Phospholipid Changes during Adaptation to Acidosis in Urinary Bladder of *Bufo marinus*[1] (42869)

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Abstract. **The purpose of this study was to determine whether phospholipids (PL) play a role in the adaptation to metabolic acidosis by toad urinary bladder epithelium. Toads were placed in an NH_4Cl acidosis for 48 hr. Quarter bladders were removed and incubated with [^{32}P]orthophosphate or [^{3}H]arachidonic acid for 1 hr at 25°C. PL were detected by thin layer chromatography, autoradiography, and quantitated by liquid scintillation counting or fractional amounts were determined from phosphate content and expressed as counts per minute per micromolar of total phosphate or as percentage of fraction of total PL. Incorporation of [^{3}H]arachidonic acid into urinary bladder PL was measured in acidotic and normal toads. There was a higher rate of arachidonic acid incorporation into several PL in acidotic animals. Phosphatidic acid and phosphatidylserine fraction in acidosis was 37,705 ± 6,821 and in normal bladders was 9,254 ± 2,652 ($P < 0.005$); phosphatidylcholine fraction in acidotic toads was 80,462 ± 16,862 and in normal bladders was 26,892 ± 5,198 ($P < 0.025$); and the phosphatidylethanolamine (PE) fraction in acidotic was 48,665 ± 10,998 and in normal animals was 17,441 ± 3,905 ($P < 0.025$). ^{32}P labeling revealed a higher rate of incorporation in bladders from acidotic toads compared with normal toads. In the acidotic bladders, the phosphatidic acid and phosphatidylserine fraction was 19,754 ± 3,597 and in normal bladders was 12,980 ± 1,394 ($P < 0.05$) and for PE acidotic bladders was 9,129 ± 1,304 and in normal bladders was 3,285 ± 416 ($P < 0.001$). Fractional PL (reported as percentage of fraction of total PL based on total lipid phosphorus) analysis in normal toads revealed phosphatidylinositol = 8.1 ± 0.6% and PE = 27 ± 1.2%, whereas for acidotic toads phosphatidylinositol = 11 ± 0.6% and PE = 32 ± 1.0% ($P < 0.01$ for both). Aldosterone, a known stimulator of acidification, had no effect on ^{32}P incorporation into PL fractions of the bladder. The increase in PL turnover following induction of acidosis is consistent with increased membrane synthesis or turnover during metabolic acidosis and this may reflect an increased transport of vesicular H^+-ATPase into the apical membrane or the result of a proliferation of acid-secreting mitochondria-rich cells or both.**

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Previously it has been shown that the urinary bladder of *Bufo marinus* has the capacity to excrete hydrogen ion and this excretion is increased by a metabolic acidosis (1–3). It has also been demonstrated that in the toad urinary bladder during chronic metabolic acidosis there is an increased number of mitochondria-rich cells and this increases the capacity of the bladder to excrete H^+ (4, 5).

Phospholipid turnover has been implicated as being important in the regulation of water permeability and the response to antidiuretic hormone (ADH) in urinary epithelia. It has been shown that the hydroosmotic response to ADH is inhibited by preincubation with phospholipase C (6) and Tarapoom *et al.* (7) have shown similarly that certain phospholipid (PL) metabolites inhibit the increase in water flow induced by ADH. Additionally, evidence has been presented that ADH can stimulate phosphoinositide turnover in amphibian urinary bladder (8, 9) and that bladder epithelial cells contain a phospholipid-dependent protein kinase that is stimulated by the PL metabolite diacylglyc-

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erol and the phorbolester, phorbol myristate acetate (8, 10). Likewise, PL turnover has been shown to be important in ion and fluid transport in various amphibian skin (11). These observations impart the significance of membrane PL in ion and fluid transport processes and also suggest that these lipids represent an important and dynamic constituent of the membrane bilayer intimately involved in regulating the permeability properties of the cell and the cells' responsiveness to hormone or physiologic changes.

The purpose of these studies was to determine whether a chronic metabolic acidosis results in an increased incorporation or change in composition of the PL of the epithelial membranes of toad urinary bladder. We also investigated the possibility that aldosterone, a known acute stimulator of acidification (12, 13), would stimulate PL turnover in the urinary bladder. If in fact the increase in H^+ excretory activity is accompanied by insertion of new H^+ carrier into the membrane or increased turnover of carrier, then we should be able to detect a difference in PL. If no change is observed, then one may eliminate PL having a role in increased H^+ excretion seen during acidosis.

Materials and Methods

The toads used in these experiments were *B. marinus* of Colombian or Mexican origin and were supplied by Carolina Biological Supply, Burlington, NC. Toads were maintained in the laboratory in an amphibian tank and fed once a week. These toads were referred to as being in normal acid-base balance. Toads were placed in metabolic acidosis by gavaging three times a day for 2 days with 10 ml of 120 m*M* NH_4Cl solution (1). The bladder was removed after double pithing the animal. The Ringer's solution used in these experiments contained in m*M*: NaCl, 114.5; KCl, 3.0; $CaCl_2$, 0.9; and sodium phosphate, 1.5; the final pH was adjusted to 6.80–7.00 by titrating the phosphate buffer with 0.12 *M* HCl or 0.12 *M* NaOH.

The experiments on [^{3}H]arachidonic acid (AA) labeling (Table I) were carried out on normal and acidotic toads. Quarter bladders were removed and incubated at 25°C in Ringer's solution containing 0.2μCi/ml of [^{3}H]AA and was allowed to label for 2 hr. The reaction was stopped by placing the tissue in 3 ml of cold, acidified acetone and then extracted as given below in the extraction method.

The experiments with ^{32}P labeling (Tables II and III) were performed on quarter bladders from normal and acidotic toads. Each quarter bladder was incubated for 1 hr at 25°C in 2 ml of Ringer's solution containing [^{32}P]orthophosphate (100 μCi/ml). The reaction was then stopped by placing the tissue in 3 ml of cold, acidified acetone and then extracted as given below. The experiments with aldosterone stimulation (Table III) were carried out with normal quarter bladders. The experimental bladder was incubated with 10^{-6} *M* aldosterone for 1 hr and then labeled as described above with [^{32}P]orthophosphate. The control bladder was incubated for 1 hr in Ringer's solution without aldosterone and then labeled in the same manner as the experimental bladder. The [^{3}H]AA and the [^{32}P]orthophosphate were obtained from New England Nuclear, Boston, MA. Aldosterone was obtained from Sigma Chemical Co., St. Louis, MO. All samples were counted to an accuracy of < 2%. The efficiency of counting was determined using an external isotope source and all samples were corrected to a standard efficiency and corrected for decay of ^{32}P before making final calculations.

Table I. Incorporation of ^{3}H into Phospholipids from AA in Normal and Acidotic Toad Urinary Bladder

Lipid fraction	Normal toads (n = 7)[a]	Acidotic toads (n = 8)[a]	P
PI	20,591 ± 5,561	36,688 ± 11,392	NS
PA + PS	9,254 ± 2,652	37,705 ± 6,821	<0.005
PC	26,892 ± 5,198	80,462 ± 16,862	<0.025
PE	17,441 ± 3,905	48,665 ± 10,988	<0.025

[a] Values represent the mean ± SEM and are reported in units of cpm/μM PO_4 × hr.

Table II. Incorporation of ^{32}P into Membrane Phospholipids in Normal and Acidotic Toad Urinary Bladder

Lipid fraction	Normal toads[a]	Acidotic toads[a]	P
PIP_2	28,014 ± 4,503	39,735 ± 9,402	NS
PIP	14,710 ± 1,718	20,843 ± 5,738	NS
PI	10,907 ± 848	13,390 ± 2,602	NS
PA + PS	12,980 ± 1,394	19,754 ± 3,597	<0.05
PC	9,546 ± 1,236	12,628 ± 2,429	NS
PE	3,285 ± 416	9,129 ± 1,304	<0.001

[a] Values are expressed as mean ± SEM of 12 experiments and are reported in units of cpm/μM PO_4 × hr.

Table III. Effect of Aldosterone on ^{32}P Incorporation into Phospholipids in Toad Urinary Bladder

Phospholipid fraction	Experimental bladders[a] (10^{-6} *M* aldosterone)[b]	Control bladders[a]	P
PIP_2	6,696 ± 728	5,771 ± 662	NS
PIP	7,036 ± 415	5,594 ± 724	NS
PI	12,892 ± 1,000	11,891 ± 1,399	NS
PA + PS	8,288 ± 447	8,609 ± 1,004	NS
PC	7,616 ± 916	8,306 ± 1,756	NS
PE	4,771 ± 284	4,781 ± 301	NS

[a] Values are expressed as mean ± SEM of eight experiments and are reported in units of cpm/μM PO_4 × hr.
[b] Experimental bladders were incubated with 10^{-6} *M* aldosterone and the control was incubated in Ringer's solution without aldosterone for 60 min before the labeling period.

Phospholipid Extraction Procedure. The tissue was homogenized using a motor-driven all glass homogenizer in 2.5 ml of chloroform:methanol:concentrated HCl (20:40:1 by volume) at 5°C, centrifuged, and the organic phase collected and washed with 3 ml of 0.1 *N* HCl. Samples were centrifuged and 1 ml of the organic phase dried under N_2 and resuspended in 50 μl of chloroform:methanol:concentrated HCl (60:30:1). Ten microliters were used for PO_4 determination and a 30-μl aliquot was spotted on silica gel thin layer chromatography plates and exposed to a two-solvent system. Plates were first placed in a solvent system of $CHCl_3$:CH_3OH:H_2O:concentrated NH_4OH (25:35:7.5:2.5) for 10 cm and then developed in a solvent of $CHCl_3$:CH_3OH:40% CH_3NH_2 (65:35:10) for an additional 8 cm. Radiolabeled PL were localized using autoradiography on Kodak Omat R x-ray film. In addition, the plates were placed in an iodine chamber to stain and localize the phospholipid fractions. PL were identified using authentic PL standards. The labeled PL were scraped from the plates, placed in a counting vial with 10 ml of phosphor (Ecolume; ICN, Irvine CA), and counted in a liquid scintillation counter (Beckman). Recovery was approximately 90% by this method. The phosphatidic acid (PA) and the phosphatidylserine (PS) fraction did not separate sufficiently in our solvent system to be able to detect two distinct moieties. Therefore, we have treated them as one fraction and refer to them as PA + PS in this study.

The experiment on changes in percentage of composition of PL (Table IV) was carried out exactly as outlined above except that there was no preincubation with ^{32}P-Ringer solution. Extraction procedures for PL were begun immediately after removing the bladder from the animal. Total tissue lipid phosphorus was determined on each sample according to the modified method of Chalvardjian and Rudnicki (14) and the results reported as counts per minute $(\mu M\ PO_4)^{-1}\ (hr)^{-1}$ or in the case of experiments in Table IV as percentage of fraction of total PL based on total lipid phosphorus. Statistics on all studies were performed using Student's *t* test.

Table IV. Phospholipid Fractions of the Urinary Bladder of *Bufo marinus*

Phospholipid fraction	Normal toads[a]	Acidotic toads[a]	*P*
PIP_2	<1.0	<1.0	
PIP	<1.0	<1.0	
PI	8.1 ± 0.6	10.5 ± 0.6	<0.01
PA + PS	10.6 ± 0.3	11.6 ± 0.6	NS
PC	37.7 ± 1.5	40.2 ± 1.4	NS
PE	27.0 ± 1.2	31.5 ± 1.0	<0.01

[a] Values are expressed as mean ± SEM of eight experiments and expressed as percentage of total phospholipids.

Results

Shown in Table I is the rate of incorporation of [^{3}H]AA into PL in toad urinary bladder. The PA + PS, phosphatidylcholine (PC), and the phosphatidylethanolamine (PE) fractions demonstrated a significantly greater turnover rate in bladders from acidotic animals. Phosphatidylinositol bisphosphate (PIP_2) and phosphatidylinositol monophosphate (PIP) are present in such small amounts that they did not label well with the [^{3}H]AA. Hence, they are not reported in this table.

Table II demonstrates the rate of incorporation of ^{32}P into membrane PL in normal and acidotic toad bladder. It is clearly seen that the PA + PS fraction and the PE fraction have a significantly higher rate of turnover during metabolic acidosis.

In Table III the effect of aldosterone on the PL turnover in toad urinary bladder is shown. This experiment was done on normal bladders that had been stimulated for 60 min with aldosterone (10^{-6} *M*). Aldosterone is known to be an acute stimulator of the acidification mechanism (12, 13). It is clear that aldosterone has no significant effect on PL turnover following short-term (60-min) stimulation.

Table IV shows the percentage of composition of membrane PL in control and acidotic toads. These values are expressed as the percentage of total PL as determined by total lipid phosphorus. There was a significant increase in the percentage of composition of the phosphatidylinositol (PI) ($P < 0.01$) and PE fractions ($P < 0.01$) during acidosis. Total lipid phosphorus in the normal group averaged 90.7 ± 2.45 μM and the acidotic group averaged 93.9 ± 1.6 μM, not significantly different ($P < 0.20$).

Discussion

Previous studies have demonstrated that PL may be involved in the transport of ions and fluid across cell membranes (15). Additionally, studies have demonstrated that PL may act as ionophores and mediate the membrane transport of a full range of ions and solutes (16). Other studies have shown that chronic NH_4Cl-induced acidosis increases H^+ and NH_4^+ excretion (1, 2). Our present study indicates that during chronic metabolic acidosis there is an increased turnover of phospholipid fractions PA + PS and PE in toad urinary bladder. There is also an increased percentage of composition of the PI and PE fractions during metabolic acidosis. These findings support the concept that during acidosis the epithelial cells of the toad urinary bladder increase H^+ and NH_4^+ excretion and this increase is associated with an increased turnover and insertion of new PL into the epithelial cell membrane. It is interesting to note that an earlier observation by Lotspeich (17) demonstrating that during NH_4Cl-induced acidosis there is an increase in total lipid content in the rat kidney.

There is also evidence to suggest that the increased H^+ excretion in the bladder observed during acidosis is a result of two different mechanisms, one acute and the other chronic. The acute response is stimulated by increased CO_2, aldosterone, and other hormones and involves membrane shuttling and insertion of new H^+ pumps into the epithelial apical membrane (18). The chronic response is brought about by an increasing number of mitochondria-rich cells, the cell responsible for H^+ excretion, and located on the apical surface of the toad bladder epithelial cells (5). Either of these responses would be compatible with our results. Insertion of new membrane and shuttling of the new H^+ pumps would result in a higher turnover rate and perhaps a change in fractional composition of the apical membrane. Likewise, an increased number of mitochondria-rich cells appearing on the apical membrane could result in an increased turnover rate of PL and a change in the total fractional composition of the membrane PL. However, since only certain phospholipid components were increased, this suggests that a general increase in total membrane could not account for our observations and, thus, the latter possibility appears highly unlikely.

Aldosterone has been shown to be a mediator of H^+ excretion in toad urinary bladder (12, 13). Aldosterone plasma levels are also known to increase in response to an acidosis (19). We therefore wanted to test the possibility that aldosterone, after a short exposure to the bladder, could stimulate the turnover of PL in toad bladder. We were unable to demonstrate an acute stimulation of turnover rates of PL fractions by aldosterone (Table III). Aldosterone, even though stimulating H^+ excretion within 60 min after exposure (13), does not appear to do so by increasing the turnover rate of PL. However, this does not rule out the possibility that a longer chronic exposure of aldosterone could bring about a change in PL turnover. Alternatively, the acidosis induced by NH_4Cl loading could be acting to increase H^+ excretion through a different mechanism than that observed following aldosterone administration. It is interesting to note that Frazier (20) has suggested two different H^+ excretory pumps in this tissue. One that is stimulated acutely and is functional at all times at low activity, and the second that is only functional after chronic stimulation by acidosis. The two pumps appear distinctly different based upon their functional characteristics. It is interesting to speculate that the chronically stimulated pump may be related to the new PL turnover in the bladder, whereas the low activity pump is not associated with changes in membrane PL.

Earlier *in vitro* studies had indicated that the primary action of aldosterone in toad urinary bladder was to alter the metabolism of membrane PL (21). Later, similar studies confirmed this finding and indicated that aldosterone specifically stimulated PL synthesis after 30 min of exposure (22). Most recent observations concerning aldosterone action have suggested that aldosterone stimulates phospholipid and protein methylation (23) with an increase in the PC to PE ratio. However, these changes in membrane PL metabolism were associated with aldosterone increases in sodium transport. In our experiments, no significant increases in ^{32}P incorporation into PL were observed following incubation with aldosterone for 60 min. The reason for this discrepancy could reflect the methods employed for detecting changes in PL turnover. The earlier studies utilized radiolabeled fatty acids or *S*-adenosylmethionine as a means to assess changes in PL composition and metabolism, whereas the current studies used [^{32}P]orthophosphate. Therefore, changes in fatty acid composition or methylation processes would not be detected.

The control data presented in Tables II and III show some variation between the two control groups. This variation in normal toads is not unexpected. It is well known that animals collected in the field show more variation than inbred laboratory strains. In addition, amphibia are known to show marked seasonal variation. It has also been reported (24) that bladders of *B. marinus* obtained from different geographical areas show different effects of aldosterone application on the Na^+ transport system. Hence, several of these features described above could account for this observed variation. It is also important to note that the experiment in Table III was done on paired hemibladders, i.e., each animal served as its own control, whereas in Table II the experimental and control animals were from the same shipment of toads but not the same shipment as animals used in the aldosterone experiment.

In the experiment using [^3H]AA to label the phospholipids, we were not able to detect AA incorporation into the PIP and PIP_2 fraction. These fractions represent a very small component of the total PL (<1%), as shown in Table IV. We therefore labeled with ^{32}P in Experiments II and III in order to be able to detect the PIP and PIP_2 fractions. It is obvious on examination of Table IV that the reported fractions do not add up to 100%. Other PL are present in the bladder which we did not isolate in our system, namely, sphingomyelin and cardiolipin (22). Presumably, the increased fractions observed in Table IV would come from one or both of these fractions since the total lipid phosphorus did not change.

We have shown that during NH_4Cl-induced acidosis there is an increased turnover rate of PA + PS, PC, and PE fractions of PL in toad urinary bladder. Additionally, during acidosis there is an increased percentage fraction of PI and PE in the toad urinary bladder. Aldosterone, a known acute stimulator of acidification in the toad bladder, did not mimic the affect of acidosis on PL metabolism. At the present time our results support the postulate that increased acidification

may reflect an increased recruitment of vesicular H^+-ATPase into the apical membrane or could result from a proliferation of acid-secreting mitochondria-rich cells with synthesis of new membrane or both.

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