Glucocorticoids have been reported to have diverse effects on glucose metabolism [20], blood pressure [12], and renal function [18]. Glucocorticoids are also important regulators of renal phosphate transporter. The primary site of action of glucocorticoids is the proximal tubular apical brush border membrane and proximal tubular inorganic phosphate reabsorption plays a key role in overall Pi homeostasis [4, 18]. However, the action of glucocorticoids on Pi transport is controversial. In rat proximal tubule and chicken kidney cells glucocorticoids decrease tubular reabsorption of phosphate [22, 30]. In contrast, dexamethasone increased Pi uptake in opossum kidney (OK) cells [17]. In addition, the mechanisms responsible for the action of glucocorticoids on Pi uptake remain to be clarified. Among signal pathways, calcium is reported to be involved in the action of glucocorticoids in several tissues. Raising serum Ca²⁺ levels stimulates phosphate absorption in the proximal tubule of parathyroidectomized rat and Ca²⁺ stimulates the sodium-dependent phosphate absorption [1, 26].

A convenient means to evaluate the effects of hormones on renal tubule epithelial cells is to use in vitro cell culture systems. A primary cultured rabbit renal proximal tubule cells (PTCs) culture system has been developed that forms confluent monolayers of polarized epithelial cells when grown in a serum-free medium supplemented with insulin and transferrin. The primary cells have been observed to retain a number of differentiated functions typical of the renal proximal tubule, including Na⁺-dependent sugar transport, Na⁺-dependent phosphate transport, and probenecid sensitive p-aminohippuric acid transport [9, 16, 33]. Therefore, the membrane transport studies, such as those establishing Pi uptake, conducted with such PTCs in hormonally defined, serum-free culture conditions have the particular advantage that the results can be directly compared with the original renal tissue [32]. Thus, we investigated the effect of DEX on the renal proximal brush border membrane transporters function and the involvement of Ca²⁺ in its action in the PTCs. The results demonstrated that DEX inhibited Pi uptake in the PTCs, which is, in part, mediated by cytosolic Ca²⁺ mobilization from the intracellular Ca²⁺ storage pool.

MATERIALS AND METHODS

Materials: Male New Zealand White rabbits (1.5–2.0 kg) were used for these experiments. Dulbecco’s Modified Eagle’s medium/Nutrient Mixture F-12 (Ham) (D-MEM/F-12, 1:1), Class IV collagenase and soybean trypsin inhibitor were purchased from Life Technologies (Grand Island, NY). Dexamethasone (DEX), testostosterone, progesterone, 17β-estradiol, α-methyl-D-glucopyranoside (α-MG), ethylene glycol-bis (β-amin ethyl ether)-N,N,N',N'-tetra acetic acid (EGTA), 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate (TMB-8), 1,2-bis(aminophenoxo)ethane-N,N,N',N'-tetra acetic acid tetrakis (acetomethyl ester) (BAPTA/AM), n-6(aminohexyl)-5-chloro-1-naphthalene-
sulfonamide (W-7), 1-[N,O-bis(5-isoquinolinesulfonfyl) -N-methyl-L-tyrosyl]-4-phenyl-piperazine (KN-62), nifedipine, methoxyverapamil, and ouabain were obtained from the Sigma Chemical Company (St. Louis, MO). 22Na, 14C-α-methyl-D-glucopyranoside (14C-α-MG), and 32P phosphate (32Pi) were purchased from Dupont/NEN. All other reagents were of the highest purity commercially available. Liquiscint was obtained from National Diagnostics (Parsippany, NY).

Isolation of rabbit renal proximal tubules and culture conditions: Primary rabbit renal proximal tubule cell cultures were prepared by the method of Chung et al. [9]. The PTCs were grown in D-MEM/F-12 medium with 15 mM HEPES and 20 mM sodium bicarbonate (pH 7.4). Immediately prior to the use of the medium, two growth supplements (5 µg/ml insulin and 5 µg/ml transferrin) were added.

Cell growth study: To determine the effects of DEX, PTCs in culture were initiated in 35 mm dishes for cell growth studies. Briefly, tubules were inoculated at one-fourth the normal inoculum (the normal inoculum being 0.3 mg protein/dish). During this time, cell counts were determined on days 5, 9, and 13 from triplicate culture plates using a Coulter Model ZF particle counter.

Marker enzymes assay: The confluent monolayers were incubated with 10–9 M DEX for 4 hr before marker enzymes assay. Alkaline phosphatase activity was assayed by the method of Green et al. using p-nitrophenylphosphate as a substrate [5]. Leucine aminopeptidase activity was assayed by the method of Green et al. using L-leucine-p-nitroanilide as a substrate [15]. γ-glutamyl transpeptidase activity was determined by the method of Tate and Meister using γ-glutamyl-p-nitroanilide as substrate and glycylglycine as the amino acid acceptor [29]. Each determination was made using triplicate dishes and was standardized with respect to protein. Protein determination was performed by the methods of Bradford using bovine serum albumin as a standard [6].

Uptake studies: The confluent monolayers were incubated with 10–9 M DEX for 4 hr before the uptake experiments. The Pi uptake experiment was conducted as described by Rabito [24]. After the culture medium was removed by aspiration, the monolayers were gently washed twice with the uptake buffer (150 mM NaCl, 1.2 mM MgSO4, 0.1 mM CaCl2, and 10 mM MES/Tris, pH 7.4). After the washing procedure, the monolayers were incubated at 37°C for 30 min in an uptake buffer containing 1.5 µCi/ml 32Pi and 1 mM unlabeled phosphate. At the end of the incubation period, the monolayers were again washed three times with ice-cold uptake buffer, and the cells were solubilized in 1 ml of 0.1% SDS. To determine the 32Pi incorporated intracellularly, 900 µl of each sample was removed and counted in a liquid scintillation counter (Beckmann Instruments, Inc., Fullerton, CA). The remainder of each sample was used for protein determination. The radioactivity counts in each sample were then normalized with respect to protein and were corrected for zero-time uptake per mg protein. All uptake measurements were made in triplicate.

Na+ uptake experiment was conducted by the method of Rindler et al. [25], and α-MG uptake experiment by the method of Sakhrani et al. [27]. Next steps were conducted as described in Pi uptake.

Statistical analysis: Results were expressed as means ± standard errors (S.E.). The difference between two mean values was analyzed with the ANOVA test. The difference was considered statistically significant when p<0.05.

RESULTS

Effects of dexamethasone on growth and brush border membrane enzyme activities: DEX has been implicated in modulating PTCs growth and function in vivo. In the first set of experiments, we examined the growth responsiveness of PTCs to DEX. Figure 1 depicts the time course growth of PTCs. PTCs were treated with DEX (10–12–10–6 M) during medium exchange every 3 days. Cell counts were determined on days 5, 9, and 13. Cell numbers increased logarithmically from day 5 to 13. On day 9, DEX did not affect the growth of PTCs (Fig. 1). In the experiments to measure the brush border membrane marker enzyme activity, alkaline phosphatase and γ-glutamyl transpeptidase activity were not significantly different from the control. However, leucine aminopeptidase exhibited significantly reduced activity (Table 1).

Effects of dexamethasone on transporters activities: Cells were incubated with DEX prior to Pi uptake. As shown in Fig. 2A, DEX inhibited Pi uptake by 17% after 4 hr, and by 21% after 8 hr. This effect was less pronounced with a more prolonged preincubation time (3% after 24 hr, p=NS). Following the treatment of DEX for 4 hr, the relationship between DEX concentration and inhibition of Pi uptake was
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Significant inhibition was achieved by DEX concentrations equal to or higher than $10^{-9}$ M (Fig. 2B). This DEX action was selective for Pi uptake since DEX did not cause changes in the activities of $\alpha$-MG uptake, Na$^+$ uptake, and $^{86}$Rb$^+$ uptake (Table 2). In order to investigate the effects of DEX or Pi uptake kinetics, the affinity ($K_m$) of Pi for Na/Pi cotransporter and the maximum velocity ($V_{max}$) of Pi uptake were calculated using a Lineweaver-Burk plot. Phosphate concentrations varied from 0.0625 to 1 mM. Figure 3 presents the results of the kinetic analysis of Pi uptake in the presence and the absence of $10^{-9}$ M DEX. Kinetic studies showed a decrease in $V_{max}$ values of Pi uptake from $234.7 \pm 17.2$ to $178.3 \pm 14.1$ nmol/mg protein/min, while apparent $K_m$ values were unchanged.

To evaluate whether the effect of DEX on Pi uptake was mimicked by other steroids, PTCs were treated with either $^{17}\beta$-estradiol, testosterone, or progesterone at a concentration of $10^{-9}$ M for 4 hr, respectively. None of these hormones affected Pi uptake (Fig. 4).

Involvement of Ca$^{2+}$ on dexamethasone-induced inhibition of Pi uptake: To examine the role of Ca$^{2+}$ on Pi uptake, the PTCs were treated with A 23187 ($10^{-6}$ M, Ca$^{2+}$ ionophore). As shown in Fig. 5, A 23187 significantly inhibited Pi uptake, mimicking the DEX-induced inhibition of Pi uptake. Therefore we examined the role of Ca$^{2+}$ or calmodulin on DEX-induced inhibition of Pi uptake. PTCs were treated with W-7 ($10^{-4}$ M, calmodulin dependent kinase inhibitor) or KN-62 ($10^{-6}$ M, Ca$^{2+}$/calmodulin dependent protein kinase II inhibitor) prior to the treatment with DEX. W-7 and KN-62 completely blocked DEX response (Fig. 5), and these results suggest that Ca$^{2+}$/calmodulin pathway is involved in the DEX-induced inhibition of Pi uptake.

Role of intracellular Ca$^{2+}$ mobilization on dexamethasone-induced inhibition of Pi uptake: To know the source of cytosolic free Ca$^{2+}$, PTCs were treated with

Table 1. Effect of dexamethasone on the brush border membrane enzyme activity of the primary cultured renal proximal tubular cells

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Control</th>
<th>Dexamethasone</th>
</tr>
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<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>16.8 ± 1.8</td>
<td>20.1 ± 2.9</td>
</tr>
<tr>
<td>(nmoles p-nitrophenyl phosphate released/mg protein/min)</td>
<td></td>
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</tr>
<tr>
<td>Leucine aminopeptidase</td>
<td>164.9 ± 5.2</td>
<td>133.0 ± 5.1*</td>
</tr>
<tr>
<td>(nmoles p-nitroanilide released/mg protein/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\gamma$-Glutamyl transpeptidase</td>
<td>23.4 ± 1.3</td>
<td>22.1 ± 1.6</td>
</tr>
<tr>
<td>(nmoles p-nitroanilide released/mg protein/min)</td>
<td></td>
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</tr>
</tbody>
</table>

Values are means ± S.E. of four to five independent experiments with triplicate dishes. * p<0.05 vs. control.

Table 2. Effects of dexamethasone on $^{32}$Pi, $^{14}$C-$\alpha$-MG, $^{22}$Na, and $^{86}$Rb uptake (pmol/mg protein/min)

<table>
<thead>
<tr>
<th></th>
<th>$^{32}$Pi</th>
<th>$^{14}$C-$\alpha$-MG</th>
<th>$^{22}$Na</th>
<th>$^{86}$Rb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>209.8 ± 9.7</td>
<td>576.2 ± 24.3</td>
<td>1382.3 ± 60.0</td>
<td>274.2 ± 9.6</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>173.3 ± 9.3*</td>
<td>530.4 ± 24.7</td>
<td>1449.9 ± 70.6</td>
<td>293.9 ± 18.5</td>
</tr>
</tbody>
</table>

Values are means ± S.E. of 12 separate experiments with triplicate dishes. * p<0.05 vs. each control.
BAPTA/AM (10^{-6} M) and TMB-8 (10^{-4} M) (intracellular Ca^{2+} mobilization blockers) alone prior to the treatment with DEX. BAPTA/AM and TMB-8 alone had no significant effect on Pi uptake. However, they completely blocked DEX-induced inhibition of Pi uptake (Fig. 6A). Therefore, as a next step, we examined the role of extracellular Ca^{2+}. PTCs were reported to have a L type Ca^{2+} channel. Thus PTCs were treated with nifedipine and methoxyverapamil (10^{-6} M, L type Ca^{2+} channel blockers) prior to the treatment with DEX. They did not block the DEX-induced inhibition of Pi uptake, and EGTA (1 mM, extracellular Ca^{2+} chelator) also did not affect the DEX-induced inhibition of Pi uptake (Fig. 6B). These results suggest that cytosolic free Ca^{2+} originating not from the extracellular Ca^{2+} influx but from the intracellular Ca^{2+} storage pool may be involved in the DEX-induced inhibition of Pi uptake.
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