds may enhance urine concentration in several ways: diminution of GFR; a consequent enhancement of ion reabsorption due to flow sensitivity of tubular transport; perhaps further activation of this reabsorption by direct action of AVT on the thick limbs; and, finally, activation of processes in the collecting ducts, perhaps involving changes in water permeability.

PTH

The P_x and probably much of the Ca in avian plasma are freely filterable at the renal glomerulus, and thus the potential exists for substantial urinary loss of these minerals. On the other hand, both physiological status (e.g., egg formation) and diet may lead to excess of these ions, particularly P_x, in the blood, requiring renal excretion even above the rate of filtration. Hence, avian renal excretion of P_x and to a lesser extent Ca, is highly variable. At both the levels of the whole kidney (47) and the individual proximal tubule (21), either net reabsorption or net secretion of P_x may be found. This variable excretion appears to be regulated, at least in most species, by PTH. Studies of homogenized (7) or dis-
persed (37) avian renal tissues indicate that the action of PTH involves formation of the second messenger cAMP.

The site of action of PTH in the avian nephron has not been elucidated, however. In the mammalian nephron, PTH stimulates adenyl cyclase activity in several cortical segments, including proximal tubule, distal tubule, and cortical thick ascending limb (28), and stimulates ion transport in cortical but not medullary thick ascending limb (44). Studies of isolated cells (e.g., 13, 22), of membrane vesicles (38), and of proximal tubules in situ (20) indicate that in birds the proximal tubules also contribute at least part of the responsiveness to PTH. However, whereas PTH did stimulate P1 secretion in proximal tubules, it was without effect on Ca reabsorption, suggesting that other segments must also respond to the hormone. The present study suggests that the loop of Henle may contribute part of this action, although it remains to be determined whether the PTH-stimulated rise in adenyl cyclase activity in this nephron region relates to Ca-Pi transport or to other processes.

Glucagon, Epinephrine, and ANP

In mammals, glucagon stimulates adenyl cyclase activity and electrolyte reabsorption in the thick ascending limb (e.g., 6, 29). This action may have physiological significance after protein feeding, when glucagon levels rise, and when its actions on the loop of Henle may interact in the urine-concentrating mechanism with the effects of increased urea excretion (2). In birds, in contrast, we are unaware of previous data suggesting a renal action of glucagon. The present studies do not support an effect in birds similar to that seen in mammals.

In mammals, release of catecholamines from the renal nerves leads to antidiuresis and antinatriuresis, effects mediated by α1-type receptors for norepinephrine (NE) that are located throughout the nephron (19). Other renal actions of NE, including effects on acid-base and electrolyte balance, may be mediated by a variety of receptor subtypes; cAMP production is stimulated in mammalian cortical thick ascending limb, distal tubule, and collecting duct by activation of β-adrenoreceptors (28, 45). In contrast, catecholamines are diuretic in birds. This response was elicited both by NE (18) and by the β2-agonist isoproterenol (35), indicating activation of β-type adrenoreceptors. Palmore et al. (36) suggested that in turkeys the diuresis was achieved primarily by an enhanced GFR, particularly by recruitment of reptilian-type nephrons to the filtering population. In ducks (Anas platyrhynchos), however, GFR was unaffected by NE, and even nonpressor doses of NE induced diuresis when introduced into the renal circulation (18). This might suggest a direct action of NE on the renal tubules or, as the authors suggest, an interaction of NE with the renal actions of other hormones. Our results, which do not indicate any action of isoproterenol on the medullary tubule elements, are more consistent with this latter interpretation. They are also consistent with the preliminary observation of Nishimura et al. (32) that isoproterenol does not influence the diffusional permeability to water in quail medullary collecting ducts.

ANP exerts diuretic and natriuretic actions on birds (14, 41). However, the mechanism of these effects, including the balance between vascular and renal tubular actions, remains unclear. In mammals, some studies report ANP induced activation of second messenger (cGMP) production in several segments of renal tubule, including thick ascending limb (23, 33) and collecting duct (49); other studies have failed to find such effects (5). In birds, the only study to examine the site of action of ANP within the kidney found that receptors for ANP were localized to the glomeruli and, less densely, to the collecting ducts of Pekin ducks (A. platyrhynchos; Ref. 41). Our inability to detect any ANP-induced increase in second messenger (cAMP or cGMP) accumulation in house sparrow thick ascending limbs is consistent with these previous findings in the duck. The lack of stimulation in collecting ducts may suggest an absence of direct effect of ANP in the renal medulla of house sparrows.

Overall Comments

A consistent finding in the present studies was that adenyl cyclase activity in the presence of forskolin was always severalfold higher than in the presence of any of the peptide hormones. This occurred despite the latter being tested at a concentration of 10^-6 M, presumably high enough to elicit a maximal response. This finding suggests the possibility that the hormones activate adenyl cyclase by independent pathways, allowing a greater stimulation in the presence of multiple hormonal stimuli than in the presence of any one hormone alone. However, this suggestion bears further investigation; in the mammalian nephron, additivity of hormonal effects on cAMP production is generally lacking, and it appears that the various peptide hormones in fact activate a common pool of adenyl cyclase (27).

We thank Don Bradshaw for providing the opportunity, and Felicity Bradshaw for providing the instruction, that allowed D. L. Goldstein to learn the single-tubule assay for cAMP production. Jill Meyer and Vignathi Atluri assisted with much of the laboratory work in this project. Latasha Naidu carried out the measurements of cGMP production.

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