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# Insulin-Mediated $H^+$ and $NH_4^+$ Excretion in the Urinary Bladder of *Bufo marinus*[1] (42683)

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*Abstract.* This study was done to determine if insulin mediates $H^+$ and $NH_4^+$ excretion in the urinary bladder of *Bufo marinus.* Acidosis was induced by gavaging with 10 ml of 120 m*M* $NH_4Cl$ 3× daily for 2 days. Hemibladders were mounted between Lucite chambers. Insulin (porcine) was added to the serosal solution of the experimental bladder ($10^2$ mU/ml). After a 15-min equilibration the flux was measured for 2 hr. $H^+$ excretion was measured from change in pH of the mucosal fluid and the $NH_4^+$ measured colorimetrically. The excretion was normalized for weight of bladder and reported in units of nanomoles (100 mg bladder)$^{-1}$ (min)$^{-1}$. Plasma insulin was determined by radioimmunoassay and glucose by the glucose oxidase method. In 14 control bladders $H^+$ excretion was 8.75 ± 1.28 and experimental was 16.35 ± 2.50 ($P < 0.025$), while $NH_4^+$ excretion in control bladder was 3.29 ± 0.95 and experimental was 6.58 ± 1.89 ($P < 0.01$). This response was absent when the insulin was heat inactivated ($P > 0.2$ and $P > 0.3$ respectively). Plasma insulin-like levels in 10 normal toads was 0.57 ± 0.16 ngm/ml and in acidotic toads 1.25 ± 0.16 ng/ml ($P < 0.025$). Plasma glucose levels in 10 normal toads were 22.0 ± 3.5 mg/dl and in 12 acidotic toads 17.8 ± 0.75 mg/dl ($P < 0.025$). We conclude that plasma insulin is increased in acidosis and that insulin stimulates excretion of $H^+$ and $NH_4^+$ in the toad urinary bladder. 

It has been shown previously that the toad urinary bladder can acidify the mucosal fluid and excrete $NH_4^+$ (1). This same study also revealed that the acidification and $NH_4^+$ excretion could be increased by an $NH_4Cl$-induced metabolic acidosis. Later studies have shown that in addition to the metabolic state of the animal, certain steroids (2) and parathyroid hormone (3) are important in regulating the excretion of $H^+$ and $NH_4^+$ in this urinary epithelium.

Herrera and co-workers (4, 5) were first to show that insulin increases the short circuit current in frog skin and toad urinary bladder. André and Crabbé confirmed a similar effect using toad skin (6). Other laboratories have since demonstrated that insulin can stimulate $Na^+$ reabsorption in toad urinary bladder under various conditions (7–9). It has also been suggested that insulin plays an important role in the handling of $Na^+$ by the mammalian nephron (10). It is well known that the toad urinary bladder is a functional analog to the mammalian distal nephron.

This study was undertaken to determine if insulin is involved in acid-base alterations in amphibians and if insulin plays a role in modifying $H^+$ and $NH_4^+$ excretion in the toad urinary bladder. Our results indicate that insulin does increase during changes in the acid-base state of the animal and may be important in controlling the $H^+$ and $NH_4^+$ excretion in the urinary epithelium of the amphibian, *Bufo marinus.*

**Materials and Methods.** The toads used in these experiments were *Bufo marinus* of Colombian or Mexican origin and were supplied by Carolina Biological Supply of Burlington, North Carolina. The routine care of toads, the solutions, the procedure of inducing acidosis, and the method of measuring $H^+$ and $NH_4^+$ excretion were as previously described (11). In all experiments, the $H^+$ excretion was calculated from change in pH and the concentration of buffer in the mucosal solution using a *p*Ka for the phosphate buffer pair of 6.50. A Radiometer Model PHM64 digital pH meter was used for all pH determinations. The concentration of $NH_4^+$ in the mucosal solution was determined colorimetrically (12). Humidified 100% $O_2$ was bubbled into the mucosal medium throughout each experiment. The Ringer's solution used contained (in m*M*): NaCl, 114.5; KCl, 3.0; $CaCl_2$, 0.9; and sodium phosphate, 1.5; the final pH was 7.00. The porcine insulin

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and aldosterone were obtained from Sigma Chemical Co. (St. Louis, MO). The insulin contained an activity of 26 units per milligram. All statistics were performed as the mean of the difference between paired hemibladders using Student's one-tailed *t* test. The one-tailed test was used because the theory being tested was that insulin stimulates $H^+$ and $NH_4^+$ excretion. If the difference was negative, regardless of the magnitude, this would reject the theory.

Heparinized blood was obtained by cardiac puncture after the animal was sacrificed. Plasma glucose was determined on a Beckman Glucose Analyzer 2 utilizing the glucose oxidase method. Insulin was assayed using a double antibody radioimmunoassay kit obtained from BioTek Research, Inc., of Lenexa, Kansas. The insulin antibody was labeled with $^{125}I$ obtained from New England Nuclear Corp. (Boston, MA).

Paired hemibladders from toads in normal acid-base balance were used except in one case where bladders from toads in metabolic acidosis were used. Acidosis was induced by gavaging with 10 ml of 120 m*M* $NH_4Cl$ 3 times per day for 2 days. The bladder was removed after double pithing the animal and hemibladders were mounted between Lucite chambers, each of which held 2 ml. The cross-sectional area of each chamber was 1.98 $cm^2$. The serosal bath of the experimental hemibladder contained the phosphate-buffered Ringer's solution to which had been added the indicated amount of porcine insulin (usually 100 mU/ml). The control hemibladder contained only the phosphate-buffered Ringer's solution in the serosal bath.

The mucosal chambers contained the same phosphate-buffered Ringer's solution as was used in the control serosal bath. In one experiment the insulin was added to the mucosal bath rather than the serosal bath. The flux period was for 120 min unless otherwise indicated. $NH_4^+$ and pH were determined on each mucosal and serosal sample both before and after the flux period. The bladders were mounted in the chambers and equilibrated 15–30 min. Insulin was added and the flux started after a 15-min equilibration period.

In the experiment measuring the time course of the insulin effect on $H^+$ and $NH_4^+$ excretion (Figs. 1 and 2) there was no equilibration period. Insulin was added to the serosal medium at time zero and the mucosal pH and $NH_4^+$ was determined every 30 min thereafter for a period of 150 min. This was done by draining the mucosal medium at each 30-min interval and replacing with fresh Ringer's solution.

In the experiment testing the effect of combining insulin and aldosterone, the aldosterone had to first be dissolved in absolute ethyl alcohol and then diluted with Ringer's solution. The final concentration of the ethyl alcohol in the serosal chamber was 1%. The paired hemibladder contained only the phosphate-buffered Ringer's solution with 1% ethyl alcohol in the serosal chamber.

**Results.** To examine the effect of insulin on $H^+$ and $NH_4^+$ excretion the bladder was exposed to insulin ($10^2$ mU/ml). Table I shows the results of porcine insulin on the $H^+$ and $NH_4^+$ excretion in toad urinary bladder. Insulin in the serosal bath increased both

TABLE I. EFFECTS OF INSULIN ON $H^+$ AND $NH_4^+$ EXCRETION IN NORMAL AND ACIDOTIC TOAD URINARY BLADDER

| State of toad | Group (*N*) | $H^+$ excretion[a] | $NH_4^+$ excretion[a] |
|---|---|---|---|
| Normal | Experimental (14)[b] | 16.35 | 6.58 |
| | Control (14) | 8.75 | 3.29 |
| | Mean difference ±SEM | 7.60 ± 2.61 | 3.29 ± 1.37 |
| | | $P < 0.025$ | $P < 0.01$ |
| Metabolic Acidosis[c] | Experimental (10)[b] | 34.22 | 3.88 |
| | Control (10) | 43.17 | 3.50 |
| | Mean difference ±SEM | −8.95 ± 4.63 | 0.378 ± 0.87 |
| | | $P > 0.10$ | $P > 0.50$ |

[a] Excretion is reported as nmole (100 mg bladder)$^{-1}$ (min)$^{-1}$.
[b] Experimental group received porcine insulin ($10^2$ mU/ml) in the serosal solution.
[c] Toads were put in metabolic acidosis by gavaging with 120 m*M* $NH_4Cl$ over a 48-hr period.

TABLE II. PLASMA GLUCOSE AND INSULIN LEVELS IN THE TOAD *BUFO MARINUS*

| State of toad ($N$) | Glucose (mg/dl) | Insulin (ng/ml) |
|---|---|---|
| Normal (10) | 22.0 ± 1.35 | 0.57 ± 0.16 |
| $NH_4Cl$-induced acidosis (12) | 17.8 ± 0.75<br>$P < 0.025$ | 1.25 ± 0.15<br>$P < 0.025$ |

$H^+$ and $NH_4^+$ excretion by approximately 100% over the normal control hemibladders. However, as also shown in Table I, when the toad was placed in an $NH_4Cl$-induced metabolic acidosis this response to insulin was absent. The assumption is made that during chronic metabolic acidosis the $H^+$ and $NH_4^+$ excretory systems are being stimulated maximally, and hence is not further responsive to insulin.

In order to determine if the insulin concentration in the toad is responsive to acid-base changes, toad plasma was assayed for insulin-like activity and for glucose. This was done both in normal toads and toads in metabolic acidosis. In Table II the results of these assays are shown. Insulin-like activity determined by radioimmunoassay in toad plasma was found to be more than doubled during metabolic acidosis ($P < 0.025$). Plasma glucose was reduced by approximately 20% during acidosis ($P < 0.025$).

The results reported in Table III indicate that the insulin stimulation is specific. When the insulin was heat inactivated (56°C for 60 min) and then placed on the bladder it had no effect on either the $H^+$ or $NH_4^+$ excretion ($P > 0.20$ and $P > 0.30$, respectively). Insulin was also without an effect when placed only on the mucosal side of the bladder as shown in Table III.

Figure 1 shows the time course for action of insulin on $H^+$ excretion and Fig. 2 shows the time course for the action of insulin on $NH_4^+$ excretion. Insulin exerts its greatest effect on $H^+$ secretion within 30 min after exposure to the bladder (40% increase above control). The insulin stimulation then decreases to about 10% over the ensuing 2 hr. As can be seen in Fig. 2 $NH_4^+$ response to insulin is delayed beyond that for $H^+$ excretion. The stimulation of $NH_4^+$ begins approximately 90 min after the exposure to insulin.

Figure 3 is the dose–response curve for the insulin stimulation of $H^+$ and $NH_4^+$ excretion. There is a significant stimulation of $H^+$ excretion at 1 μg/ml, reaching a peak response at 100 μg/ml. The $NH_4^+$ excretion was

TABLE III. RESPONSE OF $H^+$ AND $NH_4^+$ EXCRETION IN TOAD URINARY BLADDER BY "HEATED" INSULIN AND INSULIN ON THE MUCOSAL SURFACE

| Group ($N$) | $H^+$ excretion[a] | $NH_4^+$ excretion[a] |
|---|---|---|
| *"Heated" insulin* | | |
| Experimental (8)[b] | 9.68 | 1.29 |
| Control (8) | 8.70 | 1.15 |
| Mean difference ±SEM | 0.98 ± 1.49<br>$P > 0.20$ | 0.14 ± 0.27<br>$P > 0.30$ |
| *Insulin on mucosal surface* | | |
| Experimental (12)[c] | 6.68 | 2.50 |
| Control (12) | 12.75 | 2.18 |
| Mean difference ±SEM | −6.07 ± 3.39<br>$P > 0.05$ | 0.32 ± 0.44<br>$P > 0.50$ |

[a] Excretion is reported as nmole (100 mg bladder)$^{-1}$ (min)$^{-1}$.

[b] Experimental group received heated porcine insulin ($10^2$ mU/ml) in the serosal solution.

[c] Experimental group received porcine insulin ($10^2$ mU/ml) in the *mucosal* solution.

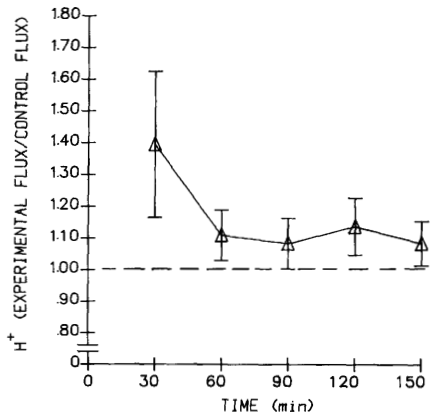


FIG. 1. Time course for the excretion of $H^+$ by the toad urinary bladder in response to insulin. Abscissa: time in minutes after insulin addition (100 mU/ml). Each point represents the average of the [experimental flux (insulin)/control flux (no insulin)] ($N = 10$) ± SEM.

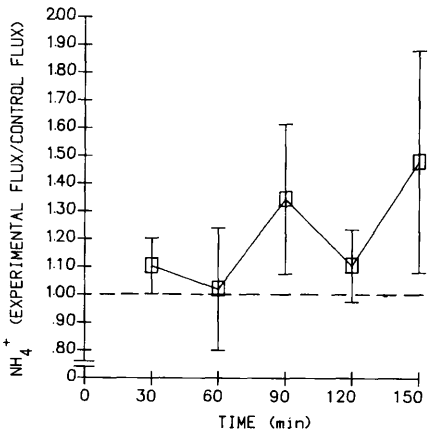


FIG. 2. Time course for the excretion of $NH_4^+$ by the toad urinary bladder in response to insulin. Legend same as for Fig. 1.

TABLE IV. THE COMBINED EFFECT OF INSULIN AND ALDOSTERONE ON $H^+$ AND $NH_4^+$ EXCRETION IN TOAD URINARY BLADDER

| Group ($N$) | $H^+$ excretion[a] | $NH_4^+$ excretion[a] |
|---|---|---|
| Experimental (12)[b] | 32.33 | 4.59 |
| Control (12)[c] | 24.83 | 2.63 |
| Mean difference ±SEM | 7.50 ± 4.00 $P < 0.05$ | 1.96 ± 0.94 $P > 0.05$ |

[a] Excretion is reported as nmole (100 mg bladder)$^{-1}$ (min)$^{-1}$.

[b] Experimental group received porcine insulin ($10^2$ mU/ml) and aldosterone $10^{-6}$ $M$ in 1% ethanol in the serosal solution.

[c] The control group contained only the 1.5 m$M$ $PO_4$ buffered Ringer solution with 1% ethanol in the serosal solution.

not stimulated until the insulin concentration was raised to 100 μg/ml.

Table IV shows the results of combining aldosterone with insulin on $H^+$ and $NH_4^+$ excretion. Aldosterone has been shown to stimulate both $H^+$ and $NH_4^+$ excretion in toad urinary bladder (2). As revealed in Table IV, insulin and aldosterone combined stimulated $H^+$ excretion ($P < 0.05$), but the stimulation did not appear to be any greater than insulin alone. $NH_4^+$ excretion was not stimulated by the combined action of insulin and aldosterone.

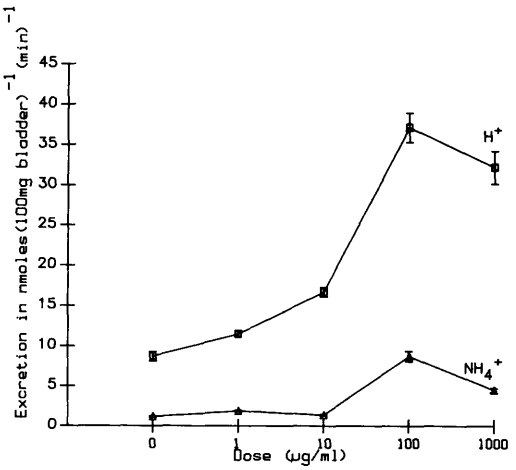


FIG. 3. Dose-response curve of $H^+$ and $NH_4^+$ excretion to varying concentrations of insulin in toad urinary bladder. Each point represents the average excretion of 10 bladders ± SEM. Zero dose represents a normal bladder with only endogenous insulin activity present. The indicated concentration of insulin was placed on the serosal surface and the excretion rate was determined over the next 2-h period as given under Materials and Methods. (Insulin activity was 26 U/mg).

**Discussion.** It has been known for some time that the urinary bladder of the toad excretes $H^+$ and $NH_4^+$ and that the excretion of both of these entities is increased by a metabolic acidosis (1). This study was an attempt to determine if insulin might be a contributing factor to causing these observed increases, and if so, if it is part of the physiological response of the animal to acidosis. Our experiments clearly demonstrate that insulin stimulates both $H^+$ and $NH_4^+$ excretion in the isolated toad urinary bladder by approximately 100%. It is also noteworthy that when the animal was maintained in a chronic metabolic acidosis (i.e., maximal excretion rates) that the stimulatory response by insulin was abolished (Table I). We assume that the rate of $H^+$ and $NH_4^+$ excretion in this state was already at maximal rates and therefore, could not be stimulated further. However, we cannot eliminate the possibility that at this decreased plasma pH the binding characteristics of the insulin receptor could be altered.

Our results in Table II showing an increased plasma insulin-like activity during chronic metabolic acidosis are evidence that this response is important in the over-all

acid-base balance of the animal. The decrease observed in blood glucose during acidosis simply reinforces the fact that plasma insulin-like activity is increased during this state.

The insulin stimulation of $H^+$ and $NH_4^+$ excretion in toad bladder does not appear to be a nonspecific effect of the protein. When the insulin was heated (Table III) the stimulatory response was abolished. This indicates that the native structure of the insulin hormone is required to bring about this stimulation. Additionally, insulin was active only in bringing about this stimulation when placed on the serosal side of the bladder. Our results indicate that insulin was without effect when placed on the mucosal surface of the bladder. This is similar to the findings on insulin stimulation of $Na^+$ transport in toad bladder (8).

In designing and carrying out the experiments in this study we chose not to run the bladders in a short-circuited state for two reasons: (i) we have previously shown that short-circuiting the bladder increased the rate of $H^+$ excretion and we did not feel that this effect should be superimposed on the hormonal response; (ii) this study was designed to determine if the observed increases in $H^+$ and $NH_4^+$ excretion could be, in part, a result of insulin. This experiment was not an attempt to elucidate the biophysical mechanism by which insulin might stimulate this system in toad bladder.

It could be argued that the effect on $H^+$ excretion reported here is secondary to the stimulating effect on $Na^+$ transport reported by others (5, 7, 14). We argue that the effect of insulin on the $H^+$ and $NH_4^+$ excretion is a direct effect and not secondary to the $Na^+$ stimulation for the following reasons: (i) it has been shown that there is not a direct coupling between either the potential difference or $Na^+$ reabsorption and $H^+$ excretion in toad bladder (15); (ii) increasing $Na^+$ transport in the toad bladder by administration of ADH has no effect on $H^+$ excretion (15); and (iii) the dose of insulin required for stimulation of $Na^+$ transport (14) is much less than we report here for stimulation of $H^+$ excretion. It should be noted that our study does not eliminate the possibility of changes in intracellular increases in $K^+$, $H^+$, or $NH_4^+$ that may be insulin dependent. Other experiments with more stringent criteria must be performed before this question can be answered.

The time course for the insulin stimulation of $H^+$ is distinctly different from that of $NH_4^+$ stimulation, indicating perhaps two different mechanisms of action. Insulin was found to stimulate $H^+$ excretion maximally at 30 min (our first sampling period). This is similar to what has been found in toad bladder for stimulation of $Na^+$ reabsorption by insulin (14). On the other hand the stimulation of $NH_4^+$ excretion did not occur until 90 min after application of insulin.

It has been shown previously that aldosterone and other related steroids stimulates $H^+$ and $NH_4^+$ excretion in toad urinary bladder (2, 16). We therefore wanted to study insulin–aldosterone-induced $H^+$ and $NH_4^+$ excretion to look at possible differences in the mechanism of action of the two hormones. If the hormones share a different mechanism of action we might expect to see an additive effect on stimulation by both together. When both hormones were applied to the serosal bath we observed a stimulation of $H^+$ excretion. The mean difference (experiment-control) was $7.49 \pm 4.0$ nmole (100 mg bladder)$^{-1}$ (min)$^{-1}$. In previous experiments the mean difference in aldosterone-treated bladders was $12.53 \pm 3.20$ (2) and insulin-treated bladders was $7.60 \pm 1.89$ (Table I). It is obvious there is not an additive effect by the two hormones. This finding suggests that aldosterone and insulin might act via the same mechanism in stimulating $H^+$ excretion. In the case of $NH_4^+$ excretion, insulin and aldosterone together failed to produce any stimulation of the system. This is a puzzling finding and is not clear from this present study what might have caused this lack of response.

In considering the overall relevance of these findings to the physiology of the acid-base balance in the toad, the following are expected to apply; (i) the dose–response curve for the effect of insulin is within the physiological range; (ii) some degree of hormonal specificity should exist; and (iii) the concentration of insulin should increase when the animal is challenged by an acid or base load. As mentioned above, we have demonstrated specificity of the insulin re-

sponse as well as an increase in insulin-like activity when the animal is in metabolic acidosis. With regard to the dose response the bladder responded in a typical log-dose manner being stimulated minimally at 1 μg/ml and maximally at 100 μg/ml. On the other hand, the actual values of plasma insulin measured in the toad during acidosis was 1.25 ng/ml, which is considerably less than the dose–response values. However, in determining plasma insulin we used a porcine insulin antibody and we do not know at this time if toad insulin differs greatly in activity or structure from porcine insulin. If indeed there is considerable difference we would be underestimating plasma insulin in the toad by this technique.

The high dose porcine insulin required to obtain a response could be due to the fact that the receptors on the toad urinary bladder might have a low binding constant for porcine insulin compared to the binding constant for endogenous insulin. Additionally, it is also possible that the porcine insulin used could be combining with other receptors in the toad urinary bladder.

It is interesting to note that insulin apparently stimulates $Na^+$ reabsorption in the toad bladder firstly by a short-term response that is independent of protein synthesis and is believed to result from a direct effect on the sodium pump. Secondly, a long-term response that is expressed after the first hour of hormone treatment and requires the synthesis of one or more specific proteins in the "granular" cell (17–19). In the toad bladder Frazier (20) has presented evidence for two $H^+$ pumps, one present in the normal toad and stimulated acutely and the other with different characteristics stimulated by a chronic acidosis in the toad. Additionally, it has been shown that in the toad urinary bladder it is the "mitochondria-rich" cell and not the granular cell that mediates $H^+$ excretion (21, 22). One could easily speculate that insulin has a twofold effect during acidosis. Insulin is increased to stimulate the granular cell to increase $Na^+$ reabsorption and the mitochondria-rich cell to increase excretion of $H^+$. This hypothesis will have to await further investigation before confirmed.

In summary, the present study has demonstrated that insulin stimulates both $H^+$ and $NH_4^+$ excretion in toad urinary bladder and this stimulation is abolished when the toad is in a chronic state of metabolic acidosis. Insulin has this stimulatory effect only when it is present on the serosal surface and has no effect when present on the mucosal surface. Finally, this response is specific for insulin and the insulin response seems to be important in the overall physiology of acid-base balance in the toad.

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