Regulation of transepithelial phosphate transport by PTH in chicken proximal tubule epithelium

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Dudas, Paul L., Alice R. Villalobos, Gayle Goeck-Sutterlin, Gary Laverty, and J. Larry Renfro. Regulation of transepithelial phosphate transport by PTH in chicken proximal tubule epithelium. Am J Physiol Regulatory Integrative Comp Physiol 282: R139–R146, 2002.—The effect of parathyroid hormone (PTH) and activation of protein kinase C (PKC) and protein kinase A (PKA) on transepithelial P_i transport was examined in monolayers of chick proximal tubule cells in primary culture (PTCs). Acute exposure of the PTCs to PTH (10^{-9} M, basolateral side) significantly decreased the net reabsorption of P_i by 66%. There was no effect after the addition of PTH to the luminal side. Activation of PKC by phorbol 12-myristate 13-acetate (PMA; 0.1 M) dramatically decreased net P_i reabsorption by 60%. Bisindolylmaleimide I (BIM; 1 M), a highly selective PKC inhibitor, prevented PMA-induced inhibition. Activation of adenylyl cyclase/PKA by forskolin (10 M) mimicked the effect of PTH by significantly reducing net P_i reabsorption by one-half. Addition of H-89 (10 M), a potent inhibitor of PKA, abolished forskolin-induced inhibition. PTH inhibition was blocked by either BIM or H-89. Tissue electrophysiology remained stable after all treatments. There was a decreased immunoreactivity of the luminal Na^+-Pi cotransporter NaPi-IIa after PTH treatment. These data indicate that PTH inhibition of P_i reabsorption in this in vitro system is mediated by PKC and PKA.

primary cultures; reabsorptive flux; secretory flux; luminal sodium-inorganic phosphate cotransporter NaPi-IIa; protein kinase C; protein kinase A

THE STUDY OF RENAL FUNCTION in birds and other nonmammalian vertebrates has provided valuable insights into the regulation of renal phosphate (P_i) excretion. In mammals, filtration and reabsorption provide sufficient control of P_i excretion; however, in birds, which generally have lower and more variable glomerular filtration rates (46), the capacity for net tubular P_i secretion has been reported (3). P_i can be excreted by the avian kidney in quantities severalfold higher than the filtered load (21). Because avian kidneys possess this additional level of control for renal P_i handling, comparative studies of its regulation should further our understanding of P_i homeostasis.

In both the avian and mammalian renal proximal tubule, P_i reabsorption occurs via secondary, Na^+-dependent electroneutral transport across the luminal membrane [brush-border membrane (BBM)] (15, 34) coupled to an undefined exit process in the basolateral membrane (BLM) (1). In both mammals and birds, parathyroid hormone (PTH) has been found to be the major regulator of this process (28, 24). PTH decreases BBM Na^+-P_i cotransport activity in both mammals and birds and, in the latter, additionally stimulates net P_i secretion (1, 34), which likely occurs through a unique Na^+-independent, voltage- and K^+-dependent transporter in the BBM (1, 35).

Studies with the opossum kidney cell line (OK cells) have shown that PTH exerts its effects through activation of both protein kinase A (PKA) and protein kinase C (PKC) (7, 32), resulting in inhibition of BBM Na^+-P_i cotransport. Other studies indicated the phosphaturic effect of PTH involves endocytic retrieval followed by lysosomal degradation of the BBM Na^+-P_i cotransporter (NaPi-II) (20). The requirement for de novo protein synthesis to recover apical Na^+-P_i cotransport activity was previously demonstrated (26). More recently, PTH activation of extracellular signal-regulated kinases has been shown to inhibit P_i uptake in OK cells but through a mechanism other than down-regulation of the Na^+-P_i cotransporter (23).

Although much has been done in mammals regarding mechanisms of PTH regulation of renal P_i transport, little is known about this process in the avian system. Methods employed in the past to study the regulation of P_i transport in birds have included BBM vesicles, renal clearance, and micropuncture (21, 34, 43). The aforementioned studies demonstrated PTH inhibition of renal proximal tubular BBM Na^+-P_i cotransport; however, they did not examine direct regulation of transepithelial P_i trans-
port by PTH nor did they examine the intracellular signaling mechanisms.

In the present study, we investigated PTH regulation of transepithelial Pi transport in avian renal proximal tubule primary monolayer cultures (PTCs) and determined the effects of the protein kinase pathways. The data indicate that PTH acts acutely and directly on the chick proximal tubule epithelium type II NaPi-2 cotransporter, inhibiting active transepithelial Pi reabsorption through activation of both PKA and PKC regulatory pathways, and has no effect on secretory Pi transport in this segment.

MATERIALS AND METHODS

Animals. Kidneys were isolated from six to eight White Leghorn chicks (domestic Gallus gallus) at 3–7 days of age for each cell culture preparation.

Solutions and chemicals. Hanks’ balanced salt solution (HBSS) was purchased from Mediatech (Herndon, VA). This medium was supplemented with 4 mM NaHCO3 (pH 7.4 with NaOH or HCl). Krebs-Henseleit buffer (2x) was purchased from Sigma Chemical (St. Louis, MO). This medium was supplemented with 4 mM NaHCO3 (pH 7.4 with NaOH or HCl). The final plating medium and maintenance medium consisted of DMEM-Ham’s F-12 supplemented with ITS premix (5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenite), 20 μM ethanolamine, 300 μM l-glutamine, and 10% fetal bovine serum (FBS). The saline solution used for Ussing chamber studies contained (in mM) 1.1 CaCl2, 4.2 KCl, 0.5 MgCl2, 0.4 MgSO4, 120 NaCl, 0.4 NaH2PO4, 0.5 Na2HPO4, 1.0 glycerine, and 25 NaHCO3 (pH 7.4 with 5% CO2-95% O2; 290 mosmol/kgH2O).

Human parathyroid hormone-(1–34) [hPTH-(1–34)], ethanolamine, l-glutamine, and all components of the saline solution were purchased from Sigma. DMEM-Ham’s F-12 was purchased from Mediatech. FBS was purchased from Fisher (Pittsburgh, PA). ITS was purchased from Collaborative Biomedical Products (Bedford, MA). Phorbol 12-myristate-13-acetate (PMA), bisindolylmaleimide I (BIM), forskolin, and medical Products (Bedford, MA). Phorbol 12-myristate-13-acetate (PMA), bisindolylmaleimide I (BIM), forskolin, and medical Products (Bedford, MA). ITS was purchased from Collaborative Biotech (Piscataway, NJ).

Preparation of chicken PTCs. Chicken renal tubule segments were isolated and dispersed as previously described by Sutterlin and Laverty (38) and modified by Dudas and Renfro (12). Briefly, kidneys were removed; rinsed in HBSS; cleaned of any blood vessels, ducts, and connective tissue; and minced. The tissue fragments were incubated in an enzyme solution containing collagenase A (0.13 U/ml) and dispase II (0.54 U/ml) at 37°C. Nephron segments were further dissociated by trituration and filtration through a stainless steel sieve (380 μm). The dissociated tissue was rinsed three times with HBSS, the last rinse containing DNase I (2,161 U/ml), and resuspended in a 1:1 Percoll:2x Krebs-Henseleit buffer.

The suspension was centrifuged at 17,500 g (12,000 rpm), and the high-density band consisting of small proximal tubule segments was removed, rinsed in HBSS, suspended in culture medium with 10% serum, and plated on native rat-tail collagen as previously described (10). After 6 days, the collagen gels were released, and after ~14 days, the floating collagen gels had been contracted by the epithelial monolayers by ~40%.

Ussing chamber studies. During days 15–29, transepithelial electrical characteristics and Pi transport were measured. The tissues were supported by 150-μm mesh and mounted in Ussing chambers as previously described (13). The temperature was maintained at 39°C, and the saline bathing the luminal and basolateral sides of the tissue was continuously gassed (95% O2-5% CO2) and stirred throughout the experiment.

Transepithelial electrical potential (VT) was determined with a pair of reference electrodes connected to the luminal and basolateral compartments via 3 M KCl-2% agar bridges. Current was passed through Ag-AgCl electrodes connected to the luminal and basolateral compartments with 3 M KCl-2% agar bridges. Electrical properties were measured with a pair of computer-controlled, high-impedance automatic dual voltage clamps (DVC 1000; World Precision Instruments, Sarasota, FL). Transepithelial electrical resistance (TER) was determined from the change in VT produced by a 10-μA current pulse and corrected for fluid resistance.

Determination of transepithelial Pi fluxes. Tissues were continuously short-circuited during flux determinations, i.e., there were no transepithelial electrical or chemical gradients. The fluxional tracer solution was delivered by the addition of 1 μCi 32P2+ (H32PO4) (ICN, Costa Mesa, CA) to the appropriate hemichamber. Duplicate 50-μl samples were taken from the unlabeled side every 30 min over a period of 1.5 h and replaced with equal volumes of unlabeled saline (see Fig. 1). The specific activity of the labeled solution was determined at the beginning and end of each experiment.

Net flux was calculated as the difference between unidirectional secretory (basolateral-to-luminal) and absorptive (luminal-to-basolateral) fluxes. A single flux experiment was done on culture mates, i.e., both control and treated tissues were paired monolayers from a single preparation. The monolayer cultures used in a given experiment were prepared from the same starting tissue at the same time and cultured under identical conditions. For statistical determinations, this is referred to as one preparation. Tissue VT, TER, and phloridzin-sensitive current (Iph, Na+-dependent glucose transport) were used to assess tissue viability and proximal tubule-like function.

SDS-PAGE and immunoblotting. PTCs (15–29 days old) were incubated in physiological saline with or without PTH (10–9 M) for 1 h. Monolayers were then placed in Kaman buffer (2.3% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.5% saturated bromphenol blue, 62.5 mM Tris-HCl, pH 6.8) and vortexed approximately 20–30 s, followed by centrifugation at 8,750 g for 5 min to remove the collagen substratum. Twenty microliters of sample were used for SDS-PAGE (4–12%) and subsequent transfer to polyvinylidene fluoride (PVDF) microporous membrane (Millipore, Bedford, MA). Nonspecific binding was blocked by incubating the PVDF membrane at room temperature for 1.5 h in PBS (in mM: 137 NaCl, 2.7 KCl, 4.3 NaH2PO4, 1.5 KH2PO4, pH 7.3 with HCl) containing 10% nonfat dry milk, 0.01% antifoam-A, 0.02% sodium azide, and 0.05% polyoxyethylene sorbitan monolaurate (Tween 20). NaPi-IIa protein was detected using polyclonal antiserum (antibody dilution, 1/4,000) raised against the NH2 terminus of the rat NaPi-IIa protein (8), kindly supplied by Dr. J. Biber, Univ. of Zurich. Twenty microliters of rat kidney cortex homogenate in Kaman buffer were used for positive control of NaPi-IIa detection. Total actin for each sample was determined with mononcional antiserum raised against mouse actin (Sigma) (antibody dilution, 1/500). Density profiles of the actin and NaPi-IIa (NH2 terminus) bands were acquired using NIH Image software. Ratios of the optical densities of NaPi-IIa bands to actin bands were used to assess changes in NaPi-IIa concentration while controlling for amount of protein loaded per lane.
with the primary antibodies took place for 1 h 15 min at room temperature. The PVDF membrane was washed two times in PBS followed by one rinse in phosphate-free buffer [in mM: 150 NaCl, 10 Tris base, 40 Tris-HCl (pH 7.5)]. The portion of PVDF membrane previously incubated with primary antibody for the NaPi-IIa NH2 terminus was incubated with a 1:10,000 dilution of goat-anti-rabbit IgG alkaline phosphatase conjugate (Sigma) in phosphate-free buffer with 10% nonfat dry milk for 1 h at room temperature. The portion of PVDF membrane previously incubated with primary antibody for actin was incubated with a 1:750 dilution of goat-anti-mouse IgG alkaline phosphatase conjugate (StressGen, Victoria, British Columbia, Canada) in phosphate-free buffer with 10% nonfat dry milk for 1 h at room temperature. The PVDF membranes were washed three times with phosphate-free buffer, and the signals were detected by 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma) according to the manufacturer’s protocol. High-range SDS-PAGE molecular weight marker proteins (Sigma) were run in parallel. Proteins were electrophoretically separated under reducing conditions, resulting in cleavage of NaPi-IIa as previously described (2).

**Statistics.** Experimental results are expressed as means ± SE. Sample means were compared with one-tailed Student’s t-tests. Differences were judged significant if $P < 0.05$.

**RESULTS**

**PTH effect on transepithelial $P_i$ transport.** Figure 1A is a representative plot illustrating net transepithelial $P_i$ reabsorption by chick PTCs under short-circuited conditions. Unidirectional fluxes were initiated by addition of $^{32}P_i$ tracer at $t = 0$. Transepithelial reabsorptive flux approached steady state at 1.5 h, reflecting a relatively long period for equilibration of isotopic label with the transportable $P_i$ pool. Secretory flux was 1–2% of reabsorptive flux and, consequently, had little effect on net reabsorptive flux. $V_T$, TER, and $I_{PHZ}$ averaged $-1.35 \pm 0.22$ mV (sign relative to luminal side), $24.1 \pm 4.05$ Ω⋅cm$^2$, and $8.28 \pm 0.98$ μA/cm$^2$, respectively. In the example shown, addition of 10$^{-9}$ M PTH to the basolateral side of paired culture mates at $t = 0$ (Fig. 1B) decreased net transepithelial $P_i$ reabsorption almost 50% (132.0 to 70.0 nmol⋅cm$^{-2}$⋅h$^{-1}$) by 1.5 h. Experimental results presented below have corresponding electrophysiological data shown in Tables 1 and 2. Hormone or drug exposures were begun at $t = 0$, or, where indicated, a 30-min preincubation was used ($t = -30$ min).

On average, addition of 10$^{-9}$ M PTH to the basolateral side of PTCs in Ussing chambers reduced the unidirectional $P_i$, reabsorptive flux by ~60% (85.18 ± 22.20 to 33.90 ± 9.09 nmol⋅cm$^{-2}$⋅h$^{-1}$), resulting in an ~66% decrease in net transepithelial $P_i$ reabsorption (77.60 ± 23.42 to 26.45 ± 11.12 nmol⋅cm$^{-2}$⋅h$^{-1}$) (Fig. 2).

**Table 1. Effect of treatments on chick PTC transepithelial electrical properties**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>$I_{PHZ}$, μA/cm$^2$</th>
<th>TER, Ω⋅cm$^2$</th>
<th>$V_T$, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPTH-B side</td>
<td>12</td>
<td>10.00 ± 0.81</td>
<td>35.33 ± 2.82</td>
<td>$-2.37 \pm 0.44^*$</td>
</tr>
<tr>
<td>Control</td>
<td>12</td>
<td>11.31 ± 0.77</td>
<td>40.77 ± 4.11</td>
<td>$-1.34 \pm 0.17$</td>
</tr>
<tr>
<td>hPTH-L side</td>
<td>15</td>
<td>15.49 ± 2.03</td>
<td>30.37 ± 3.05</td>
<td>$-1.45 \pm 0.15$</td>
</tr>
<tr>
<td>Control</td>
<td>16</td>
<td>16.07 ± 2.92</td>
<td>28.23 ± 2.73</td>
<td>$-1.24 \pm 0.14$</td>
</tr>
<tr>
<td>PMA</td>
<td>8</td>
<td>18.64 ± 2.18</td>
<td>21.24 ± 2.53</td>
<td>$-1.29 \pm 0.17$</td>
</tr>
<tr>
<td>PM + BIM</td>
<td>8</td>
<td>20.40 ± 1.96</td>
<td>20.65 ± 2.25</td>
<td>$-1.66 \pm 0.13^*$</td>
</tr>
<tr>
<td>BIM</td>
<td>8</td>
<td>16.88 ± 1.13</td>
<td>19.54 ± 2.83</td>
<td>$-1.28 \pm 0.09$</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>20.21 ± 2.53</td>
<td>23.68 ± 3.53</td>
<td>$-1.58 \pm 0.23$</td>
</tr>
<tr>
<td>Forskolin</td>
<td>8</td>
<td>11.79 ± 1.87^*</td>
<td>26.78 ± 4.45</td>
<td>$-1.75 \pm 0.14^*$</td>
</tr>
<tr>
<td>Forskolin + H-89</td>
<td>8</td>
<td>14.75 ± 1.32</td>
<td>27.18 ± 1.60^*</td>
<td>$-1.86 \pm 0.17^*$</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>15.14 ± 2.37</td>
<td>25.31 ± 3.03</td>
<td>$-1.41 \pm 0.13^*$</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>16.30 ± 2.09</td>
<td>22.23 ± 1.68</td>
<td>$-1.35 \pm 0.15$</td>
</tr>
</tbody>
</table>

Values are means ± SE. Treatments: human parathyroid hormone-(1–34) (hPTH)-basolateral (B) side, 10$^{-9}$ M at $t = 0$; hPTH-luminal (L) side, 10$^{-9}$ M at $t = 0$; porphyrin-12-myristate 13-acetate (PMA), 10$^{-7}$ M, B and L sides at $t = 0$; bisindolylmaleimide I (BIM), 10$^{-6}$ M, B and L sides at $t = -30$ min; forskolin, 10$^{-6}$ M, B and L sides at $t = 0$; Na₂-[b-(p-bromocinnamylamino)ethyl]-5-isouquinolinesulfonamide, 2 HCl (H-89), 10$^{-5}$ M, B and L sides at $t = -30$ min. $I_{PHZ}$, phlorizin-sensitive current (Na$^+$-dependent glucose transport); TER, transepithelial electrical resistance; $V_T$, transepithelial electrical potential (lumen negative); PTC, proximal tubule cell in primary culture. Values shown were obtained at $t = 1.5$ h. *$P < 0.05$ compared with controls. †$P < 0.05$ compared with PMA- or forskolin-treated tissues. Significant differences were determined by paired and unpaired $t$-test comparisons.

**Table 2. Effect of treatments on chick PTC transepithelial electrical properties**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>$I_{PHZ}$, μA/cm$^2$</th>
<th>TER, Ω⋅cm$^2$</th>
<th>$V_T$, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPTH</td>
<td>8</td>
<td>13.09 ± 1.60</td>
<td>27.79 ± 2.66^*</td>
<td>$-1.93 \pm 0.06^*$</td>
</tr>
<tr>
<td>hPTH + BIM</td>
<td>7</td>
<td>10.47 ± 1.61</td>
<td>26.09 ± 3.82</td>
<td>$-1.50 \pm 0.16^*$</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>14.03 ± 1.53</td>
<td>21.54 ± 2.28</td>
<td>$-1.28 \pm 0.13$</td>
</tr>
<tr>
<td>hPTH</td>
<td>6</td>
<td>12.72 ± 2.16</td>
<td>27.88 ± 3.63</td>
<td>$-1.88 \pm 0.07^*$</td>
</tr>
<tr>
<td>hPTH + H-89</td>
<td>6</td>
<td>13.13 ± 2.16</td>
<td>27.43 ± 1.48</td>
<td>$-1.97 \pm 0.24^*$</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>14.40 ± 1.39</td>
<td>23.47 ± 2.27</td>
<td>$-1.37 \pm 0.05$</td>
</tr>
</tbody>
</table>

Values are means ± SE determined at $t = 1.5$ h. Treatments: hPTH, B side, 10$^{-9}$ M at $t = 0$; BIM, B and L sides, 10$^{-6}$ M at $t = -30$ min; H-89, B and L sides, 10$^{-5}$ M at $t = -30$ min. *Significantly different at $P < 0.05$ compared with controls. †Significantly different at $P < 0.05$ compared with hPTH-treated tissues. Significant differences were determined by paired and unpaired $t$-test comparisons.
Fig. 2. A: effect of basolateral addition of PTH on steady-state unidirectional L-to-B, B-to-L, and net Pi fluxes (see conditions in Table 1 legend). B: effect of luminal addition of PTH on steady-state unidirectional L-to-B, B-to-L, and net Pi fluxes (see conditions in Table 1 legend). Bars are means; vertical lines are 1 SE of n = 6 preparations. *Significantly different from paired controls at P < 0.05.

Fig. 3. Effects of phorbol 12-myristate 13-acetate (PMA) and bisindolylmaleimide I (BIM) on transepithelial reabsorptive (L to B), secretory (B to L), and net Pi transport by chick proximal tubule cells in Ussing chambers (see Table 1 legend). Addition of 10⁻⁶ M BIM prevented PMA inhibition. Each bar is the mean + 1 SE of n = 4 preparations. †Significantly different from controls (P < 0.05). ‡Significantly different from PMA-treated tissues (P < 0.05).

2A). Secretory flux was unaffected by PTH addition, indicating an influence only on the reabsorptive (active) transport mechanisms. The same 90-min exposure to PTH on only the luminal side had no effect on Pi transport (Fig. 2B). Table 1 shows that PTH significantly increased $V_T$ on basolateral-side exposure in paired tissues but had no effect on $I_{PHZ}$ or TER. In the Ussing chambers, the mode of exposure to PTH is identical on the two sides of PTCs with the exception that PTH must permeate a thin collagen (99% water) layer on the basolateral side.

Effect of PKC activation on Pi transport. PMA at a concentration of 10⁻⁷ M is generally considered specific for activation of several isoforms of PKC (39). Figure 3 shows that addition of 10⁻⁷ M PMA to the basolateral and luminal sides of PTCs in Ussing chambers significantly decreased unidirectional Pi reabsorptive flux by ~50% (176.82 ± 27.82 to 87.97 ± 20.50 nmol·cm⁻²·h⁻¹) and net transepithelial Pi reabsorption by ~60% (170.26 ± 27.79 to 69.44 ± 26.89 nmol·cm⁻²·h⁻¹). PMA inhibition was entirely through inhibition of Pi reabsorption with Pi secretory flux remaining unchanged. A 30-min preincubation with 10⁻⁶ M BIM, a PKC inhibitor, in the basolateral and luminal compartments prevented PMA inhibition of the unidirectional reabsorptive and net transepithelial Pi fluxes (Fig. 3). BIM alone had no effect. A small secretory flux was also further diminished by the combination of PMA and BIM but was otherwise unaffected by treatment. PMA alone and BIM alone had no effect on electrical properties (Table 1). BIM slightly increased $V_T$ in PMA-treated tissues; however, $I_{PHZ}$ and TER remained unchanged. These data show that PMA activation of PKC precisely mimicked the basolateral-side PTH effect.

Effect of PKA activation on Pi transport. In mammals, PKA is also implicated (7) in control of Pi transport, and, similar to PTH, its activation significantly reduces Pi reabsorption. Forskolin is a known activator of adenylate cyclase in the chick (14). Figure 4 shows that addition of 10⁻⁵ M forskolin to the basolateral and luminal sides of PTCs in Ussing chambers significantly decreased unidirectional reabsorptive flux by ~50% (138.93 ± 18.29 to 72.01 ± 4.07 nmol·cm⁻²·h⁻¹) and net transepithelial Pi reabsorption by ~50% (132.43 ± 16.98 to 69.14 ± 4.85 nmol·cm⁻²·h⁻¹). This effect was prevented by 30-min preincubation with 10⁻⁵ M H-89, a PKA inhibitor, in the luminal and basolateral compartments. This concentration of H-89 is threefold below the inhibition constant for PKC. Thus the above data support the possible involvement of both PKC and PKA. The low secretory flux was unaffected by forskolin, but the combination of forskolin and H-89 further reduced this flux (Fig. 4). H-89 alone had no significant effect compared with paired controls but caused fluxes to vary more than normal. Forskolin significantly increased $V_T$ and significantly decreased $I_{PHZ}$ but had no effect on TER (Table 1). The combination of forskolin and H-89 significantly increased $V_T$ and TER. H-89
alone had no effect on any of the electrical properties compared with control but significantly reduced the effect of forskolin on $I_{PHZ}$ and $V_T$. These data show that PKA activation as a result of forskolin treatment closely resembled the PTH (basolateral side) effect.

**Relationship of PTH and kinase activation.** Figure 5A shows that addition of $10^{-6}$ M BIM ($t = -30$ min, basolateral and luminal sides) prevented PTH (basolateral side) inhibition of unidirectional $P_i$ reabsorptive $P_i$, reabsorptive flux and net $P_i$ reabsorption with no effect on secretory flux. BIM also decreased the effect of PTH on $V_T$ (Table 2). These data indicate that PTH activation of PKC is part of its action in regulation of proximal tubular transepithelial $P_i$ transport in the chick. Figure 5B shows that $10^{-5}$ M H-89 ($t = -30$ min, basolateral and luminal sides) also prevented PTH (basolateral side) inhibition of net transepithelial $P_i$ reabsorption.

**NaPi-IIa transporter levels in response to PTH.** The effect of PTH on the type II $Na^+/Pi$ cotransporter protein content was investigated by immunoblotting. Polyclonal antibodies raised against the N-terminal sequence of the rat-type IIa $Na^+/Pi$-cotransporter were used for detection under reducing conditions. Figure 6 shows that exposure to $10^{-9}$ M PTH for 60 min resulted in decreased NaPi-IIa cotransporter expression. The amount of actin was determined for each sample to control for total protein loaded per lane. The average ratio of optical densities for the NaPi-IIa bands to the actin bands for control chick PTCs was 0.16 and for PTH-treated chick PTCs was 0.05, indicating a 70% decrease in NaPi-IIa abundance. Rat kidney cortex is shown as a positive control for antibody detection. The ratio of optical densities for the NaPi-IIa band to actin band for the rat control was 0.24.

**DISCUSSION**

It has previously been demonstrated that treatment of birds with PTH inhibited renal $P_i$ reabsorption (5, 24). These studies primarily used renal clearance techniques to characterize the phosphaturic effect of PTH.

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![Fig. 4](http://ajpregu.physiology.org/)

![Fig. 5A](http://ajpregu.physiology.org/)

![Fig. 5B](http://ajpregu.physiology.org/)

![Fig. 6](http://ajpregu.physiology.org/)
on avian renal function. A later study by Renfro and Clark (34) utilizing isolated proximal tubule luminal membrane vesicles (BBM vesicles) provided strong evidence that pretreatment of chicks with PTH resulted in a decrease in both apparent affinity and maximal velocity for Na\(^+\)-dependent P\(_i\) transport in the BBM. The data reported in the present study, however, demonstrate that PTH acts directly on avian renal proximal tubule epithelium and inhibits active transepithelial P\(_i\) reabsorption. Direct action of PTH on the proximal tubule has been shown previously for the mammal (11), and the direct effect in an avian system indicates a highly conserved response.

Exposing the basolateral side of chick PTCs to PTH resulted in substantially reduced unidirectional reabsorptive and net fluxes; both were reduced to less than one-half of paired controls. These experiments assessed P\(_i\) fluxes under biophysically precise conditions, providing a clear demonstration that PTH acted directly on the proximal tubule cell to inhibit the mechanisms of active P\(_i\) reabsorption. Prior studies in chickens and starlings had shown that PTH also induced tubular P\(_i\) secretion (43, 44). The present data show no change in the P\(_i\) secretory flux in response to PTH treatment. The reason for this lack of effect is not currently known; however, avian kidneys contain renal tubules of both the reptilian (no loop of Henle) and mammalian type (loop of Henle) (4), and micropuncture experiments on starlings showed that PTH administration induced P\(_i\) secretion by the proximal tubules of superficial, reptilian-type nephrons (22). If the P\(_i\)-secretory capacity in response to PTH is present more abundantly in this nephron type, the primary culture method may have selected against secretory-competent segments. These earlier studies did not show that P\(_i\) secretion was a result of the direct action of PTH on proximal tubule epithelium. It is possible that the increase in secretion and excretion of P\(_i\) in response to PTH was indirect. No information is available regarding P\(_i\) transport in other segments of the superficial reptilian-type or in the deeper reptilian-type or mammalian-type nephrons in birds.

Previous studies using isolated rat renal cortical BBM and BLM vesicles and OK cells have shown receptors for PTH/PTH-related peptide (PTHrp) localized to both luminal and basolateral membranes and provided evidence for functional differences between them (18, 36, 37). Intact PTH and its fragments are removed from the circulation through glomerular filtration (9, 16, 27). The filtered PTH may then interact with receptors on the luminal membrane of proximal tubule cells. PTH inhibition of P\(_i\) transport through activation of luminal PTH receptors was demonstrated in OK cell monolayers (36, 37) and later in perfused murine proximal tubules (40). In perfused proximal tubules of mice, PTH-(1–34) strongly downregulated NaPi-IIa in the luminal membrane from both luminal and basolateral sides. PTH-(3–34) was effective only from the luminal side (40). The hormone concentration, however, was exceptionally high (10\(^{-6}\) M) in the aforementioned studies. In the present study, there was no effect of 10\(^{-9}\) M PTH on P\(_i\) secretion or reabsorption when applied to the luminal side of the chick PTCs but more than 50% inhibition when applied to the basolateral side. Whereas culturing conditions could influence receptor location and abundance, there is no evidence at this time for selective downregulation of PTH/PTHrp receptor isotypes.

Another factor that could influence sidedness of the PTH effect in the chick system is our use of synthetic human (h) PTH-(1–34). In the chicken, this peptide has previously been demonstrated as effective as bovine parathyroid extract and synthetic bovine PTH-(1–34) in several parameters, including effects on glomerular filtration rate, renal plasma flow rates, urine pH, and fractional excretion of sodium, potassium, chloride, calcium, magnesium, and P\(_i\) (45). Fractional excretion of P\(_i\) was greatest with hPTH compared with the other peptides. Several other studies have used hPTH with chicken kidney preparations, demonstrating its effectiveness in inducing a phosphaturic response (1, 34, 42). PTH-(1–34) from human and chick had equal potencies in several chick bioassay systems (25).

In the mammalian OK cell line, which retains proximal tubular transport function, it is well established that PTH activates both the adenylate cyclase/PKA and a phospholipase C/PKC regulatory pathway, resulting in inhibition of P\(_i\) transport (6, 7, 17, 33). Later studies demonstrated a membrane retrieval and degradation of the BBM type II Na\(^+\)-P\(_i\) cotransporter in response to PTH activation of PKA and PKC (32). Much less has been done in avian species characterizing second messenger signaling in renal epithelial cells in response to PTH. There is strong evidence for activation of PKA (31); however, there is little information implicating the concurrent activation of PKC. Additionally, prior studies investigating PTH-induced second messenger and kinase activation in the bird were performed on whole kidney cell cultures or isolated renal plasma membranes (14, 30, 31). Here, we have shown activation of both PKA and PKC in response to PTH in a pure culture of avian renal proximal tubule epithelium.

The phorbol ester PMA was used to investigate PKC activation and its effect on P\(_i\) transport in chick PTCs. PKC activation resulted in a significant decrease in the unidirectional P\(_i\) reabsorptive flux and net P\(_i\) reabsorption. This effect was prevented by BIM, a potent PKC inhibitor. PMA is very specific for the activation of PKC. This, combined with the BIM inhibition of the PMA effect, clearly demonstrates PKC involvement.

Forskolin, an effective activator of adenylate cyclase in chicken kidney cells (14), was used to activate PKA in this study. As shown above, forskolin significantly reduced the unidirectional P\(_i\) reabsorptive flux and net transepithelial P\(_i\) reabsorption. This inhibitory effect was blocked by the selective PKA inhibitor H-89, indicating a probable PKA effect. There were small but significant changes in tissue electrophysiology with forskolin addition.
The addition of PMA and BIM together and forskolin and H-89 together resulted in a decrease in the unidirectional P_i secretory flux. The reason for this is unclear but may indicate the presence of a very small but mediated secretory flux or may be due to variability in tissue responses.

Inhibition of P_i reabsorption by PKA and PKC activation clearly implicates these signaling pathways as mechanisms by which PTH elicits its effect on P_i transport in chick PTCs. In support of this, BIM and H-89 were shown to prevent the inhibitory effect of PTH.

It has been demonstrated in the mammal that PTH provoked a decrease of type II Na^+–P_i cotransporters in the luminal membrane that was paralleled by a change of BBM Na^+–P_i cotransport (19). In the present study, PTH also works through PKA and PKC activation and also decreases the amount of NaPi-IIa. It is clear that there is an immunoreactivity of the mammalian antibody we used with the NH_2-terminal portion of the chicken type II Na^+–P_i cotransporter and a decrease in abundance of this transporter in the chick PTCs in response to PTH. On SDS-PAGE under nonreducing conditions, a protein of ~85 kDa corresponding with the intact transporter (8) was detected. However, on SDS-PAGE under reducing conditions, as in the present study, the transport protein was cleaved, producing bands of molecular mass 50 kDa (detected with the anti-NH_2-terminal antibody) and 40 kDa (detected with the anti-COOH-terminal antibody; data not presented) as was previously described for the mammal (2).

In summary, examination of transepithelial P_i transport by primary cultures of chicken proximal tubule epithelium as monolayers in Ussing chambers has provided important evidence that 1) transepithelial P_i transport is active, i.e., occurs under short-circuited conditions and 2) PTH can act directly on the epithelium to inhibit reabsorption of P_i. These data clearly indicate a very similar mechanism of action of PTH in the bird and mammal for inhibition of P_i reabsorption in the renal proximal tubule. PTH regulation of P_i transport through dual kinase activation may seem redundant, but as has been proposed for the mammal, different kinases may be functioning at different points in the membrane retrieval and degradative pathways for the transporter (29). These data therefore provide clear indications of those events occurring in vivo mediating avian renal proximal tubular P_i transport.

Perspectives

This examination of renal P_i transport by chick PTCs makes possible valuable predictions about the regulation of this transport system in the intact bird while also demonstrating a technique potentially useful for examining the mechanisms of transepithelial P_i transport. Access to both the basolateral and luminal sides of the cell monolayer allowed for thorough examination of the direct regulation of P_i transport in the avian proximal tubule by PTH and its effect on the transepithelial transport of P_i rather than cellular uptake alone.

Of further note is the similarity in the regulation of renal P_i transport by PTH in the bird and mammal. Dual kinase control in these different species and the presence of the PTH-regulated Na^+-P_i cotransporter NaPi-IIa support the evidence for a common origin and conservation of the transport protein (41). However, an interesting difference was discovered in this culture system. As discussed previously, after luminal exposure to PTH, there was no effect in the chick PTCs on P_i transport, whereas a prior study in perfused murine proximal tubules showed a downregulation of NaPi-IIa. These data suggest that if PTH/PTHrP receptors are present in the luminal membrane of the bird, they do not respond to hPTH(1–34). Is this due to culturing conditions and downregulation of the luminal receptors or species differences? At present, the data obtained from this culture system would seem to be the best indicator of events regulating the transepithelial transport of P_i in the avian renal proximal tubule.

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