STIMULATION OF Na:H EXCHANGE BY INSULIN

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ABSTRACT In frog skeletal muscle, the increase of intracellular pH (pH_i) induced by insulin is correlated with an increase in intracellular Na^+ when the sodium pump is inhibited by ouabain. Reversing the Na^+ free energy gradient by substituting either Mg^{2+} or choline for extracellular Na^+ converts the effect of insulin to a decrease in pH_i, indicating that the action of insulin upon pH_i is determined by the Na^+ free energy gradient. Moreover, estimates of the Na^+ free energy gradient indicate that both the direction and magnitude satisfy the hypothesis that this is the source of energy for the observed changes in pH_i. Both the increase in intracellular pH induced by insulin and the associated increase in intracellular Na^+ produced by this hormone in the presence of ouabain are blocked by amiloride. This drug also blocks the decrease in pH_i by insulin when Mg^{2+} is substituted for Na^+ in the Ringer. In Ringer containing Na^+, the increase in pH_i by insulin occurs when both metabolic and atmospheric sources of CO_2 are eliminated by using a 100% N_2 atmosphere. Thus, the mechanism stimulated by insulin is not a Na^+-CO_3^{2-} cotransport system, but is either an Na:H exchange or a Na^+-OH^- cotransport system which can be inhibited by amiloride. The suggestion is advanced that the Na:H exchange mechanism is part of the membrane transduction system for insulin.

INTRODUCTION

In frog skeletal muscle, insulin increases intracellular pH (pH_i) by 0.1 to 0.2 units (1). Since both the effect of insulin upon pH_i and the action of this hormone upon glycolysis are blocked by either amiloride or lowered extracellular Na^+ (2), it was suggested that insulin activates an Na:H exchange system in the plasma membrane. If this hypothesis is correct, the following should be observed: (*a*) The elevation of pH_i should be associated with an increased influx of Na^+. In the presence of sufficient ouabain to inhibit the stimulation of the Na pump by insulin (3), this increased Na^+ influx would result in an increase in intracellular Na^+ (Na_i^+), which should be correlated with the increase in pH_i produced by insulin. (*b*) Since reversing the Na^+ free energy gradient across the membrane would reverse the direction of Na:H exchange, removing extracellular Na^+ should convert the action of insulin from an increase to a decrease in pH_i. (*c*) If amiloride does indeed block Na:H exchange, this drug should block all the above effects of insulin.

The present studies verify the above predictions. Moreover, they demonstrate that insulin also elevates pH_i in Ringer containing Na^+, but free of CO_3^{2-} ions, indicating that the mechanism stimulated is not a Na^+-CO_3^{2-} cotransport mechanism, but is either a Na^+-OH^- cotransport or an Na:H exchange system.

METHODS

All experiments were conducted at a constant temperature of 20 to 21 °C. Paired sartorius muscles from the frog *Rana pipiens* were used throughout. Muscles weighing < 100 mg and usually 40–60 mg were

removed from healthy frogs which had been stored at 22 °C and force fed liver three times per week. After passing microscopic checks for damage, the muscles were mounted at rest length on platinum frames (4) and kept in Na-Ringer at room temperature for 2–3 h before each experiment.

Na-Ringer contained 104 mM Na^+, 2.5 mM K^+, 2 mM Ca^{2+}, and 1.6 mM HPO_4^{2-}; the remaining anion was Cl^-. Na-free Ringer was prepared by substituting 104 mM Li^+, 104 mM choline, or 75 mM Mg^{2+} for Na^+; 50 μM *d*-tubocurarine was added to the choline Ringer to prevent twitching. All Ringer was glucose-free and was titrated to a pH of 7.40 ± 0.03. When ouabain was used, its concentration was 1 mM; when amiloride was used, its concentration was 0.5 mM. To avoid any complications which might be due to Cl^-:HCO_3^- exchange (5–7), $H_2PO_4^-/HPO_4^{2-}$ was chosen as the only buffer. In the experiment where lactate production was determined, the Ringer also contained 10 μM l-epinephrine bitartarate to increase glycogen breakdown to ensure sufficient substrate for glycolysis.

In those experiments using ouabain or amiloride, the dissected muscles were first placed in Ringer containing the drug for 20 min before addition of insulin to the experimental muscle; the drug was present in the Ringer of all subsequent steps. In those experiments using Na-free Ringer, muscles were first placed in the Na-free Ringer for 1 min, followed by 10 min in a second tube to clear most of the Na^+ from the extracellular space.

For all subsequent steps (except where explicitly indicated), all solutions in which the experimental muscles were placed contained 250 mU/ml insulin. The control muscles were treated identically except for the absence of insulin. The experimental muscles were equilibrated with insulin in Ringer for 20 min before placing them, and their paired controls, in Ringer containing [^{14}C]5,5-dimethyl-2,4-oxazolidinedione (DMO) and [^{3}H]sucrose for 90 min to prepare both muscles for determination of pH_i by the method of Waddell and Butler (8). Sufficient unlabeled DMO was present to bring the total concentration of DMO to 1 mM. The [^{14}C]DMO and [^{3}H]sucrose were then washed out of the muscle by transfer through a series of three tubes of Ringer containing the same concentration of ions and hormone as the loading solutions, over a period of 140 min, which removes more than 99% of the [^{14}C]DMO (9).

In those experiments using Na-Ringer, immediately after the isotope washout period, the muscles were placed in Na-free Ringer containing ouabain (10) without insulin for an additional 20 min to clear the extracellular space of Na^+. Muscles were then blotted and weighed. Dry weights were determined by reweighing after drying the muscles for 14 h at 105 °C. The dried muscles were then ashed and intracellular Na^+ was assayed by flame emission as described previously (10).

Aliquots of Ringer from the washout tubes were counted by liquid scintillation as described in reference 1, and pH_i was calculated from the equation in reference 8.

Except where indicated, experiments were aerobic. The anaerobic experiments were conducted in 100% N_2 in glove bags. Lactate production was determined using lactic dehydrogenase (E.C. 1.1.1.27) to convert lactic acid and NAD^+ to NADH and pyruvic acid as described previously (2). All results are presented as the mean ± the standard error.

The insulin used was porcine insulin (0.00% Zn; 25.9 U/mg), which was a gift from Eli Lilly and Company (Indianapolis, Ind.). Ouabain, *d*-tubocurarine, and l-epinephrine bitartarate were obtained from Sigma Chemical Co. (St. Louis, Mo.). [^{14}C]DMO and [^{3}H] sucrose were obtained from New England Nuclear (Boston, Mass.). Amiloride was a gift from Merck, Sharp and Dohme Research Laboratories (West Point, Pa.).

RESULTS

The diuretic drug amiloride (3,5-diamino-6-chloropyrazinoyl-guanidine) has no effect upon the Na pump (11), but evidence suggests that it blocks Na:H exchange (12, 13). In the presence of 5% CO_2/30 mM HCO_3^-, 0.5 mM amiloride blocks the effect of insulin upon pH_i (2). To test whether amiloride still blocks the elevation of pH_i by insulin in the absence of this buffer system, both muscles of a pair were placed in Ringer containing 0.5 mM amiloride for 20 min before addition of insulin to the experimental muscle. The results shown in Table I

TABLE I

EFFECT OF INSULIN ON INTRACELLULAR pH AND INTRACELLULAR SODIUM

Ringer	n	Intracellular pH			Intracellular sodium Na_i^+ (*mmol/kg wet wt*) {α^i_{Na} (*mM*)}			Calculated α^i_{Na} for $\langle \Delta G \rangle_{Na:H} = 0$	
		Control	Effect of insulin	P	Control	With insulin	P	Control	With insulin
								(mM)	
Na^+ with amiloride	8	7.33 ± 0.04	+0.020 ± 0.017	>0.25	9.49 ± 0.61 {<10.8}	7.81 ± 0.82 {<8.8}	<0.1	98	62
Na^+ with ouabain	13	7.40 ± 0.03	+0.069 ± 0.018	<0.01	39.95 ± 2.83 {<45}	51.64 ± 2.04 {<58}	<0.001	83	71
Li^+	10	7.28 ± 0.02	+0.146 ± 0.021	<0.005	2.21 ± 0.21 {>2.5}	1.59 ± 0.08 {>1.8}	<0.01		
Mg^{2+}	10	7.41 ± 0.07	−0.082 ± 0.014	<0.005	1.55 ± 0.10 {>1.7}	1.18 ± 0.16 {>1.3}	<0.05	0.09	0.11
choline	10	7.30 ± 0.04	−0.058 ± 0.014	<0.005	2.79 ± 0.41 {>3.1}	1.66 ± 0.13 {>1.8}	<0.025	0.12	0.14
Mg^{2+} with amiloride	12	7.19 ± 0.03	+0.018 ± 0.021	>0.25	3.05 ± 0.81 {>3.4}	2.32 ± 0.44 {>2.6}		0.16	0.15
Na^+ under nitrogen atmosphere	6	7.56 ± 0.02	+0.118 ± 0.035	<0.025					

The main cation in the Ringer is indicated in the left-hand column. n is the number of pairs of muscles observed for the given experimental condition. The effect is the difference between pH_i of the experimental muscle and its paired control muscle. Na_i^+ is expressed in millimoles Na^+ per Kilogram wet weight. α^i_{Na} is estimated by converting Na_i^+ to $[Na^+]_i$ using the average dry weight in these experiments (18%), and an estimate for muscles of this size of extracellular space (24%) (10), and then multiplying by an intracellular activity coefficient, γ^i_{Na} of 0.66 (15). In calculating the minimum value of α^i_{Na} which would make $\langle \Delta G \rangle_{Na:H} = 0$ by Eq. 1 in the Na-free Ringer, a value of 0.12 mM for $[Na^+]_o$ was used. This represents the mean of the Na^+ levels in the Ringer, determined by flame photometry, due to contaminants, and the value of 0.2 mM, due to addition of Na^+ diffusing from the muscles during the 90-min [^{14}C]DMO equilibration period. A value of 0.8 was used for γ^o_{Na} (16). In Na-Ringer, values of α^i_{Na} less than those listed in the table and in Na-free Ringer, values of α^i_{Na} greater than those listed in the table would result in a negative free energy change for operation of a $1Na^+:1H^+$ exchange system in the direction required to produce the observed change in pH_i. The t-test for ΔpH_i is for the mean of paired samples and for ΔNa_i^+ is for the difference of the means.

demonstrate that, in Ringer lacking CO_2/HCO_3^-, amiloride still blocks the action of insulin upon pH_i. In an identical Ringer lacking amiloride, insulin significantly (P <0.001) increased pH_i by 0.096 ± 0.016 (1). This is consistent with the hypothesis that the elevation of pH_i by insulin may be due to an Na:H exchange mechanism.

If the elevation of pH_i is driven by an influx of Na^+, this influx will produce an increase in Na_i^+, provided the Na pump is inhibited to prevent this Na^+ from being immediately pumped back out of the cell. 1 mM ouabain inhibits the Na pump even in the presence of insulin (3). To test whether ouabain itself affects pH_i, a series of experiments were performed in which 1 mM ouabain was added to one muscle of each pair. In 14 such pairs, the average difference in pH_i between the ouabain-treated muscle and its paired control, −0.019 ± 0.017, was not significant (P >0.25). This result is the same that Roos (14) obtained for the effect of ouabain upon rat skeletal muscle.

In the presence of ouabain, insulin still produced a significant increase in pH_i (Table I). In these same 13 pairs, where both muscles were exposed to ouabain, the total exposure to insulin (250 min; see the legend of Fig. 1 for a discussion) produced an average increase of 11.7 ± 2.3

mmol Na^+/kg in intracellular Na_i^+ ($P < 0.005$) compared with the paired controls. Fig. 1 illustrates the change in pH_i produced by insulin in each muscle plotted against the elevation in Na_i^+ produced by the hormone in the same muscle. The elevation in pH_i is positively correlated ($r = 0.689$, $P < 0.01$) with the increment of Na_i^+ produced by insulin, and the slope by a least squares fit, $(5.3 \pm 2.3) \times 10^{-3}$ pH units•(mmol Na^+/kg)$^{-1}$, is significantly greater than zero ($P < 0.05$).

Since amiloride blocks the increase in pH_i produced by insulin, it follows that it should also block the observed increase in Na_i^+ if this increase is also due to activation of an Na:H exchange mechanism. In nine experiments in which both muscles of a pair were exposed to 1 mM ouabain and 0.5 mM amiloride for 20 min before addition of insulin to the experimental muscle, the increase in Na_i^+ upon exposure of the muscles to the hormone for 90 min in the presence of both ouabain and amiloride was blocked ($P < 0.005$), producing an average increase of only 0.5 ± 3.0 mmol Na^+/kg ($P > 0.5$) compared with the paired controls, thus confirming this prediction.

If the efflux of acid is driven by the free energy gradient for Na^+ across the plasma membrane, the effect of insulin on acid transport should be reversed in a Na-free Ringer. However, when Na^+ in the Ringer was replaced by Li^+, insulin still produced an increase in pH_i, which was significantly greater ($P < 0.05$) than the elevation observed in Na-Ringer. This indicates either that Li^+ can substitute for Na^+ in the Na:H exchange, or that some other mechanism is involved.

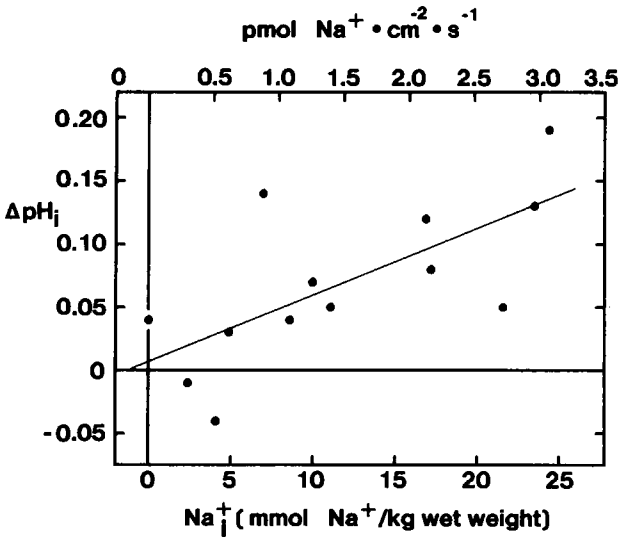

FIGURE 1 Change in pH_i versus elevation of Na_i^+ produced by insulin in Ringer containing 1 mM ouabain. The increase in Na_i^+ is in millimoles per Kilogram (per 250 min) on the bottom abscissa. Using a figure of 552 cm^2/g muscle wet weight (17), this is converted to pmol × $cm^{-2}s^{-1}$ on the top abscissa. The plotted line is a linear regression of the data (see text). It might seem that the slope, $(5.3 \pm 2.3) \times 10^{-3}$ pH units•(mmol Na^+/kg)$^{-1}$, could be used to estimate intracellular buffering power, assuming a 1:1 coupling between Na^+ influx and H^+ efflux. However, because of the experimental protocol (see Methods), the ΔpH_i is determined 110 min after addition of insulin, whereas ΔNa_i^+ is determined 250 min after insulin addition. Moreover, as determined by ^{31}P NMR, ΔpH_i may have reached a steady state within 40 min (18). It is therefore not possible to reliably use the present data to estimate intracellular buffering power.

When either Mg^{2+} or choline was used to replace the Na^+ in the Ringer, the effect of insulin upon pH_i was converted to a statistically significant decrease (Table I). It might be argued that the decrease in pH_i in these experimental conditions was due to insulin stimulation of glycolysis with a resulting increase in metabolic production of protons. However, in seven pairs of muscles using the Mg-Ringer under identical experimental conditions, except for a 100% N_2 atmosphere, anaerobic lactate production was decreased 49% ± 13% ($P < 0.01$) by insulin. Therefore, the decrease in pH_i most probably is due to an influx of protons into the cell. This reversal of the proton flux caused by reversing the Na^+ free energy gradient further supports the hypothesis that the change in pH_i is due to operation of an Na:H exchange system in the plasma membrane.

If this hypothesis is true, and if amiloride blocks Na:H exchange, the effect of insulin in Na-free Ringer to depress pH_i should also be blocked by this drug. As seen in Table I, the presence of 0.5 mM amiloride in Mg-Ringer inhibited the effect of insulin upon pH_i.

The results observed in the present study could be due to the operation of a Na^+-CO_3^{2-} cotransport system (19). Although HCO_3^- was not added to the Ringer in the above experiments, some bicarbonate, and therefore CO_3^{2-}, would be present due to atmospheric and metabolic CO_2. To eliminate these sources of CO_3^{2-}, the effect of insulin in Na-Ringer in the presence of a 100% N_2 atmosphere was determined. As seen in Table I, even in the virtual absence of CO_2, where only trace amounts of CO_3^{2-} should be present, insulin still produced a significant increase in pH_i, thus ruling out the possibility that the effect is mediated by a Na^+-CO_3^{2-} cotransport system. A similar elevation of pH_i in a N_2 atmosphere has been observed using a Ringer of the same composition, except for the addition of 1.2 mM Mg^{2+}, 1.9 mM SO_4^{2-}, and 1 mM ascorbate (2).

DISCUSSION

To conclude that the observed changes in pH_i are indeed due to an Na:H exchange system rather than some other mechanism which requires Na^+, one must demonstrate that, under these experimental conditions, sufficient energy is available from the Na^+ free energy gradient across the plasma membrane to produce the observed changes in pH_i. If the coupling ratio between protons and Na^+ is assumed to be $1Na^+:1H^+$, the average free-energy change for an Na:H exchange which couples Na^+ influx to proton efflux is described by:

$$\langle \Delta G \rangle_{Na:H} = kT \ln (\alpha^i_{Na} / \alpha^o_{Na}) - kT \ln (\alpha^i_H / \alpha^o_H), \quad (1)$$

where k is the Boltzmann constant, T is the absolute temperature, α^i_{Na} (α^o_{Na}) is the intracellular (extracellular) Na^+ activity, and α^i_H (α^o_H) is the intracellular (extracellular) H^+ activity.

This mechanism will transport protons outward when $\langle \Delta G \rangle_{Na:H} < 0$, and will transport protons inward when $\langle \Delta G \rangle_{Na:H} > 0$. Thus, determination of the experimental variables in Eq. 1 must yield negative values of $\langle \Delta G \rangle_{Na:H}$ for those experiments in Na-Ringer where insulin increases pH_i and positive values for those using Na-free Ringer where insulin decreases pH_i. Put another way, it must be verified that in 104 mM Na^+, when pH_i is increased by insulin, α^i_{Na} is sufficiently low for $\langle \Delta G \rangle_{Na:H}$ to be negative; and that in Na-free Ringer, when pH_i is decreased by insulin, α^i_{Na} is sufficiently high for $\langle \Delta G \rangle_{Na:H}$ to be positive.

Because Na_i^+ could not be determined until 140 min after determination of pH_i (see

Methods), the measured values of Na_i^+ are expected to be erroneously high estimates of the Na_i^+ present at the time pH_i was actually determined in Na-Ringer containing ouabain. Similarly, the measured Na_i^+ will be erroneously low estimates in those experiments conducted in Na-free Ringer. Both errors would tend to lead to underestimates of the magnitude of $<\Delta G>_{Na:H}$ available to drive the ΔpH_i in the direction observed. In the absence of detailed information concerning compartmentalization of intracellular protons, α_H^i, as determined by [^{14}C]DMO, is substituted for α_H^i in Eq. 1. In Table I it is seen that in Na-Ringer with or without ouabain, where insulin elevated pH_i, the estimated values of α_{Na}^i based upon measured Na_i^+ are less than those calculated from Eq. 1, assuming $<\Delta G>_{Na:H} = 0$; and in both Mg-Ringer and choline Ringer, where insulin decreased pH_i, the estimated values of α_{Na}^i based upon measured Na_i^+ are considerably greater than those calculated, assuming $<\Delta G>_{Na:H} = 0$. In other words, for the observed flux of protons, the calculated value of α_{Na}^i is of the proper magnitude for the free energy change of an Na:H exchange process to be negative in each case, indicating that no energy source other than the concentration, or activity, gradient for Na_i^+ is required to account for all experimentally observed changes in pH_i.

Therefore it may be concluded that the change in pH_i produced by insulin is driven by the Na^+ concentration gradient across the plasma membrane since: (*a*) The increase in pH_i is associated with an influx of Na^+ and the magnitudes of the two changes are correlated. (*b*) The reversal of the Na^+ concentration gradient reverses the direction of the pH_i change. (*c*)

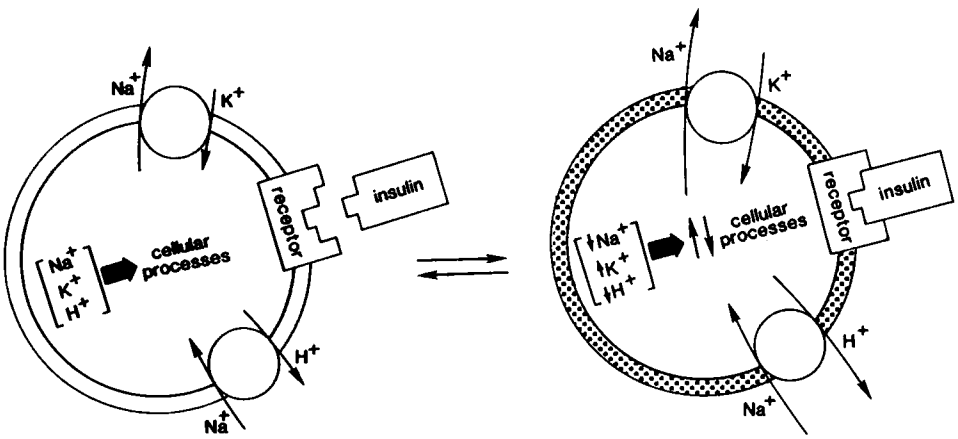

FIGURE 2 The model for the insulin transduction system to mediate the effect of insulin upon glycolysis and perhaps upon other intracellular processes. In this model, the binding of insulin to its receptors on the plasma membrane sends a signal, presumably intramembrane, to stimulate (presumably by decreasing the activation energy) the Na:H exchange system. This system utilizes the free energy gradient for Na^+ to transport protons (or OH^-) against their free energy gradient. Under physiological conditions, the Na^+ flux through the exchange system is inward and supplies free energy to transport protons outward against their free energy gradient. Na_i^+ is prevented from increasing, and indeed may decrease (see Table I), by the simultaneous stimulation of the Na pump by insulin (3, 10). To observe the increased Na^+ influx, ouabain was used in the present experiments to prevent Na^+ from being extruded by the Na pump back out of the cell. By the simple expedient of removing Na_o^+, the free energy gradient for Na^+ is reversed, which should result in reversal of the Na:H exchange mechanism. Under these conditions, activation of this system by insulin results in a net proton influx rather than efflux and thus decreases, rather than increases, pH_i.

Amiloride blocks both (*a*) and (*b*). (*d*) $<\Delta G>_{Na:H}$ has the appropriate sign to produce the observed change in pH_i, as is indicated by comparison of estimates of α^i_{Na} (based upon measured values of Na_i^+) with values calculated from Eq. 1 with $<\Delta G>_{Na:H} = 0$.

The change in pH_i produced by insulin in the present experiments is not due to a Na^+-CO_3^{2-} cotransport system, since the change in pH_i is not affected by the virtual absence of the CO_3^{2-} ion. Therefore, these results would seem to demonstrate the operation of an Na:H exchange system (or a Na^+-OH^- cotransport system, which in these experiments would be operationally indistinguishable) as opposed to a Na^+-CO_3^{2-} cotransport system.

There is evidence that the elevation of pH_i produced by insulin may be a signal whereby this hormone stimulates glycolysis (2). The finding that insulin produces a decrease in glycolysis under experimental conditions (Mg-Ringer) where the effect of the hormone also decreases pH_i supports this hypothesis for insulin action. This suggests that the Na:H exchange mechanism, together with the Na pump, may constitute part of the membrane transduction system for this hormone. In this model (Fig. 2), the two systems work in series. Stimulation of the Na pump by insulin prevents Na_i^+ from rising secondary to the stimulation of the Na:H exchange process. Thus, the free energy gradient required to operate the Na:H exchange mechanism is maintained and pH_i is elevated.

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