Stimulation of Membrane Phospholipid Metabolism by Agents that Increase Acidification in Toad Urinary Bladder[1] (43248)

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Abstract. **Previous reports have indicated that metabolic acidosis stimulates H^+ excretion, and this excretion is accompanied by an increased turnover of phospholipids (PL) in toad urinary bladder. The purpose of this experiment was to determine if other known stimulators of H^+ excretion [insulin, deoxycorticosterone acetate (DOCA), epinephrine, parathyroid hormone, and CO_2] might also stimulate PL turnover in the toad urinary bladder. Quarter bladders from normal toads were removed, weighed, and then incubated with [^{32}P]orthophosphate for 2 hr at 25°C. PL were extracted, separated, and detected using thin layer chromatography and autoradiography, and quantitated by liquid scintillation counting. Results were expressed in cpm (100 mg bladder)$^{-1}$ (hr)$^{-1}$. One quarter bladder received insulin (100 milliunits/ml), DOCA (10^{-6} *M*), epinephrine (50 m*M*), parathyroid hormone (100 μg/ml), or 5% CO_2 during the incubation, whereas the paired quarter bladder received no treatment. Phosphatidylcholine (PC) and phosphatidylinositol turnover were increased by insulin ($P < 0.025$ and < 0.05, respectively). DOCA had no effect on PL turnover, but stimulated the percentage fraction of PC ($P < 0.05$) expressed as percentage fraction of total lipids. Five percent CO_2 in the bath resulted in an increased rate of turnover of the PL fractions phosphatidylinositol ($P < 0.05$), and the phosphatidic acid plus phosphatidyl-serine ($P < 0.01$). Epinephrine and parathyroid hormone were both without effect on PL metabolism. We conclude that insulin, DOCA, and CO_2 may stimulated H^+ excretion in toad bladder in part by increasing turnover of membrane PL, PC, and phosphatidylinositol, and in the case of CO_2, phosphatidic acid plus phosphatidylserine as well, but not PC.** [P.S.E.B.M. 1991, Vol 197]

It is well known that the urinary bladder of the toad *Bufo marinus* can acidify the mucosal fluid and that this acidification is increased by a metabolic acidosis (1). In addition, it has been shown that during metabolic acidosis phospholipid (PL) turnover is increased in toad urinary bladder (2). Specifically, the turnover rate of phosphatidic acid (PA) plus phosphatidylserine (PS) and phosphatidylethanolamine (PE) were increased during acidosis. Also, the percentage fraction of the PL, phosphatidylinositol (PI) and PE, were both increased during metabolic acidosis (2). These findings indicated that the adaptive response of the urinary bladder to acidosis to increase H^+ excretion somehow results in a change in PL metabolism. It has also been shown that certain hormones are increased during acidosis and may stimulate H^+ excretion in the bladder, hence functioning as modulators of this adaptive response. Insulin (3), aldosterone, and deoxycorticosterone acetate (DOCA) (4, 5), epinephrine (6), parathyroid hormone (PTH) (7), and CO_2 (8) have all been reported to be important in this adaptive response by the bladder to acidosis.

Changes in PL metabolism have also been implicated in several H^+ secreting tissues, namely gastric mucosa (9, 10) and renal epithelia (11). Both of these studies implicated the changes in PL metabolism with the H^+-K^+ transporting ATPase activity being related to its lipid environment or domain.

The purpose of this experiment was to determine if any of the above known stimulators of acidification in toad bladder would increase turnover rate and/or increase the percentage fraction of membrane PL in

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the toad urinary bladder. Several of these agents were found to change PL metabolism in the toad urinary bladder. The results and some possible implications of how this change in PL metabolism may alter H^+ excretion are discussed.

Materials and Methods

The toads used in these experiments were *Bufo marinus* of Mexican origin and were supplied by Carolina Biological Supply, Burlington, NC. The Ringer's solution used in these studies 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 Ringer's solution used in the CO_2 experiments had 24 m*M* $NaHCO_3^-$ instead of the sodium phosphate. The NaCl was adjusted to maintain the Na^+ concentration at 116 m*M*. This HCO_3^- buffered Ringer's was equilibrated with 5% CO_2-95% O_2 mixture, which resulted in a final pH of 7.28–7.31. The control CO_2 experiments used the same solution but without the 5% CO_2 equilibration, and the final pH of this solution was 8.0. After double pithing the toad, the bladder was removed and placed in the indicated Ringer's solution.

In the experiments with ^{32}P labeling, paired whole quarter-bladders were isolated and blotted on filter paper, wet weight was obtained, and they were incubated for 2 hr at 25°C in 2 ml of Ringer's solution containing [^{32}P]orthophosphate (100 μCi/ml) obtained from ICN Radiochemicals, Irvine, CA. The reaction was then stopped by the addition of 3 ml of cold acidified acetone and then extracted as given below. In each experiment, one quarter-bladder was the control and the other was the experimental quarter-bladder. The experimental bladder received one of the following agents in the incubation solution simultaneously with the ^{32}P labeling; insulin, 100 milliunits/ml; DOCA, 10^{-6} *M*; epinephrine, 50 μ*M*; or PTH, 100 μg/ml. All of these hormonal agents were obtained from Sigma Chemical Co., St. Louis, MO. The experimental bladder in the CO_2 studies received a Ringer's solution equilibrated with 5% CO_2. The paired control bladders received none of the agents during the ^{32}P labeling.

The experiments on changes in percentage of composition of PL were carried out exactly as outlined above, except that there was no incubation with ^{32}P-labeled Ringer's solution. Extraction procedures for PL were begun after the 2-hr incubation with the indicated agent. Total tissue lipid phosphorus was determined on each sample according to the modified method of Chalvardjian and Rudnicki (12), and the results reported as percentage fraction of total PL based on total lipid phosphorus.

Phospholipid Extraction Procedure. The tissue was homogenized using an 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 was collected and washed with 3 ml of 0.1 *N* HCl. Samples were centrifuged, and 1 ml of the organic phase was removed, 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 (25:30:7.5:2.5) for 10 cm and then developed in a solvent of $CHCl_3$:CH_3OH:40% CH_3NH_2 (65:35:10) for an addition 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 Radiochemicals, Irvine, CA), and counted in a liquid scintillation counter (Beckman). Recovery was approximately 90% by this method. All samples were counted to an accuracy of <2% and corrected for decay of ^{32}P and expressed as cpm (100 mg bladder)$^{-1}$(hr)$^{-1}$. Statistics were performed using the paired *t* test.

Results

Table I shows the effect of insulin (100 milliunits/ml) on PL turnover in urinary bladder. Phosphatidylcholine (PC) and PI were stimulated significantly by insulin ($P < 0.01$ and 0.05, respectively). Insulin, however, did not result in any changes in the percentage of composition of the PL fractions as shown in Table II.

Table III shows the effect of DOCA (10^{-6} *M*) on PL turnover in toad urinary bladder. There was no significant stimulation found of turnover of PL fractions by DOCA. However, as shown in Table IV, there was a significant stimulation of the PC fraction as percentage of total phospholipid by DOCA ($P < 0.05$).

Shown in Table V is the effect of exogenous CO_2 (5%) on turnover of the PL fractions. In the presence of CO_2, the turnover of PI and PA plus PS was increased significantly ($P < 0.005$ and $P < 0.01$, respectively). Accompanying these increases, there was also a significant decrease observed in the turnover of PC ($P < 0.05$). The reasons for this decrease in PC turnover are not apparent from these studies. Table VI shows that exogenous CO_2 had no effect on any of the PL fractions in terms of their percentage of composition. The PA and the 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.

Table VII shows the results of experiments in which the effects of CO_2 on PL metabolism in the presence of ouabain were determined. These experiments were

Table I. Insulin Stimulation of Phospholipid Turnover in Toad Urinary Bladder[a]

Phospholipid fraction	Experimental quarter-bladder (insulin, 100 milliunits/ml)	Control quarter-bladder (no exogenous insulin)	Mean difference (experimental − control ± SEM)	*P*
PIP_2[b]	64	58	6 ± 9.2	NS
PIP	146	108	38 ± 34.9	NS
PI	701	574	127 ± 43.7	<0.05
PA + PS	1400	1267	133 ± 89.8	NS
PC	1442	951	491 ± 148.2	<0.01
PE	509	461	48 ± 81.5	NS

[a] Each value represents the average of 10 paired quarter-bladders ± SEM. Turnover is reported as cpm (100 mg bladder)$^{-1}$ (hr)$^{-1}$. Each quarter-bladder was incubated for 2 hr at 25°C in 2 ml of Ringer's solution containing 100 μCi/ml of ^{32}P. NS, not significant at the 0.05 level. *P* value calculated using Student's paired *t* test.
[b] PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate.

Table II. Phospholipid Fractions in Control and Insulin-Treated Bladders[a]

Phospholipid fraction	Insulin-treated bladders (100 milliunits/ml)	Control bladders	*P*
PIP_2	<1.0	<1.0	
PIP	<1.0	<1.0	
PI	17.3 ± 0.8	17.7 ± 1.3	NS
PA + PS	11.7 ± 0.4	12.3 ± 0.4	NS
PC	34.0 ± 1.3	35.6 ± 1.3	NS
PE	27.9 ± 1.3	28.1 ± 1.9	NS

[a] Each value represents the average of seven paired quarter-bladders ± SEM. The values reported are given as the percentage of total phospholipid present. See footnotes to Table I.

Table III. Effect of DOCA on Phospholipid Turnover in Toad Urinary Bladder[a]

Phospholipid fraction	Experimental quarter-bladder (DOCA, 10^{-6} *M*)	Control quarter-bladder	*P*
PIP_2	216 ± 62	216 ± 49	NS
PIP	279 ± 88	305 ± 55	NS
PI	704 ± 250	778 ± 184	NS
PA + PS	1598 ± 433	1544 ± 236	NS
PC	1432 ± 354	1480 ± 292	NS
PE	746 ± 133	686 ± 140	NS

[a] Each value represents the average of nine paired quarter-bladders ± SEM. Turnover is reported as cpm (100 mg bladder)$^{-1}$ (hr)$^{-1}$. See footnotes to Table I.

done to eliminate the effects of Na^+ transport on PL turnover. The results show that when Na^+ transport is blocked with ouabain, CO_2 still produces a significant increase in turnover of PI and PA + PS ($P < 0.01$ in each case).

Identical experiments were performed as above using epinephrine and PTH, known stimulators of acidification in toad urinary bladder (6, 7). Epinephrine and PTH were both found to be without a significant effect on PL turnover or stimulation of changes in the percentage fraction of total PL.

Discussion

Reports as early as 1959 (13) have implicated membrane PL in the transport of ions and fluid across membranes. Green *et al.* (14) have demonstrated that PL may act as ionophores and mediate the membrane transport of a full range of ions and solute. Similar observations have been made showing that PL are important in ion and fluid transport in various amphibian skin (15). It has also recently been demonstrated that during NH_4Cl induced acidosis, a condition that increases H^+ excretion in the toad urinary bladder, there is a stimulation of PL turnover in the PA + PS and PE fractions and an increased percentage of composition of the PI and PE fractions (2). These findings suggest that during acidosis the epithelial cells of the toad urinary bladder increase H^+ excretion, and this increase is associated with an increased turnover and insertion of new PL into the epithelial cell membrane. Our present studies further support this concept. In addition, our studies suggest that the hormonal control of acidification in this tissue by insulin, DOCA, and CO_2 is mediated by changes brought about by increased turnover or compositional changes in the membrane PL. It is also noteworthy in this regard that an early observation in the rat kidney demonstrated that NH_4Cl-induced acidosis resulted in an increase in the total lipid content (11).

Dixon *et al.* (16) have presented evidence that the acute response of increased H^+ excretion in the bladder by CO_2, aldosterone, and other hormones involved membrane shuttling and insertion of new H^+ pumps into the epithelial apical membrane. Our present findings would support this concept. Insertion of new membrane and shuttling of the new H^+ pumps could result in a higher turnover rate and/or a change in fractional composition of the apical membrane. However, this is only speculation at the present time, and it should be pointed out that our results only indicate that the

Table IV. Phospholipid Fractions in Control and DOCA-Treated Bladders[a]

Phospholipid fraction	DOCA-treated bladders (10^{-6} *M*)	Control bladders	Mean difference (experimental − control ± SEM)	*P*
PIP_2	<1.0	<1.0		
PIP	<1.0	<1.0		
PI	19.2	18.6	0.6 ± 0.9	NS
PA + PS	15.3	13.4	1.9 ± 1.2	NS
PC	24.8	21.8	3.0 ± 1.2	<0.05
PE	21.2	19.0	2.2 ± 1.5	NS

[a] Each value represents the average of seven paired quarter-bladders ± SEM. The values reported are given as the percentage of total phospholipids. See footnotes to Table I.

Table V. Membrane Phospholipid Turnover in the Presence and Absence of 5% CO_2 in Toad Urinary Bladder[a]

Phospholipid fraction	Experimental quarter-bladder (5% CO_2)	Control quarter-bladder (no exogenous CO_2)	Mean difference (experimental − control ± SEM)	*P*
PIP_2	113	121	−8 ± 21.5	NS
PIP	227	297	−70 ± 49.4	NS
PI	698	567	131 ± 68.1	<0.05
PA + PS	1883	1340	543 ± 103.7	<0.01
PC	1566	2114	−548 ± 221.6	<0.05
PE	445	399	46 ± 49.4	NS

[a] Turnover is reported as cpm (100 mg bladder)$^{-1}$ (hr)$^{-1}$. Each value represents the average of eight paired experiments. See footnotes to Table I.

Table VI. Phospholipid Fractions in Toad Urinary Bladder in the Presence and Absence of 5% CO_2[a]

Phospholipid fraction	Experimental quarter-bladder (5% CO_2)	Control quarter-bladder (no exogenous CO_2)	*P*
PIP_2	<1.0	<1.0	
PIP	<1.0	<1.0	
PI	21.2 ± 2.4	19.4 ± 2.1	NS
PA + PS	13.1 ± 1.2	11.4 ± 0.9	NS
PC	36.2 ± 1.8	33.0 ± 2.4	NS
PE	29.6 ± 2.2	27.4 ± 1.8	NS

[a] Each value represents the average of eight paired quarter-bladders ± SEM. The values given are reported as the percentage of total phospholipids present. See footnotes to Table I.

increase in H^+ excretion caused by acidosis runs parallel to the change seen in PL metabolism. The exact mechanism by which the changes in PL are correlated to H^+ excretion remains uncertain.

Our present findings, indicating that DOCA stimulates an increase in the percentage fraction of PC, are consistent with earlier reports by Goodman *et al.* (17, 18). Their studies indicated that the primary action of aldosterone on toad urinary bladder, with regard to Na^+ transport, was to stimulate PL synthesis after a 30-min exposure. Later observation (19) concerning aldosterone action have indicated that aldosterone stimulates PL and protein methylation with an increase in the PC and PE ratio.

It is also true that the two agents, insulin and DOCA, are both known stimulators of Na^+ transport in the toad urinary bladder (20, 21). We cannot exclude the possibility that insulin and DOCA are producing these changes observed in PL metabolism in relationship to increasing Na^+ transport. Likewise, it has been reported that CO_2 or acidification of the mucosal fluid may also stimulate Na^+ transport in the urinary bladder (22). This increased Na^+ transport due to one of these agents could then conceivably raise H^+ excretion by increasing endogenous CO_2 levels in the cell. We believe that this is not a likely possibility since we have reported in earlier studies (8) that in the normal toad there is small H^+ secretion in the bladder that is Na^+ dependent, but when the animal is placed in acidosis the H^+ excretory system that is stimulated is independent of Na^+. In addition, the experiment in Table VII with ouabain clearly indicates that the increased turnover of PL by CO_2 is not directly related to Na^+ transport but is a specific response to increased H^+ excretion. Previous studies (23) have shown that in the presence of CO_2, ouabain (10^{-4} *M*) blocks Na^+ transport while having no effect on H^+ excretion.

Our present study indicates that insulin stimulates PC turnover, and DOCA was found to increase the percentage fraction of PC. On the other hand, CO_2 was found to decrease the turnover of PC. This seems paradoxical at first, but considering that both PI and PA + PS fractional turnover were increased signifi-

Table VII. Stimulation of Membrane Phospholipid Turnover by CO_2 in the Presence of Ouabain (10^{-4} *M*)[a]

Phospholipid fraction	Experimental quarter-bladder (5% CO_2)	Control quarter-bladder (no exogenous CO_2)	Mean difference (experimental − control ± SEM)	*P*
PIP_2	224	128	96 ± 45.9	NS
PIP	335	232	103 ± 73.3	NS
PI	1050	489	561 ± 83.1	<0.01
PA + PS	2073	1116	957 ± 196.8	<0.01
PC	2094	2491	−397 ± 525.3	NS
PE	780	881	−101 ± 161.7	NS

[a] Turnover is reported as cpm (100 mg bladder)$^{-1}$ (hr)$^{-1}$. Each value represents the average of 10 paired experiments. See footnotes to Table I. Experiment was carried out exactly as that in Table V, except that ouabain was present at all times to inhibit Na^+ transport.

cantly by CO_2, it could be that PC is serving as a source of supply for the increased cycling of PI and PA + PS. It is also interesting to note that a recent report (24) indicates that phospholipase A_2 and prostaglandin synthesis are both increased in the urinary bladder during metabolic acidosis. This could potentially lead to a decrease in the PC fraction if the PC were being cycled as a precursor of the arachidonic acid component.

Epinephrine and PTH were both found to be without an effect on PL metabolism in the toad urinary bladder. We assume that the action of epinephrine and PTH on stimulating H^+ excretion in this tissue would have to be via other intracellular signals, such as cyclic AMP or Ca^{2+}. In this regard, it is important to note that both epinephrine and PTH are reported to stimulate cyclic AMP in the toad urinary bladder (6, 7).

Table VIII is a summary giving all of the effects of various agents used in this experiment and others on PL metabolism. Also given is the effect of these same agents on H^+ excretion and their relative potency. It is clear from Table VIII that acidosis is the most potent stimulus to H^+ excretion. NH_4Cl (metabolic acidosis) and CO_2 (respiratory acidosis) stimulate maximum levels of H^+ excretion. Also, these two metabolic states result in increased turnover rates of PA + PS, PE, and PI. On a physiologic basis, one would expect these two changes in the metabolic state to have the greatest effect on H^+ excretion and turnover rate of PL. On the other hand, insulin and DOCA stimulate H^+ excretion to a much lesser degree (2×) than does a change in the acid-base state of the animal. Therefore, we saw only changes in the PI and PC fraction when stimulated with insulin or DOCA. This could be due to the fact that insulin and DOCA during this period of time are not stimulating the phospholipid cascade maximally. It is not clear from these studies why CO_2 decreased PC turnover in the presence of Na^+ transport (Table V) and yet had no effect on PC turnover in the presence of ouabain (Table VII). This will have to await delineation in future studies.

This study has presented evidence that insulin, DOCA, and increased CO_2 stimulate PL metabolism in the toad urinary bladder. Insulin, DOCA, and CO_2 stimulate H^+ excretion in the bladder, and evidence suggests that this stimulation may be via increasing turnover rate and insertion or deletion of new PL fractions (PI, PC, PA + PS) into the membrane, or by direct stimulation of the proton-ATPase.

Table VIII. Summary of Changes in H^+ Excretion and Membrane Phospholipid Turnover or Composition by Various Metabolic or Hormonal Pertubations in Toad Urinary Bladder

Treatment and time	H^+ excretion[a] (X-fold increase)		Phospholipid fractions[b]					
			PE	PC	PA + PS	PI	PIP[c]	PIP_2
Metabolic acidosis (48 hr)	↑ 6–8X	(Turnover)	↑[d]	↑	↑	NC	NC	NC
		(%)	↑[e]	NC	NC	↑	NC	NC
Insulin (100 milliunits/ml, 2 hr)	↑ 2X	(Turnover)	NC	↑	NC	↑	NC	NC
		(%)	NC	NC	NC	NC	NC	NC
DOCA (10^{-6} *M*, 2 hr)	↑ 2X	(Turnover)	NC	NC	NC	NC	NC	NC
		(%)	NC	↑	NC	NC	NC	NC
5% CO_2 (2 hr)	↑ 4–6X	(Turnover)	NC	↓	↑	↑	NC	NC
		(%)	NC	NC	NC	NC	NC	NC

[a] Taken from Refs. 3, 8, and 25.
[b] Data come from present study and Ref. 2.
[c] PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; NC = no change.
[d] ↑, Increased turnover was measured from incorporation of ^{32}P.
[e] ↑, Increased percent was measured as percentage of total lipid phosphate.

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