Type 1 diabetes leads to cytoskeleton changes that are reflected in insulin action on rat cardiac $K^+$ currents

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Shimon, Y., and J. B. Rattner. Type 1 diabetes leads to cytoskeleton changes that are reflected in insulin action on rat cardiac $K^+$ currents. Am J Physiol Endocrinol Metab 281: E575–E585, 2001.—A sustained $K^+$ current ($I_{wa}$) is attenuated in ventricular cells from streptozotocin (STZ)-induced diabetic rats. The in vitro addition of insulin to isolated cells augments $I_{wa}$ in a process that is blocked by disrupting either actin microfilaments (with cytochalasin D) or microtubules (with colchicine). When these agents are added at progressively later times, the effect of insulin becomes evident in a time-dependent manner. $I_{wa}$ is also augmented by insulin in control cells in a cytoskeleton-dependent manner. However, in contrast to diabetic cells, cytoskeleton-dependent augmentation of $I_{wa}$ by insulin occurs at a considerably faster rate in control cells. Immunofluorescent labeling shows a reduced density of $\beta$-tubulin in diabetic cells, particularly in perinuclear regions. In vitro insulin replacement or in vivo insulin injections given to STZ-treated rats enhances $\beta$-tubulin density. These results suggest an impairment of cytoskeleton function and structure under insulin-deficient conditions, which may have implications for cardiac function.

Cardiac potassium channels; microtubules; actin microfilaments.

Cardiac contraction is initiated by an electrical signal, the action potential. A number of ionic currents that underlie the action potential determine its configuration and duration (22). Several outward $K^+$ currents are responsible for termination of the action potential plateau and repolarization of the membrane potential (2). It is now recognized that variations in action potential repolarization are a major source of cardiac arrhythmias (35). Furthermore, variations in action potential duration (such as those caused by changes in $K^+$ currents) can indirectly affect the force of contraction by determining the duration of Ca$^{2+}$ influx (5). Because only very small currents flow during the plateau phase of the action potential, even slight changes in current magnitude can have a dramatic impact on action potential duration (22). In the rat heart, there is a dynamic interplay of several conductances that contribute to the repolarization of the ventricular action potentials. Although the currents involved have been well characterized (2, 9), the exact contribution of each current to the repolarization process is unclear. Potentially, a reduction in any of the outward currents may prolong the action potential.

Diabetes mellitus is an increasingly common disease, with a $>5\%$ incidence in the adult population in Western countries (15). Cardiovascular complications are common and are the leading cause of diabetes-related mortality (15, 39). Among the common complications are changes in the electrocardiogram (1) and the development of cardiac arrhythmias (26). Underlying these changes are changes in the action potential and some of the ion currents associated with it (11, 16, 33).

We and others have reported (11, 16, 31–33) the attenuation of two cardiac $K^+$ currents in ventricular myocytes from insulin-deficient (type 1) diabetic rats. This attenuation leads to a prolongation of the action potential (16, 33), which may underlie the prolongation of the QT interval observed in the electrocardiogram of humans with type 1 diabetes (1). These $K^+$ currents can be restored to normal by in vitro incubation ($>5$ h) of isolated myocytes with insulin. The working hypothesis consistent with this and previous data by us and others (11, 16, 32) is that insulin has a tonic regulatory effect on the density of two Ca$^{2+}$-independent $K^+$ currents: a transient, inactivating current ($I_t$) and a sustained, noninactivating current ($I_{wa}$) (2). Insulin deficiency leads to a decline in the density of these currents, which can be reversed by adding insulin to cells in vitro. This effect occurs after a lag period of $>5$ h and can be blocked by inhibiting protein synthesis with cycloheximide or by disrupting the actin microfilaments with cytochalasin or the microtubules with colchicine (32). This suggests that addition of insulin to isolated cardiac cells from insulin-deficient diabetic rats triggers the synthesis of new channels. These are then transported to the cell membrane with the aid of the actin and microtubular networks. These two networks are known to interact (8) with protein transport to the cell membrane requiring both components (7). This is consistent with data obtained by Nagaya and Papazian (19) indicating...
that channel assembly occurs in the endoplasmic reticulum before transport of the channel to the membrane.

Insulin has been suggested to play a key regulatory role in the functional organization of actin microfilaments (14, 37). The actin network is an essential mediator in the action of insulin, leading to recruitment of preformed glucose transporters and their translocation to the cell membrane (38).

The microtubules are also targets of insulin (3, 12, 24). Thus many cellular effects of insulin may depend on the functional integrity and organization of the cytoskeleton, as indeed has been suggested (32, 38). A chronic insulin deficiency could lead to impairment in the organization of the cytoskeleton, as has been suggested previously (17, 27, 28). This could entail a compromised or slower action of insulin in cells from chronically insulin-deficient animals on reexposure to insulin.

The present work was designed to test the hypothesis that cytoskeleton-dependent effects of insulin on K⁺ currents follow a different time course in cardiac myocytes from control and diabetic rats. Because in control myocytes insulin (added in vitro) was found (32) to enhance only $I_{\text{ss}}$ (but not $I_{\text{f}}$), the comparison between normal and diabetic conditions was done by measuring the timing of insulin effects on $I_{\text{ss}}$. The time course of insulin action was measured by disrupting the cytoskeleton at progressively later times after the addition of insulin.

The results show that the cytoskeleton-dependent action of insulin occurs much more rapidly in control myocytes. Immunofluorescent labeling of $\beta$-tubulin, a major component of microtubules, indicates that insulin deficiency results in a marked reduction in its density and distribution. In combination, these results suggest that changes in cytoskeleton structure and function occur during chronic insulin deficiency. This affects the expression and function of membrane ion channels and may underlie some of the complications associated with type 1 diabetes.

**METHODS**

All experiments were done in accordance with the guidelines of the Animal Care Committee of the University of Calgary.

**Animals.** Sprague-Dawley rats (200–250 g) were used as controls or after a single injection of streptozotocin (STZ, 100 mg/kg iv) 6–12 days before the experiments. STZ destroys pancreatic $\beta$-cells and leads to insulin deficiency and hyperglycemia (31–33). Another group of rats received insulin replacement (6 U/kg se daily, from day 1 of STZ injection). Measurements of plasma levels of glucose and insulin verified the diabetic status of the rats.

**Cell isolation.** Single right ventricular myocytes were prepared by enzymatic dispersion in the following manner. Rats were heparinized (2,400 U/kg ip) and anesthetized by methoxyflurane inhalation. After cervical dislocation, the hearts were removed and cannulated on a Langendorff apparatus. Coronary perfusion was performed (at 37°C, 70 cmH₂O pressure) initially with a solution consisting of (in mM) 121 NaCl, 5.4 KCl, 2.8 sodium acetate, 1 MgCl₂, 1 CaCl₂, 5 Na₂HPO₄, 24 NaHCO₃, and 5 glucose bubbled with 95% O₂-5% CO₂. After 5 min, the solution was changed to a similar solution with Ca²⁺ omitted. This was followed after 10 min by the same Ca²⁺-free solution containing 20 mM taurine, 40 μM CaCl₂, 10 U/ml collagenase (Yakult Honsha, Tokyo, Japan), and 0.01 mg/ml protease (type XIV, Sigma). After 7–8 min in the enzyme-containing solution, the free wall of the right ventricle was dissected and cut into smaller pieces for further incubation in the Ca²⁺-free solution, also containing collagenase (50 U/ml), protease (0.1 mg/ml), 0.1 mM CaCl₂, and albumin (5 mg/ml). After incubation in a shaker bath at 37°C, cells were collected over the next 20–40 min and stored in the Ca²⁺-free solution, which contained 20 mM taurine, 0.1 mM CaCl₂, and 5 mg/ml albumin (no enzymes). Cell viability was assessed by appearance (rod-shaped, striated, clear borders).

![Fig. 1](http://ajpendo.physiology.org/)

**Fig. 1.** Stability of insulin effect on sustained nonactivating current ($I_{\text{ss}}$) in myocytes from diabetic (A) and control (B) rats. Current density measurements were grouped into 2 separate periods: 5–7 and 7.5–11 h after addition of insulin. Open bars, without insulin (−ins; $n$ = 29 and 20 for diabetic and control, respectively); hatched bars, 5–7 h of insulin treatment ($n$ = 88 and 55 for diabetic and control, respectively); cross-hatched bars, 7.5–11 h of insulin treatment ($n$ = 53 and 41 for diabetic and control, respectively). Values are means ± SE. In control and diabetic cells, insulin augmentation of $I_{\text{ss}}$ is complete by 5 h and stable thereafter, with no significant differences between $I_{\text{ss}}$ density measured 5–7 and 7.5–11 h after insulin addition. ANOVA showed that $I_{\text{ss}}$ density was significantly larger in the presence than in the absence of insulin ($P < 0.01$) for both exposure periods, with no differences ($P > 0.05$) between insulin effects at different exposure times.
and function (stability in normal physiological $\text{Ca}^{2+}$: poorly functioning cells are leaky and contract in 1 mM $\text{Ca}^{2+}$). The yield of viable cells was typically 60–80% of that of cells from control and diabetic rats.

Current recordings. Aliquots of cells were placed in a 1-ml chamber on the stage of an inverted microscope and superfused with a solution containing (in mM) 150 NaCl, 5.4 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 5 HEPES, and 5.5 glucose, with pH adjusted to 7.4 with NaOH. CdCl$_2$ (0.3 mM) was added to inhibit the L-type $\text{Ca}^{2+}$ current. Currents were recorded (at 20–22°C) using the whole cell suction electrode method. Pipettes were filled with a solution containing (in mM) 110 potassium aspartate, 30 KCl, 4 Na$_2$ATP, 1 MgCl$_2$, 10 EGTA, 1 CaCl$_2$, and 5 HEPES, with pH adjusted to 7.2 with KOH. Electrode resistance was 2–4 MΩ. Correction was made for the liquid junction potential created in these conditions ($\sim$10 mV). Series resistance was kept to a minimum by using low-resistance electrodes and by electronic compensation.

The focus of these experiments was the sustained, quasi-low-resistance electrodes and by electronic compensation. Whereas the exact molecular nature of $I_{1}$ remains elusive (21). A third $K^+$ current, elicited by pulses to potentials ranging from $-100$ to $-40$ mV, is the background inwardly rectifying current ($I_{K1}$), which was also measured at the end of 500-ms pulses. Because insulin does not affect this current, it can be used as a monitor of changes in series resistance during recordings. An increase in series resistance during recording will reduce the amplitude of this current. When the change was $>10\%$, the cell was discarded.

Indirect immunohistochemistry. Cells were deposited on coverslips by aliquoting a small volume of solution into a Plexiglas chamber mounted with wax on the coverslip. The chamber-coverslip unit was then placed in a tabletop centrifuge operated at 3,003 g for 1 min. Subsequently, the chamber was removed, and the coverslip containing the sample was fixed in methanol for 10 min and air-dried. The samples were then rehydrated in phosphate-buffered saline (PBS) and incubated for 30 min at 37°C with primary antibody (β-tubulin; Sigma). The samples were then washed in PBS and incubated for an additional 30 min at 37°C with a Cy3-conjugated donkey anti-mouse IgG (H + L) secondary antibody (Jackson Labs, Mississauga, ON, Canada). Specimens were counterstained with 4',6-diamidino-2-phenylindole, mounted in 90% glycerol containing paraphenylenediamine, and observed using a Zeiss Axiohot fluorescence microscope. Images were recorded on Ilford HP-5 film, using an FX-35A camera (×40 objective). Exposure times were set automatically by a Nikon HFX-II exposure meter. Differences in fluorescence between cells are thus a function of reactivity with the antibody and not experimental artifacts.

**Fig. 2.** Time dependence of cytochalasin D (CD) inhibition of insulin effects in cells from a streptozotocin (STZ)-diabetic rat. A: sample current traces obtained in response to 500-ms pulses given from $-80$ to $-110$ mV (downward trace, eliciting an inwardly rectifying current ($I_{K1}$) and to potentials ranging from $-10$ to $+50$ mV (eliciting a transient inactivating current ($I_{t}$) and $I_{w}$). Examples are from 3 cells in the absence of insulin (left) or with insulin and cytochalasin D added 1 h (middle) or 5 h (right) after insulin. In the last case, cytochalasin D is ineffective in blocking insulin action, whereas the early addition of cytochalasin D completely blocks insulin effects. Insulin was present for 5.5 and 10.5 h in the cells shown in the middle and right panels, respectively. Line to left of traces denotes zero current level. B: current-voltage relationship for steady-state currents measured at the end of 500-ms pulses given to potentials ranging from $-110$ to $+50$ mV. Current densities between $-30$ and $+50$ mV represent $I_{w}$, and those between $-40$ and $-110$ mV represent $I_{K1}$ (which is unaffected by insulin). Values are means ± SE. Whereas total inhibition of insulin effects on $I_{w}$ is evident at 2 h, there is a gradual removal of inhibition with the later times of cytochalasin D addition.
This method did not enable a precise quantification of β-tubulin content. However, a large number of cells were visually divided into three categories on the basis of the amount of β-tubulin surrounding the nucleus, which was easily identifiable with the 4',6-diamidino-2-phenylindole staining. The three categories included cells in which the nuclei were completely surrounded by tubulin, cells in which the nuclei were partially surrounded, or cells with nuclei without surrounding tubulin. These categories allowed a clear distinction among cells from the different groups (see RESULTS).

Drugs. Drugs were prepared as stock solutions and frozen until the final dilution before use. Cytochalasin D, STZ, and insulin (from bovine pancreas) were purchased from Sigma. Novolin ultralente insulin (Novo Nordisk, Bagsvaerd, Denmark) was used for in vivo insulin injections.

Statistics. A two-tailed t-test was used for comparison of two experimental groups. \( P < 0.05 \) was considered statistically significant. When three groups were compared, one-way ANOVA was done, using a Bonferroni multiple comparisons test.

RESULTS

Experimental protocols. In all experiments, cells were divided into untreated and treated groups, with insulin added to the appropriate groups soon after cell isolation. In these experiments we used 100 nM insulin, although we have shown that current augmentation occurs with 1 and 10 nM insulin as well (32). Current magnitudes from individual cells are variable. Thus, for every treatment group, recordings must be made from many cells, and the current magnitudes must be averaged. Current amplitudes are normalized for cell size by dividing by cell capacitance, so that current densities are used for comparison. The augmentation of \( K^+ \) currents by insulin can be measured in individual cells only 5 h (or more) subsequent to its addition. Because of current amplitude variability, recordings must be made for several hours (>5 h) to collect data from a sufficient number of cells. Typically, these measurements were made between 5 and 11 h after insulin addition. The protocols described below involved recordings from cells exposed to insulin (as well as other agents) over prolonged periods. Current magnitudes in some cells were measured at shorter times of exposure to insulin than others. Thus it was important to ensure that once insulin augmented \( I_{ss} \) (after >5 h), the current amplitudes reached a plateau and were stable thereafter. Figure 1 shows that cells exposed to insulin for 5–7 or 7.5–11 h exhibited a similar degree of current augmentation by insulin. This was true for diabetic and control cells, although the currents (with and without insulin) were smaller under diabetic conditions.

A further series of experiments confirmed the stability of \( I_{ss} \) in control myocytes over prolonged recording durations with no insulin treatment. In 66 control, untreated cells, \( I_{ss} \) density (at +50 mV) was 8.39 ± 0.25 (SE) pA/pF when recorded 1–5 h after cell isolation. In 19 untreated cells in which \( I_{ss} \) was recorded 5–9 h after cell isolation, \( I_{ss} \) density was 8.74 ± 0.50 pA/pF. This was true for diabetic and control cells, although the currents (with and without insulin) were smaller under diabetic conditions.

Fig. 3. Time dependence of cytochalasin D effects on insulin action in control cells. A: sample current traces (same protocol described in Fig. 2 legend) from cells exposed to 100 nM insulin and treated with 1 μM cytochalasin D 0.5 h before (left) or 2 h after (right) insulin. In the former case, insulin effects are blocked, indicating a dependence on the actin cytoskeleton in control as well as diabetic cells. When added 2 h after insulin, cytochalasin D is ineffective in blocking the augmentation of \( I_{ss} \) by insulin, in marked contrast to the case in diabetic cells (Fig. 2). Line to left of traces denotes zero current level. B: current-voltage relationships, with \( I_{ss} \) density (mean ± SE) plotted against membrane potential, obtained in control cells without insulin (circles) or with insulin (>5 h) and treated with cytochalasin D 0.5 h before insulin, 2 h after insulin, or 4 and 5 h (combined data) after insulin. The earliest addition of cytochalasin D completely blocks the increase in \( I_{ss} \) by insulin, whereas later addition leads to a gradual development of the effects of insulin.
I work, we found that, in control cells, insulin augments with those described above in diabetic cells. In previous experiments compared the effects of insulin in control cells which have normal insulin levels. The following experiments investigated whether the cytoskeleton mediates insulin action on K⁺ currents in control cells, as it does in diabetic cells. The rate of insulin action in both groups was compared as well. Cytochalasin D (1 μM) was added at different times after insulin, and currents were measured ≥5 h after insulin addition and ≥1 h after other drugs.

Diabetic conditions. In earlier experiments, we showed (32) that, under insulin-deficient diabetic conditions, addition of 1 μM cytochalasin D 1 h after insulin (100 nM) completely abolishes the enhancement of $I_t$ and $I_{ss}$ by insulin. The present experiments showed that the addition of cytochalasin D at increasingly later times after insulin enabled the cells to develop insulin effects (augmentation of $I_{ss}$ and $I_t$) in a time-dependent manner. Figure 2A shows that, whereas at 1 h after insulin cytochalasin D is completely effective in preventing any current augmentation by insulin, addition of cytochalasin D 5 h after insulin results in an enhancement of $I_t$ and $I_{ss}$ by insulin to the same extent as with insulin alone. Figure 2B shows the current densities for $I_{ss}$ before insulin addition and with insulin in the presence of cytochalasin D added 2, 4, and 5 h after insulin (in all cases, insulin was present for 5–11 h and cytochalasin D for ≥1 h). A clear progression of the increase in current density by insulin can be seen for later additions of cytochalasin D.

Control cells. Insulin is an important regulator of cytoskeleton organization and function. We therefore postulated that a chronic deficiency in insulin would alter the function of cytoskeleton components. Because insulin augments cardiac K⁺ currents in a cytoskeleton-dependent manner, we tested whether the kinetics of insulin action differ in cells from control animals, which have normal insulin levels. The following experiments compared the effects of insulin in control cells with those described above in diabetic cells. In previous work, we found that, in control cells, insulin augments $I_{ss}$ but not $I_t$. Presumably, $I_t$ saturates at lower concentrations of insulin than $I_{ss}$, so that, in myocytes from normal rats, insulin can still augment $I_{ss}$, but not $I_t$. Thus the comparison of control and diabetic conditions focused on $I_{ss}$.

The cycloheximide dependence of the effect of insulin on $I_{ss}$ (implying induction of channel protein synthesis) was previously shown only in STZ-diabetic rats but had not been established in control cells. In the present experiments, the effects of cycloheximide on insulin action were also tested in control cells. Cycloheximide (2 μM) was added 30 min before insulin, and $I_{ss}$ was measured ≥5 h after incubation with insulin. Measurements of $I_{ss}$ under these conditions showed that $I_{ss}$ augmentation by insulin was totally blocked by cycloheximide. The mean $I_{ss}$ densities at +50 mV were 7.8 ± 0.2 pA/pF without insulin (n = 37), 9.7 ± 0.3 pA/pF after incubation with insulin (n = 27), and 8.2 ± 0.5 pA/pF with insulin and cycloheximide (n = 14). ANOVA showed the effects of insulin to be significantly larger than both control (P < 0.001) and insulin + cycloheximide (P < 0.01). Current density for the insulin + cycloheximide group was not different from control (P > 0.05). These results show that the augmentation of $I_{ss}$ by insulin in control cells is also dependent on protein synthesis.

The following experiments investigated whether the cytoskeleton mediates insulin action on K⁺ currents in control cells, as it does in diabetic cells. The rate of insulin action in both groups was compared as well. Cytochalasin D (1 μM) was added at different times after insulin, and currents were measured ≥5 h after insulin addition. The results showed that the effects of insulin are inhibited by cytochalasin D, but in a shifted time frame. Addition of cytochalasin D 1 or 2 h after insulin proved to be ineffective, with a complete augmentation of $I_{ss}$ by insulin. This effect was significantly (P < 0.05) larger than the effect of cytochalasin D given...
and 2 h after insulin in the diabetic cells. Only by adding cytochalasin D 1 h or 30 min before insulin could the augmentation of \( I_{ss} \) be blocked. These results are shown in Fig. 3. Figure 3A shows current traces from cells exposed to insulin + cytochalasin D added 0.5 h before or 2 h after insulin. In the former, no enhancement of \( I_{ss} \) was observed; in the latter, a large augmentation is seen. Figure 3B shows the current-voltage relationships for the steady-state currents without insulin and with cytochalasin D added at different times. These curves show the time dependence of the inhibition of insulin action by cytochalasin D in control cardiac myocytes.

A summary of the time frame for cytochalasin D action and a comparison between cells from control and insulin-deficient diabetic rats is shown in Fig. 4. The plots show the increase in \( I_{ss} \) at +50 mV, plotted as the ratios of \( I_{ss} \) magnitude after insulin to that before insulin addition against the time of cytochalasin D addition. In all cases, currents were measured ≥5 h after insulin addition and ≥1 h after cytochalasin D addition. Figure 4 clearly illustrates that insulin action is faster in control cells. Moreover, Fig. 4 emphasizes that it is the cytoskeleton-dependent action of insulin that is faster in control cells.

Similar results were obtained when colchicine (5 \( \mu \)M) was added after insulin. As reported earlier, the addition of colchicine 1 h after insulin completely inhibited the augmentation of \( I_{ss} \) in cells from STZ-diabetic rats (32). However, when colchicine was added 5 h after insulin, \( I_{ss} \) augmentation by insulin was complete. At 2 h there was an intermediate effect (Fig. 5A). Figure 5A shows current traces obtained from myocytes isolated from a diabetic rat with insulin added 7 h before recording. Colchicine was added 1 or 5 h after insulin. Colchicine added 1 h after insulin prevents insulin action; colchicine added 5 h after insulin enables current augmentation by insulin. Figure 5B shows summary data obtained by adding colchicine 1, 2, and 5 h after insulin.

In marked contrast, adding colchicine 1 or 2 h after insulin in control cells showed a complete augmentation of \( I_{ss} \) by insulin. Adding colchicine 1 h after insulin had significantly different (\( P < 0.02 \)) effects in control and diabetic cells. Only by adding colchicine 1 h after insulin was the inhibition of \( I_{ss} \) augmentation evident in control cells. The difference between the effects of insulin on \( I_{ss} \) when colchicine was added 1 h before or 1 h after insulin was also significantly different (\( P < 0.05 \)). These results are shown in Fig. 5C, which highlights the faster action of insulin in control than in diabetic cells.

These results suggest that some functional aspects of the cytoskeleton are profoundly different under condi-

![Fig. 5. Time dependence of the effects of colchicine (colch) on \( I_{ss} \) enhancement by insulin. A: sample current traces in myocytes from diabetic rats exposed to 100 nM insulin, with 5 \( \mu \)M colchicine added 1 or 5 h after insulin. When colchicine is added 1 h after insulin, it blocks insulin action. When added 5 h after insulin, colchicine no longer blocks \( I_{ss} \) augmentation by insulin. Line to left of traces denotes zero current level. B: mean values of \( I_{ss} \) enhancement by insulin in STZ-diabetic rats, plotted as a ratio of the magnitude (at +50 mV) after insulin to that before insulin. X-axis, time of colchicine addition; right-hand bar, effect of insulin alone. C: time dependence of colchicine effect in control cells. Note the faster action in control cells, as indicated by a complete effect of insulin when colchicine is added 1 or 2 h after insulin. This is in contrast to an inhibition of the effect of insulin in diabetic cells, when colchicine is added 1 or 2 h after insulin.](http://ajpendo.physiology.org/)

AJP-Endocrinol Metab • VOL 281 • SEPTEMBER 2001 • www.ajpendo.org
tions of chronic insulin deficiency. Diabetes-related changes in the cytoskeleton have been reported previously, mainly in conjunction with neuropathy (17, 28). Changes in cytoskeletal proteins could be measured within 1–2 wk (18, 27) and were tissue specific (18). These changes are probably due to the fact that insulin itself plays a tonic regulatory role in controlling the actin microfilament network (14), as well as the microtubules (3, 12, 13). To test whether structural changes can be identified in the cytoskeleton of cardiac cells in our experimental conditions, we set out to label elements of the cytoskeleton. Labeling of the cytoskeleton-linked actin in a contractile cell, where most of the actin is in the contractile apparatus, was not feasible. We therefore used antibodies against β-tubulin, a major component of the microtubule system (10), to test whether insulin deficiency results in alterations in this component of the cytoskeleton. A comparison of the microtubule network in control and STZ-diabetic rats showed markedly different patterns of β-tubulin distribution. Figure 6 shows examples from several cells.

In addition to an overall reduction in the density of β-tubulin in the diabetic cells (Fig. 6, B, D, and F), there is also a marked reduction in β-tubulin around the cell nucleus.

To establish that these changes are due to insulin deficiency (rather than toxic effects of STZ on the microtubules), another group of rats was given daily injections of insulin (8 U/kg, slow release) together with STZ. After 7–8 days, cells were isolated and tested for tubulin immunofluorescence. In addition, cells from STZ-diabetic rats were incubated for 6–9 h with 100 nM insulin, and the β-tubulin fluorescence was compared with that in untreated cells. In both cases, the addition of insulin restored β-tubulin density to normal, indicating that the alterations in β-tubulin fluorescence in the diabetic state are mainly due to insulin deficiency. Examples of cells from an untreated STZ-diabetic rat (with very little perinuclear labeling) and from cells from the same rat after addition of insulin (in vitro) for 6 h are shown in Fig. 7; cells from

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Fig. 6. β-Tubulin immunofluorescence in cells (magnified ×1,200) from untreated (A, C, and E) and STZ-treated rats (B, D, and F). Note the less intense fluorescent signal in cells from diabetic rats, which is particularly prominent in the perinuclear region.
STZ-treated rats receiving insulin for 9 days are also shown.

Cells could be roughly categorized according to whether the nuclei (seen as dark ovals) were completely surrounded by fluorescence (Fig. 6, C and E), were partly surrounded (Fig. 6, A and D), or had very little perinuclear staining (Fig. 6, B and F). It was thus possible to obtain a semiquantitative comparison between cells from control and STZ-treated rats. Although 52.1% of control cells (n = 71) showed nuclei completely surrounded, only 10.3% of diabetic cells (n = 214) were in this category; 12.7% of normal cells had no perinuclear staining, whereas 51.4% of STZ-treated cells fell into this category. With in vitro insulin addition, 28.0% of cells (n = 82) had nuclei completely surrounded by tubulin. With in vivo insulin replacement, 50.3% of cells (n = 153) fell into this category. These results are shown in Fig. 8.

These results support the suggestion that insulin maintains the normal distribution of microtubules and that insulin replacement can restore the changes in function and morphology of STZ-treated rats (23, 25). The marked differences in cytoskeleton structure or organization between cells from control and diabetic rats may underlie the slower action of insulin in the diabetic cells. It is possible that, in these cells, effects of insulin on K⁺ currents require a reorganization of the cytoskeleton as an additional early step, thus accounting for the slower action of insulin than in control cells.

These results illustrate an important difference in the action of insulin in control and diabetic cells, which is presumably due to differences in the regulation of the cytoskeleton.

**DISCUSSION**

**Summary of findings.** The results presented here demonstrate several novel findings. First, it is shown that, by timed intervention with agents that disrupt different elements of the cytoskeleton, it is possible to follow the progression of insulin action on the functional expression of a protein (K⁺ channel) at the cell membrane. Thus Figs. 2-5 show that, under control and insulin-deficient diabetic conditions, the augmentation of a K⁺ current by insulin is blocked by cytochalasin D or colchicine but that this inhibition can be relieved by adding these agents at increasingly later times after insulin. Second, this approach enabled the
distinction of differences between normal and pathological (diabetic) conditions. As shown in Figs. 4 and 5, the enhancement of $I_{ss}$ by insulin occurs much more rapidly in normal myocytes. This is true for the effects of insulin with cytochalasin D and colchicine. The slower effects of insulin in cells from insulin-deficient rats may be linked to the reduced density of microtubules, as illustrated by β-tubulin labeling (Figs. 6–8).

Significance and implications. These results greatly support earlier suggestions that insulin plays a major role in maintaining the structure and function of the microtubule system (12, 13, 24). Insulin deficiency has been suggested to reduce levels of tubulin mRNA and reduce tubulin density in axons (17, 18, 28). Because a major function of the microtubules is to facilitate intracellular trafficking, an insulin deficiency is expected to impair translocation between cellular compartments. This was indeed shown to occur in diabetic neuropathy, in which axonal transport is impaired (34). This effect, which is reversible and preventable by insulin treatment (34), has been linked to a reduction in the amount and density of microtubules (28). The present results show a reduction in microtubule density in cardiac myocytes as well and suggest that the translocation of newly synthesized ion channels to the cell membrane is also impaired in insulin-deficient conditions. Addition of insulin (in vivo or in vitro) restores current magnitude as well as microtubule density.

The enhancement of $K^+$ currents by insulin was suggested earlier to involve the synthesis of new channel protein and its translocation to the membrane in a process dependent on elements of the cytoskeleton (but see Limitations). The present results are in agreement with recent work by Nagaya and Papazian (19), who showed that $K^+$ channel α- and β-subunits assemble in the endoplasmic reticulum before transfer to the Golgi apparatus and subsequent transport to the cell membrane. Interestingly, β-subunits, which have been suggested to promote the surface expression of $K^+$ channels (29), have also been shown to be associated with the cytoskeleton (20). This may provide a mechanism whereby an impaired cytoskeleton in diabetic (or other pathological) conditions will delay or attenuate the expression of new channels at the cell surface. Consistent with the present findings are results showing that STZ-induced diabetes alters the expression of different components of the cytoskeleton (18, 27, 28). These changes may not occur in all tissues (6, 18). However, functional consequences of altered cytoskeletal ultrastructure have been described in skeletal muscle (4) and in sensory neurons (28), as well as the present results, which demonstrate modifications in the cytoskeleton-dependent actions of insulin on $K^+$ channels. The approach taken in this work could be utilized in future studies of processing and posttranslational modifications of channel proteins in normal and pathological conditions. It is now recognized that several pathologies involve impairment of protein processing or trafficking (30), as well as alterations in the cytoskeleton (36). The present results support this concept by demonstrating that the action of insulin is

Fig. 8. Distribution of β-tubulin labeling on the basis of categorization of cells into 3 groups. Cells were divided into these groups on the basis of complete, partial, or missing perinuclear fluorescence. Seventy-one control cells (A) were assessed, with 52.1% appearing in the 1st category. Only 10.4% of cells ($n = 214$) from diabetic rats were in this category (B). Insulin replacement partially or completely restored the normal distribution of perinuclear staining in STZ-treated rats. C: in the former, insulin was added in vitro (100 nM for 6–8 h, $n = 82$). In vivo replacement of insulin was performed by daily injection of insulin (7 days) to STZ-treated rats. D: distribution of staining in these cells ($n = 158$) shows a pattern similar to control cells. Thus the changes in STZ-treated rats are due to insulin deficiency and not to STZ per se.
slower than normal in conditions of chronic insulin deficiency. This is due, at least partly, to impairment of the cytoskeleton-dependent component of insulin action.

The relatively small effect of insulin (25–30% increase in I_{ss}) should be considered. During the plateau phase of the cardiac action potential, all currents are small, so that repolarization is greatly affected by very small changes in any current. Interestingly, in clinical settings, small variations in insulin have been found to affect the dispersion of action potential repolarization (40), which is a key factor in the onset of cardiac arrhythmias (35). Thus the present results are important in the context of understanding the development of arrhythmias in the setting of diabetes.

Limitations. We do not yet have direct evidence for an increase in channel protein by insulin. The effects of insulin are blocked by cycloheximide, which inhibits all protein synthesis: it is possible that it is accessory proteins that are stimulated by insulin, or cytoskeleton components, rather than channel proteins. The fact that ≥6 h are required before current augmentation is seen suggests that transcription and translation are involved, but this is not yet proved. One limitation in addressing this issue in regard to I_{ss} is that this current is complex, consisting of several components (9), and not very amenable to pharmacological manipulation (2). It is also not yet known which K_+ channel isoforms contribute to the macroscopic current (21). This impedes the investigation of detailed mechanisms involved in insulin effects on the individual channel components that underlie I_{ss}. Another possible interpretation for the difference in the rate of insulin action is that colchicine and cytochalasin D permeate control pretreatment for the difference in the rate of insulin action.

REFERENCES


