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ELEVATION OF INTRACELLULAR pH BY INSULIN IN FROG SKELETAL MUSCLE

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Summary. Insulin produces a statistically significant elevation of intracellular pH in frog sartorius muscle. Ouabain, 1 mM, does not inhibit the elevation of intracellular pH by insulin. Neither serum albumin nor growth hormone, at the same concentration as insulin, produces a significant effect upon intracellular pH.

Introduction. Although it is generally accepted that insulin specifically binds only to the surface of target cells, to date there is no general consensus as to the identity of a second messenger which could mediate all the known actions of insulin (1). It was pointed out (2) that if insulin does indeed elevate intracellular pH, "it is apparent that this might be one mechanism whereby insulin could regulate intracellular events".

The experiments reported here confirm the prediction that insulin elevates pH_i in frog skeletal muscle.

Methods. All experiments were conducted at a constant temperature of 20-21 ^{o}C. Paired sartorius muscles from the frog Rana pipiens were used throughout. Muscles weighing less than 100 mg and usually 40-60 mg were removed from healthy frogs stored at 22 ^{o}C and force fed liver 3 times per week. After passing microscopic checks for damage, the muscles were mounted at rest length on platinum frames (3) and kept in Ringer at room temperature for 2-3 hr before each experiment.

Ringer contained 104 mM Na^+, 2.5 mM K^+, 2.0 mM Ca^{2+}, 0.8 mM $H_2PO_4^-$, and 0.8 mM HPO_4^{2-}, and was glucose-free. The remaining anion was Cl^-. The Ringer was titrated to a pH of 7.40 $\pm$ 0.03 except for one series of experiments where the pH was adjusted to values ranging from 6.83 to 7.00. In experiments using ouabain, Ringer contained 1 mM ouabain, and Na^+ was substituted for the 2.5 mM K^+. To avoid any complications arising from relatively permeant buffers (4,5), $H_2PO_4^-/HPO_4^{2-}$ was chosen as buffer rather than CO_2/HCO_3^-.

After the experimental muscles were equilibrated with the appropriate hormone, such as insulin, both experimental and control muscles were loaded with [^{14}C]DMO and [^{3}H]sucrose for 90 min, a time sufficient for both the effect of insulin and the equilibration of labeled compound to reach a steady state. Sufficient unlabeled DMO was added to the loading solution to bring the total concentration of DMO to 1 mM. The [^{14}C]DMO and [^{3}H]sucrose were

Abbreviations: DMO, 5,5-dimethyl-2,4-oxazolidinedione; pH_i, intracellular pH; pH_o, extracellular pH.

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washed out of the muscles over a period of 140 min by transfer through a series of tubes containing Ringer with the same concentration of ions and hormone as the loading solutions. The wet weight of the muscles was then determined, and the muscles were soaked in the same Ringer for an additional 22 hr to remove all traces of [^{14}C]DMO. This procedure removes well over 99% of [^{14}C]DMO from the muscles (6). Muscles were then dried overnight at 105 °C and reweighed to determine water content. Aliquots of Ringer from the washout tubes were dissolved in Aquasol (New England Nuclear) and counted in a liquid scintillation counter to an accuracy of 3% (SD). Back addition of the counts gave the amounts of [^{14}C]DMO and [^{3}H]sucrose present at the beginning of washout. The extracellular space (ECS) determined by [^{3}H]sucrose was used to calculate the intracellular [^{14}C]DMO in the muscle before washout, from which pH_i was calculated as in (7). All results are presented as the mean ± the standard error (SE).

Insulin was porcine insulin (0.00% Zn), 25.9 U/mg, and was a gift from Eli Lilly and Company. Albumin was bovine serum albumin (<.005% fatty acid) obtained from Sigma. Bovine growth hormone was obtained from the NIH through Emory University. Ouabain was obtained from Sigma. [^{14}C]DMO and [^{3}H]sucrose were obtained from New England Nuclear.

Results. In eight of nine muscles incubated with insulin (250 mU/ml) in Ringer of pH 7.4, pH_i was greater than that of their paired controls incubated without insulin. The average difference in pH_i produced by insulin was +0.096 ± 0.016 ($P<0.001$) (see Table 1). This result can be compared to the average difference, 0.008 ± 0.034, in a group of 11 paired muscles where both members were exposed only to Ringer free of any hormone.

The rate and magnitude of the recovery of pH_i after acid loading is proportional to the magnitude of the initial acidification (8,9). This

Table 1. Effect of Insulin upon Intracellular pH.

Experimental Condition	n	pH_o	control pH_i (mean ± SE)	effect (mean ± SE)	P
250 mU/ml insulin	9	7.4	7.30 ± 0.03	+ 0.096 ± 0.016 **	<0.001
250 mU/ml insulin	14	6.8-7.0	7.08 *	+ 0.163 ± 0.032	<0.001
250 mU/ml insulin + 1 mM ouabain	11	7.4	7.39 ± 0.02	+ 0.079 ± 0.019 **	<0.002
2 μM growth hormone	13	7.4	7.40 ± 0.03	- 0.022 ± 0.011	>0.05
2 μM albumin	4	7.4	7.55 ± 0.01	+ 0.006 ± 0.040	>0.5

* Since pH_o in this series varied widely (see text), pH_i also varied widely, from 6.80 to 7.37, decreasing as pH_o decreased.

** These effects do not differ significantly from each other ($P>0.5$).

suggests that the action of insulin on pH_i might be affected by an acidotic state. Decreasing the extracellular pH results in a decrease in pH_i (10,11). When pH_o was 7.40, pH_i averaged 7.30 (see Table 1). In 14 experiments, before addition of insulin, pH_i was decreased to an average of 7.08 by lowering pH_o to values ranging between 6.83 and 7.00. At this lower range of pH_i and pH_o, the elevation of pH_i produced by insulin was increased to 0.163 ± 0.032. Figure 1 shows the results of both groups of experiments. Although the difference between the average of the two groups is not significant ($P>0.10$), from inspection of Figure 1 it is evident that the elevation in pH_i due to insulin increases as the control pH_i decreases. A linear regression of the data in Figure 1 yields a slope which is significantly less than zero ($P<0.05$).

The experimental analysis which led to the prediction that insulin might elevate pH_i suggested that this effect would be mediated via the Na pump. To test this hypothesis, the Na pump was inhibited (2,12) by placing the muscles in K-free Ringer containing 10^{-3} M ouabain. Figure 2 shows that the presence of ouabain did not prevent an average elevation of pH_i by insulin of $0.079 \pm$

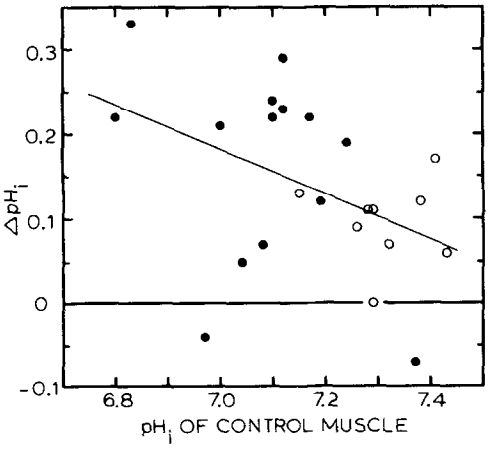

Figure 1. Effect of insulin on intracellular pH of frog sartorius. The change in intracellular pH produced by insulin is plotted against the intracellular pH of the paired controls. Open circles: pH of the Ringer was 7.4. Solid points: pH of the Ringer ranged between 6.8 and 7.0. Plotted line is a linear regression to all the data.

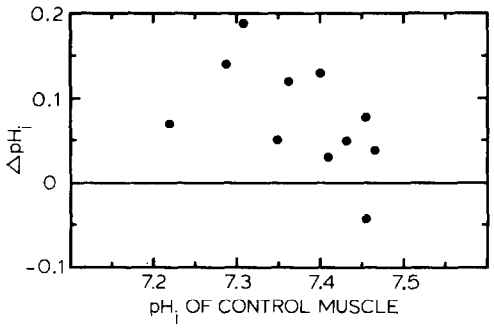

Figure 2. Change in intracellular pH produced by insulin in the presence of 1 mM ouabain plotted against the intracellular pH of the paired control muscle.

0.021 ($P<0.002$). Moreover, the effect of insulin in the presence of ouabain was not significantly different from its effect without ouabain ($P>0.5$). Figure 2 again suggests that the effect of insulin is greater when the initial pH_i is lower.

The concentration of insulin used in these experiments, 250 mU/ml, is that required to produce many other effects of insulin in frog skeletal muscle (2). In order to rule out a non-specific protein effect, or a generalized hormone effect, both albumin and growth hormone were tested at the same concentration, 2×10^{-6} M, as the insulin. As seen in Table 1, neither of these proteins produced a significant effect upon pH_i.

Discussion. Insulin clearly increases intracellular pH as determined by [^{14}C]DMO, although this increase appears not to be mediated directly by the sodium pump as originally predicted (2). The effect of insulin upon pH_i appears to become greater if the cell is made slightly acidic. This is consistent with the observation (8,9) that the restoration of pH_i after acid loading is proportional to the induced depression of pH_i, and suggests that the same membrane ionic transport mechanisms (i.e., Na:H exchange and/or HCO_3^-:Cl^- exchange) may be responsible.

Most investigators are convinced that the effects of insulin are not mediated by cyclic nucleotides (1). In frog skeletal muscle, the stimulation of glycolysis by insulin probably results from an enhancement (13,14) of the

activity of phosphofructokinase (E.C. 2.7.1.11), a key rate-limiting enzyme of glycolysis. Nearly maximal activation of phosphofructokinase extracted from frog skeletal muscle can be produced by pH elevations as small as 0.1-0.2 units (15). Thus, the small insulin-induced increase in pH_i reported here is of sufficient magnitude to have an important stimulatory effect upon the activity of phosphofructokinase.

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