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The Role of Cyclic AMP in Parathyroid Hormone Action in the Toad Bladder

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Summary. Parathyroid hormone (PTH) inhibited active transport of inorganic phosphate and stimulated an increase in cyclic AMP concentration in the urinary bladder of the toad, *Bufo marinus.* Active transport of phosphate in the toad bladder was also inhibited by an analog of cyclic AMP (dibutyryl cyclic AMP) and by other drugs (pitressin and theophylline) which increase toad bladder intracellular cyclic AMP concentration. These data support the concept that cyclic AMP may be the mediator of PTH-induced phosphate transport inhibition in the toad bladder.

Parathyroid hormone (PTH) inhibits reabsorption of inorganic phosphate in the mammalian kidney (Greenwald & Gross, 1925; Aurbach & Heath, 1974) and in the isolated toad urinary bladder (Sellers *et al.*, 1977). In the mammalian kidney this action appears to be mediated by the release of cyclic AMP which inhibits reabsorption of phosphate from the tubular lumen to the interstitium. The role of cyclic AMP in the action of PTH in the toad urinary bladder is the subject of this report.

Materials and Methods

Phosphate Flux

Phosphate flux was measured as described in the preceding paper. Following the two control periods, the bathing solutions were altered by the addition of (i) 2.0 mM dibutyryl cyclic AMP, (ii) 10 mM theophylline, or (iii) 100 mU/ml of pitressin (ADH) to the serosal solutions. One hour later, phosphate flux was measured for two additional 45-min periods. SCC and PD were measured every 5 min until they stabilized, then every 15 min.

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Water Flow

Urinary bladders of the toad *Bufo marinus* were excised, and each hemibladder was mounted as a sac, serosal side outward, at the end of a hollow glass tube filled with 3 ml of Ringer's solution diluted 1:5 (Bentley, 1958). The sac was then immersed in a bathing medium of 20 ml isotonic Ringer's solution. At the beginning of the experiment one hemibladder was designated as the control sac and the other hemibladder from the same toad was designated as the experimental sac. The sacs and their contents were weighed initially, then at one hour (*Period 1*). The weight loss was used as a measure of the amount of water which had moved from the sac along the osmotic gradient. If the sac had a control water flow greater than 1.5 μl/min, it was discarded along with its pair.

After the control period, 25 mU/ml of PTH were added to the serosal solutions of the experimental sacs. Sacs were then incubated for 1 hr and were reweighed (*Period 2*). The effect of PTH on water flow was calculated as *Period 2 – Period 1* and expressed in μl/min.

Cyclic AMP Concentrations

Urinary bladders of the toad *Bufo marinus* were excised and hemibladders divided into two segments. Each quarterbladder was placed in an Erlenmeyer flask containing Ringer's solution and gassed with 97% O_2, 3% CO_2. Ten mM theophylline was added to the control quarterbladders. Ten mM theophylline and 50 mU/ml parathyroid hormone (PTH) were added to the experimental quarterbladders. After 1 hr, the quarterbladders were removed from the solutions and placed mucosal side up on a glass plate. Mucosal epithelial cells were scraped from the stroma with a glass slide and immediately frozen in liquid nitrogen. Scraping and freezing was completed in less than 1 min. The frozen tissue was ground to a fine powder with mortar and pestle. The powder was thawed at 4 °C by mixing with 1 ml of 5% trichloroacetic acid (TCA) containing ^{3}H-cyclic AMP to monitor recovery. After 30 min the samples were centrifuged at $700 \times g$ for 20 min at 4 °C. 0.1 ml 1 N HCl was added to the supernatant which was then extracted 5 times with water-saturated ether to remove excess TCA. Residual ether was removed by heating the sample in a water bath at 80 °C. Samples were lyophilized and stored frozen. The lyophilized powder was diluted with 1 ml of distilled water and 50-μl aliquots were assayed by radioimmunoassay.

The TCA-precipitate was dissolved in 0.4 N NaOH and the protein concentration determined colorimetrically (Lowry *et al.*, 1951). Cyclic AMP concentrations are reported as pmoles/mg protein.

Theophylline was from Nutritional Biochemical Corporation, (Cleveland, Ohio); dibutyryl cyclic AMP from Calbiochem (La Jolla, Calif.); parathyroid hormone from Wilson Pharmaceutical and Chemical Corp. (Park Forest South, Ill.) and contained 971 U/mg by rat bioassay; antidiuretic hormone (Pitressin) from Parke-Davis (Detroit, Mich.); ^{3}H Cyclic AMP from New England Nuclear Corp., the cyclic AMP radio-immunoassay kit (^{125}I) from Schwarz/Mann Division, Becton, Dickenson and Co., (Orangeburg, N.Y.). Since neither the parathyroid hormone nor the antidiuretic hormone was a synthetic preparation, it is possible that impurities may have played a role in the observed changes.

The data in this report were evaluated using Student's *t*-test and are presented as the mean ± SE.

Results

Phosphate Flux (Table 1)

Net transport of inorganic phosphate in the toad urinary bladder was significantly inhibited by dibutyryl cyclic AMP, theophylline, or antidiuretic hormone (ADH). After each agent $S \rightarrow M$ phosphate flux increased significantly. There was no significant change in $M \rightarrow S$ phosphate flux. We have previously reported that phosphate flux does not change significantly in control bladders during the 4 hr required for

Table 1

		Phosphate flux nmoles/cm^2/hr		P (Control *vs.* Exptl.)
		Control	Experimental	
A. Control ($n=21$)	$M \rightarrow S$	0.49 ± 0.11	0.64 ± 0.17	<0.01
	$S \rightarrow M$	0.20 ± 0.03	0.29 ± 0.03	<0.02
	Net	0.29 ± 0.11	0.35 ± 0.17	>0.5
		$P<0.025$	$P<0.05$	
B. Parathyroid hormone ($n=25$)	$M \rightarrow S$	0.70 ± 0.13	0.58 ± 0.14	>0.05
	$S \rightarrow M$	0.46 ± 0.08	0.56 ± 0.11	<0.05
	Net	0.24 ± 0.08	0.02 ± 0.06	<0.02
		$P<0.005$	$P>0.5$	
C. Dibutyryl cyclic AMP ($n=20$)	$M \rightarrow S$	0.89 ± 0.17	0.54 ± 0.08	<0.01
	$S \rightarrow M$	0.47 ± 0.06	0.87 ± 0.19	<0.05
	Net	0.42 ± 0.20	-0.32 ± 0.21	<0.01
		$P<0.05$	$P>0.1$	
D. Theophylline ($n=20$)	$M \rightarrow S$	0.25 ± 0.03	0.32 ± 0.03	<0.025
	$S \rightarrow M$	0.13 ± 0.02	0.29 ± 0.03	<0.001
	Net	0.11 ± 0.03	0.02 ± 0.03	<0.005
		$P<0.001$	$P>0.4$	
E. Antidiuretic hormone ($n=22$)	$M \rightarrow S$	0.49 ± 0.10	0.36 ± 0.03	>0.1
	$S \rightarrow M$	0.25 ± 0.04	0.52 ± 0.06	<0.001
	Net	0.24 ± 0.11	-0.16 ± 0.08	<0.005
		$P<0.01$	$P>0.05$	

n=number of bladders. Phosphate flux was measured for two control flux periods of 45 min each which began 1 hr after the addition of 10 µCi of $KH_2\,^{32}PO_4$ to the source sides. The mean flux of these two periods was calculated for each bladder. The bathing solutions were then not changed (control) or altered by addition of 25 mU/ml parathyroid hormone (PTH), 2.0 mM dibutyryl cyclic AMP, 10 mM theophylline, or 100 mU/ml pitressin (ADH) to the serosal solutions. After 1 hr for re-equilibration, phosphate transport was measured for two additional 45-min flux periods. The mean flux for these two periods was calculated for each bladder. Data are expressed as mean ± SE.

these studies and that PTH inhibits active phosphate transport (Sellers *et al.,* 1977). For ease of comparison these data have been included in Table 1.

A transient increase in the SCC was observed following the addition of theophylline, dibutyryl cyclic AMP, and ADH. This increase usually peaked and began diminishing within 15 min. SCC was at or near baseline levels at 1 hr when the phosphate flux periods were begun.

Water Flow

When 25 mU/ml of PTH were added to the serosal bathing solution there was no significant change in osmotic water flow. *Period 2 – Period 1* for control bladders was 0.07 ± 0.2 µl/min ($P > 0.5$, $n = 5$). In the bladders to which PTH was added in the experimental period, *Period 2 – Period 1* was 0.03 ± 0.1 µl/min ($P > 0.5$, $n = 5$).

Parathyroid Hormone Stimulated Cyclic AMP

Following the addition of PTH, a significant increase in the cyclic AMP concentration of scraped mucosal epithelial cells occurred. In bladder segments incubated with 10 mM theophylline, cyclic AMP levels were 18.0 ± 2.6 pmoles/mg protein ($n = 16$). In bladder segments incubated with 10 mM theophylline and 50 mU/ml PTH, cyclic AMP levels were 29.9 ± 4.2 pmoles/mg protein ($n = 16$). The increase in cyclic AMP concentration after incubation with PTH was statistically significant ($P < 0.025$).

Discussion

Parathyroid hormone (PTH) inhibits reabsorption of inorganic phosphate (P_i) in the mammalian kidney (Greenwald & Gross, 1925; Aurbach & Heath, 1974) and in the isolated toad urinary bladder (Sellers *et al.,* 1977). The phosphaturic effects of PTH in the proximal tubule are well established (Samiy, Hirsch & Ramsay, 1965; Beck & Goldberg, 1974). Distal nephron P_i transport and the effects of PTH on the distal nephron are controversial (Staum, Hamburger & Goldberg, 1972; Agus *et al.,* 1973; Goldfarb *et al.,* 1973; Dennis, Bello-Reuss & Robinson, 1977; Gregor *et al.,* 1977). Considerable evidence supports the concept that

the action of PTH in the mammalian kidney is mediated by cyclic AMP (Aurbach & Heath, 1974). In the toad bladder the postulate that cyclic AMP may be the mediator of PTH action is supported by the observations that: (i) PTH stimulates an increase in the concentration of cyclic AMP; and (ii) the action of PTH in inhibition of phosphate transport can be simulated by addition of an analog of cyclic AMP or by inhibition of cyclic nucleotide phosphodiesterase by theophylline.

The mechanism by which cyclic AMP effects phosphate reabsorption in the mammalian nephron is unknown. There is evidence that cyclic AMP may alter the permeability characteristics of the mammalian proximal tubule (Lorentz, 1974). In the toad bladder it is likely that $S \rightarrow M$ P_i flux occurs by passive movement and that $M \rightarrow S$ P_i flux represents the sum of passive movement and active transport. In control bladders, both $M \rightarrow S$ and $S \rightarrow M$ fluxes increased with time without a significant change in the net P_i flux (Sellers *et al.*, 1977). These data are repeated in Table 1. Presumably this reflects an increase with time in the passive movement of P_i across the toad bladder. Following the addition of PTH (Sellers *et al.*, 1977) and in the present experiments following the addition of theophylline, dibutyryl cyclic AMP or ADH, $S \rightarrow M$ P_i flux increased to an extent similar to the increase in the control experiments while $M \rightarrow S$ P_i flux did not change consistently. Since a change in passive movement of P_i across the bladder should affect the $M \rightarrow S$ and $S \rightarrow M$ flux equally, the increase in $S \rightarrow M$ P_i flux in the absence of a consistent change in $M \rightarrow S$ P_i flux has been interpreted as inhibition of the active P_i transport along with an increase in passive P_i movement. Since passive $M \rightarrow S$ P_i flux is increasing while active $M \rightarrow S$ P_i flux is inhibited, it is not surprising that total $M \rightarrow S$ P_i flux during the experimental period might increase (theophylline), decrease (dibutyryl cyclic AMP), or not change (PTH and ADH).

The phosphate flux experiments were begun 1 hr after the addition of theophylline, dibutyryl cyclic AMP, ADH or PTH to allow the short-circuit current to stabilize. Tissue cyclic AMP concentrations were measured 1 hr after the addition of PTH to make the experiments comparable to the flux studies. Since hormones which stimulate adenylate cyclase often act quickly, the changes may represent an underestimate of the effect of PTH.

Pitressin (ADH) inhibits P_i reabsorption in the mammalian kidney and in the toad bladder. ADH-induced phosphaturia in the absence of PTH has been reported in man (Eisinger *et al.*, 1970) and in other mammals (Kalonsek, 1968; Wen, 1974). Since other effects of ADH

are mediated by cyclic AMP, it is possible that this phosphaturia is cyclic AMP mediated. In the toad bladder both PTH and ADH inhibit P_i transport and increase cyclic AMP concentrations. It is speculated that PTH and ADH both act by stimulating an adenylate cyclase, increasing intracellular cyclic AMP concentration which inhibits P_i transport by an unknown mechanism. Several investigators have suggested that active sodium transport and osmotic water flow across the toad bladder are mediated by separate pools of ADH-stimulated cyclic AMP (Petersen & Edelman, 1964; Lipson & Sharp, 1971; Flores *et al.,* 1975). Since PTH has no effect on Na transport or osmotic water flow, the cyclic AMP stimulated by PTH must be separate from the pools of cyclic AMP which regulate these phenomena. Whether ADH or PTH stimulate the same adenylate cyclase and thus the same pool of cyclic AMP, or whether ADH stimulates cyclic AMP in a separate pool which can spill over into the P_i transport cyclic AMP pool is unknown.

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