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INHIBITION OF RENAL BRUSH BORDER PHOSPHATE TRANSPORT AND STIMULATION OF RENAL GLUCONEOGENESIS BY CYCLIC AMP AND PARATHYROID HORMONE

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Abstract—The aims of the study were to determine whether 8-bromo-cyclic AMP (8BcAMP) *in vivo* mimics the inhibitory action of parathyroid hormone (PTH) on phosphate transport across the brush border membrane (BBM) of the renal proximal tubule, and to examine whether changes in BBM transport are accompanied by changes in the rate of renal gluconeogenesis. Thyroparathyroidectomized dogs were anesthetized and equilibrated, and control urine collections were obtained prior to removing the left kidney. Subsequent intravenous infusion of 8BcAMP at 50 mg/hr for 2 hr increased fractional excretion of phosphate from 4 ± 1 (controls) to 29 ± 4% ($P < 0.001$) without changing glomerular filtration. In BBM vesicles isolated from the renal cortex, the initial Na^+-dependent transport of phosphate was decreased from 747 ± 135 (controls) to 564 ± 126 pmoles per mg per 0.25 min after 8BcAMP ($P < 0.025$), but Na^+-independent phosphate uptake and Na^+-dependent L-proline uptake were not changed significantly. Renal gluconeogenesis in the same animals was increased from 2.5 ± 0.3 (controls) to 5.3 ± 0.5 μmoles glucose per g tissue per hr after infusion of 8BcAMP ($P < 0.001$). Infusion of PTH, like 8BcAMP, inhibited BBM phosphate transport and stimulated renal gluconeogenesis. We conclude that the inhibitory action of cyclic AMP and PTH on BBM phosphate transport is accompanied by stimulation of gluconeogenesis which suggests, indirectly, that changes in gluconeogenesis may be part of the intracellular mechanism for regulating BBM phosphate uptake in response to certain stimuli.

Gluconeogenesis in the mammalian kidney is localized almost exclusively in the proximal tubule [1–3], the site where most of the phosphate in the glomerular filtrate is reabsorbed [4]. The initial step in transtubular reabsorption of phosphate is entry into the proximal tubule cell across the luminal brush border membrane (BBM)†, and this BBM transport process is modified by various stimuli which alter renal phosphate handling. Parathyroid hormone (PTH) is a phosphaturic stimulus which *in vivo* inhibits renal BBM transport of phosphate [5, 6] and which *in vitro* stimulates gluconeogenesis in renal tubule suspensions [7–9]. In contrast, low phosphorus diet is an antiphosphaturic stimulus which stimulates renal BBM phosphate transport [10–12] and inhibits renal gluconeogenesis [13]. Taken together, these findings from different laboratories point to a possible role for gluconeogenesis in the intracellular regulation of BBM transport of phosphate in the proximal tubule.

The relationship between gluconeogenesis and phosphate transport was examined in the present study by determining whether gluconeogenesis is stimulated after *in vivo* administration of PTH in a dose which inhibits BBM transport of phosphate. Since cyclic AMP, which also stimulates renal gluconeogenesis *in vitro* [7, 8, 14, 15], may be the intracellular mediator of the phosphaturia induced by the action of PTH [16], another aim of the present study was to determine whether infusion of cyclic AMP can reproduce the effects of PTH on renal gluconeogenesis and BBM phosphate transport.

MATERIALS AND METHODS

Female mongrel dogs (15–25 kg) were thyroparathyroidectomized at least 48 hr, and usually 1 week, prior to study. Thyroid hormone was replaced as Synthroid (0.1–0.3 mg/day). The animals were anesthetized with Nembutal (25 mg/kg), and supplemental doses were given as required. An endotracheal tube was inserted and the dogs were ventilated with a Harvard respirator. Stroke volume was adjusted according to arterial blood pH and pCO_2 which were measured with an Astrup apparatus (BMS-3, London Co., Westlake, OH). Intravenous infusions were given through a saphenous vein, and blood sampling and blood pressure determinations were performed utilizing a cannula inserted in a femoral artery. A priming dose of inulin and *p*-aminohippurate was given, and a sustaining infusion of these substances was begun in saline at 1 ml/min. Volume expansion (2.5% of body weight) with normal saline solution (containing 1.5 m-equiv. calcium

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† Abbreviations: BBM, brush border membrane; 8BcAMP, 8-bromo-cyclic AMP; PTH, parathyroid hormone; and HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

gluconate) was accomplished, and the infusion rate was then set at 1–3 ml/min for the remainder of the experiment. Following an equilibration period of 1 hr, two to three control urine collections (15–30 min in duration) were obtained, and the left kidney (control) was removed. A sample of the renal cortex was excised immediately, snap-frozen with tongs precooled in liquid nitrogen, and stored frozen for later extraction and measurement of NAD^+ and NADH. The remaining cortex was placed in ice-cold 154 mM NaCl, 1 mM Tris–HEPES (pH 7.5) and was processed at once for studies on BBM transport and gluconeogenesis. The dogs were given 1 additional hr of equilibration; then two to three control urine collections were taken and the animals were given either 8BcAMP (Sigma Chemical Co., St. Louis, MO) by infusion of 50 mg/hr for 2 hr [17] or synthetic 1-34 PTH (Beckman Instruments Inc., Palo Alto, CA) in a priming dose of 2 μg/kg body weight followed by a sustaining infusion of the same dose per hour for 2 hr [8]. Urine collections were continued and, at the end of the infusion period, the right kidney (experimental) was removed and processed in the same way as described above for the left kidney. Blood was obtained at the beginning, middle, and end of each control period and every 40 min throughout the study. Both blood and urine were analyzed for inulin, *p*-aminohippurate, and phosphorus by methodology described previously [17, 18].

BBM fractions were prepared from homogenized renal cortex by the calcium precipitation procedure described in detail in previous studies [5, 6, 11, 19, 20]. The identity and purity of the BBM vesicle preparations were established routinely by assaying the activities of appropriate marker enzymes for subcellular organelles [6, 11] and were not different between control and experimental kidneys. The activities of alkaline phosphatase and gamma-glutamyl transpeptidase, typical BBM enzymes, were increased 8- to 10-fold in the BBM fraction compared to the cortical homogenate, as reported previously for the dog [6]. The activity of the basolateral membrane enzyme $(Na^+ + K^+)$-ATPase was decreased in the BBM fraction to one-half of the activity in the homogenate. Details of the method for measuring transport by BBM vesicles are described elsewhere [11, 19, 20]. Briefly, Na^+ gradient-dependent uptake of phosphate or L-proline was determined by incubating BBM vesicles at 20° in a medium of (final concentrations) 100 mM NaCl, 100 mM mannitol, 5 mM Tris–HEPES (pH 8.5) containing either 0.1 mM $K_2H^{32}PO_4$ or 0.025 mM L-[^{3}H]proline. The uptake was terminated at various times by rapid addition of a large volume of ice-cold 135 mM NaCl, 10 mM arsenate, 5 mM Tris–HEPES (pH 8.5) followed by filtration through a Millipore filter (0.65 μm). The Na^+-independent transport was measured by replacing NaCl in the incubation medium with 100 mM KCl. Protein in tissue fractions was assayed by the method of Lowry *et al.* [21], and enzyme activities were determined as described previously [11, 19].

Gluconeogenesis in renal cortex was determined as the rate of glucose production by thin slices of cortex incubated in the presence of an appropriate substance [22, 23]. Rinsed tissue slices were shaken at 37° for 60 min in continuously oxygenated KRB containing 127.2 mM NaCl, 4.0 mM KCl, 1.0 mM CaCl, 1.4 mM $MgCl_2$, 2.4 mM NaH_2PO_4 (pH 7.4). At the end of this time the slices were blotted dry, weighed, and distributed into flasks (250 mg tissue/flask) containing 25 ml fresh KRB with or without 10 mM α-ketoglutarate. The slices were incubated as before for 60 min and then samples (2 ml) of the incubation medium were deproteinized by addition of perchloric acid. The acid extracts were neutralized and analyzed for glucose by a colorimetric procedure using glucose oxidase [23]. The data presented for gluconeogenesis are the values obtained after subtraction of the rate of endogenous glucose production measured in the absence of α-ketoglutarate.

Acidic and alkaline extracts of snap-frozen cortex were prepared using perchloric acid and alcoholic KOH respectively [19, 23, 24]. NAD^+ in the acid extract and NADH in the alkaline extract were assayed immediately after neutralization. The assay method for NAD^+ is based on the reduction of NAD^+ by alcohol, and the assay of NADH utilizes oxidation of NADH by dihydroxyacetone phosphate [23, 24].

[^{32}P]Phosphate and L-[2,3,4,5-^{3}H]proline were from the New England Nuclear Corp. (Boston, MA). All enzymes and biochemicals were from the Sigma Chemical Co. (St. Louis, MO). Control and experimental data were evaluated statistically by Student's *t*-test for paired comparisons, and values of $P > 0.05$ were considered not significantly different (NS).

RESULTS

Administration of 8BcAMP or PTH to dogs led to significant increases in both the absolute and fractional phosphate excretion rate (Table 1). The glomerular filtration rate and the renal plasma flow did not change significantly, indicating that the increases in phosphate excretion were due primarily to decreased tubular reabsorption of phosphate. The slight decrease in serum ultrafiltrable phosphate after 8BcAMP treatment may reflect the increase in renal phosphate excretion.

The Na^+ gradient-dependent transport of phosphate by isolated BBM vesicles was decreased significantly at the initial and peak phases of the transient overshoot after treatment with 8BcAMP (Fig. 1 and Table 2), a change in parallel with the decreased phosphate reabsorption. The uptake at the equilibrium point (steady state) at 120 min was not different from controls (Fig. 1), indicating it is unlikely that the decreased uptake at earlier time points was due to an affect of 8BcAMP on intravesicular space. Infusion of 8BcAMP failed to decrease BBM phosphate transport only in experiment No. 3 (Table 2). In all other experiments, 8BcAMP decreased phosphate uptake at 0.25 min by 2–59%, with a mean decrease of 29%. The Na^+-independent transport of phosphate, which probably represents a passive diffusion process, was 8 ± 1 pmoles per mg protein per 0.5 min (mean ± S.E.) in control BBM vesicles compared to 15 ± 5 pmoles per mg protein per 0.5 min in vesicles after 8BcAMP administration (NS, paired *t*-test), indi-

Table 1. Effect of 8-bromo-cAMP and parathyroid hormone on renal function and renal phosphate excretion*

		C_{In} (ml/min)	C_{PAH} (ml/min)	SUF_P m-equiv./l	U_PV (μmoles/min)	% EP
8-bromo-cAMP	C	22 ± 3	61 ± 9	1.2 ± 0.2	1.1 ± 0.5	3.9 ± 1.4
(N = 7)	E	26 ± 5	58 ± 10	0.9 ± 0.1†	6.8 ± 2.1†	29.4 ± 4.3†
PTH	C	26 ± 2	66 ± 6	1.0 ± 0.1	0.3 ± 0.1	1.4 ± 0.4
(N = 5)	E	30 ± 2	82 ± 15	1.0 ± 0.1	5.1 ± 0.7†	17.8 ± 2.7†

* One dog was used in each experiment, and data are the means ± S.E. from five to seven animals. The left kidney was removed after the control (C) collections, and the drugs were administered intravenously during the experimental (E) period. Abbreviations: C_{In}, glomerular filtration rate (inulin clearance); C_{PAH}, renal plasma flow (clearance of *p*-aminohippurate); SUF_P, serum ultrafilterable phosphate level; U_PV, rate of urinary phosphate excretion; % EP, fractional excretion of phosphate (% of filtered load); PTH, parathyroid hormone; and N, number of animals.

† Significantly different from control ($P < 0.02$, or higher level of significance, paired *t*-test).

cating that inhibition of Na^+ gradient-dependent phosphate transport is not due simply to an effect of 8BcAMP on BBM permeability to phosphate.

The change in BBM phosphate transport was specific as illustrated by the finding that there was no decrease in the transport of L-proline, another Na^+ gradient-dependent transport process in BBM (Fig. 1). In fact, in every experimental animal (Table 2), L-proline uptake at 0.25 min tended to increase after 8BcAMP treatment, in contrast to the decrease in phosphate uptake. These data also exclude the possibility that inhibition of Na^+ gradient-dependent BBM phosphate transport by 8BcAMP is due to faster dissipation of the transmembrane Na^+ gradient and suggest that the primary action of 8BcAMP, like PTH [5, 6, 25], is directly on the phosphate transport system. The Na^+-independent uptake of L-proline

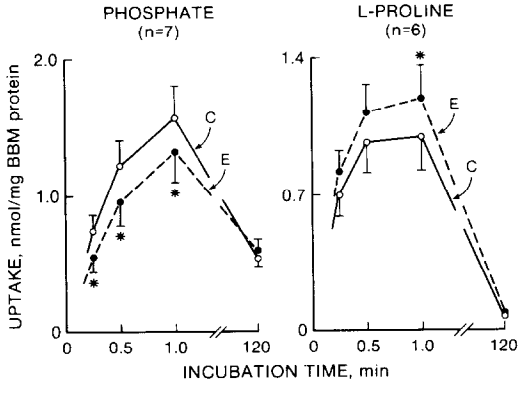

Fig. 1. Na^+ gradient-dependent transport of phosphate and L-proline by dog renal brush border membrane (BBM) vesicles. In each dog, the control (C) kidney (○——○) was removed prior to administration of 8-bromo-cAMP, and the remaining experimental (E) kidney (●——●) was removed after intravenous infusion of the drug. Both kidneys were processed immediately for preparation of a BBM fraction. Na^+ gradient-dependent uptake by BBM vesicles was measured at the initial (0.25 and 0.5 min) and peak (1.0 min) phases of uptake and at the equilibrium point at 120 min. The data at each time point represent the mean ± S.E. from several animals, and N is the number of animals. An asterisk (*) indicates a significant difference ($P < 0.05$, or higher level of significance, paired *t*-test) compared to the uptake at the same time in controls.

was 6 ± 2 pmoles per mg protein per 0.5 min (mean ± S.E.) in controls compared to 8 ± 3 pmoles per mg protein per 0.5 min after 8BcAMP (NS, paired *t*-test).

The specificity of the change in Na^+ gradient-dependent phosphate uptake by BBM vesicles is apparent also when the uptake data are expressed relative to the equilibrium uptake at 120 min, an analysis which is often used [5, 10, 25, 26] to minimize the effects of variations in absolute uptake or intravesicular space in different BBM preparations. The relative uptake of phosphate at 0.25, 0.5 and 1.0 min was decreased significantly after treatment with 8BcAMP, but there was no significant change in the relative uptake of L-proline at the same time points. For example, the relative uptake of phosphate at 0.25 min was 1.4 ± 0.1 (mean ± S.E.) in controls compared to 0.9 ± 0.1 in BBM vesicles from experimental kidneys ($P < 0.005$, paired *t*-test, N = 7), whereas the relative uptake of L-proline at 0.25 min was 11.5 ± 1.4 in controls compared to 10.3 ± 0.9 in vesicles from experimental kidneys (NS, paired *t*-test, N = 6).

In each animal in this group, the *in vivo* administration of 8BcAMP increased the rate of gluconeogenesis determined in slices of renal cortex (Table 2).

The effects of PTH on BBM transport of phosphate and renal gluconeogenesis were similar to 8BcAMP, and only the mean data from all experiments are listed in Table 3. The initial Na^+ gradient-dependent phosphate uptake by BBM vesicles was decreased after infusion of PTH, but there was no significant change in phosphate uptake at the equilibrium point. The extent of the decrease in phosphate transport at 0.5 min was 20%, comparable to the change in uptake at this time induced by 8BcAMP (Fig. 1). Na^+ gradient-dependent transport of L-proline at 0.5 min tended to increase from 968 pmoles/mg protein in controls to 1178 pmoles/mg protein after PTH treatment (mean values from two experiments), indicating the specificity of the decrease in phosphate uptake. The decrease in BBM transport of phosphate in these animals was accompanied by a stimulation in the rate of gluconeogenesis measured in slices of renal cortex (Table 3).

Table 2. Effect of 8-bromo-cAMP administered *in vivo* on Na^+-dependent transport by renal brush border vesicles and on gluconeogenesis in renal cortical slices*

	Exp. No.							
	1	2	3	4	5	6	7	Mean ± S.E.
	Phosphate transport (pmoles/mg protein/0.25 min)							
C	821	606	584	1514	660	609	435	747 ± 135
E	446	507	599	1286	272	409	427	564 ± 126†
	L-Proline transport (pmoles/mg protein/0.25 min)							
C	786	588	673	1243		494	381	694 ± 124
E	1057	680	938	1203		540	509	821 ± 117
	Gluconeogenesis (μmoles glucose/g tissue/hr)							
C		3.02	1.85	1.58	2.16	3.21	3.42	2.54 ± 0.32
E		4.89	3.78	4.39	6.13	6.70	5.60	5.25 ± 0.45†

* One dog was used in each experiment, and data are given for both the control (C) and experimental (E) kidney. The control kidney was removed prior to infusion of 8-bromo-cAMP. The values for each kidney are the mean of measurements in triplicate. This group of animals is the same as that in Table 1.

† Significantly different from controls ($P < 0.025$, or higher level of significance, paired *t*-test).

The NAD^+/NADH ratio in snap-frozen renal cortical tissue was 1.4 ± 0.2 (mean ± S.E., N = 7) in controls and was not significantly different (paired *t*-test) compared to the value of 1.2 ± 0.2 after infusion of 8BcAMP. The total content of NAD^+ + NADH also was not changed. The values were 515 ± 49 in controls and 495 ± 44 nmoles/g wet weight after 8BcAMP. Infusion of PTH, like 8BcAMP, did not alter the NAD^+/NADH ratio or the total content of NAD^+ + NADH in renal cortex.

DISCUSSION

These findings confirm the observations [5, 6] that administration of a phosphaturic dose of PTH leads to inhibition of Na^+ gradient-dependent phosphate transport across renal BBM. The degree of inhibition of the initial phosphate uptake was comparable to that in previous studies [5, 25]. The detailed experiments with 8BcAMP show that infusion of a phosphaturic dose of this nucleotide, like PTH, specifically inhibits phosphate transport across the renal BBM, a finding reported previously only in preliminary form [5]. Furthermore, the present studies indicate that inhibition of renal BBM transport of phosphate by both PTH and 8BcAMP is accompanied by stimulation of renal gluconeogenesis.

The amount of 8BcAMP administered was similar to the dose of dibutyryl cyclic AMP used in previous experiments on renal phosphate handling in the rat [27] and dog [17, 28]. Since neither cyclic AMP nor its derivatives penetrate cell membranes readily, a relatively high circulating concentration must be maintained in order to increase the intracellular concentration of these nucleotides to a level which is physiologically effective. Although the phosphaturia produced by dibutyryl cyclic AMP develops rapidly [27], it can be maintained for at least 12 hr by continued infusion of the drug [27]. We used long-term administration of 8BcAMP because this procedure had been used successfully to uncover PTH effects on BBM phosphate transport [6], and because this protocol allows the study of renal phosphate transport both *in vivo* with clearance techniques and *in vitro* using BBM vesicles prepared subsequently from the kidneys of the same animals.

Na^+ gradient-dependent L-proline transport by BBM vesicles was measured to determine the specificity of the decrease in BBM transport of phosphate.

Table 3. Effect of parathyroid hormone administered *in vivo* on Na^+-dependent phosphate transport by renal brush border vesicles and on gluconeogenesis in renal cortical slices*

	Phosphate transport (pmoles/mg protein)		Gluconeogenesis (μmoles glucose/g tissue/hr)
	0.5 min	120 min	
C	1738 ± 440	729 ± 306	2.90 ± 1.25
E	1390 ± 322†	690 ± 290	4.60 ± 1.43†

* One dog was used in each experiment, and data are given for both the control (C) and experimental (E) kidney. The control kidney was removed prior to infusion of parathyroid hormone. Each value is the mean ± S.E. from five animals. This group of animals is the same as that in Table 1.

† Significantly different from control ($P < 0.05$, or higher level of significance, paired *t*-test).

While the initial uptake of L-proline by BBM vesicles tended to increase after treatment with 8BcAMP (Fig. 1 and Table 2), the differences reached statistical significance only at the 1.0 min point. When the initial uptake was expressed relative to the equilibrium uptake at 120 min, there were no significant differences in L-proline transport at any time point. There have been no previous reports of the action of 8BcAMP or PTH on renal BBM transport of L-proline, and the present findings do not demonstrate unequivocally that BBM transport of L-proline is stimulated by 8BcAMP.

The data in this study are consistent with the concept that cyclic AMP is the intracellular mediator of the action of PTH on Na^+ gradient-dependent transport of phosphate across the renal BBM. The mechanism by which cyclic AMP inhibits BBM phosphate transport is unlikely to involve a direct effect of intracellular cyclic AMP on the BBM transport system. Addition of dibutyryl cyclic AMP to isolated BBM vesicles [5, 29] inhibited Na^+ gradient-dependent phosphate uptake only when the concentration of the nucleotide exceeded 0.1 mM, and at least part of the inhibition may be due to the butyryl moiety of the molecule [29]. It is more likely that regulation of BBM phosphate transport by cyclic AMP requires intermediate steps, and recent *in vitro* studies suggest that cyclic AMP-dependent phosphorylation of specific BBM proteins may be involved [30]. Another possibility to be considered is that there may be a causal relationship between gluconeogenesis and BBM transport of phosphate in the proximal tubule [19, 22] and that cyclic AMP, by increasing the level of cytosolic Ca^{2+} [7, 8], may stimulate the rate of gluconeogenesis. This may lead to an increase in the content of NAD^+ in cytosol and allow increased interaction of NAD^+ with the BBM and inhibition of the phosphate transport system [19, 22] possibly via ADP-ribosylation of BBM proteins [31, 32]. The present observations that inhibition of BBM transport of phosphate was accompanied by an increase in the rate of gluconeogenesis are consistent with the concept of a causal relationship between these processes, although final proof will require detailed studies comparing the time of onset and dose-dependency of the changes. Additional support for this mechanism is provided by the findings from other laboratories that infusion of PTH into rats increased both the NAD^+ content and the NAD^+/NADH ratio in renal cortex [33, 34]. In the studies on dogs, neither PTH nor 8BcAMP caused an increase in the cortical NAD^+ content or in the total NAD^+/NADH ratio; the reasons for this disparity are not known at the present time.

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