Insulin Hyperpolarizes Rat Myotube Primary Culture Without Stimulating Glucose Uptake

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SUMMARY

Insulin (100 μU/ml) increased the electrical potential difference between the inside and outside of fused myotubes from fetal rat hindlimbs 5–8 days after primary culture. Myotubes were hyperpolarized by 3.5 ± 1.2 mV (mean ± SE). Tetrodotoxin (TTX) eliminated spontaneous twitching. With TTX, membrane potentials of myotubes became more negative both in the absence and presence of insulin, and insulin hyperpolarized myotubes by 6.6 ± 1.7 mV, which is at least as great as the hyperpolarization this concentration of insulin produced in muscles excised from young adult rats. However, insulin did not significantly stimulate uptake of the glucose analogue 2-deoxy-D-glucose, although there was basal D-glucose transport. Specific insulin receptors were present, demonstrated by radioinsulin binding. In summary, in primary cultures of rat myotubes, there are insulin receptors, insulin hyperpolarizes at least as much as it hyperpolarizes muscle excised from young adult rats, but insulin does not stimulate D-glucose transport.

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Insulin increases the electrical potential difference between the inside and outside of skeletal muscle cells (1–10). The largest insulin-induced hyperpolarization (IIH) is by 8–10 mV in rat caudofemoralis muscle (10). Half-maximum IIH occurs in response to an insulin concentration of 100 μU/ml, ~0.7 nM (10). We proposed that IIH might be a link in the transduction chain between insulin-receptor association and stimulation of specific D-glucose transport (11). This hypothesis was supported by our observations that IIH occurred more rapidly (in <1 s) than any other reported response to insulin (10), that hyperpolarization produced electrically in the absence of insulin stimulated stereospecific D-glucose transport (11), and that when hyperpolarization was blocked (without blocking insulin binding to its receptor), there was a correlated decrease in insulin-stimulated glucose uptake (12). We wanted to challenge the hypothesis further by seeing whether there is a quantitative relationship between electrically produced hyperpolarization and stimulated D-glucose uptake. For this purpose we sought a cell suitable for voltage clamping and used primary cultures of rat myotubes with the expectation that eventually we would use rat myoballs,* which are suitable for voltage clamping by the whole-cell clamp technique. I report herein the existence of insulin receptors on rat myotubes in primary culture, that insulin hyperpolarizes them quantitatively by at least as much as it does excised mature muscle, but that insulin does not demonstrably stimulate glucose uptake in these cells under the conditions employed.

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MATERIALS AND METHODS

Primary cultures of hindlimb muscle were prepared from embryos from CD rats (Charles River, Wilmington, MA) on gestational day 20, according to established methods (13), with a few modifications used by Dr. A. Sastre (Department of Physiology, The Johns Hopkins University School of Medicine, Baltimore, MD), who generously advised me and allowed use of his equipment. Minced hindlimb muscles were pooled in flasks containing Dulbecco's modified Eagle's medium (DMEM) (3 parts) and medium 199 (Gibco, Grand Island, NY; 1 part) to which were added collagenase type CLS III (Cooperbiomedical, Malvern, PA; 400 U/ml), penicillin, and streptomycin. The medium contained 10% fetal calf serum. After a series of three 5- to 10-min incubations at 37°, the suspension was centrifuged, the pellet was triturated, fibroblasts were allowed to settle out, and the remaining cells were transferred to 35-mm culture dishes and incubated.

*Myoballs are spherical cells produced by treating early myotubes with colchicine.

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Medium was changed every 3rd day, and fetal calf serum was reduced to 5%. On day 3, cytosine arabinoside (10 μM) was added. The definitive experiments were carried out on days 5–8 of culture.

Solutions. The standard solution used in definitive experiments was a modified Krebs-Ringer HEPES (KRH) unless stated otherwise. Its composition was 128 mM Na, 4.8 mM K, 2.6 mM Ca, 1.2 mM Mg, 136.7 mM Cl, 1.35 mM phosphate, 1.2 mM sulfate, 20 mM HEPES, and 1 mM pyruvate. At 24°, the pH was 7.4. Osmolality was 282 mosmol/kg.

Membrane potentials. Membrane potentials were measured by impaling a myotube with a conventional glass electrode filled with 3 M KCl, contacting an Ag-AgCl pellet, and led to an electrometer (WPI KS700). The criterion for accepting a result of an impalement was that the voltage signal had to jump sharply, nearly instantaneously, from baseline to a constant value that was maintained for at least 10 s.

Glucose uptake. Glucose uptake was assessed by comparing the distribution space of [^{3}H]-2-deoxy-D-glucose (2-DG) to that of [^{14}C]sucrose, a marker of extracellular space. For this purpose, cultures were washed three times with KRH solution. Finally, the solution was replaced by a KRH solution containing the two tracers. Insulin (100 μU/ml) was added to two of every three culture dishes so that two dishes containing insulin were paired with a control. One set was held at room temperature for 10 min; a second set was held at room temperature for 30 min. At the end of the incubation, samples of the bathing solution were taken for determination of extracellular radioactivity. Dishes were washed fives times with isotonic NaCl solution. Cells were then digested by 0.05 N NaOH solution for 2 h at room temperature. Aliquots of the digest were taken for determination of protein by the Coomassie blue method (14) and for determination of ^{3}H and ^{14}C content by scintillation counting. Extracellular contamination of "cell" ^{3}H counts was detected by the amount of ^{14}C.

Inhibition of glucose transport by cytochalasin B was used as a test of presence of specific D-glucose transporters (15). In these experiments, cultures were serum starved for 4 h, during which they were incubated at 37° in medium 199. They were then washed 3 times with KRH solution and incubated for 15 min at room temperature in KRH containing tracer quantities of [^{14}C]sucrose and [^{3}H]-2-DG. Two of the solutions in each set of four cultures contained 100 μU/ml insulin. Cytochalasin B was added to one control and one insulin culture in each set of four to give a final concentration of 1 μM. At the end of the 15-min period a sample of solution was taken for scintillation counting. The culture wells were then washed with isotonic NaCl, and the cells were digested in 0.05 N NaOH as described above. Samples of the digest were taken for scintillation counting and protein assay.

Insulin binding. Presence of insulin receptors was assessed by estimates of specific binding of ^{125}I-labeled insulin (New England Nuclear, Boston, MA). Four sets of experiments were carried out; each set used 24 dishes of cultured myotubes from fetal rats from one mother. Culture dishes were washed four times with KRH solution. Myotubes from four dishes were then scraped into a polypropylene tube to which an insulin solution was added. The insulin solutions were made up in KRH solution containing ~0.25% bovine serum albumin. The three concentrations of labeled insulin per set were 3×10^{-11}, 3×10^{-10}, and 3×10^{-9} M or 10^{-10}, 10^{-9}, and 10^{-8} M. There were two sets of tubes per experiment, one set of which contained 10^{-5} M unlabeled insulin. After 1 h at room temperature (21°) the tubes were centrifuged at 1000 rpm for 5 min. Supernatant was removed and held for analysis of free iodoinsulin. Precipitated cells were treated with 0.2 ml of 0.05 N NaOH for 2 h at room temperature. Aliquots were then taken for protein assay and determination of ^{125}I content. Supernatants and cell extracts were counted in a γ-counter.

RESULTS

Membrane potentials. There were three protocols. The first (group A) served as a control for the effect of time; a culture dish contained either no insulin or 100 μU/ml insulin throughout the period of measurement of membrane potentials (V_m). The second (group B) neglected possible effects of time but provided paired measurements (with or without insulin) on muscle fibers in the same culture dish. The third (group C) was like the second protocol except that 1 μM tetrodotoxin (TTX) was present throughout the period of measurement. TTX was added to prevent the frequent spontaneous twitches occurring in its absence, which seemed to be the cause of spuriously small potentials and unsatisfactory maintenance of impalement (Table 1).

Fused myotubes in nine culture dishes, 5–8 days after plating (mean 6.4 days), were studied without insulin. V_m was recorded from 20 muscle fibers in each dish. Mean V_m was −45.2 ± 2.3 mV (±SE). (Note, for assistance in interpreting

TABLE 1
Effects of insulin on myotube membrane potential in presence and absence of tetrodotoxin (TTX)

Conditions	Membrane potential (mV)		
	Control	Insulin treated	Insulin effect
Group A: no TTX	−45.2 ± 2.3 (9)	−51.7 ± 1.7 (8)	−6.5 ± 2.8
Group B: no TTX (10)	−50.0 ± 1.9	−53.6 ± 2.0	−3.6 ± 1.2
Group C: TTX (6)	−57.4 ± 2.2	−63.9 ± 2.6	−6.6 ± 1.7

Group A served as control for effect of time elapsed after removal from incubator. Control (no insulin) potentials were measured in one set of dishes; insulin (100 μU/ml) was added to separate set of dishes. Insulin effect was analyzed from unpaired differences. In group B, after measurements were made on 10 myotubes in absence of insulin, insulin was added to each dish, and potentials were measured on another 10 myotubes/dish. Insulin effect was analyzed from paired differences. Group C was treated like group B except that TTX was present throughout. Measurements are means ± SE. Number of culture dishes is given in parentheses for group A; numbers of pairs of dishes are in parentheses for groups B and C. Insulin effect is significant at $P \leq .05$ in all groups.

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TABLE 2
Effects of 100 μU/ml insulin and serum starvation on 2-deoxy-D-glucose (2-DG) uptake (pmol/mg protein) by rat myotubes

Serum starved	10-min incubation		30-min incubation	
	Control	Insulin	Control	Insulin
Yes	2.3 ± 0.80	2.9 ± 0.95	7.7 ± 1.9	8.2 ± 2.1
No	5.0 ± 1.57	5.1 ± 1.67	25.0 ± 9.7	21.3 ± 5.8

2-DG uptake given as means ± SE after 10 and 30 min of incubation. There were 3 sets of experiments on cultures not serum starved and 4 sets on cultures serum starved for 4 or 12 h.

data from studies of groups B and C, that there was no difference in V_m between the mean from the first 10 impalements and that of the second 10.)

Insulin (100 μU/ml) was added to each of eight culture dishes 5 min before the first impalement. Each dish to which insulin was added was from the same batch of embryonic muscle and was studied on the same postplating day as a control. Mean V_m in the presence of insulin was −51.7 ± 1.7 mV. Analyzed as an unpaired difference, insulin hyperpolarized by 6.5 ± 2.8 mV, an effect different from zero with $P < .05$.

Ten culture dishes, 6–8 days after plating (mean 6.6 days), were used in group B. In each dish there were 10 impalements, after which insulin was added, and 5 min later there was another series of 10 impalements. V_m was more negative in this series of controls than it had been in group A. In the presence of insulin, V_m was even more negative. Analyzed as paired differences, insulin hyperpolarized by 3.5 ± 1.2 mV ($P < .02$).

Group C consisted of six culture dishes 5 or 6 days after plating (mean 5.5 days). Measurements were technically better in the presence of TTX, and V_m was substantially more negative in controls and in the presence of insulin than in experiments without TTX. Insulin hyperpolarized by 6.6 ± 1.7 mV ($P < .02$).

2-DG uptake. There were seven sets of experiments. The first three were carried out exactly as described in MATERIALS AND METHODS. When it was apparent that the response to insulin was small and probably not significant, I considered the possibility that the fetal calf serum in the medium in which cells were cultured might have supplied sufficient insulin to stimulate glucose uptake by control cells, as suggested by Klip et al. (16). They reported that, in the L_6 muscle cell line, serum depletion led to reduced basal 2-DG uptake (presumably because there was sufficient insulin in serum to stimulate 2-DG uptake so that there was little further response to additional insulin), permitting an effect of additional insulin. Accordingly, a series of experiments was carried out in which the cells were starved of serum and incubated at 37° only in medium 199 (in which glucose concentration is 5.5 mM) for either 4 or 12 h before addition of tracers.

In the three experiments in which cells were not serum starved, cells exposed to insulin took up 28 ± 12.7% more 2-DG in 10 min than did controls (not a significant insulin effect) and only 5 ± 2.3% more in 30 min (Table 2). There was no difference in 2-DG uptake between cultures serum starved for 4 h and those serum starved for 12 h. The results of all serum-starvation experiments are pooled. There was a possibility (although none of the differences is significant) of greater 2-DG uptake by serum-starved cells both in controls and insulin-treated cells. The increases in 2-DG uptake by serum-starved cells over 10 min were 2.7 ± 1.7 pmol/mg protein in controls and 2.2 ± 1.9 pmol/mg protein with insulin; over 30 min the increases were 17.3 ± 7.3 pmol/mg protein in controls and 13.2 ± 6.1 pmol/mg protein with insulin. Insulin did not stimulate 2-DG uptake in serum-starved cells.

In another series, cells were incubated with or without insulin and cytochalasin B to see whether the observed 2-DG uptake was inhibited by cytochalasin B and hence presumably due to specific D-glucose transporters. Results appear in Table 3. Again, insulin had no effect on 2-DG uptake. Cytochalasin B decreased 2-DG uptake by 79 ± 0.6% in the absence of insulin and by 74 ± 1.7% in its presence. From this, we conclude that at least 75% of the observed 2-DG uptake was by way of specific transporters.

It is difficult to compare these results with those reported previously. There appears to be no report of glucose uptake by myotubes in primary culture of rat skeletal muscle. In the L_6 muscle cell line, Klip et al. (16) reported 2-DG uptake as 24 pmol · min^{-1} · mg^{-1} protein, but this was in the presence of 5.5 mM D-glucose. In primary culture of chick embryo skeletal muscle, Schudt et al. (17) reported a linear relation between 3-*O*-methyl-D-glucose uptake and concentration in the bathing solution for myoblasts but not for fused myotubes. Schudt et al. estimated values for K_m and V_{max} for glucose transport in fused chick myotubes. From these values the glucose uptake expected in the presence of the carrier-free concentration of radiolabeled 2-DG in these experiments (3.3×10^{-7} M) was calculated. The expected uptake is 1 pmol · min^{-1} · mg^{-1} protein, which is 2–4 times that actually found (Tables 2 and 3), not surprisingly different in view of the fact that the K_m and V_{max} for rodent muscle glucose uptake are not likely to be the same as for chick.

Insulin receptors. Addition of 10^{-5} M unlabeled insulin decreased the amount of bound labeled insulin in every case. The difference between bound labeled insulin in the presence and absence of unlabeled insulin was designated specifically bound insulin (mol/mg protein). A saturation plot of specifically bound insulin appears in Fig. 1. No analysis of the data by Scatchard plot (bound insulin/free insulin vs. bound insulin) was done because there were only three points per experiment and the data from different experiments fell on different curves. The data displayed in the saturation plot were analyzed according to three models (Fig. 1). The first was in terms of the classic binding

TABLE 3
Effects of 100 μU/ml insulin and 1 μM cytochalasin B on 2-deoxy-D-glucose (2-DG) uptake by rat myotubes

Insulin	Cytochalasin B	2-DG uptake (pmol · 15 min^{-1} · mg^{-1} protein)
−	−	6.8 ± 0.44
+	−	6.7 ± 0.47
−	+	1.5 ± 0.065
+	+	1.8 ± 0.18

2-DG uptake is given as means ± SE of experiments on 6 cultures, each after 15 min of incubation. +, Present; −, absent.

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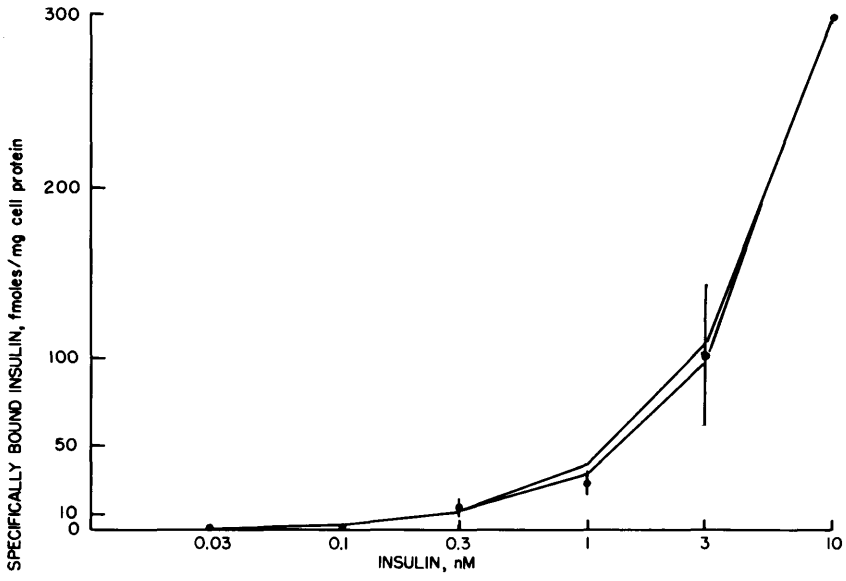

FIG. 1. Insulin receptors in rat myotubes. Bound ^{131}I-labeled insulin, corrected for nonspecific binding (fmol/mg cell protein), as a function of the concentration of insulin added to the cells at time 0 (nM) is plotted on logarithmic scale. ●, Experimental data ± SEM. *Continuous lines* indicate upper and lower extremes of values predicted by binding parameters (capacities and K_D) to which the 4 models were fitted. See text for details of those parameters.

polynomial with two or three binding sites (18) B = N (numerator)/D (denominator), where $N = (K_1F + 2K_1K_2F^2 + 3K_1K_2K_3F^3)R$, $D = 1 + K_1F + K_1K_2F^2 + K_1K_2K_3F^3$, B is moles of bound insulin per milligram of protein, F is the concentration of free insulin, R is moles of receptor per milligram protein, and K's are association constants. Second, the data were analyzed as the sum of the two hyperbolic terms used commonly in analysis of nonlinear Scatchard plots (19) $B = (N_1/D_1) + (N_2/D_2)$, where $N_j = K_jFR_j$ and $D_j = 1 + K_jF$. Finally, the data were analyzed as a combination of the two. In the last case, there were also two terms of the general form given above for B, but the first term was a function of a K_1 and a K_2 (i.e., the numerator and denominator were second-degree polynomials in F), and the second term was first degree as given above.

The models described by these equations differ. The binding polynomial describes a system in which there is only one species of binding molecule and that molecule has *n* binding sites. The commonly used sum of two hyperbolic terms describes a system with two species of binding molecules, each with only one binding site. Finally, the mixed model describes a system with two kinds of binding molecules, one with multiple binding sites, the other with just one site. This last model was used because it has been reported that the insulin receptor may have a capacity of ~1.5 molecules of insulin per receptor molecule (20), a situation that cannot occur if the insulin receptors are entirely homogeneous with respect to numbers of binding sites.

A number of values of K and R fitted the data almost equally well. The point of the analysis was not to choose among models; the data are not good enough for that. The point was simply to see if these cells contained insulin receptors at a density associated in other kinds of cells with a biological response.

The two-site polynomial was fitted by either of the following sets of parameters: $R = 1.2 \times 10^{-12}$ mol/mg protein, K_{D1} (dissociation constant, reciprocal of K_1) $= 3 \times 10^{-8}$ M, and $K_{D2} = 1.15 \times 10^{-6}$ M; $R = 2.25 \times 10^{-13}$, $K_{D1} = 10^{-8}$, and $K_{D2} = 4 \times 10^{-9}$. For this model, binding capacity is 2R. The first set of parameter values describes binding with negative cooperativity; the second set describes binding with positive cooperativity. Addition of a third set did not improve the fit to the data.

Analysis according to the second model, two kinds of insulin-binding molecules, each with only one binding site (sum of two first-degree hyperbolic terms), gave the following, which yielded a fit with about the same residual sum of squares as obtained with the polynomial models. For the high-affinity site these were $R = 5 \times 10^{-13}$ mol/mg protein and $K_D = 3 \times 10^{-8}$ M; for the low-affinity site these were $R = 2 \times 10^{-11}$ mol/mg protein and $K_D = 1.15 \times 10^{-6}$ M.

The composite model (two species of insulin-binding molecules, one with two binding sites, the other with one) was fitted almost equally well by several sets of parameters. In one set, $R_1 = R_2 = 8.7 \times 10^{-13}$ mol/mg protein; for the two-site molecule, $K_{D1} = 4.8 \times 10^{-8}$ M and $K_{D2} = 4.8 \times 10^{-6}$ M; for the one-site molecule, $K_D = 4.8 \times 10^{-8}$ M. In another set, $R = 1.7 \times 10^{-13}$ mol/mg protein; for the two-site molecule, $K_{D1} = 3.5 \times 10^{-8}$ M and $K_{D2} = 2 \times 10^{-9}$ M; for the one-site molecule, $K_D = 7 \times 10^{-9}$ M. In these cases, total binding capacity is 3R. Of all models tested, the second set of parameters for the composite model gave the smallest residual sum of squares, although not significantly a better fit than any of the other models.

In summary, there were specific insulin receptors. Estimates of their density varied from 2.25×10^{-13} to 1.74×10^{-12} mol/mg protein. Estimates of K_D for the low-affinity binding site varied from 35 nM to 5 μM, and for the high-affinity site it varied from 2 to 48 nM. These values are within the ranges reported by others and summarized by Klip et al. (21).

DISCUSSION

Skeletal muscle is the largest mass of target tissue for insulin. The density of receptors for insulin on the surface of skeletal muscle cells is relatively small compared with that in some other target cells (21). I studied responsiveness of primary culture of rat hindlimb muscle to insulin. In fused myotubes there are insulin receptors. The amount of specifically bound

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insulin per milligram of myotube protein at any given concentration of insulin was quite variable, perhaps because the density of receptors and affinity for insulin may change with time [as reported by Klip et al. for L_6 muscle cells (21)] and may vary from culture to culture, and, perhaps for technical reasons simply because the amount of protein was small, may vary from dish to dish and increase with the age of the culture. This variability of bound insulin accommodated analysis according to several models equally well. The range of values of binding parameters extracted from these analyses included values reported by Yu and Gould (22) and Le Marchand-Brustel et al. (23) for isolated soleus muscle and Klip et al. (21) for fused myotubes of L_6 cells.

Insulin exerted an effect on fused myotubes in primary culture. It hyperpolarized them by at least as much as it hyperpolarizes isolated rat caudofemoralis muscle (10). Note, particularly for its implications about mechanisms underlying insulin-induced hyperpolarization, that insulin hyperpolarized even in the presence of TTX at a concentration adequate to abolish spontaneous twitches, an observation first made by Lantz et al. in cultured chick heart cells (24).

In view of the facts that there were insulin receptors and that insulin caused hyperpolarization and the expectation that embryonic cells metabolize glucose, it was surprising to find that insulin did not stimulate transport of 2-DG into fused myotubes. It was also surprising because it has been reported in other kinds of muscle cells in culture, although not in normal mammalian cells in primary culture, that insulin stimulated glucose transport (16,17). The reported responses to insulin were definite but not as impressive as in whole muscle. For chick cells, glucose transport increased by ~70% in response to a large concentration of insulin, ~500 mU/ml. For L_6 cells, glucose transport could be slightly more than doubled, with half-maximum response to insulin at ~7.5 mU/ml. Note that in their successful demonstration of insulin-stimulated glucose transport in L_6 cells, Klip et al. (16) kept the preparation at room temperature, not at 37°. This, plus the fact that we have regularly found insulin-stimulated glucose transport at room temperature in whole muscle excised from rats, suggests that it is unlikely that failure of insulin to stimulate glucose transport in this study was attributable to the fact that cells were held at room temperature rather than at 37°.

Failure of embryonic rat muscle cells in primary culture to respond to insulin with an increase in glucose transport was not due to total absence of specific D-glucose transporters, because there were transporters, which were measured by the decrease in 2-DG transport in the presence of cytochalasin B. If insulin-induced hyperpolarization is a step in the transduction chain between activation of the receptor and stimulation of glucose transport, as has been suggested (25), then failure to find increased glucose transport implies a block in the transduction chain distal to hyperpolarization.

We considered the possibility that there might be a deficiency in the mechanism of recruitment of glucose transporters from the intracellular pool (26). To this end, we carried out a number of experiments based on the glucose transporter cytochalasin B–binding assay of Wardzala et al. (24) and Cushman and Wardzala (25). However, the amount of material was too small to permit separation into plasma membrane and microsomal fractions, and it was too small to give quantitatively reliable estimates of D-glucose–displaceable cytochalasin B binding.

Note that fused myotubes in primary culture of rat hindlimb muscle respond to insulin by hyperpolarizing quantitatively, like normal isolated rat skeletal muscle, but that insulin does not stimulate D-glucose uptake under the conditions of these experiments. When it was first reported that insulin hyperpolarizes rat skeletal muscle, it was specified that the effect was independent of the presence or absence of glucose in the bathing solution (8,9); that is, insulin-induced hyperpolarization was not a consequence of glucose transport or metabolism. The same conclusion can be reached from the observations of insulin action on fused myotubes reported here. Although the results confirm that insulin-induced hyperpolarization does not depend on glucose transport, they do not test the hypothesis that hyperpolarization is a step in the transduction chain leading to insulin-stimulated D-glucose transport.

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