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# CORRELATION BETWEEN INSULIN ACTION UPON GLYCOLYSIS AND CHANGE IN INTRACELLULAR pH

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Summary. In the presence of $CO_2/HCO_3^-$, insulin both increases intracellular pH and stimulates glycolysis in frog skeletal muscle. When the action of insulin upon intracellular pH is blocked, either by amiloride or by decreasing extracellular sodium fifteen fold, the effect of the hormone upon glycolysis is also blocked.

Introduction. Independently of its effect upon glucose transport, insulin stimulates glycolysis in skeletal muscle probably by activating phosphofructokinase, the rate limiting enzyme of glycolysis (1,2). The molecular mechanism whereby insulin affects glycolysis or other intracellular events is not known, but it is generally conceded that the effect of insulin is not mediated by cyclic nucleotides (3). In the previous paper (4), it was demonstrated that insulin increases intracellular pH in frog skeletal muscle. In view of the reported high sensitivity to pH of PFK (5), the suggestion was made that the increase in $pH_i$ may be a mediator for the action of insulin upon glycolysis (4,6,7).

The hypothesis that $pH_i$ is the mediator of the insulin effect upon glycolysis is tested by observing the effect of insulin upon glycolysis under conditions which block the hormone's effect upon $pH_i$.

Materials and Methods. Paired sartorius muscles from the frog Rana pipiens were used throughout. Muscles were dissected and mounted as described previously (4). The mounted muscles were placed overnight in 25 ml of Ringer previously bubbled with 5% $CO_2$ in $O_2$ at 4 °C in separate stoppered tubes. The composition of the Ringer, which was glucose-free, was 104 mM $Na^+$, 2.2 mM $K^+$, 2.0 mM $Ca^{2+}$, 1.6 mM $HPO_4^{2-}$, 1.2 mM $Mg^{2+}$, 1.9 mM $SO_4^{2-}$, and 30.0 mM $HCO_3^-$ at pH 7.4; the remaining anion was $Cl^-$. In the low $Na^+$ experiments, choline was used to replace all but 6.8 mM $Na^+$ (a 15 fold reduction) and d-tubocurarine, 50 μM,

Abbreviations: PFK, phosphofructokinase (E.C. 2.7.1.11); $pH_i$, intracellular pH.

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was added to prevent twitching. Experiments were conducted under anaerobic conditions (100% $N_2$, or 95% $N_2$ and 5% $CO_2$) in a glove bag in order to prevent pyruvate from entering the Krebs cycle, thus ensuring that lactate production reflects the rate of glycolysis. In $CO_2/HCO_3^-$ free experiments, $Cl^-$ was substituted for $HCO_3^-$. Because glucose was absent, 0.5 μM l-epinephrine bitartarate was used to increase glycogen breakdown to provide sufficient substrate to ensure the rate-limiting condition of the PFK reaction (1). Ascorbate, 1 mM, was added to inhibit auto-oxidation of the epinephrine.

All experiments were conducted at constant temperature between 20.5 and 22.5 °C. After overnight storage, muscles were allowed to equilibrate anaerobically for 30 min and then were incubated for 40 min in the appropriate Ringer, with the experimental muscles also exposed to 400 mU insulin/ml.

Muscles were placed in 2.5 ml Ringer for 90 min. Lactate was then extracted from the muscles using a perchloric acid extraction technique (8). Activated charcoal, 15 mg, was added prior to the precipitation of perchlorate in order to eliminate material from the muscle causing an interference peak at 290 nm in the resulting supernatant without affecting the peak due to lactate. Lactate was measured in both the muscle extract and in the efflux tubes by a modified enzymatic technique of Hohorst (9) using lactic dehydrogenase (E.C. 1.1.1.27) to convert lactic acid and $NAD^+$ to NADH and pyruvic acid with the latter trapped by hydrazine. In computing the rate of lactate production, it is assumed that the 40 min preincubation period is sufficient for the lactate content of the controls to reach a steady state (8). Accordingly, the glycolytic rate of the controls is obtained from the lactate effluxed during the 90 min in 2.5 ml Ringer. Since the lactate content of the insulin-treated muscles changes, at least relative to the controls, during the experiments, this change is added to the lactate effluxed before dividing by 90 min to give the rate of lactate production for the experimental muscles.

Intracellular pH was determined as described in the previous paper (4).

Insulin was Zn-free porcine sodium insulin (26.5 U/mg) and was a gift from Eli Lilly and Co. l-epinephrine bitartarate and d-tubocurarine were obtained from Sigma. [$^{14}$C]5,5-dimethyl-2,4-oxazolidinedione and [$^3$H]sucrose were obtained from New England Nuclear. Amiloride was a gift from Merck, Sharp & Dohme Research Laboratories. All data is presented as the mean ± the standard error.

Results. To avoid any ambiguity of interpretation due to effects on glucose transport, all Ringer was, as in the previous paper (4), glucose-free. In the previous paper (4), $CO_2/HCO_3^-$ was absent from the Ringer in order to avoid any complications in interpretation arising from the presence of relatively permeant buffers (10,11). However, in a $CO_2/HCO_3^-$ free Ringer under anaerobic conditions, insulin had no effect upon glycolysis (see Table 1) although insulin still elevated $pH_i$ by 0.16 ± 0.02 units ($P<0.001$). This lack of effect of insulin upon glycolysis in a bicarbonate-free medium has been observed in the rat hemidiaphragm by Shaw and Stadie (12,13) who found that addition of bicarbonate restored the insulin effect. When $CO_2/HCO_3^-$ was added to the Ringer, insulin significantly stimulated glycolysis by an average of 42.7 ± 4.0 % while still producing a significant increase in $pH_i$ which

Table 1. Effect of insulin upon intracellular pH and anaerobic lactate production.

| Conditions | Intracellular pH | | | | | Anaerobic Lactate Production (nmole $min^{-1}$/g wet weight) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | With Insulin | Change | n | P | Control | With Insulin | Percent Change | n | P |
| $HCO_3^-$ Free Ringer | 7.27 ± .02 | 7.43 ± .03 | +.16 ± .02 | 8 | <.001 | 36.4 ± 1.8 | 36.4 ± 1.3 | +1.9 ± 5.0 | 11 | >.5 |
| Ringer | 6.98 ± .03 | 7.11 ± .03 | +.13 ± .02 | 6 | <.002 | 28.0 ± 2.0 | 39.6 ± 2.5 | +42.7 ± 4.0 | 8 | <.001 |
| Low Sodium Ringer | 6.75 ± .04 | 6.74 ± .10 | -.01 ± .08 | 6 | >.5 | 23.8 ± 3.1 | 22.5 ± 2.1 | -2.0 ± 6.6 | 7 | >.5 |
| Ringer + Amiloride | 7.03 ± .03 | 7.05 ± .03 | +.02 ± .01 | 6 | >.1 | 28.4 ± 2.7 | 28.5 ± 3.0 | +1.3 ± 7.0 | 7 | >.5 |

averaged 0.13 ± 0.02 units and was not significantly different ($P>0.25$) from the increase produced by insulin in the $CO_2/HCO_3^-$ free Ringer (Table 1).

The elevation of $pH_i$ by insulin appears not to be directly mediated by the Na pump since $10^{-3}$ M ouabain fails to block this effect (4). Considerable evidence indicates $pH_i$ can be regulated by two other systems, an ATP dependent $HCO_3^-/Cl^-$ exchange mechanism (14) and a system which couples $Na^+$ influx to either $H^+$ efflux (15) or to $CO_3^{2-}$ influx (16). The latter mechanism, which requires an inward driving force for $Na^+$, could be neutralized by reducing the inward driving force for $Na^+$ by eliminating its concentration gradient, since either mechanism involving $Na^+$ is presumably electrically neutral. To minimize the $Na^+$ driving force, a 15-fold reduction in the external $Na^+$ concentration to 6.8 mM was tested and found to block the effect of insulin upon $pH_i$, the change, -0.01 ± 0.08, being not significant (Table 1). Moreover, insulin stimulation of glycolysis was also blocked, the effect, -2.0 ± 6.6 %, not being significant and the decrease from 42.7 ± 4.0 % being significant ($P<0.001$).

The diuretic drug amiloride (3,5-diamino-6-chloropyrazinoyl-guanidine) has no effect upon the Na pump (17) but has been reported to block Na:H exchange (15,18). Addition of $5 \times 10^{-4}$ M amiloride blocked ($P<0.005$) the effect of insulin upon $pH_i$, the increase, 0.02 ± 0.01, being not significant (Table 1). This same concentration of amiloride decreased ($P<0.001$) the stimulation of glycolysis from 42.7 % to 1.3 %, the effect of insulin in the presence of amiloride being not significant.

Discussion. The finding that simple replacement of part of the extracellular $Na^+$ inhibits both the elevation of $pH_i$ and the stimulation of glycolysis by insulin provides support for the hypothesis that $pH_i$ plays a role in the action of this hormone upon glycolysis. In these experiments, no molecular inhibitors were involved, thus eliminating the possibility of direct action of inhibitor upon intracellular enzymes.

As discussed previously (4), the activation of glycolysis by increase in $pH_i$ may be due to the sharp pH profile of PFK. Depending upon the concentrations of other effectors, this sharp activation of PFK, by changes in pH as small as 0.1 unit, can occur anywhere (5) in the range of pH usually found within the cell, about 6.7 to 7.4.

The results also strongly suggest that the rise in $pH_i$ produced by insulin is mediated by the same type of membrane ion transport mechanisms which raise $pH_i$ after an acid load (15,19). That either lowering extracellular $Na^+$ or addition of amiloride blocks the effect of insulin upon $pH_i$ suggests that the mechanism may be a Na:H exchange (15).

In the absence of $CO_2/HCO_3^-$, the failure of insulin to increase anaerobic lactate production, in spite of an effect on $pH_i$, seems to present a problem for the hypothesis tested here. However, reduction of $CO_2/HCO_3^-$ increases $pH_i$ (19,20). This increase in $pH_i$ in the absence of $CO_2/HCO_3^-$ is seen in the control (without insulin) values in Table 1, where it is also observed that, in the absence of $CO_2/HCO_3^-$, anaerobic lactate production increases to a level not significantly ($P>0.3$) different from that observed during insulin stimulation in the presence of $CO_2/HCO_3^-$. This suggests that the increase in $pH_i$ resulting from the absence of $CO_2/HCO_3^-$ increases anaerobic lactate production to its maximum rate, even without insulin. This observation offers a possible explanation for the previously observed (12) requirement for the presence of $CO_2/HCO_3^-$ for insulin to affect carbohydrate metabolism.

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