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Ouabain-resistant hyperpolarization induced by insulin in aggregates of embryonic heart cells

(membrane potential/Na^+,K^+-ATPase/specific ion channels/pacemaker current/receptors)

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ABSTRACT **Spheroidal aggregates formed from trypsin-dissociated 14-day embryonic chicken hearts after 48 hr of rotation on a gyratory shaker. Intracellularly recorded resting membrane potentials of aggregates bathed in 1.3 mM K^+ balanced salt solution had a mean (± SD) of 64 ± 4 mV. After a stable potential was achieved, addition of 1–100 nM sodium bovine insulin caused a slow hyperpolarization of up to 19 mV after 4–5 min, followed, in some cases, by a further, more rapid, shift to a potential near E_K. Equivalent hyperpolarizations were observed when insulin was added in the presence of 10 mM ouabain, indicating that enhanced Na^+,K^+ pump activity was not responsible for the change in membrane potential. The concentration of insulin that produced half-maximal hyperpolarization (2 nM) corresponded to the association constant of a high-affinity insulin receptor, suggesting that binding to this class of receptors led to the change in membrane potential. Steady-state current–voltage curves from current clamp experiments suggested that insulin produced an increase in slope conductance at potentials near rest by inducing an outward current with an apparent potential negative to −90 mV.**

Insulin is known to alter the distribution of Na^+ and K^+ across the plasma membrane of susceptible cells (1, 2) and to cause membrane hyperpolarization (1, 3). There are two alternative mechanisms to which these effects might be attributed. The hormone may have a direct effect on membrane permeability. Zierler (1) argued that insulin's primary action is to alter fixed charges within the membrane, thereby decreasing permeability to both Na^+ and K^+ but in different amounts as to decrease the sodium/potassium permeability ratio (P_{Na}/P_K). He demonstrated such a shift in rat skeletal muscle adequate to account for both the measured increase in K_i and in transmembrane potential.

An alternative mechanism postulated for insulin's effect is that the hormone enhances the activity of the transport enzyme complex Na–K–ATPase without altering passive permeability. Insulin-induced increases in activity of this enzyme have been reported in membrane preparations isolated from frog skeletal muscle (4, 5) and rat brain (4). Indeed, Gavin *et al.* (4) suggested that Na–K–ATPase was the major transducer between the insulin receptor on the plasma membrane and the cellular response. Evidence supporting this concept comes from studies of Na^+,K^+-ATPase from the salt gland of marine birds (6) which showed that the purified soluble enzyme did not respond to insulin with increased activity, whereas intact gland cell preparations did. Cheng *et al.* (7) have suggested that catechols and their *o*-quinones increased Na^+,K^+-ATPase activity in insulin-binding muscle membrane vesicles by altering the configuration of the enzyme in the membrane.

Early evidence from excitable cell preparations (nerve, muscle, heart) suggested that active ion transport was an electroneutral process (8). More recent studies have indicated that the Na^+,K^+ pump is more often, and perhaps always, "electrogenic." The Na^+,K-ATPase extrudes 3 mol of Na^+ per mol of ATP degraded while <3 mol of K^+ is pumped into the cell. Thus, the total pump reaction induces a net outward (hyperpolarizing) current and the cell interior is made more negative (8, 9).

Agents that diminish Na^+,K^+-ATPase activity, such as cardiac glycosides (10, 11) or Li^+ (12), cause an immediate depolarization of 10–20 mV due to a decrease in the electrogenic current. A more gradual further decrease in membrane potential is produced as Na^+ and K^+ diffuse passively down their respective electrochemical transmembrane gradients. Agents that augment pump activity such as insulin, catechols (7), or chemotactic factor tripeptide (13) should hyperpolarize the cell and cause a net increase in steady state K_i. Recent results with mammalian Purkinje fiber and ventricular tissue (14) and with frog sartorius muscle (15) indicated that insulin produced a small hyperpolarization (2–8 mV) that was blocked by acetylstrophanthidin and was not associated with a change in membrane resistance (R_{in}) or ionic permeabilities.

It has been demonstrated that all of the cell membrane in aggregates of embryonic heart cells (16) is virtually isopotential at voltages near rest (17, 18). Moreover, these cells contained insulin receptors (19) and responded to that hormone with augmented amino acid transport (19, 20) and ion movements. We report here that the insulin-induced hyperpolarization (IIH) in heart cell aggregates is associated with an increase in net outward current and is not ouabain-sensitive (21). In addition, we show that the IIH is correlated with occupancy of a specific receptor site.

METHODS

Preparation of Cultures. After incubation at 38°C for 14 days, White Leghorn chicken embryos were harvested in amniotic fluid and decapitated. Hearts were excised, trimmed of extraneous tissue, and snipped with iridectomy scissors to release trapped blood. The whole hearts were dissociated into single cells by a multiple-cycle trypsinization procedure now standard in this laboratory. Spheroidal aggregates were prepared from dissociated cells as described (16). Briefly, an inoculum of 1×10^7 cells was added to 10 ml of medium 21212 in 50-ml erlenmeyer flasks. The flasks were gassed with 5% CO_2/10% O_2/85% N_2, sealed with silicone rubber stoppers, and placed on a gyratory shaker. Aggregation took place at 37°C during 48 hr of gyration at 60 rpm.

Aliquots of aggregates were removed from the flasks and placed in Falcon plastic tissue culture dishes (35 mm) containing

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Abbreviations: IIH, insulin-induced hyperpolarization; I–V, current–voltage.

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3 ml of fresh medium 21212. After incubation for 30 min to allow the aggregates to attach to the bottom of the dish, the medium was replaced by a balanced salt solution containing bovine serum albumin (0.1%). The aggregates were repeatedly washed with this solution over the next 3 hr to remove all serum that was present in the original medium. Aggregates were maintained at 37°C in a 5% CO_2/10% O_2/85% N_2 atmosphere.

Media and Solutions. Aggregates were prepared in medium 21212. This medium contains (by volume) 68.5% medium F-12 without potassium (GIBCO), 25% M199 (GIBCO), 4% fetal calf serum (GIBCO), 2% heat-inactivated horse serum (Colorado Serum, Denver, CO), and 0.5% gentamycin. The potassium concentration of the final medium was brought to 1.3 mM. For electrophysiological studies, aggregates were washed in balanced salt solution containing (in mM): NaCl, 116.0; KCl, 1.3; $MgSO_4$, 0.8; NaH_2PO_4, 0.9; $CaCl_2$, 1.7; $NaHCO_3$, 26.2; glucose, 5.5. It also contained 0.1% fatty acid-free bovine serum albumin (fraction V, Sigma). Chicken insulin was isolated from fresh frozen pancreas and its purity was confirmed by $NaDodSO_4$ gel electrophoresis and isoelectric focusing by described methods (19). Sodium bovine insulin (lot 1DG 64-94-195; 26 units/mg) was a gift from R. L. Jackson (Lilly). After dilution from stock concentrations, insulin was stored and transferred in polypropylene ware to avoid nonspecific binding to glass. Ouabain (strophantin G) and tetrodotoxin were purchased from Calbiochem.

Electrophysiological Measurements. Aggregates 150–200 μm in diameter were washed in balanced salt/albumin solution for 3 hr and transferred to a microscope warm stage. Klearol, a nontoxic mineral oil, was layered over the solution to decrease evaporation, and a toroidal gassing ring was placed around the plate to maintain a 5% CO_2/10% O_2/85% N_2 atmosphere and a solution pH of 7.3. Tetrodotoxin (10 μg/ml), an inhibitor of the fast sodium current, was added to abolish spontaneous action potentials. Transmembrane potentials of cells in aggregates were recorded with intracellular glass micropipettes (Omega Dot, Frederick Haer, Brunswick, ME) filled with 3 M KCl. Direct current resistance of the electrodes ranged from 20 to 40 MΩ. The intracellular electrode was coupled to a capacity-compensated high-input-impedance field effect transistor (FET) amplifier, and the extracellular solution was connected to ground by an agar bridge.

The amplifier circuit used for measurement of intracellular potential and current injection was similar in design to that of Wilson and Goldner (22). This circuit design (discontinuous current injection) allows a single electrode to be used for simultaneous voltage recording and current injection within specified limits of current passage and frequency response (23). No differences in input impedance were observed between the discontinuous current injection method and a two-electrode measurement performed simultaneously in the same aggregate, thus confirming the low intercellular resistance in aggregates (18).

Voltages and current signals were monitored on a storage oscilloscope (Tektronix, Beaverton, OR) and the data were recorded at $^{15}/_{16}$ inch/sec on an instrumentation recorder (Hewlett-Packard, model 3964A). Simultaneously, the membrane potential was recorded on a strip chart recorder (Cole-Palmer).

RESULTS

Insulin-Induced Hyperpolarization. The mean (± SD) resting potential of 20 aggregates measured in 1.3 mM K^+ and 30 μM tetrodotoxin was 63.9 ± 3.9 mV. After the membrane potential had stabilized (less than ± 2 mV drift over 5 min or more), insulin was added to the plate as a bolus. In about half the cases, within 1–2 min after addition of the hormone the membrane potential gradually began to hyperpolarize and achieved a new resting potential at a level 10–19 mV more negative which remained stable for many minutes (Fig. 1*a*). In the remaining aggregates (Fig. 1*b*), a similar slow hyperpolarization began but was followed by a more rapid shift to a potential near E_K. In the case shown, the aggregate came to rest at −113 mV.

The increased slow spike activity seen in Fig. 1 *a* and *b* was typical of voltage recordings made during these experiments. The cause is unknown but might be related to the serum-free solution in which the aggregates were washed or to spasmodic action of an electrogenic pump.

Mechanism of Action. The contribution of the Na^+,K^+-ATPase system to the IIH was determined by addition of 10 μM or 50 μM ouabain to the plates. At even lower concentrations (0.1–1 μM), ouabain blocked ^{42}K uptake and ^{24}Na efflux in cultured monolayers of embryonic ventricle cells (24); and in aggregates, 10 μM ouabain caused ionic redistributions over a period of 3 hr which were consistent with an immediate and prolonged inhibition of the Na,K pump (2). After the preparation had been exposed to ouabain for 20 min, insulin produced hyperpolarizations that were equivalent to those without ouabain. A typical response to a level near E_K is shown in Fig. 1*c*.

In order to investigate the ionic mechanism underlying the IIH, steady-state current–voltage (I–V) curves were measured

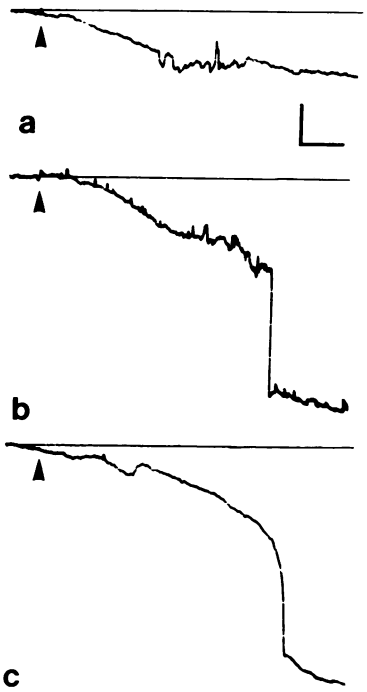

FIG. 1. IIH. A stable intracellular potential (E_r = horizontal line) was obtained in balanced salt/albumin containing 1.3 mM K_o^+ and 3 μM tetrodotoxin. Sodium bovine insulin (17 nM) was then added to the bathing solution as a bolus (arrow). (Scale = 10 mV, 100 sec.) (*a*) E_r = −61 mV; within 1–2 min after insulin was added, the aggregate began to hyperpolarize and reached a second stable potential at −74 mV after about 5 min. (*b*) E_r = −57 mV; in an alternative response seen in about half the cases, the membrane began to hyperpolarize slowly, remained for about 4 min at a potential that fluctuated around −75 mV, and then precipitously hyperpolarized further to a new negative level, coming to rest at −113 mV. (*c*) E_r = −61 mV; 20 min after addition of 10 μM ouabain, the hyperpolarizing response to insulin (17.4 nM) was similar to that in *b* although the second hyperpolarizing step (to −118 mV) was less abrupt.

at potentials near rest before and after application of insulin by injecting a very slow current ramp ($dI/dt = 1 \times 10^{-11}$ A/sec) and measuring the resulting voltage change with the discontinuous-current single electrode circuit. Fig. 2 shows that insulin (1.0 nM) shifted the I–V curve to the left; that is, the hormone hyperpolarized and increased the slope of the steady-state I–V relationship. The slope of these curves (I/V) at any given voltage (slope conductance) is a direct measure of the net steady-state membrane permeability to ions at that voltage; the inverse slope (V/I) is the input resistance (R_i). The aggregate represented in Fig. 2 rested at −67 mV, where R_i was 23.15 MΩ. After exposure to insulin, an injected current of 0.53 nA was required to return the membrane to the original rest potential (−67 mV) and R_i at that potential was 4.7 MΩ.

The current induced by the application of the insulin in Fig. 2 could be determined by subtracting the magnitude of the total current under control conditions from that after 15 min of IIH at each voltage. The insulin-induced current (Fig. 3) was a net outward current that was largest near rest and tended toward zero at a voltage more negative than −90 mV.

Dose–Response. Embryonic chicken heart cells are thought to have two classes of insulin binding sites (19) based on the quantitative correlation of functional responses with receptor occupancy. At 30°C, one class of receptors has a high affinity with K_a of 1.6×10^9 M^{-1} and a low capacity (≈600 per cell) and the other has a low affinity with K_a of 3×10^7 M^{-1} and a high capacity (9000 per cell). Percentage occupancy of these two sites as a function of insulin concentration is shown in Fig. 4 by the solid lines. The two lines for each site represent the extent of the error bars. The lines were redrawn from Santora *et al.* (19).

Exposure of aggregates to four concentrations of insulin from 0.1 to 100 nM resulted in the hyperpolarizations represented by X on Fig. 4. Maximal hyperpolarization observed in these experiments was 19 mV. The size of the hyperpolarization response corresponded closely to the high-affinity receptor occupancy. This suggested that hormone binding to this class of receptors produced the changes in membrane potential. Aggregates that exhibited a precipitous shift to potentials near E_K (as in Fig. 1 *b* and *c*) were excluded from these experiments. The magnitude of that shift was apparently not dose-dependent.

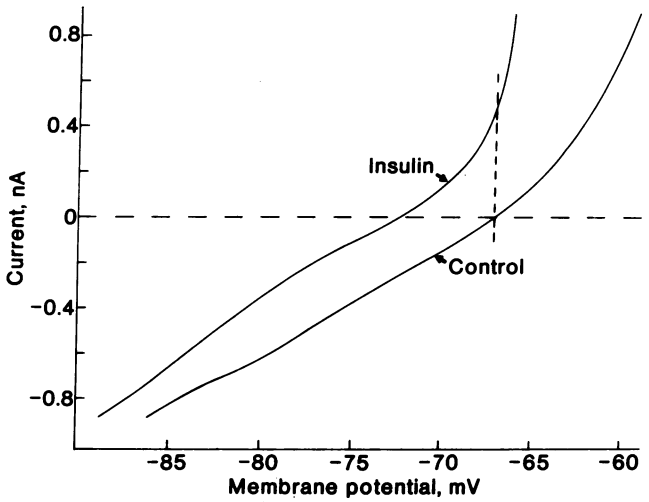

FIG. 2. Steady-state I–V curves before (control) and after introduction of 1 nM sodium bovine insulin (insulin). The curves, produced by injecting a slow current ramp and measuring the resulting voltage change, have been redrawn from the original data. The vertical broken line at control V_{rest} (zero current) crosses the insulin I–V curve at 0.53 nA.

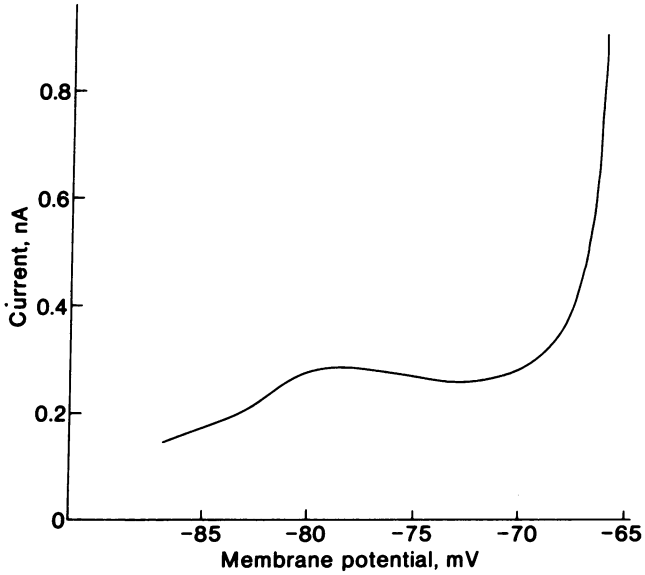

FIG. 3. Insulin-induced current. Net current was calculated from the data in Fig. 2 by subtracting the magnitude of the control current from the insulin current at each voltage. The current had a net outward direction which tended toward reversal at a potential more negative than −90 mV.

Specificity. Additional evidence correlating high-affinity binding and hyperpolarization was sought by comparing specificity of action of several hormones. Santora *et al.* (19) found that chicken insulin bound most specifically to the high-affinity site. Bovine insulin was one-fifth to one-half as potent and mouse epidermal growth factor did not bind at all (19). In electrophysiological experiments, chicken insulin gave comparable hyperpolarization to bovine insulin at only one-third the concentration; epidermal growth factor did not produce hyperpolarizations even at very high doses (100 μg/ml).

DISCUSSION

We have shown that insulin produces a hyperpolarization in embryonic heart cell aggregates in a concentration range (1–20 nM) that corresponds to hormone binding to a high-affinity site. There are at least two alternative mechanisms to which the IIH

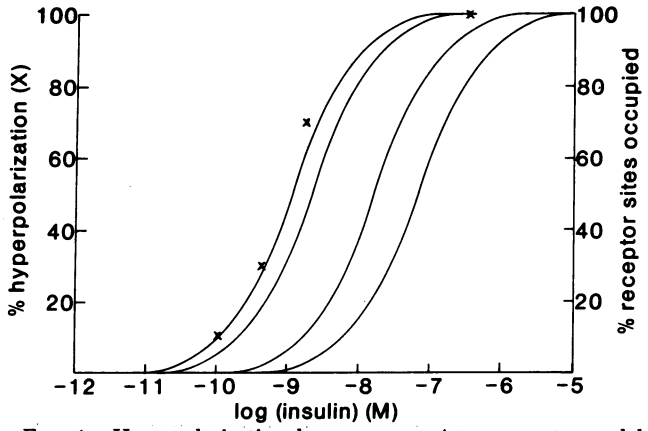

FIG. 4. Hyperpolarization dose–response. A two-receptor model was proposed for the aggregate system (19). The two pairs of S-shaped functions represent the envelope of the error bars of the percentage occupancy of the two receptor sites proposed for the heart cell aggregates (19). The percentage hyperpolarization (X) closely correlates with occupancy of the proposed high-affinity site. Maximal hyperpolarization was −19 mV for application of 100 nM bovine insulin in these experiments.

may be attributed (15, 25): (*i*) the hormone may increase the activity of an electrogenic Na^+,K^+-ATPase pump (4, 5); or (*ii*) the hormone may have a direct effect on membrane ionic permeabilities (1). Zierler (26) postulated that the effect was due to a direct action on membrane permeability. Using radioactive flux measurements, he found that insulin (10–100 milliunits/ml) decreased the sodium and potassium permeabilities of rat skeletal muscle. But, because sodium permeability was decreased more than potassium permeability, a net hyperpolarization occurred. Similar experiments on frog sartorius muscle have recently been performed by Moore and Rabovsky (15). With large doses of insulin (250 milliunits/ml = 1 mM) these authors found that the IIH was abolished by ouabain and was not associated with alterations in the P_K/P_{Na} ratio. DeMello (3), who also used supraphysiological doses of insulin (0.32 mM), found that the IIH he observed in frog sartorius muscle was associated with an increase in "effective resistance" of the preparation.

These were not the results we have found in heart cell aggregates. Here, IIH was associated with a decrease in slope resistance and a net outward current that was largest near resting potential and declined at more negative voltages. The reversal potential of the insulin-induced current was not measured directly, but extrapolation of the curve in Fig. 3 suggested that the intercept was at a level more negative than −90 mV. Among the ions that determine membrane resting potentials (Na^+,K^+, Ca^{2+}, Cl^-) two have negative equilibrium potentials: $E_{Cl} = -40$ mV, and $E_K = -120$ mV§. These findings suggest that, for heart cell aggregates, the hyperpolarization was most likely due to an increase in membrane permeability to potassium. The precipitous shift of potential to near E_K seen in some aggregates (Fig. 1 *b* and *c*) could be explained by a further abrupt opening of all K^+ channels or by the virtual disappearance of conductance to other ionic species or both. Moreover, the correlation between hyperpolarization and insulin binding to a high-affinity site suggested that hormone occupancy of these receptors, at concentrations only slightly greater than physiological levels in the embryonic chicken (19, 27), caused the changes in membrane potential. Because the embryonic heart cells that comprise the aggregates used in these studies had about 400 μm^2 of surface (28), the estimate of 600 high-affinity insulin binding sites per cell converts to a receptor density of about $1.5/\mu m^2$. It is interesting that from fluctuation analysis of the voltage noise recorded from heart cell aggregates we could estimate a K channel density of about $1/\mu m^2$ (18). Chloride has been implicated in a thyroid-stimulating hormone hyperpolarization of membrane vesicles prepared from thyroid tissue (29), but a change in Cl^- conductance was ruled out as a cause for IIH in frog sartorius muscle (3). This ion could not be the primary charge carrier in the heart cell preparation if the estimate of E_{Cl} is correct.

Although the results obtained with ouabain eliminated an increase in ouabain-sensitive Na^+,K^+-ATPase activity as the mechanism of action, there are still other pump mechanisms that may be electrogenic and could contribute to the IIH. For example, Mullins (30) reviewed the evidence that the Na/Ca exchange is electrogenic. This appears to be an unlikely candidate for the IIH because the apparent reversal potential for the pump would occur at about −40 mV. However, the I–V relationship for the carrier-mediated Ca^{2+} influx (I_C) bears some resemblance in the range −60 to −100 mV to that shown for insulin in Fig. 2. This illustrates the need for caution in interpreting the ionic mechanism involved in the IIH.

§ Calculation based on the following ion distributions: $Cl_i^- = 30$ mM; $Cl_o^- = 120$ mM; $K_i^+ = 120$ mM; $K_o^+ = 1.3$ mM.

A more intriguing possibility arises from the recent observation that insulin inhibited a high-affinity Ca^{2+}-ATPase in isolated adipocyte membranes (31). Such inhibition would result in decreased levels of Ca^{2+} extrusion and increased Ca_i. There is ample evidence that Ca^{2+} influences the outward ionic conductance mechanisms (32, 33). Increasing Ca_o caused hyperpolarization of Purkinje fibers, resulting either from a direct increase in P_K (34) or from more indirect mechanisms (35). In neuroblastoma cells, increased Ca_o caused an inward Ca current and the activation of a slow outward K current (36). Increasing Ca_i by direct injection of the ion into Purkinje fibers had a similar effect (37, 38). These results prompted Isenberg (38) to propose that K conductance is regulated by Ca_i rather than by membrane potential. Thus, if insulin modulates the Ca content of embryonic heart cells, it could explain the IIH observed here.

Is it possible that some of the more commonly recognized effects of insulin are secondary to the role this hormone plays in regulating membrane potential? Zierler (1) proposed that transmembrane potential changes caused by insulin may affect membrane transport, and Kaback (39) has discussed the effect of total electromotive force on bacterial membrane transport according to the chemosmotic theory of Mitchell (40). Because our own data indicated that specific amino acid transport across the heart cell membrane is augmented by insulin (19, 20), it is reasonable to assume that hyperpolarization may be involved. There are at least two mechanisms whereby increased electronegativity could augment transport of nonelectrolytes. First, hyperpolarization might increase the driving force across the plasma membrane for positively charged substances and thereby increase the cotransport of amino acids. Zierler and Rogus (41) demonstrated recently that a 10-mV hyperpolarization in rat muscle resulted in 30% increase in uptake of 2-deoxy[^{3}H]glucose. A second mechanism might be to induce conformational changes in membrane-associated proteins through an increased transmembrane voltage difference. This could lower the K_m for substrate binding or increase the capacity for uptake by increasing the number of available binding sites. In addition to direct effects, ion redistribution caused by IIH may lead to alteration of intracellular enzyme activities as previously postulated (2). Further efforts to correlate biochemical and electrophysiological measurements should provide valuable information on the basic mechanisms of insulin action.

The discontinuous current injection amplifier used in these experiments was constructed by Dr. Richard Weigel. We thank Dr. Weigel for his assistance in using the amplifier, Dr. L. J. DeFelice for helpful discussions of this work, and Mr. Tom Fisk for technical expertise in preparation of the aggregate cultures. This work was supported by National Institutes of Health Grants HL16567 (to R.L.D. and L.J.E.) and L-T32-AM07298-01 (Emory Endocrinology Training Program).

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