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Active Phosphate Transport Across the Urinary Bladder of the Toad, *Bufo marinus**

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Summary. Net transport of inorganic phosphate occurs in the absence of an electrochemical gradient from the mucosal to the serosal bathing solution in the isolated toad urinary bladder. This transport can be inhibited by metabolic inhibitors. The magnitude of this transport can be altered by changes in phosphate concentration or by the addition of parathyroid hormone.

The mechanisms by which the excretion of phosphate is regulated have been studied for many years. In spite of a number of important recent studies [1, 3, 4, 7, 11, 13], many aspects of this regulation remain unclear. In the present studies, active transport of phosphate from the mucosal to the serosal bathing solution has been demonstrated in the isolated urinary bladder of the toad, *Bufo marinus*. Phosphate transport can be inhibited by addition of metabolic inhibitors to the bathing solutions or by bubbling the bathing solutions with nitrogen. The magnitude of the phosphate transport can be altered by changes in phosphate concentration or by the addition of parathyroid hormone.

Materials and Methods

Phosphate Flux

The toads, *Bufo marinus,* were of Colombian origin. They were stored on San-i-cel (Paxton Processing Company, Inc., Paxton, Illinois) moistened with tap water. On the

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day of the experiments, the toads were double-pithed and the bladders excised and placed in amphibian Ringer's solution containing (mM): 90 NaCl; 25 $NaHCO_3$; 3 KCl; 1 $CaCl_2$; 0.5 KH_2PO_4; 0.5 $MgSO_4$; and 5.5 glucose. The solution was gassed with 97% O_2 and 3% CO_2 maintaining a PH of 7.6 to 7.8. The short-circuit current was measured by modification of the method of Ussing and Zerahn [14] as previously described [10]. Hemibladders were mounted between halves of plastic chambers which were divided by vertical bars. This arrangement permitted the study of two contiguous areas of the same hemibladder. The cross-sectional area of each bladder area was 3.2 cm^2. Each bathing solution had a volume of 4 ml.

Bladders with initial potential differences less than 10 mV were discarded. Bladders were kept short-circuited continuously except for brief periods during which the potential differences were measured.

When the short-circuit current was stable (less than 10% variation in a 30-min period), 10 μCi of carrier-free $KH_2{}^{32}PO_4$ were added to the mucosal bathing solution of one bladder area and to the serosal bathing solution of the contiguous bladder area. The radioisotope was added 1 hr before the beginning of the initial flux period to allow isotopic equilibration. After 1 hr the bathing solutions were sampled, and control phosphate flux was measured for two consecutive 45-min periods.

Following the two control periods, the bathing solutions were altered as described below. After a suitable time for re-equilibration, phosphate fluxes were measured for two additional 45-min periods.

The 50 μl samples were added to 7 ml of scintillation counting mixture (Toulene-Triton X-100, 3:1 with 4 g Omnifluor per liter) and counted in a Packard Model 3375 Liquid Scintillation Counter (Packard Instrument Co., Chicago, Ill.). Net phosphate flux was calculated as the difference between the mucosal-to-serosal flux and the serosal-to-mucosal flux. The phosphate fluxes are expressed in nanomoles per square centimeter per hour. For ease of comparison, short-circuit current has been converted to μequiv/cm^2/h.

Statistics were calculated using Student's *t*-test. In all statistical calculations, the mean flux for the control and experimental periods was obtained for each bladder. These mean values were used to calculate the statistics for each group of data. Data are expressed as mean ± standard error.

In a small number of experiments the concentration of isotope on the recipient side exceeded 0.6% of the concentration on the source side at the time the bathing solutions were first sampled. These high initial isotope concentrations were usually associated with visible holes in the bladder area at the end of the experiment. For this reason, all experiments in which such a high initial recipient side isotopic phosphorus concentration occurred were discarded.

Control Phosphate Transport

Following the initial two control periods, the bladders were incubated for 1 hr without changing experimental conditions. Phosphate fluxes were then measured for two additional 45-min periods.

Metabolic Inhibition

Following the two control periods, the bathing solutions were altered by 1) gassing with 97% N_2–3% CO_2, or 2) adding 0.1 mM sodium iodoacetate to both mucosal and serosal bathing solutions. Following 1 hr for re-equilibration, phosphate fluxes were measured for two additional 45-min periods.

Varying Phosphate Concentration

Following the initial two control periods, the phosphate concentration of both the mucosal and serosal bathing solutions was increased by rinsing each side of the bladder three times with high phosphate toad Ringer's solution. NaH_2PO_4 was substituted isotonically for NaCl to give 5.0, 10.0, or 25.0 mM phosphate solutions with a pH of 7.6 and osmolality of 230 mOsm/kg H_2O. Phosphate fluxes were measured for two 45-min periods beginning 1 hr after the change in phosphate concentration.

Parathyroid Hormone Addition

Following the initial control periods, 25 mU/ml of highly purified parathyroid hormone, (Wilson Pharmaceutical and Chemical Corp., Park Forest South, Ill., by rat bioassay 971 U/mg) were added to the serosal bathing solutions. Short-circuit current was measured immediately and every 5 min for 30 min, then as in the control periods. Phosphate fluxes were measured for two 45-min periods beginning 1 hr after the addition of parathyroid hormone.

Inorganic Phosphate Isolation

Inorganic phosphate was precipitated using a modification of the Embden-Fetter method described by Peters and Van Slyke [12]. At the conclusion of the flux experiment, the bathing media were aspirated and saved. Molybdate solution was made by dissolving 50 g of ammonium molybdate in 150 cc of warm water. This solution was added to 450 cc of nitric acid (2 volumes concentrated acid/1 volume water); and 3.92 g of strychnine sulfate were dissolved in 250 cc water. Strychnine molybdate solution was prepared by adding one volume of the strychnine sulfate solution to 3 columes of the molybdate solution. The strychnine molybdate solution was filtered after 24 hr. The bathing media (0.5 cc) were added to 2 cc of water and 2.5 cc of 10% trichloroacetic acid; 2.5 cc of this mixture were diluted to 6 ml with water and 2 cc of the strychnine molybdate solution were added. This mixture was allowed to stand at room temperature for 1 hr with occasional shaking and then was filtered. The precipitate was washed with 2.5 cc of ice cold strychnine molybdate solution diluted 1:5 followed by 2.5 cc of ice cold water. Samples of the original solution, the filtrate, and the filter paper containing the precipitate were counted to determine the percentage of the isotope existing as inorganic phosphate.

Carrier-free $KH_2{}^{32}PO_4$ and Omnifluor were obtained from New England Nuclear, Boston, Massachusetts. Strychnine sulfate was purchased from Sigma Chemical Company, St. Louis, Missouri; ammonium molybdate from Mallinckrodt Chemical Works; parathyroid hormone from Wilson Pharmaceutical and Chemical Corp., Park Forest South, Illinois.

Results

Phosphate Transport (Table 1)

Net transport of phosphate from the mucosal bathing solution to the serosal bathing solution occurred in the absence of an electrochemical gradient. Net transport of phosphate was determined in two consecutive

Table 1. Measurement of phosphate transport across toad bladder

Flux direction	Phosphate flux (nmoles/cm^2/h)	
	Periods 1 and 2	Periods 3 and 4
M→S (n=21)	0.49±0.11	0.64±0.17
S→M (n=21)	0.20±0.03	0.29±0.03
Net (n=21)	0.29±0.11	0.35±0.17
	($p<0.025$)	($p<0.05$)

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 for these two periods was calculated for each bladder. One hour later phosphate flux 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.

45-min periods and did not change during that time. In this series of experiments, the mucosal-to-serosal phosphate flux exceeded serosal-to-mucosal flux by a ratio of 2.5:1. The net mucosal-to-serosal flux averaged 0.29±0.11 nmoles/cm^2/hr ($p<0.025$, $n=21$). After 1-hr incubation period, phosphate fluxes were again measured. In the next two 45-min periods, net transport of phosphate again occurred. Mucosal-to-serosal flux exceeded serosal-to-mucosal flux by a ratio of 2.3:1. Net mucosal-to-serosal flux averaged 0.35±0.17 nmoles/cm^2 ($p<0.05$, $n=21$).

The short-circuit current of the bladder areas used to measure mucosal-to-serosal phosphate flux averaged 0.63±0.07 μequiv/cm^2/hr while the short-circuit current of the bladder areas used to measure serosal-to-mucosal phosphate flux averaged 0.60±0.07 μequiv/cm^2/hr. This difference was not statistically significant ($p>0.5$). This indicates that the observed net phosphate flux did not result from a systematic difference in stretching of the two bladder areas.

Metabolic Inhibition (Table 2)

Net mucosal-to-serosal flux of inorganic phosphate was significantly inhibited by gassing with 97% N_2–3% CO_2 ($p<0.01$) or by addition of iodoacetate ($p=0.01$). This inhibition was the result of both a decrease in the mucosal-to-serosal phosphate flux and an increase in the serosal-to-mucosal phosphate flux. Short-circuit current was inhibited 93% by gassing with 97% N_2–3% CO_2 and 84% by addition of iodoacetate.

Table 2. Metabolic inhibition

		Phosphate flux (nmoles/cm^2/hr)	
		Control	Experimental
97% N_2–3% CO_2	M→S	0.80±0.15	0.58±0.14
(n=19)	S→M	0.46±0.08	0.64±0.06
	Net	0.34±0.14	−0.06±0.13
		$p<0.05$	$p>0.5$
Iodoacetate	M→S	0.89±0.13	0.79±0.11
(n=19)	S→M	0.57±0.06	0.86±0.10
	Net	0.32±0.13	−0.07±0.14
		$p<0.025$	$p>0.5$

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 for these two periods was calculated for each bladder. The bathing solutions were then altered by gassing with 97% N_2−3% CO_2 or by addition of 0.1 mM sodium iodoacetate. One hour later 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.

Varying Phosphate Concentration (Table 3)

Phosphate concentration was increased from 0.5 to 5.0, 10, and 25.0 mM. Net phosphate flux increased over a range of phosphate concentration from 0.5 to 10.0 mM. Phosphate transport was statistically significantly greater than control when phosphate concentration was 5 mM ($p<0.025$) and 10 mM ($p<0.05$). At a higher phosphate concentration (25.0 mM) no statistically significant net phosphate flux was observed.

Following the substitution of 25.0 mM phosphate Ringer's solution, short-circuit current decreased from 0.90±0.06 μequiv/cm^2/hr in controls to 0.49±0.04 μequiv/cm^{2}hr ($p<0.001$), n=64). No gross precipitate was observed in the 25 mM phosphate Ringer's solution.

Parathyroid Hormone Addition (Table 4)

Following the addition of 25 mU/ml of parathyroid hormone to the serosal bathing medium, net mucosal-to-serosal phosphate flux was significantly inhibited. Both a decrease in the M→S flux and an increase in S→M flux were noted in the presence of parathyroid hormone.

Table 3. Varying phosphate concentration

Phosphate concentration	Net phosphate flux (nmoles/cm^2/hr)	
	Controls	Experimental
5.0 mM ($n=13$)	0.24 ± 0.08 ($p<0.025$)	2.6 ± 0.9 ($p<0.025$)
10.0 mM ($n=16$)	0.37 ± 0.16 ($p<0.05$)	9.6 ± 4.1 ($p<0.05$)
25.0 mM ($n=32$)	0.67 ± 0.22 ($p=0.005$)	4.4 ± 13.8 ($p>0.5$)

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 for these two periods was calculated for each bladder. Phosphate concentration of the bathing media was then increased to 5.0, 10.0, or 25.0 mM by rinsing each side of the chamber three times with the high phosphate Ringer's solution. One hour later, 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.

Table 4. Parathyroid hormone addition

Flux direction	Phosphate flux (nmoles/cm^2/hr)	
	Control	With PTH
M→S ($n=25$)	0.70 ± 0.13	0.58 ± 0.14
S→M ($n=25$)	0.46 ± 0.08	0.56 ± 0.11
Net ($n=25$)	0.24 ± 0.08 ($p<0.005$)	0.02 ± 0.06 ($p>0.5$)

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 for these two periods was calculated for each bladder. Parathyroid hormone (25 mU/ml) was then added to the serosal bathing solution. One hour later 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.

Addition of 25 mU/ml parathyroid hormone to the serosal bathing solution caused no change in the net sodium transport as estimated by the short-circuit current. Short-circuit current in controls was 0.77 ± 0.05 μequiv/cm^2/hr. Fifteen to 20 min after parathyroid hormone addition, short-circuit current was 0.78 ± 0.04 μequiv/cm^2/hr ($p>0.5$, $n=$

50). There was no statistically significant effect of PTH on the short-circuit current at any time during the 90-min period in which net phosphate flux was measured.

Phosphate Isolation

Inorganic phosphate was precipitated as strychnine molybdophosphate. Eighty-five $\pm 5\%$ of the counts on both the source and recipient sides were precipitated, and $8 \pm 1.3\%$ of the isotope remained in the supernatant. This indicated that the observed net phosphate flux did not result from the incorporation of labelled phosphate into organic compounds which then leaked preferentially across the serosal border of the cell.

Discussion

The toad bladder transports inorganic phosphate from the mucosal to the serosal bathing medium in the absence of an electrochemical gradient. The magnitude of the active phosphate transport is less than 0.1% of the magnitude of the sodium transport which was estimated by the measurement of the short-circuit current. Net sodium transport has been shown to equal short-circuit current in the toad bladder [8, 10, 14]. Since the net phosphate transport is relatively small, it would not disturb the apparent equality between measured net sodium transport and short circuit current. The small magnitude of the net phosphate transport does not indicate that it is physiologically insignificant, however. The concentration of sodium in the bathing medium is ordinarily 230 times as high as the concentration of phosphate. It is therefore not unexpected that net phosphate transport is a small fraction of net sodium transport.

By definition, active transport is an energy-requiring process. The demonstration of the energy dependence of phosphate transport in the toad bladder is compatible with the conclusion that there is active phosphate transport by the tissue.

Net transport of phosphate from the mucosal to the serosal bathing solution can be inhibited by the addition of an inhibitor of glycolysis (iodoacetate), or by inhibition of oxidative metabolism (anaerobiosis). Similar results have been obtained by numerous authors with regard to sodium transport in the toad bladder [5, 9, 10]. From the data

presented, no specific conclusions can be made with regard to the mechanism by which the net transport of phosphate is linked to metabolism. The existence of net phosphate transport in the absence of an electrochemical gradient which is dependent upon metabolism is highly suggestive of a specific active transport mechanism for phosphate.

Active transport of phosphate increases with an increase in the phosphate concentration in the bathing medium over a range of phosphate concentrations from 0.5 to 10 mM. Above this concentration, it was not possible to demonstrate active phosphate transport. There are two possible explanations for this observation. First, it is possible that active phosphate transport does occur at high medium phosphate concentrations, but that it is masked by the large amount of passive backflux of phosphate at these high concentrations. Second, it is possible that high phosphate concentrations inhibit active phosphate transport. The fact that sodium transport, as measured by short-circuit current, was inhibited at these high phosphate concentrations is compatible with this hypothesis. One possible explanation for this inhibition is that binding of calcium and phosphate occurred, lowering the concentration of ionized calcium in the bathing media. The absence of a gross precipitate does not invalidate this hypothesis. It has previously been shown that the toad bladder epithelial cells detach from each other and from their supporting tissue when calcium is removed from the bathing medium [6]. In addition, short-circuit current falls to near zero in the absence of calcium [2, 6].

Active phosphate transport is inhibited by parathyroid hormone. Parathyroid hormone causes phosphaturia in mammals. This has been demonstrated to occur by a decrease in phosphate reabsorption in both the proximal and distal tubules [4, 7, 11]. Phosphate reabsorption by the toad bladder is also decreased by parathyroid hormone.

There appears to be no direct relationship between the active transport of sodium and the active transport of phosphate. Increasing the concentration of phosphate in the bathing medium from 0.5 to 10.0 mM had no effect on the active sodium transport as estimated by the short-circuit current, but markedly increased the active transport of phosphate. On the other hand, parathyroid hormone markedly inhibited active phosphate transport without changing active sodium transport. These observations do not eliminate the possibility that phosphate transport is linked to a small portion of net sodium transport. This possibility is currently under investigation in our laboratory.

In summary, net phosphate transport from the mucosal to the serosal bathing solution in the absence of an electrochemical gradient has been reported in the urinary bladder of the toad, *Bufo marinus*. This active transport continues for at least 4 hr *in vitro*. The phosphate transport can be inhibited by metabolic inhibitors. This transport increases with an increase in the concentration of inorganic phosphate in the bathing solution over a range of 0.5 to 10.0 mM. Active phosphate transport in the toad bladder is inhibited by parathyroid hormone.

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