Insulin Depolarization of Skeletal Muscle in Absence of External Na^+

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Three mechanisms have been proposed by which insulin might increase the electrical potential difference across the cell membrane of some of its main target cells: stimulation of an electrogenic pump; increased permeability to K^+ (P_K); and decreased ratio of permeability to Na^+ (P_{Na}) compared to P_K, with an absolute decrease in permeability to both ions. Our laboratory has reported that insulin-induced hyperpolarization (IIH) of rat skeletal muscle is not due to stimulation of a ouabain-inhibitable pump and that insulin decreases ^{42}K efflux, apparently eliminating the first two candidate mechanisms. If the remaining hypothesis is correct, when Na^+ is removed from the bathing solution, insulin should depolarize, not hyperpolarize. It did. With Tris or *N*-methyl-D-glucamine substituted for Na^+, insulin depolarized by ~3 mV. Ouabain had no effect. P_{Na} decreased by >90%; P_K was reduced by <40%. The main component of the immediate mechanism of IIH is the near elimination of P_{Na}. Furthermore, when a poorly permeable cation was substituted for Na^+, muscles hyperpolarized in the absence of insulin. This gave us an opportunity to test the hypothesis that hyperpolarization is a link in the insulin-transduction chain. Consistent with this hypothesis, rat muscles hyperpolarized in this manner in the absence of insulin took up more glucose than paired controls in normal Na^+ solution. *Diabetes* 38:333–37, 1989

Insulin hyperpolarizes skeletal muscle (1–7), cardiac muscle (8,9), and adipocytes (10–12). The immediate mechanism has been disputed.

Insulin-induced hyperpolarization (IIH) has been attributed to stimulation of a ouabain-inhibitable electrogenic Na^+-K^+–exchange pump (3,13), although others (4,8,14) find that ouabain does not alter IIH. Our laboratory reported that when ouabain was added to solutions bathing rat skeletal muscle at a concentration and for a length of time adequate to prevent completely the hyperpolarization produced by isoproterenol, there was no effect on IIH (14). Thus, in rat skeletal muscle bathed in a Krebs-Ringer HCO_3 solution with normal K^+ concentration, IIH cannot be attributed to stimulation of a ouabain-inhibitable electrogenic pump. We suggested that in cases in which IIH has been reported to be blocked or reversed by ouabain, the investigators used too much ouabain for too long and were observing secondary effects. [We stipulate that this conclusion rests on studies in which extracellular [K^+] was normal, because the evidence is otherwise when rat caudofemoralis muscle was bathed in a [K^+] incompatible with life, 38.4 mM (15). At this high [K^+], insulin hyperpolarized to a value more negative than the K^+ equilibrium potential, a result that can be explained only by stimulation of an electrogenic pump, but this occurred only if muscles were treated with insulin while they were still held in normal [K^+] solution, before exposure to high [K^+]. The relevance of this observation to the mechanism of IIH at normal [K^+] is not apparent. It has not been reported that in normal [K^+] insulin hyperpolarized to a value more negative than the K^+ equilibrium potential (E_K).]

IIH has also been attributed to increased K^+ conductance in chick embryonic heart cells (8), but others have data interpreted as showing decreased K^+ conductance in frog muscle (7) or decreased permeability to K^+. Data from our laboratory showed that insulin decreased $^{42}K^+$ efflux and permeability of mammalian skeletal muscle to K^+ (P_K) (16).

Finally, IIH has been explained on the basis of a relatively greater decrease in permeability to Na^+ (P_{Na}) than to K^+ (17); i.e., IIH is caused by reduction of the ratio P_{Na}/P_K with a consequent increase in electrical polarization toward, but not exceeding, E_K. Owing to the complicated distribution of

Glucose 1 mM = 18 mg/dl	Insulin 1 pM = 0.139 μU/ml

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Na^+ in muscle and the probable lack of uniformity of its intracellular concentration (18), we have not been able to estimate P_{Na} from experiments in which radiosodium fluxes were measured. Therefore, we have turned to other methods.

If insulin decreases the ratio P_{Na}/P_K, then when muscles are bathed in a Na^+-free solution, insulin should depolarize, provided that at least one other ion in which permeability is not affected by insulin contributes to resting potential (V_m) in accordance with the following scheme, based on the classic Goldman-Hodgkin-Katz formulation

$$V_m = -(RT/F)\ln(N/D)$$

where $N = P_K[K^+]_i + P_{Na}[Na^+]_i + P_{Cl}[Cl^-]_o + P_B[B^+]_i$, $D = P_K[K^+]_o + P_{Cl}[Cl^-]_i + P_B[B^+]_o$, R is the gas constant, T the absolute temperature, F the Faraday constant, subscripts i and o refer to intra- and extracellular concentrations, respectively, and B^+ is the cation substituted for Na^+.

It has been generally but not universally held, largely on the basis of experiments on frog muscle, that there is no evidence of a Cl^- pump in skeletal muscle; Cl^- is said to be distributed passively between extra- and intramuscular solution (19); i.e., the Cl^- equilibrium potential is determined by the membrane potential. Therefore, the modified Goldman-Hodgkin-Katz equation can be, and often is, rewritten for skeletal muscle with the Cl^- terms omitted. If $P_B = 0$, membrane potential would be essentially the K^+ equilibrium potential, and no matter what insulin does to P_K, insulin would not alter the membrane potential. However, if P_B is not 0 and if insulin has no effect on P_B but does decrease P_K, insulin should depolarize. On the other hand, if insulin were to increase P_K, then there should still be hyperpolarization. Although we know from previous experiments on rat skeletal muscle that insulin does not stimulate the ouabain-inhibitable pump, for the sake of argument, let us suppose that it does; then, because $[K]_o$ is normal, if the removal of $[Na]_o$ did not alter $[Na]_i$, insulin would still hyperpolarize, and if removal of $[Na]_o$ led to depletion of $[Na]_i$, then because the pump ratio (Na^+ out/K^+ in) can be sensitive to $[Na]_i$, insulin might have no effect on membrane potential if the pump ratio becomes unity or might depolarize if the pump ratio inverts. We report here that insulin depolarized muscles bathed in Na^+-free solutions.

The equation also tells us that if P_B is less than P_{Na}, the membrane potential should be more polarized in Na^+-free solution than in normal Na^+ solution. When we found that this was the case, we were provided an opportunity to test the hypothesis that hyperpolarization is a step in the insulin-transduction chain leading to glucose uptake (17). We found that the muscles hyperpolarized in Na^+-free solution in the absence of insulin took up more glucose than paired controls in normal Na^+ solution, consonant with our hypothesis and previous experiments (20).

MATERIALS AND METHODS

Experiments were carried out on excised caudofemoralis muscles (20) from Sprague-Dawley male rats (90–160 g, Charles River, Wilmington, MA) by measurement of V_m with conventional glass pipettes filled with 3 M KCl, leading to Ag-AgCl electrodes and to a WPI electrometer. Pipette resistance was 5–30 MΩ. For the Na^+-free Tris [tris(hydroxymethyl)aminomethane; Sigma, St. Louis, MO] experiments, freshly dissected muscles were placed in a normal Na^+-Tris solution, pH 7.4, at 23–24° and gassed with 100% O_2 until ready for mounting in the chamber. The solution was changed to Na^+ free, the muscle was gassed with 100% O_2, and after ~25 min, impalements and recording began and continued for ~10 min. For the Na^+-free *N*-methyl-D-glucamine (Sigma) experiments, freshly dissected muscles were placed immediately in the Na^+-free solution, where they equilibrated for 30 min before impalements began. From then on, protocols were similar for both types of Na^+-free solution. The solution was either not changed or changed to one of the same composition plus either valinomycin (Sigma), 9-anthracenecarboxylic acid (Aldrich, Milwaukee, WI), tetrodotoxin, α-bungarotoxin (both generous gifts of A. Sastre, Dept. of Physiology, The Johns Hopkins Univ. School of Medicine, Baltimore, MD), or insulin (porcine crystalline zinc insulin, 26 U/mg; generous gift of Lilly, Indianapolis, IN). The muscle was gassed for ~5 min. Impalements and recording of membrane potentials continued for another ~10 min. More than a year after the first series of experiments, we repeated them in part and also modified the protocol to add a third experimental period to test possible effects of ouabain (ouabain octahydrate, Sigma) on the changes in membrane potential produced by Na^+-free solution and insulin. Details are given in the text.

Normal Na^+-Tris solution had the following composition: 135 mM NaCl, 3.5 mM KCl, 1.3 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 5 mM glucose, 10 mM Tris base, and 6.9 mM HCl, pH 7.4. The Na^+-free Tris solution had the following composition: 3.5 mM KCl, 1.3 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 145 mM Tris base, 108 mM HCl (with which Tris base was titrated to pH 7.4), 5 mM glucose, and 18 mM sucrose (to yield a final osmolality of 276 mosmol/kg). The Na^+-free *N*-methyl-D-glucamine solution was the same except that 145 mM *N*-methyl-D-glucamine was substituted for Tris and pH was adjusted to 7.4 with HCl. No sucrose was required. Final osmolality was 280 mosmol/kg.

Approximately 20 measurements of V_m were made in each period. Estimates of standard error of the mean were based on the number of muscles, not on the total number of impalements. Data from first and second periods were analyzed as paired differences.

Glucose uptake was measured as follows. Extensor digitorum longus (EDL) muscles were excised from rats killed by guillotine immediately before dissection. (Caudofemoralis muscles could not be used for these experiments because the distal tendon, which attaches to the knee, is too short to accept a ligature, and part of the knee is removed with the muscle to preserve the attachment. Because the preparation includes bone, it is unsuitable for studies of glucose uptake by muscle.) Muscles were placed in a chamber at room temperature (25°C) in one of four solutions (normal Na^+-Tris, normal Na^+-Tris plus insulin, Na^+-free Tris, or Na^+-free Tris plus insulin). The two muscles from each rat were placed in different solutions so that the results could be paired. After ~1 h, the solutions were removed and replaced by fresh solution of the same composition to which were added tracer quantities of 2-deoxy-D-[^{3}H]glucose (2-DG) and [^{14}C]sucrose (both from New England Nuclear, Boston, MA), so that each isotope provided ~2×10^6 dpm/ml of solution. After another

TABLE 1
Effect of insulin on membrane potential of rat caudofemoralis muscles in Na^+-free solution

Na^+ substitute	Addition in 2nd period	Resting membrane potential (mV)		
		1st period	2nd period	Paired difference
Tris	None	−82.3 ± 1.8 (4)	−82.6 ± 2.5	NS
Tris	Valinomycin	−83.2 ± 0.6 (6)	−89.6 ± 0.4	−6.4 ± 0.56
Tris	9-ACA	−82.5 ± 0.8 (7)	−81.9 ± 0.7	NS
Tris	Insulin	−82.8 ± 0.9 (8)	−79.3 ± 0.8	3.5 ± 0.86
NMG	Insulin	−84.5 ± 0.6 (6)	−81.7 ± 0.3	2.8 ± 0.60

Values are means ± SE. Number of muscles in parentheses. SE is based on number of muscles, not number of fibers impaled. Each period was ~10 min, and during each there were ~20 measurements of membrane potential. Na^+ substitute was either Tris or *N*-methylglucamine (NMG). There were no additions during the 1st period. Concentrations of additions during the 2nd period were 60 nM valinomycin, 1 mU/ml insulin, or 100 μM 9-anthracenecarboxylic acid (9-ACA). NS, not significant.

1 h, samples of solutions were removed for counting, and the rest of the solution was removed from each chamber. Muscles were washed quickly with the corresponding tracer-free solution, then removed from the chamber, tendons cut off, blotted dry, weighed rapidly on a torsion balance, placed in 1 ml distilled water, and held in a cold room for 15–24 h. This procedure extracts intracellular 2-DG quantitatively (20). After extraction of intracellular 2-DG, 0.5-ml samples of the water were added to 5 ml Aquasol-2 (New England Nuclear) in minivials, and ^3H and ^{14}C were measured in a liquid-scintillation counter. The [^{14}C]sucrose counts were used to correct ^3H dpm for extracellular 2-DG. Total glucose uptake in picomoles per minute per milligram muscle wet weight was calculated on the assumption that 2-DG uptake traced D-glucose uptake.

RESULTS

Data on V_m in Na^+-free solutions appear in Table 1. In Na^+-free Tris solutions, there was no effect of time in the absence of insulin. V_ms during the first period in the Na^+-free Tris solution were the same as those during the second period, averaging −82.4 mV. This is hyperpolarized with respect to the V_m our laboratory has reported previously (−78 mV) for rat caudofemoralis muscle in Krebs-Ringer bicarbonate solution with normal Na^+ concentration. The increase in polarization is attributable to absence of extracellular Na^+.

However, V_m was less polarized than E_K, as shown by the response to valinomycin, an agent that increases permeability to K^+ to such an extent that V_m becomes E_K. After measurements of V_m were made in a series of six muscles during the first period in Na^+-free solution, 60 nM valinomycin was added, and a second period of V_m measurement followed. In the first period, V_m was −83.2 mV, similar to the other values of V_m under these conditions. During the first 5 min after valinomycin, V_m was −89.6 mV, indistinguishable from the value we have found in another series of experiments on effects of valinomycin in Krebs-Ringer bicarbonate solution with normal Na^+ concentration and in agreement with the predicted E_K for this muscle. The fact that V_m in Na^+-free Tris solution was less polarized than E_K implies that permeability of the membrane to some ion besides K^+ contributes to the observed V_m and that the equilibrium potential of this other ion is less polarized than the observed V_m. The other ion or ions might be $Tris^+$ or Cl^-, although Cl^- is usually thought to be distributed passively in skeletal muscle, driven only by its electrochemical potential gradient.

The possibility that Cl^- was responsible for making V_m less polarized than E_K was rendered unlikely by results of a series of experiments on seven muscles in which the Cl^--channel blocker 9-anthracenecarboxylic acid was added to the Na^+-free Tris solution at a concentration that completely blocked Cl^- transport in rat diaphragm (21). V_m was unchanged by addition of the blocker. Therefore, we assume that V_m did not reach E_K in Na^+-free Tris solution because Tris must have entered sarcoplasm, and the Tris gradient across the membrane may then have tended to keep V_m less polarized than E_K.

There have been several reports that Tris enters cells, including skeletal muscle (22–24). Mechanism and sites of entry are unknown. We sought some information as to whether Tris might enter by way of channels used by Na^+. In experiments with the protocol given in Table 1, we found the following results. Addition of 5 μg/ml α-bungarotoxin during the second period of incubation in Na^+-free Tris had no effect. In six muscles, the paired difference in V_m was −0.5 ± 0.97 mV. Therefore, in the absence of activation by acetylcholine, the end-plate Na^+ channel is not the likely site through which Tris penetrates. Addition of 10^{-5} M tetrodotoxin, a concentration that completely blocks the voltage-

TABLE 2
Lack of effect of ouabain on membrane potential of rat caudofemoralis muscle in Na^+-free Tris solution

Bath time (min)	Additions in 2nd and 3rd periods	Membrane potential (mV)				
		1st period	2nd period	Paired difference	3rd period	Paired difference
35	Ouabain	−83.1 ± 0.5	−83.0 ± 0.6	+0.15 ± 0.26		
25	Insulin plus ouabain	−83.4 ± 0.6	−80.2 ± 0.8	+3.21 ± 0.64	−80.0 ± 0.4	+0.20 ± 0.85

Bath time is equilibration period in Na^+-free solution before 1st period.

sensitive Na^+ channel in rat skeletal muscle, had no effect. In six muscles the paired difference in V_m was 0.8 ± 0.6 mV.

Given the likelihood that Tris penetrates the muscle fibers, although not by routes inhibitable by α-bungarotoxin or tetrodotoxin in the resting state, and the observation from $^{42}K^+$ flux studies that insulin decreases permeability to K^+ (16), the hypothesis predicts that insulin will depolarize muscle bathed in Na^+-free Tris solution. In a series of eight rat muscles incubated in Na^+-free solution without insulin during the first period and incubated in Na^+-free solution with insulin during the second period, insulin depolarized significantly, by 3.5 mV.

In six muscles bathed in the Na^+-free *N*-methylglucamine solution, V_m was −84.5 mV, slightly but significantly more polarized than in Na^+-free Tris and significantly less polarized than E_K (Table 1). As was the case for Tris, this strongly suggests that *N*-methylglucamine is at least a slightly permeant cation, although its permeability coefficient appears to be less than that of Tris. When insulin was added to the Na^+-free *N*-methylglucamine solution, there was significant depolarization, by 2.8 mV.

It is possible that soaking in Na^+-free solution may have so reduced muscle Na^+ concentration as to reverse the pump ratio (pump more K^+ in than Na^+ out), and that if insulin stimulates the Na^+-K^+–exchange pump under these conditions, there would be depolarization. To study this possibility, we carried out two additional series of experiments (Table 2).

Caudofemoralis muscles were bathed in Na^+-free Tris solution for 35 min or 25 min, at the end of which ~10 fibers were impaled during the next 5–7 min (Table 2). Membrane potentials were indistinguishable from those obtained in the earlier set of experiments (Table 1). Ouabain was added to the muscles bathed for 35 min, and 10 min later there was a second set of impalements. Ouabain, at a concentration and over a period we have shown adequate to inhibit completely the hyperpolarization induced by isoproterenol (14), had no effect on the hyperpolarization observed in Na^+-free Tris (Table 2). In muscles bathed for 25 min, after the control set of impalements was complete, insulin was added. Ten minutes later there was a second set of measurements; insulin depolarized. This insulin effect was indistinguishable from that observed earlier (Table 1). With insulin still present, ouabain was added, and 10 min later there was a third set of measurements. Ouabain had no effect on the depolarization produced by insulin (Table 2).

TABLE 3
Effect of Na^+-free Tris solution on glucose uptake and insulin-stimulated glucose uptake by rat extensor digitorum longus muscle

Bath solution	Glucose uptake (pmol · min^{-1} · mg^{-1} muscle wet wt)	Paired difference (%)
Normal Na^+	45.2 ± 2.67 (20)	
Plus 1 mU/ml insulin	81.8 ± 3.56 (10)	90 ± 11
Na^+-free Tris	65.9 ± 3.95 (15)	
Plus 1 mU/ml insulin	79.2 ± 10.50 (5)	17 ± 8
Normal Na^+	45.9 ± 5.62 (10)	
Na^+-free Tris	65.3 ± 7.82 (10)	47 ± 9

Number of muscles is indicated in parentheses.

On the assumption that insulin does not affect membrane permeability to Tris or *N*-methylglucamine, we calculated insulin's effect on P_K. From the equation, neglecting intracellular Na^+ and Tris concentrations, the ratio P_B/P_K is calculated for an observed V_m. In the absence of insulin, in Na^+-free Tris, V_m was −83 mV, and calculated P_B/P_K ratio is 0.010. Insulin depolarized in Na^+-free Tris to −79.5 mV; the calculated P_B/P_K ratio became 0.016. Then, if P_B was not altered, (P_K in the presence of insulin)/(P_K in its absence) = 0.010/0.016 = 0.62, from which we conclude that insulin decreased P_K by ~38%.

With the V_m measurements in hand, we examined effects of Na^+-free solutions and insulin on glucose uptake. It has been our hypothesis that hyperpolarization is one of the steps in the chain linking insulin-receptor binding to stimulated glucose uptake (17), and we have presented experimental support for this conjecture (2,20,25). The hypothesis predicts that glucose uptake by hyperpolarized muscles in Na^+-free solution in the absence of insulin should be greater than that in normal Na^+ solutions, and that insulin should fail to stimulate glucose uptake in Na^+-free solutions.

In normal Na^+ solution, 1 mU/ml insulin nearly doubled glucose uptake by rat EDL muscles, but in Na^+-free Tris solution, it had no significant effect (Table 3). In 10 pairs of EDL muscles in the absence of insulin, glucose uptake was ~50% greater in Na^+-free Tris than in normal Na^+ solution (Table 3).

DISCUSSION

The facts that insulin depolarizes, instead of hyperpolarizing, when either $Tris^+$ or *N*-methylglucamine replaces external Na^+ and that ouabain has no effect on this insulin-induced depolarization appear incompatible with the hypothesis that insulin normally hyperpolarizes by increasing permeability to K^+ or that it hyperpolarizes by stimulating an electrogenic Na^+-K^+–exchange pump, unless circumstances caused inversion of the pump ratio. We considered the latter an unlikely scenario for two reasons: *1*) we had already reported that ouabain has no effect on IIH when muscles are bathed in normal $[K^+]_o$, and *2*) if $[Na^+]_i$, were depleted, it would likely be replaced by increased $[K^+]_i$, which would increase E_K, but we found no increase in E_K (Table 1, for valinomycin addition). We tested the effect of ouabain on insulin-induced depolarization and found none, strong evidence that insulin did not alter membrane potential by stimulation of the ouabain-sensitive electrogenic Na^+-K^+–exchange pump. If the hypothesis were correct that insulin normally hyperpolarized by increasing permeability to K^+, we would expect the substitution to have no effect on IIH. If no other information is considered, these insulin-induced depolarizations might be attributed to a relative increase in permeability to Tris and to *N*-methylglucamine rather than to, or along with, a decrease in K^+ permeability. We have no data to test a possible effect of insulin on Tris and *N*-methylglucamine distribution. However, when the results of previous studies of IIH of mammalian skeletal muscle and insulin-induced decrease in K^+ permeability (16) are put together with our results, the inference is strong that depolarization occurred because insulin decreased P_K. The result is consistent with the hypothesis that insulin hyperpolarizes muscle in normal extracellular Na^+ solutions by decreasing the ratio P_{Na}/P_K, with an absolute decrease in both permeability coefficients.

On the assumption that insulin had no effect on permeability to Tris, we calculated that insulin decreased P_K by 38%. This estimate, together with estimates of insulin effect on P_{Na}/P_K ratio in previous experiments, leads to an estimate of insulin effect on P_{Na}. In previous experiments in which rat muscles were bathed in normal Na^+ Krebs-Ringer-HCO_3 solutions (14), at $V_m = -78$ mV, $P_{Na}/P_K = 0.019$. With 100 μU/ml insulin, $V_m = -82$ mV and $P_{Na}/P_K = 0.010$. With 1000 μU/ml insulin, $V_m = -88$ mV and $P_{Na}/P_K = 0.0024$. That is, P_{Na} in controls = $0.019 \cdot P_K$ in controls, P_{Na} in insulin = $0.0015 \cdot P_K$ in controls, and P_{Na} in insulin = $0.077 \cdot P_{Na}$ in controls. Thus, whereas insulin decreases P_K by 38%, it decreases P_{Na} by 93%. Insulin almost eliminates permeability of resting muscle to Na^+.

In a study of effects of electrical hyper- and depolarization on glucose uptake by rat muscle, our laboratory reported that in the absence of insulin, small hyperpolarization increased D-glucose uptake but depolarization by the same amount had no effect (20). It is not clear whether to expect that insulin should decrease glucose uptake in Na^+-free Tris or have no effect, but it should not increase glucose uptake if the hypothesis is correct. There was no significant effect on glucose uptake. It might be argued that the absolute rate of glucose uptake in Na^+-free Tris in the presence of insulin was about as great as in normal Na^+-Tris in the presence of the same maximum concentration of insulin, and that no further uptake could be generated. For that reason, it is not yet possible to state on the basis of this set of experiments alone that hyperpolarization is a necessary step in the insulin-transduction chain.

The fact that rat skeletal muscle hyperpolarized in Na^+-free solutions gave us an opportunity to test the hypothesis that hyperpolarization is a step in the chain of events linking the insulin-receptor complex to stimulation of D-glucose uptake. If the hypothesis is correct, muscles hyperpolarized in Na^+-free Tris in the absence of insulin should take up more glucose than muscles in normal Na^+-Tris. Glucose uptake was nearly 50% greater in muscles bathed in Na^+-free Tris. In normal Na^+-Tris, insulin at what is ordinarily a concentration that produced maximum response nearly doubled glucose uptake. The effect of Na^+-free Tris on glucose uptake is about what is expected from a half-maximum concentration of insulin, and the result is consistent with the hypothesis that hyperpolarization is a step in the insulin-transduction chain leading to glucose uptake.

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