Insulin Does Not Act by Causing a Change in Membrane Potential or Intracellular Free Sodium and Potassium Concentration of Adipocytes

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SUMMARY

The influence of insulin on the intracellular free sodium and potassium ion concentrations ($[Na^+]_i$, $[K^+]_i$) and resting membrane potential of rat epididymal adipocytes was examined to determine its potential for mediating insulin's action on other cellular processes. Direct intracellular measurements of $[Na^+]_i$, $[K^+]_i$, and the resting membrane potential were made using ion-selective and conventional microelectrodes. The use of these microelectrodes enabled us to continuously monitor these parameters in the same cell before, during, and after periods of insulin stimulation of up to 20-min duration. The electrical potentials measured in these experiments remained unchanged when the cells were stimulated with insulin (0.01, 0.1, or 1.0 mU/ml). Varying the extracellular glucose concentration had no effect on these results. Our results provide the first direct measurement of $[Na^+]_i$ and $[K^+]_i$ in adipose tissue and clearly demonstrate that the response to insulin's association with its receptors on the cell surface and subsequent action on hexose transport and cellular metabolism does not involve a change in membrane potential or intracellular sodium and potassium ions. DIABETES 29:1040–1043, December 1980.

Insulin has been reported to stimulate an increase in the resting membrane potential (E_m) of insulin-sensitive cells while increasing the cells' permeability to glucose. The insulin-induced hyperpolarization was reported to be 20–35 mV in rat epididymal adipose tissue[1-3] and 3–10 mV in skeletal muscle.[4-8] In the same studies, insulin's effect on the membrane potential was shown to be completely independent of the level of extracellular glucose.

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Received for publication 8 September 1980.

An increase in the resting membrane potential would be expected to result from either a decrease in the intracellular free sodium ion concentration ($[Na^+]_i$), an increase in the intracellular potassium free ion concentration ($[K^+]_i$), or a combination of both. The numerous investigations examining the role of insulin in stimulating glucose transport and changes in intracellular ion concentrations have been extensively reviewed.[9-12] In general, insulin has been reported to stimulate an increase in Na^+ efflux and subsequently in K^+ influx.[9] However, the mechanism by which insulin initiates these changes and the role of Na^+ and K^+ in mediating insulin's action on glucose transport and cellular metabolism remains uncertain. In rat muscle, the change in intracellular Na^+ and K^+ in response to insulin was reported to result from a decrease in the ratio of Na^+ to K^+ permeability (P_{Na}/P_K).[9,13] In those studies, insulin was found to have no effect on membrane Na-K ATPase activity. In contrast, insulin has been demonstrated to increase Na-K ATPase activity and stimulate the outward current generated by the sodium pump in frog sartorius muscle.[8,14] In addition, Bihler[15] has concluded from studies on rat diaphragmatic muscle that while hexose transport was dependent on the level of $[Na^+]_i$, it could not be directly related to either the rate of Na^+ efflux or the activity of the sodium pump. The main reason for the confusion concerning the mechanism of insulin action has been the inability to accurately measure the normally low intracellular sodium activities. Even though the large potential changes in response to insulin observed in adipocytes would be expected to produce highly demonstrable changes in intracellular Na^+ and K^+, the technical difficulties involved in measuring intracellular Na^+ in these cells has until now prevented any such determinations. However, with the recent development in this laboratory of a liquid ion-exchanger microelectrode that is highly selective for sodium[16] and the availability of a similar K^+-selective microelectrode,[17,18] it is now possible to accurately measure the changes in the intracellular activities of these ions during insulin stimulation. We have used these ion-selective microelectrodes and conventional membrane microelectrodes to continuously measure the membrane potential

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(E_m) and osmotically active intracellular concentrations of Na^+ and K^+ during insulin stimulation of rat adipocytes to determine the nature of insulin's action at the cell membrane and subsequent enhancement of glucose transport.

MATERIALS AND METHODS

Segments of epididymal adipose tissue weighing 50–100 mg were excised from normal Sprague-Dawley rats (50–150 g) and transferred to a plexiglas perfusion chamber. The tissue was firmly pinned to a layer of silicone rubber lining the floor of the chamber and blackened with carbon to aid in visualizing the adipocytes. The mounted tissue was maintained at a constant temperature of 37°C and continuously perfused with a mammalian Ringer's solution containing 143 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.5 mM $MgCl_2$, 2.8 mM glucose, and 1 mM NaH_2PO_4 adjusted to pH 7.4 with 0.1 N NaOH. The Ringer's solution was supplied to the chamber containing the adipocytes by a simple gravitational system from one of two reservoirs at a rate of 3–4 ml/min. One of the reservoirs contained the basic Ringer's solution and the other contained Ringer's plus 0.01, 0.1, or 1.0 mU/ml of porcine insulin (28.5 U/mg; provided as a gift from Eli Lilly and Company, Indianapolis, Indiana). A specially designed valve located on the inflow tube of the chamber was used to rapidly and smoothly change from one solution to the other while maintaining a steady, uninterrupted flow of Ringer's solution to the chamber. The chamber volume was 0.5 ml. When a 0.001% solution of methylene blue was added to the Ringer's solution, it was observed that after a solution change over, the dye began to enter the chamber in 10–20 s, the contents of the chamber were completely turned over in 20–40 s, and the adipocytes began to accummulate the dye in approximately 30 s. Therefore, under these conditions the mounted cells were fully exposed to the insulin within 20–30 s after a solution change over.

Methods of impalement and recording. The chamber containing the mounted tissue was enclosed in a well-shielded Faraday cage and the adipocytes viewed with a Zeiss SR stereomicroscope at a magnification of 50×, allowing impalement of the cells under direct visual control. Due to the large size of the adipocytes (50–120 μm), it was possible, after impalement, to visually determine the position of the electrode tip within the cell. The microelectrodes were advanced into the adipocyte and their tips positioned within the cell with a Narishige remote hydraulic microdrive located outside of the cage and attached to a micromanipulator within the cage. The microelectrodes were connected via an electrode holder containing a Ag/AgCl half-cell to a specially designed high impedence buffer. The electrode potentials were monitored on a digital voltmeter and simultaneously recorded on a Gould-Brush 2200 oscillographic recorder and a Tektronix 5111 oscilloscope equipped with storage. The Ringer's solution in the chamber was grounded via a 3M KCl agar bridge and a second Ag/AgCl half-cell.

Fabrication and calibration of the microelectrodes. Conventional glass microelectrodes were used to measure the membrane potential of the adipocytes, and K^+- and Na^+-selective liquid ion-exchanger microelectrodes were used to measure the intracellular K^+ and Na^+ ionic activities (tip diameters < 0.4 μm). Corning 477317 K^+ liquid ion-exchanger was used in the K^+-selective electrodes and a liquid ion-exchanger incorporating the neutral ionophore 1,1,1-tris [1¹-(2¹-oxa-4¹-oxo-5¹-methyl) dodecanyl] propane was used in the Na^+-selective electrodes. Detailed descriptions of the preparation, properties, and calibration of these ion-selective microelectrodes have been reported previously.[16–20]

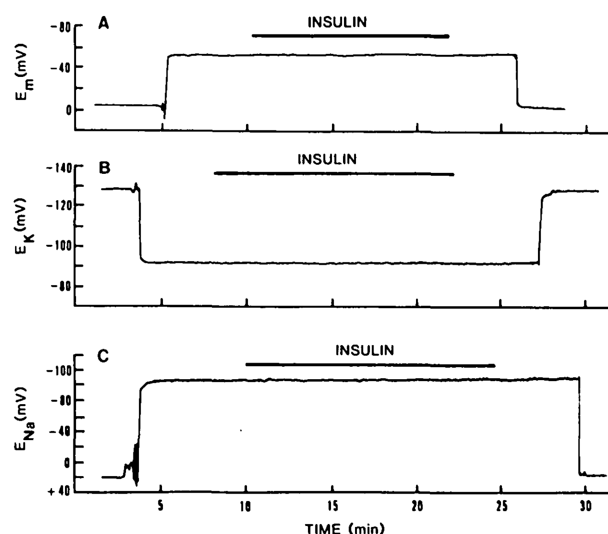
The K^+- and Na^+-selective microelectrodes were calibrated in standard KCl and NaCl solutions and the potentials plotted as a function of log a_K or log a_{Na}. The average slope (S_K) of the K^+-selective microelectrodes was 60.2 ± 1.1 mV (N = 19) and the mean K^+/Na^+ selectivity ratio was 60:1. The Na^+-selective electrodes had a mean S_{Na} of 58.6 ± 0.9 mV (N = 14), a Na^+/K^+ selectivity of 125:1, and a Na^+/Ca^{2+} selectivity ratio of 1:4.8.

The values of a_K^i and a_{Na}^i were calculated from an extended form of the Nicolsky-Eisenman equation.[19] All mean values are given ± the SEM. Statistical significance was determined by Student's *t* test.

RESULTS

Impalement of adipocytes with conventional microelectrodes generally resulted in a rapid negative increase in potential to establish a steady-state membrane potential (E_m) that could be maintained for up to 25 min (Figure 1A). In some experiments, the potential generated upon impalement of the cell tended to be larger than normal and highly unstable, producing significant fluctuations in E_m. In most of these cases, direct visual observation indicated that the tip of the electrode had extended into the large neutral lipid drop that filled most of the center of the cell. Partial withdrawal of the electrode back into the more superficial cytoplasmic layer often produced a typical steady-state condition. As a result, only impalements producing potentials that remained stable within 1–2 mV for a minimum of 3 min were used in these studies. The mean value for E_m in 38 such measurements was −46.1 ± 0.9 mV. The measurements of ionic activities (Na^+ and K^+) were made from single impalements of adipocytes with the appropriate ion-selective mi-

FIGURE 1. Representative oscillographic recordings of the resting membrane potential (A), K^+-selective microelectrode potential (B), and Na^+-selective microelectrode potential (C) made during impalements of rat epididymal adipocytes. The bars over each recording represent the period the cells were stimulated with 0.1 mU/ml insulin.



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croelectrode. The mean value of E_m was used to calculate a_{Na}^i and a_K^i. Impalement with a K^+-selective microelectrode produced a positive change in potential, which again reached a steady-state (Figure 1B). The mean value of a_K^i calculated from 28 measurements was 108.1 ± 9.6 mM. This corresponds to an intracellular K^+ concentration of 144 ± 11.5 mM. Similar measurements (N = 30)of a_{Na}^i and $[Na^+]_i$ using Na^+-selective microelectrodes (Figure 1C) produced values of 6.1 ± 0.6 mM and 8.1 ± 0.8 mM, respectively.

Figure 1 illustrates the type of results obtained when insulin was added to the Ringer's solution bathing the adipocytes. This figure shows three oscillographic recordings made with a conventional (1A), a K^+-selective (1B), and a Na^+-selective (1C) microelectrode in which insulin (0.1 mU/ml) was added to the Ringer's solution after a steady-state potential had been established. It can be seen from these recordings that even after 15 min of exposure to the insulin, there was no significant change in membrane potential or intracellular K^+ and Na^+ activities.

In none of the experiments in which insulin (0.1 mU/ml) was added to the Ringer's solution for periods ranging from 4–20 min during continuous measurement of E_m (N = 12), a_K^i (N = 7), or a_{Na}^i (N = 10) was there any significant change in any of these values. Neither changing the concentration of insulin to 0.01 or 1.0 mU/ml nor increasing the glucose concentration in the Ringer's solution to 5.5 or 10.8 mM have any effect on these results. In no case did insulin stimulation produce a significant change in E_m, a_K^i, or a_{Na}^i ($P > 0.5$).

DISCUSSION

To properly understand the action of insulin on its target cells (fat, muscle, liver, etc.), it is essential to identify the events occurring in immediate response to insulin's association with its receptors on the cell surface. Zierler[9] has proposed that the insulin-induced hyperpolarization of the membrane potential observed in both muscle and adipose tissue results from a decrease in intracellular Na^+ and subsequent increase in intracellular K^+, and suggests that these electrochemical changes (particularly the decrease in $[Na^+]_i$) may serve to initiate hexose transport and changes in insulin-regulated metabolic pathways. However, because direct measurement of the cytosolic concentration of Na^+ and K^+ in these cells had not been possible until recently, the mechanism of insulin's action on the membrane potential and hexose transport has remained unclear and produced a number of conflicting observations (see Clausen[10] and Czech[11] for detailed discussion).

Our measurements of intracellular sodium and potassium in rat adipocytes using ion-selective microelectrodes represent the first accurate determinations of the cytosolic concentration of these ions in adipocytes. Because these electrodes provide direct accurate measurement of these ions, they avoid the difficulty of estimating the amount of cytoplasmic water, a major problem in adipose tissue with its large intracellular lipid droplets.

Our results clearly demonstrate that administration of insulin to white adipose tissue has no effect on the intracellular concentration of osmotically active Na^+ and K^+. These results were not what was predicted by the large (20–35 mV) hyperpolarization of the membrane in response to insulin reported by Beigelman and Hollander.[1–3] However, in those studies, each measurement was made in a different cell and for short durations (up to 13 impalements/min) at various points during insulin stimulation. Their measurements showed a great amount of variability between one experiment and another under similar conditions. In seven equivalent experiments, the *mean* resting potentials of unstimulated cells measured in each experiment ranged from −17 to −69 mV and in stimulated cells from −47 to −76 mV with mean hyperpolarizations of between 18 and 35 mV after 10 min of stimulation.[1] In larger rats (300–450 g), generally having larger cells and more lipid per cell, no hyperpolarization was reported.[2] This high degree of variability, particularly in light of the small volume of active cytoplasm maintained near the cell surface and the large area of neutral lipid in the center of these cells, points to the need to make these measurements continuously in the same cell. In fact, when we measured the membrane potential continuously in the same adipocyte and stimulated the cells with insulin in concentrations previously reported to have a maximal hyperpolarizing effect,[1,2] we never observed a hyperpolarization or any change in the potential even after 20 min of stimulation. Similar results have also been reported for ventricular heart muscle.[21] The insulin-induced membrane hyperpolarizations reported in frog[8] and rat skeletal muscle[4–7] were also the result of discontinuous studies using different fibers and muscles. We have found that in rat soleus muscle, as in adipose tissue, insulin did not produce any significant change in membrane potential, $[Na^+]_i$, or $[K^+]_i$ when continuously measured in the same fiber.[22] Similarly, Moore and Rabovsky[8] have demonstrated in the frog sartorius muscle that there is no significant change in $[Na^+]_i$, $[K^+]_i$, or the sodium/potassium permeability ratio during insulin stimulation. They claimed that insulin hyperpolarized the muscle fiber by stimulating Na/K pump activity. However, they fail to explain how this is accomplished in the absence of a change in intracellular Na^+ or K^+.

In a very carefully constructed series of experiments measuring Na^+ flux in rat diaphragm muscle, Creese[23] concluded that there was no net change in Na^+ efflux or P_{Na} in response to insulin. This evidence, when combined with our direct continuous measurements of E_m, $[Na^+]_i$, and $[K^+]_i$ during insulin stimulation in adipocytes, formulates the conclusion that insulin in association with its membrane receptors must increase glucose transport and initiate changes in intermediary metabolism by some mechanism that is not coupled to a change in membrane potential or change in intracellular sodium and potassium.

ACKNOWLEDGMENTS

We thank Dr. R. Chance of Eli Lilly and Company for generously providing the insulin used in these experiments.

This investigation was supported by NIH grant AM 26246, grants from the American Diabetes Association and its Indiana Affiliate, and faculty grants to RJS from the Purdue Research Foundation and Indiana University Research Committee.

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