Stimulation of Phosphoinositides by Agents that Stimulate Proton Secretion in Toad Urinary Bladder[1] (43652)

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Abstract. **The urinary bladder of *Bufo marinus* excretes H^+ and this excretion is increased by metabolic acidosis (MA), insulin (IN), prostaglandin E_2 (PGE_2), $\uparrow CO_2$, and aldosterone. The purpose of this experiment was to determine whether MA, IN, PGE_2, CO_2, and aldosterone stimulate inositol phosphate's (IP) formation in isolated cells of toad urinary bladder. Cells were prepared by treating bladder sacs with collagenase. Cells were obtained from 10 toads in MA and 10 normal toads, suspended in 2 ml of Ringer's solution containing LiCl (10 m*M*), *myo*-inositol (5 m*M*), and [^{3}H]*myo*-inositol (10 μCi), and then incubated for 2 hr at 25°C. Cells were homogenized and the IP fractions quantitated by column chromatography and liquid scintillation counting. The results were expressed as dpm $(\mu MPO_4)^{-1}$ $(hr)^{-1}$. The IP in MA cells was 44,202 ± 4,646 and in normal toad cells it was 31,637 ± 3,613 ($P < 0.05$). In a separate experiment, cells from 10 paired hemibladders were isolated from normal toads. The cells were treated exactly as above except there was no LiCl in the bath. LiCl was added to all baths after 2 hr and the experimental cells were challenged with IN, PGE_2, $\uparrow CO_2$, and aldosterone for 20 min. The IP were quantitated as above. IN treatment stimulated inositol bisphosphate and inositol triphosphate ($P < 0.01$). PGE_2 and $\uparrow CO_2$ also stimulated inositol triphosphate ($P < 0.05$). Aldosterone did not alter formation of any of the IP fractions. We conclude that MA, IN, PGE_2, and $\uparrow CO_2$ stimulate IP formation in cells of toad urinary bladder and inositol triphosphate may be an important second messenger in mediating the response of MA, IN, PGE_2, and $\uparrow CO_2$.** [P.S.E.B.M. 1993, Vol 204]

The original work of Hokin and Hokin (1) demonstrated that acetylcholine stimulated the incorporation of ^{32}P into phosphatidylinositol and phosphatidic acid in pancreatic slices. Since then, a wide variety of receptors have been identified as being linked to the metabolism of polyphosphoinositides and the generation of a calcium signal (2, 3). It is now firmly established in many cells that membrane phospholipids serve as a source for receptor signaling in cells and that the products of phospholipid metabolism have second messenger functions.

It is well known that the urinary bladder of the toad *Bufo marinus* can acidify the mucosal fluids and that this acidification is increased by a metabolic acidosis (4, 5). In addition, it has been shown that during metabolic acidosis, phospholipid (PL) turnover is increased in toad urinary bladder (6). These findings suggest that the adaptative response of the urinary bladder to acidosis to increase H^+ excretion is correlated with a change in PL metabolism of the cell membranes. Insulin, aldosterone, $\uparrow CO_2$, and prostaglandin E_2 (PGE_2) are also known stimulators of H^+ excretion in the toad bladder and have also been shown to stimulate PL turnover in this same tissue (7).

The purpose of this experiment was to determine whether acidosis, insulin, aldosterone, CO_2, and PGE_2 can stimulate formation of inositol phosphates in toad bladder. If inositol phosphates are stimulated, it would be evidence that these compounds might serve as intracellular second messengers in mediating the adaptive response to acidosis and increased secretion of proton.

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Materials and Methods

The toads used in these experiments were *B. marinus* of Mexican origin and were supplied by Carolina Biological Supply, Burlington, NC. The PGE_2, insulin, ouabain, and aldosterone used in this study were all obtained from Sigma Chemical Co., St. Louis, MO, and the collagenase used was obtained from Worthington Biochemical Co., Freehold, NJ (170 units/mg). [^{3}H] *myo*-Inositol was used in these experiments and was obtained from Amersham Corp., Arlington Heights, IL. Toads were sacrificed by double pithing and the urinary bladders were removed and hemibladder sacs were prepared. Urinary bladders from normal toads (normal acid-based balance) were used throughout the study except for one experiment. In this experiment, the toads were placed in metabolic acidosis by gavaging three times a day for 2 days with 120 m*M* NH_4Cl. This method of inducing acidosis produces a marked acidosis as reported earlier (8). After this treatment in a group of 10 toads, the plasma [HCO_3^-] was 8.3 ± 0.7 mEq/liter compared with normal toads of 21.2 mEq/liter and the plasma pH was 7.35 ± 0.03 compared with the normal group of 7.70 ± 0.02.

Epithelial cells were isolated from the bladder by treating the hemibladder sacs with Ca^{2+}-free media and exposing the bladder to collagenase (100 units/ml) for 45 min. After gently massaging the bladder sacs, the mucosal fluid containing the cells was collected and centrifuged at 1000*g* for 15 min. The cells were washed in 4% bovine serum albumin (Sigma), centrifuged, and resuspended in 2 ml of Ringer's solution containing [^{3}H]*myo*-inositol (10 μCi). The Ringer's solution contained in m*M*: NaCl, 114.5; KCl, 3.0; $CaCl_2$, 0.9; inositol, 5.0; and sodium phosphate, 1.5; the final pH was adjusted to 6.80–7.00. This procedure of obtaining isolated epithelial cells from the bladder has been shown to have greater than 90% viability using trypan blue.

The cells were incubated for 2 hr at 25°C in the above-indicated solution. After the 2-hr labeling period, the cells were centrifuged, washed, and resuspended in 2 ml of nonlabeled Ringer's solution containing LiCl (10 m*M*). Cells from one hemibladder of each bladder served as the control cells, while cells from the other hemibladder were the experimental group. PGE_2, 5% CO_2, insulin, insulin plus ouabain, or aldosterone was then added to the indicated experimental group and the cells were challenged for 20 min. Twenty minutes was chosen as the time of challenge because this was the earliest time we could see a measurable increase in H^+ excretion (5) and a concurrent measurable amount of inositol triphosphate (9). The control cells, i.e., cells from the paired hemibladder, received no treatment. At the end of this 20-min period, the cells were extracted and fractions were obtained as given below. In one experiment, cells from toads in metabolic acidosis were used as the experimental group and no agonist was added. The control for this experiment was cells from normal toads.

At the end of the incubation period with the agonist (PGE_2, 5% CO_2, insulin, insulin plus ouabain, or aldosterone), the reaction was stopped by adding 2 ml of 20:40:1 of chloroform:methanol:HCl. The tissue was then homogenized and 0.75 ml of chloroform and 0.254 ml of water added and the solution was centrifuged (500*g* for 10 min). One milliliter of the organic phase was collected for PO_4 determination. Additionally, 1.5 ml of the water soluble phase were collected. This water-soluble phase was placed on a Dowex-Ag-1 × 8 column (formate form), 200–400 mesh in 2 *M* ammonium formate. Fractions of phosphoinositides (glycerophosphoinositol, inositol phosphate [IP], inositol bisphosphate [IP_2], inositol triphosphate [IP_3]) were then eluted from the column with varying concentrations of formic acid/ammonium formate as given by Berridge *et al.* (9). Three milliliters of each sample were then placed in a scintillation vial with 10 ml of phosphor (Ecolume; ICN, Irvine, CA) and counted in a Beckman liquid scintillation counter. All samples were counted to an accuracy of <2%. The 1-ml organic phase sample was dried under 100% N and reconstituted in 50 μl of chloroform:methanol:concentrated HCl (60:30:1). Ten microliters of this solution were then used to determine total lipid PO_4 by the method of Chalvardjian and Rudnicki (10). Results were calculated as dpm $(\mu M\ PO_4)^{-1}\ (hr)^{-1}$ for the metabolic acidosis study and as dpm $(\mu M\ PO_4)^{-1}\ (min)^{-1}$ for all other experiments. Differences in fractions between the experimental and control groups were compared using the Student's *t* test for paired data and the metabolic acidosis experiment for the grouped data (nonpaired test).

Results

Shown in Table I is the effect of chronic metabolic acidosis on inositol phosphates in toad bladder. In this experiment the Li^{2+} was present during the entire labeling period. After 2 hr of incubation in the presence of LiCl, it can be seen that there was a greater turnover rate in the cells from metabolic acidotic animals than in normal animals. The IP fraction was increased significantly over control animals ($P < 0.05$). The fact that IP was the fraction with the highest turnover is not surprising because LiCl blocks the conversion of IP back to inositol and during the 2-hr incubation most of the IP_2 and IP_3 would have been converted to IP.

Table II shows the effect of insulin on the IP fractions. After labeling, the cells were challenged for 20 min with insulin. This resulted in a significant increase in both IP_2 and IP_3 fractions ($P < 0.01$ in both cases). This same experiment was repeated at a later date, but done in the presence of ouabain (10^{-4} *M*). The results are shown in Table III. Even in the presence

Table I. Inositol Phosphates in Isolated Cells of the Urinary Bladder of *Bufo marinus* (reported in units of dpm $(\mu MPO_4)^{-1}$ $(hr)^{-1}$)

Inositol phosphate fraction	State of toad		P^b
	Normal[a]	Metabolic acidosis[a]	
Glycerophosphoinositol	20,557 ± 3,941	24,892 ± 4,767	NS
Inositol phosphate	31,637 ± 3,613	44,202 ± 4,646	<0.05
Inositol bisphosphate	3,271 ± 509	4,282 ± 1,263	NS
Inositol triphosphate	528 ± 73	694 ± 95	NS

[a] Each value represents the average of 10 experiments ± SE.
[b] P calculated by Student's t test for grouped data. NS, nonsignificant.

Table II. Inositol Phosphates in Isolated Cells of Toad Urinary Bladder after Stimulation with Insulin (reported in units of dpm $(\mu MPO_4)^{-1}$ $(min)^{-1}$)

IP fraction	Control	Insulin treated (100 mU/ml)	Mean difference[a] ± SE	P^b
Glycerophosphoinositol	2359	3011	651 ± 355	NS
Inositol phosphate	1488	2232	744 ± 342	NS
Inositol bisphosphate	312	409	97 ± 26.1	<0.01
Inositol triphosphate	84	116	32 ± 7.4	<0.01

[a] Represents mean difference of 12 paired experiments.
[b] Calculated from the Student's paired t test. NS, not significant.

Table III. Inositol Phosphates in Isolated Cells of Toad Urinary Bladder after Stimulation with Insulin in the Presence of Ouabain (reported in units of dpm $(\mu PO_4)^{-1}$ $(min)^{-1}$

IP fraction	Control	Insulin (100 mU/ml) + ouabain (10^{-4} M)	Mean difference[a] ± SE	P^b
Glycerophosphoinositol	704	565	−139 ± 94	NS
Inositol phosphate	463	515	52 ± 35	NS
Inositol bisphosphate	141	235	94 ± 51	NS
Inositol triphosphate	68	110	42 ± 17	<0.05

[a] Represents mean difference of eight paired experiments.
[b] Calculated from the Student's paired t test. NS, not significant.

of ouabain, the increase in IP_3 was still observed. This would indicate that the observed stimulated response of IP_3 was not related to Na^+ metabolism.

Table IV shows the effect of challenging [^{3}H]*myo*-inositol-labeled cells with 5% CO_2 for 20 min. It is clear that this treatment stimulates formation of IP_3 in toad bladder cells ($P < 0.05$). It is not clear from the present study why 5% CO_2 stimulated only IP_3 and insulin stimulated both IP_3 and IP_2 fractions.

PGE_2, another agent that stimulates proton secretion in toad urinary bladder, was also tested for its affect on IP_3. Shown in Table V are the results of this experiment. It can be seen that PGE_2 (10^{-5} M) stimulates IP_3 after the 20-min application ($P < 0.05$).

Aldosterone is also a known stimulator of acidification in toad bladder. However, it is believed to act via a cytoplasmic receptor and not with a membrane receptor (11). We tested the effect of aldosterone (10^{-6} M) on the metabolism of phosphatidylinositides. When challenged with aldosterone (Table VI), there was no stimulation of any of the four inositide fractions, with $P > 0.10$ in all cases ($n = 8$).

Table IV. Inositol Phosphates in Isolated Cells of Toad Urinary Bladder after Stimulation with 5% CO_2 (reported in units of dpm $(\mu MPO_4)^{-1}$ $(min)^{-1}$)

IP fraction	Control	5% CO_2 treated	Mean difference[a] ± SE	P^b
Glycerophosphoinositol	964	1139	175 ± 162	NS
Inositol phosphate	1431	1758	327 ± 161	NS
Inositol bisphosphate	422	510	88 ± 44.1	NS
Inositol triphosphate	126	163	37 ± 13.2	<0.05

[a] Represents mean difference of 15 paired experiments.
[b] Calculated from the Student's paired t test. NS, not significant.

Table V. Inositol Phosphates in Isolated Cells of Toad Urinary Bladder after Treatment with PGE_2 (10^{-5} M) (reported in units of dpm $(\mu MPO_4)^{-1}$ $(min)^{-1}$)

IP fraction	Control	PGE_2 treated (10^{-5} M)	Mean difference[a] ± SE	P^b
Glycerophosphoinositol	4639	7054	2415 ± 1615	NS
Inositol phosphate	3304	4925	1621 ± 1122	NS
Inositol bisphosphate	1814	2777	962 ± 679	NS
Inositol triphosphate	636	951	315 ± 134	<0.05

[a] Represents mean difference of nine paired experiments ± SE.
[b] Calculated from the Student's paired t test. NS, not significant.

Table VI. Inositol Phosphates in Isolated Cells of Toad Urinary Bladder after Treatment with Aldosterone (10^{-6} *M*) (reported in units of dpm $(\mu MPO_4)^{-1}$ $(min)^{-1}$)

IP fraction	Control	Aldosterone treated (10^{-6} *M*)	Mean difference[a] ± SE	*P*[b]
Glycerophosphoinositol	1018	751	267 ± 241	NS
Inositol phosphate	1810	1538	271 ± 149	NS
Inositol bisphosphate	551	545	6 ± 61	NS
Inositol triphosphate	170	141	29 ± 21	NS

[a] Represents mean difference of eight paired experiments ± SE.
[b] Calculated from the Student's paired *t* test. NS, not significant.

Discussion

Our present studies have indicated that the hormonal agents CO_2, insulin, and PGE_2, known to stimulate acidification in toad urinary bladder, also stimulate formation of IP_3 concurrent with this process. This is evidence that the control of H^+ excretion in this tissue may be mediated by the IP_3 second messenger system. Previous studies from our laboratory have shown that CO_2 and insulin (7), as well as metabolic acidosis (6), stimulate membrane PL turnover. This increased PL turnover would be consistent with our current findings that these agents additionally stimulate IP_3 formation in cells of toad urinary bladder. Reports as early as 1959 (12) have implicated membrane PL in the transport of ions and fluid across membranes. Green *et al.* (13) have demonstrated that PL may act as ionophores and mediate the membrane transport of a full range of ions and solute. Our current findings suggest yet another possible role of PL (phosphoinositides) as mediators and/or second messengers in cell signaling for specific ion transport systems, specifically H^+ excretion in this tissue.

It has been reported by Dixon *et al.* (14) that the acute response of increased H^+ excretion in the bladder by CO_2 and other hormones involves membrane shuttling and insertion of new H^+ pumps into the epithelial apical membrane. Our present findings would indirectly support this concept. The increased rate of IP_3 formation after hormone stimulation could be the signal for increased insertion of new H^+ pumps in the apical membrane as illustrated in Figure 1. However, this is only speculation at the present time, and it should be mentioned that the increase in H^+ excretion caused by acidosis runs parallel to the change seen in phosphoinositide metabolism. The exact mechanisms by which the changes in IP_3 are correlated to H^+ excretion remains uncertain and will have to await other experiments in the future.

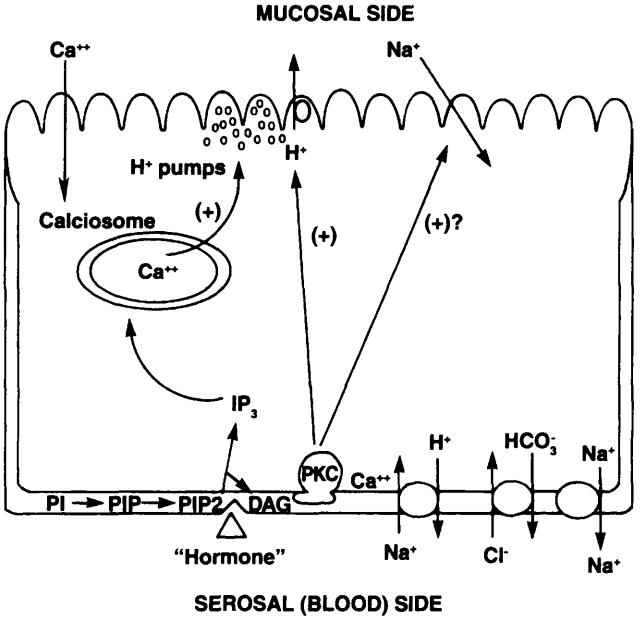

Figure 1. A hypothetical model suggesting possible hormonal control involved in the regulation of H^+ excretion and intracellular pH in the epithelial cell of toad urinary bladder. "Hormone" could be designated as insulin, PGE_2, or CO_2. Phosphoinositide breakdown would also result in the formation of diacylglycerol (DAG), an endogenous stimulator of protein kinase C (PKC).

The question arises from our results of whether anything that could stimulate IP_3 in the cell results in increased H^+ excretion. This may, in fact, be a correct assumption. However, the control of H^+ excretion in this tissue may be more specific and related to compartmentilization in the cell, whereby a particular hormone-receptor response would activate a specific pool of IP_3, hence leading to the appropriate stimulation of H^+ excretion. One must also consider the possibility that in the toad urinary bladder, we are dealing with a heterogenous cell population, and only one cell type, the mitochondria-rich cell, excretes H^+ (15). The hormonal stimulation of IP_3 and H^+ excretion may be selectively limited to this particular cell type. We have no direct evidence of this at the present time, but future studies should help elucidate this mechanism of action.

There was some variation in the extent of labeling of the phosphoinositides between control groups (Tables II–V). Part of this variation in labeling could be explained by the fact that each of these experiments were done several months apart and on different groups of toads. It is a well-established fact that there is great seasonal as well as geographical variation in amphibians to different physiologic states and responses (16). However, even though we observed different labeling between various experiments, it should not affect our conclusions, since we were looking at the effect of various hormones on paired groups of cells.

It should be mentioned that the two agents, insulin and PGE_2, are both known stimulators of Na^+ transport

in the toad urinary bladder (17–19). We cannot entirely exclude the possibility that insulin and PGE_2 are producing these changes observed in phosphoinositide metabolism in relation 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 (20). 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 because our laboratory (5) has shown that in the normal toad there is a 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 III with ouabain clearly indicates that the increased turnover of IP_3 by insulin is not directly related to Na^+ transport, but is a specific response to increased H^+ excretion. Additionally, previous studies (21) have shown that in the presence of CO_2, ouabain (10^{-4} *M*) blocks Na^+ transport, while having no effect on H^+ excretion.

We were not able to demonstrate any effect of aldosterone on phosphoinositide turnover. This was a little surprising because we had reported earlier (7) that deoxycorticosterone acetate stimulated phosphatidylcholine percent fraction in membrane preparations. Earlier reports by Goodman *et al.* (22, 23) 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. Our exposure time for our experiments was only 20 min, which could explain this lack of affect by aldosterone. It is also well known that the phosphoinositide-IP_3 system is linked to a membrane receptor, whereas it is generally agreed that aldosterone acts through a cytoplasmic and nuclear receptor (10).

Figure 1 shows a hypothetical model of a mitochondria-rich cell of the toad urinary bladder. This is a representation of how we believe the IP_3 system could signal the cell, after hormone activation by CO_2, insulin, or PGE_2. The activation of IP_3 from the membrane PL would then lead to an increase in intracellular calcium ($[Ca^{2+}]_i$). This increase in $[Ca^{2+}]_i$ could come from cell stores (calciosomes) or from extracellular stores and activate the insertion of new H^+ pumps into the apical membrane. Preliminary reports (24, 25) have indicated that CO_2, metabolic acidosis, insulin, and PGE_2 all stimulate $[Ca^{2+}]_i$ within 5 min of application. The $[Ca^{2+}]_i$, as well as diacylglycerol, may also stimulate the H^+ pumps directly. The exact mechanism by which increased $[Ca^{2+}]_i$ is involved in H^+ excretion will have to await future studies.

In summary, our studies have shown that CO_2, metabolic acidosis, insulin, and PGE_2 result in increased turnover of phosphoinositides, particularly IP_3, in toad urinary bladder. This evidence supports the concept that stimulation of H^+ excretion by CO_2, metabolic acidosis, insulin, and PGE_2 may be mediated by the intracellular messenger IP_3 in this renal epithelia.

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