

Insulin stimulation of rat ventricular K⁺ currents depends on the integrity of the cytoskeleton

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(Received 12 August 1998; accepted after revision 31 October 1998)

1. The effect of insulin on K⁺ currents was studied with enzymatically dispersed ventricular myocytes from insulin-deficient (type I) diabetic rats. Diabetic conditions were induced by a single intravenous injection of streptozotocin (100 mg kg⁻¹) given 8–13 days before the experiments. Measurements of plasma glucose and insulin levels confirmed the diabetic status of the animals.
2. A Ca²⁺-independent transient outward K⁺ current, I_t , and a slowly inactivating, quasi-steady-state current, I_{ss} , which are depressed in diabetic myocytes, could be restored by exposure to 1, 10 or 100 nM insulin. This was only observed after a delay of 5–6 h, although an insulin exposure of only 1 h was sufficient to initiate its stimulatory action on I_t and I_{ss} . The stimulatory effect of insulin on these K⁺ currents was prevented by 2 μM cycloheximide, which in itself had no direct effect on these currents.
3. Disruption of the actin microfilament network with 1 μM cytochalasin D (CD) also prevented the stimulatory effect of 100 nM insulin on both I_t and I_{ss} . Since CD was added 1 h after insulin, inhibitory effects on insulin signalling were ruled out. Adding CD (1 μM) 5–9 h after insulin, when currents were already augmented, had no effect (up to 50 min exposure). Incubating control cells for 6–10 h with 1 μM CD had no effect on any of the currents measured.
4. Stabilization of the actin network by pre-exposure to 2.5 μM phalloidin restored the stimulatory effect of insulin, in the continued presence of CD, ruling out any effects of CD on components other than the cytoskeleton.
5. The stimulatory effect of insulin was also prevented by incubating cells with insulin in the presence of the microtubule-disrupting agent colchicine (5 μM).
6. These results suggest that the insulin-mediated augmentation of K⁺ currents in diabetic myocytes requires protein synthesis, possibly of K⁺ channels, as well as an intact cytoskeleton. The possibility that newly formed channels translocate to the plasma membrane in a process dependent on different elements of the cytoskeleton is discussed.

We have recently reported (Shimoni *et al.* 1998) that insulin reverses the depression of ventricular K⁺ currents which occurs in an insulin-deficient model of diabetes. The stimulating effect of insulin requires 5–6 h and is blocked by inhibition of mitogen-activated protein (MAP) kinase, a key enzyme regulating transcription (Saltiel, 1996). This suggests that new K⁺ channels are synthesized in myocytes from insulin-deficient diabetic rats, upon re-exposure to insulin.

A key issue in the study of cellular functions is the intracellular sorting, processing and targeting of proteins involved in a variety of cellular activities (Griffiths & Simons, 1986; Rothman, 1994; Rothman & Wieland, 1996), including ion transfer. For example, it has recently been reported (Makhina & Nichols, 1998) that regulatory subunits

of the ATP-dependent K⁺ channel and the channel itself show independent trafficking to the plasma membrane. It has also been shown (Nagaya & Papazian, 1997) that voltage-dependent K⁺ channel subunits complete their assembly in the endoplasmic reticulum, prior to being transported to the plasma membrane. Furthermore, it has been suggested that alternative splicing leads to ion channel isoforms containing (different) membrane targeting signals (Ponce *et al.* 1997). However, the mechanisms whereby ion channels reach the plasma membrane following their synthesis and assembly are unknown.

The trafficking of newly formed proteins from the *trans*-Golgi network to the cell membrane has been suggested to depend on two components of the cytoskeleton, the actin

microfilament system and the microtubule network (Nelson, 1992). These may serve a protein-transporting role either in parallel (Nelson, 1992) or in sequence (Fath *et al.* 1993). Our results demonstrating an enhancement of K^+ currents in diabetic myocytes within hours of exposure to insulin (Shimoni *et al.* 1998) enabled us experimentally to address some of these issues.

The on-going activity of many ion channels has been shown to depend on a normal cytoskeleton, based on the sensitivity of channel activity to disruption of different elements of the cytoskeleton. Cytochalasin D, a fungal toxin which specifically inhibits actin polymerization (Cooper, 1987) and thus disrupts microfilament organization, can affect Na^+ channel gating (Undrovinas *et al.* 1995), inward rectification of K^+ channels (Mazzanti *et al.* 1996), and several aspects of K_{ATP} channel function (Brady *et al.* 1996; Terzic & Kurachi, 1996; Furukawa *et al.* 1996). Disruption of the microtubules with colchicine has also been found to affect the function of L-type calcium channels (Johnson & Byerly, 1993; Galli & DeFelice, 1994). This sensitivity to cytoskeletal disruption is assumed to reflect 'architectural' requirements of channel function, based on the known interactions between the cytoskeleton and the plasma membrane (Luna & Hitt, 1992). This interaction presumably controls the correct spatial orientation of the channels and their components. In addition, there is also evidence suggesting that the delivery, expression or clustering of ion channels in the cell membrane can be affected by changes in the cytoskeleton (reviewed in Janney, 1998).

In the present study we set out to investigate whether the stimulatory effect of insulin on potassium currents in ventricular myocytes from diabetic rat hearts (Shimoni *et al.* 1998) is dependent on actin microfilaments and the microtubular system. We find that both cytochalasin D and colchicine prevent the stimulation of these potassium currents by insulin. Our results, therefore, demonstrate for the first time that an insulin-stimulated augmentation of ion currents, which presumably leads to synthesis of new channels, is dependent on the integrity of the cytoskeleton.

METHODS

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

Experimental model

Ionic currents were measured from ventricular myocytes obtained either from control Sprague–Dawley (200–250 g) rats or from rats made diabetic by a single i.v. injection of streptozotocin (STZ, 100 mg kg^{-1}), given 8–13 days prior to cell isolation. Measurements of plasma glucose and insulin levels confirmed the diabetic status of these animals.

Cell isolation

Animals were heparinized (2400 units kg^{-1} i.p.), anaesthetized by methoxyflurane inhalation and then killed by cervical dislocation. The hearts were removed and the aortas cannulated on a

Langendorff apparatus. The hearts were then perfused (at 37 °C; 70 cmH₂O pressure) for 5 min with a solution containing (mM): 121 NaCl, 5.4 KCl, 2.8 sodium acetate, 1 MgCl₂, 1 CaCl₂, 5 Na₂HPO₄, 24 NaHCO₃ and 5 glucose. This was followed by a 10 min perfusion with the same solution with CaCl₂ omitted, followed by 7–8 min in the same calcium-free solution to which the following were added: 40 μ M CaCl₂, 10 units ml^{-1} collagenase (Yakult Honsha, Tokyo), 0.01 mg ml^{-1} protease (Sigma Type XIV) and 20 mM taurine.

The free wall of the right ventricle was then dissected and cut into smaller pieces for further incubation (at 37 °C) in calcium-free solution to which was added 0.1 mM CaCl₂, 50 units ml^{-1} collagenase, 0.1 mg ml^{-1} protease, 20 mM taurine and 10 mg ml^{-1} albumin. Cells were collected over the next 30–60 min, and stored in calcium-free solution with no enzymes to which was added 0.1 mM CaCl₂, 20 mM taurine and 5 mg ml^{-1} albumin.

Recording and measurements

Cells were placed in a small chamber on the stage of an inverted microscope, and perfused with a solution containing (mM): 150 NaCl, 5.4 KCl, 1.0 CaCl₂, 1.0 MgCl₂, 5 Hepes and 5.5 glucose, adjusted to pH 7.4 with NaOH. Currents were recorded (at 20–22 °C) using conventional whole-cell suction electrode voltage clamp methods. Electrode resistance was 2–5 M Ω . The filling solution contained (mM): 110 potassium aspartate, 30 KCl, 4 Na₂ATP, 1 MgCl₂, 10 EGTA, 1 CaCl₂ and 5 Hepes, adjusted to pH 7.2 with KOH. This solution produces a junction potential of ~10 mV, which was corrected for. Series resistance (R_s) was minimized by using low resistance electrodes, and by (30–50%) compensation. Recordings with R_s of over 10 M Ω were discarded. For one series of experiments longer recording times were required. Since R_s tends to change with time, leading to changes in the magnitude of the currents being monitored, it was important to control for such drifts in R_s . This was done by interspersing test pulses periodically to monitor for changes. Small drifts in R_s could be corrected by slight adjustments of the negative pressure used in this recording method. Changes of more than 10% led to discarding of the data.

Three potassium currents were measured in all experiments: a calcium-independent transient outward current I_t , which was measured in the presence of 0.3 mM CdCl₂ in the perfusate and 10 mM EGTA in the pipette solution, to ensure inhibition of the calcium-dependent outward current; a slowly inactivating quasi-steady-state current I_{ss} ; and the background inward-rectifier current I_{K1} . I_t was measured as the peak outward current, rather than as the difference current between peak and steady-state values. This is justified based on the following: I_{ss} activates much more slowly than I_t (Apkon & Nerbonne, 1991) so that little I_{ss} is present when peak current is measured; our earlier work (Shimoni *et al.* 1995) showed that the same results were obtained by measuring I_t as either peak current or as the difference current; in addition, I_t and I_{ss} are both altered in insulin-deficient conditions and in response to insulin. These changes are not always present to the same degree, so that relating I_t to the steady-state level may be erroneous. Unfortunately there is no suitable pharmacological tool for inhibiting I_{ss} , in order to separate it from I_t . The comparative statistics were done for current densities at +50 mV, at which no interference by the sodium current is present. I_{K1} was measured as the current elicited by voltage steps to –110 mV. Since this current was found to be unaffected by any of the treatments used, pulses to –110 mV could be used as indicators of drifts in R_s . An increase in R_s produced a reduction in the current elicited by these negative

pulses. When this reduction was larger than 10% (in the longer duration recordings) the cell was abandoned. In all cases current magnitudes were divided by cell capacitance, giving current densities, enabling comparisons between cells of different sizes.

Drugs and reagents

Cycloheximide, cytochalasin D, colchicine and phalloidin were purchased from Sigma. Concentrated stocks were made and aliquots were frozen. Cytochalasin D was dissolved in DMSO and diluted to a final concentration of $1 \mu\text{M}$. This contained 0.03% DMSO, which had no effect on its own. The other reagents were water soluble.

Statistics

One-way ANOVA (followed by a Student–Newman–Keuls multiple comparisons test) or Student's unpaired t test were used for statistical evaluation of differences between groups, with $P < 0.05$ considered significant. It should be noted that due to variation in the degree of insulin deficiency induced by STZ treatment, each group of treatments contained its own control (untreated) group. On each experimental day, recordings were made from several untreated cells, followed by recordings from cells subjected to a given treatment. Data obtained on different days were pooled, for a given treatment.

RESULTS

Effects of insulin

Our earlier work (Shimoni *et al.* 1998) showed that when myocytes from diabetic animals are exposed to 100 nM insulin for 5–9 h, the attenuation in I_t is fully reversed, and that of I_{ss} partially reversed. Since higher concentrations of insulin may produce effects by stimulating insulin-like growth factor receptors in addition to insulin receptors (Steele-Perkins *et al.* 1988), it was important to repeat our results with a lower insulin concentration. In our first experiments (results not shown), we repeated the same protocols using 1 or 10 nM insulin. In 14 cells exposed to 1 nM insulin for 5–9 h, I_t density was $28.2 \pm 2.4 \text{ pA pF}^{-1}$ (mean \pm s.e.m.), and I_{ss} density was $8.8 \pm 0.5 \text{ pA pF}^{-1}$. These values are significantly ($P < 0.05$) larger than in untreated cells, in which mean I_t density was $17.6 \pm 1.1 \text{ pA pF}^{-1}$ ($n = 30$ cells), and I_{ss} density was $6.0 \pm 0.2 \text{ pA pF}^{-1}$.

In addition, in the context of understanding the mechanism by which insulin increases functional ion current density at the cell membrane (see below), we investigated the required

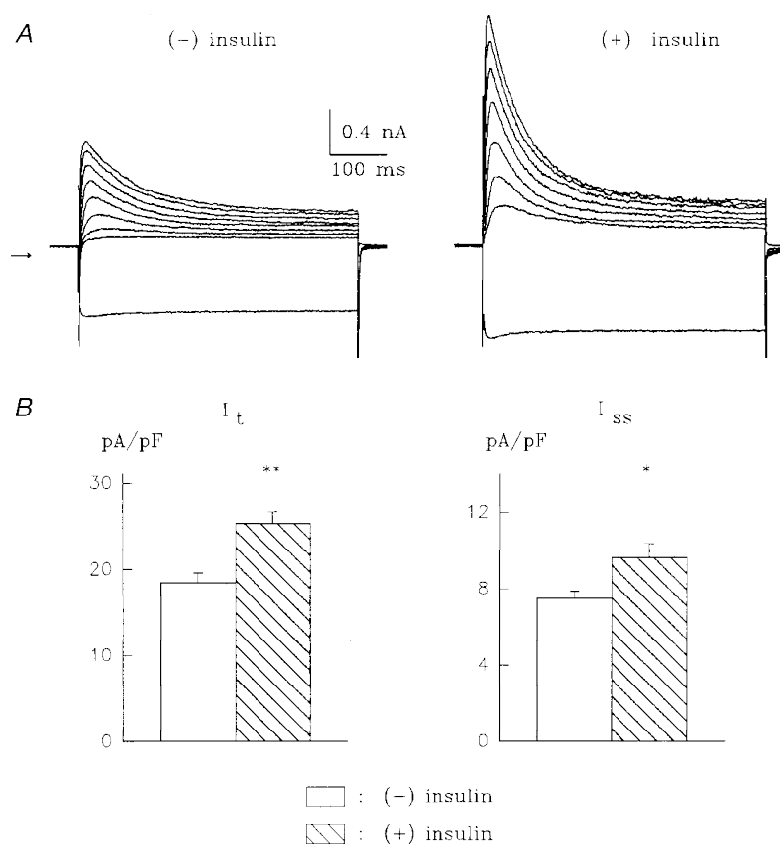


Figure 1. Effects of 10 nM insulin on K^+ currents in ventricular myocytes from STZ-diabetic rats

Insulin was applied for 1 h and subsequently removed by centrifugation and resuspension of myocytes in insulin-free solution. *A*, current traces in response to voltage steps from -80 to -110 mV (lowest trace) and from -80 to potentials ranging from -20 to $+50$ mV (in 10 mV steps) before (left, (-) insulin) and 6 h after (right, (+) insulin) the exposure to insulin. Zero current level is indicated by the arrow to the left. Cell capacitances were 65.8 (left) and 62.8 pF (right). *B*, mean \pm s.e.m. for I_t (left) and I_{ss} (right) obtained from 26 cells in the absence of insulin and from 22 cells recorded 5–9 h after a 1 h exposure to 10 nM insulin. * $P < 0.01$, ** $P < 0.001$.

duration of exposure to insulin. We found that if cells were exposed to 10 nM insulin for 1 h, followed by removal of insulin (by centrifugation, removal of the supernatant and resuspension of the cells in insulin-free storage solution), I_t and I_{ss} were still significantly ($P < 0.001$) augmented, when measured 6–10 h after the initial insulin exposure. Thus, at +50 mV, I_t density (in diabetic myocytes) was 18.4 ± 1.2 pA pF⁻¹ ($n = 26$) in the absence of insulin, and 25.3 ± 1.4 pA pF⁻¹ ($n = 22$) after a 1 h exposure to 10 nM insulin (recorded 6–10 h after exposure). The corresponding values for I_{ss} were 7.5 ± 0.4 and 9.6 ± 0.7 pA pF⁻¹. An example from two cells is shown in Fig. 1.

This result was of importance, since it enabled us to apply several pharmacological agents (see below) 1 h after exposure to insulin, thereby eliminating concerns about possible interference of these agents with insulin binding and activation of the signalling cascade which is responsible for augmentation of I_t and I_{ss} density.

Involvement of protein synthesis

Insulin is known to stimulate protein synthesis in ventricular myocytes from diabetic rats (Braun & Severson,

1992). Thus, the stimulatory effect of insulin on K⁺ currents (Fig. 1) may be due to the synthesis of new channels (for both currents). This notion arose from our previous findings (Shimoni *et al.* 1998) which showed that at least 6 h must elapse before insulin stimulates the K⁺ currents and that this stimulatory effect can be blocked by inhibiting the activation of MAP kinase, a key mediator of transcriptional control by insulin (Saltiel, 1996). In order to test this hypothesis more directly, we examined the effect of insulin after exposing cells to 2 μ M cycloheximide, an inhibitor of protein synthesis.

Cycloheximide (2 μ M) was added 1 h after the addition of 100 nM insulin, and currents were measured 5–9 h later. In 24 cells, it was found that cycloheximide completely abolished the enhancement of both I_t and I_{ss} by insulin. In this group, I_t density at +50 mV was 17.2 ± 0.9 pA pF⁻¹ in untreated cells ($n = 42$), 20.7 ± 1.1 pA pF⁻¹ in insulin-treated cells (significantly larger, $P < 0.05$, $n = 35$) and 17.3 ± 1.2 pA pF⁻¹ in cells exposed to insulin and cycloheximide ($n = 24$). The corresponding values for I_{ss} were 6.7 ± 0.3 , 8.5 ± 0.5 and 6.9 ± 0.5 pA pF⁻¹. Exposure of cells to 2 μ M cycloheximide alone did not alter any of the K⁺

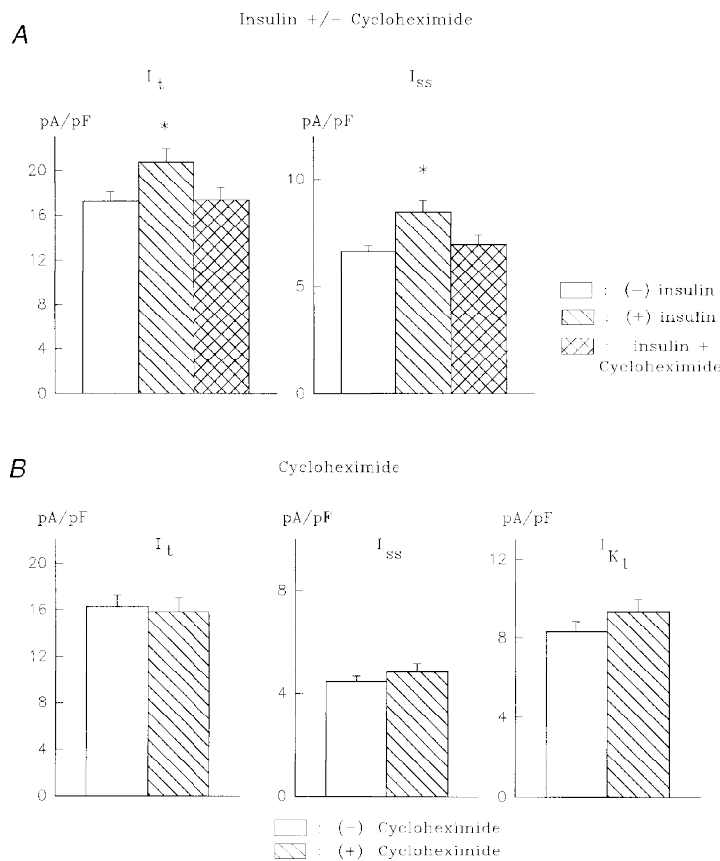


Figure 2. Abolition of the stimulatory effects of insulin on K⁺ currents by cycloheximide

A, mean + s.e.m. of I_t (left) and I_{ss} (right) in the absence of insulin ($n = 42$), after 5–9 h with 100 nM insulin ($n = 35$), and in the presence of 100 nM insulin and 2 μ M cycloheximide ($n = 24$). Cycloheximide was added 1 h after insulin. * $P < 0.05$. B, cycloheximide (2 μ M) alone (for 5–8 h, $n = 14$) has no effect on I_t (left), I_{ss} (centre) and I_{K1} (right), in comparison to control ($n = 16$). Absolute values are given for I_{K1} here and in other figures.

currents measured. The mean values in the absence ($n = 16$) and presence ($n = 14$) of cycloheximide, respectively, were: I_t , 16.3 ± 1.0 and 15.8 ± 1.2 pA pF $^{-1}$; I_{ss} , 4.5 ± 0.2 and 4.8 ± 0.4 pA pF $^{-1}$; I_{K1} , -8.3 ± 0.6 and -9.3 ± 0.6 pA pF $^{-1}$. These results, shown in Fig. 2, show that the stimulatory effect of insulin on I_t and I_{ss} is dependent on protein synthesis.

Involvement of the cytoskeleton

We next examined whether the enhancement of I_t and I_{ss} current density by insulin requires the participation of the actin microfilament network, which has been implicated in the control of ion channel function (Undrovinas *et al.* 1995; Brady *et al.* 1996; Mazzanti *et al.* 1996). Cytochalasin D inhibits actin polymerization and thereby shifts the dynamic balance existing between monomeric and filamentous actin towards the monomeric form (Cooper, 1987). Glucose transporters (GLUT-4) translocate to the cell membrane within minutes after insulin exposure. This translocation process depends on actin microfilaments of the cytoskeleton (Tsakiridis *et al.* 1994). The disruption of this intracellular actin network with cytochalasin D prevents GLUT-4 translocation in response to insulin, without affecting basal glucose transport.

Cytochalasin D elicits maximal effects on disassembly of the actin network in L6 muscle cells within 1–2 h after exposure, at concentrations of 0.1–10 μ M (Tsakiridis *et al.* 1994), although other work suggests that cytochalasin D may act within minutes in some cells (Gottlieb *et al.* 1993).

In our experiments, we added 1 μ M cytochalasin D 1 h after the addition of insulin, ensuring no interference with insulin binding or signal transduction. In 18 cells, the addition of cytochalasin D completely blocked the effects of insulin on both I_t and I_{ss} . The current densities for I_t in the absence of insulin, with insulin alone, and with insulin and cytochalasin D were 18.4 ± 1.0 , 25.4 ± 1.5 and 17.7 ± 1.6 pA pF $^{-1}$, respectively. The corresponding values for I_{ss} were 6.0 ± 0.2 , 7.9 ± 0.4 and 5.9 ± 0.3 pA pF $^{-1}$. Insulin had no effect on I_{K1} , and the combination of insulin and cytochalasin D also did not alter I_{K1} , suggesting that there are no non-selective effects of cytochalasin D in these myocytes (such as increased membrane leakiness). These results are shown in Fig. 3, which also shows the current–voltage relationship for I_t in the presence of insulin, with and without cytochalasin D.

Our protocol, in which cytochalasin D was added 1 h after insulin, allowed us to infer that cytochalasin D did not

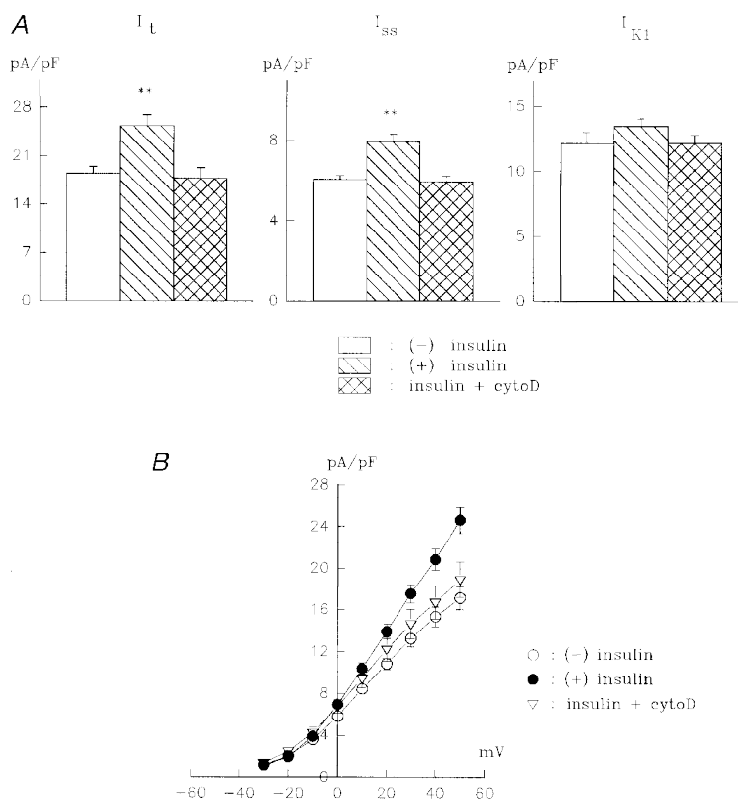


Figure 3. Inhibition of the effects of insulin by cytochalasin D

A, means \pm S.E.M. for I_t (left), I_{ss} (centre) and I_{K1} (right) are shown in the absence of insulin ($n = 45$), after 5–9 h in 10 nM insulin ($n = 16$), and after 5–10 h in 10 nM insulin + 1 μ M cytochalasin D (cytoD, $n = 18$). Cytochalasin D was added 1 h after insulin (** $P < 0.001$). Note that cytochalasin D abolishes the effects of insulin on I_t and I_{ss} , yet does not affect I_{K1} . B, a current–voltage relationship for I_t (peak outward current) in the absence of insulin ($n = 34$), with 10 nM insulin ($n = 23$), and with insulin and cytochalasin D ($n = 17$).

interfere with any of the early stages of insulin signalling. However, it is still possible that cytochalasin D could have interfered with transcription or synthesis of the K^+ channels, in addition to disruption of the cytoskeleton, which may occur in some circumstances (Cooper, 1987). In order to rule out such possible additional effects of cytochalasin D, we carried out experiments in which the action of cytochalasin D is pre-empted by phalloidin, a compound that stabilizes the cytoskeleton (Cooper, 1987). It has been shown that phalloidin can prevent the action of cytochalasins on K^+ channel activity in the collecting duct (Wang *et al.* 1994). Since phalloidin enters cells only poorly (Cooper, 1987), our experiments consisted of a prolonged (4 h) pre-exposure to phalloidin ($2.5 \mu\text{M}$), followed by 1 h exposure to insulin, and then by addition of $1 \mu\text{M}$ cytochalasin D. All recordings were done at least 5 h after exposure of cells to insulin. The results show that phalloidin prevented the inhibitory action of cytochalasin D on the stimulation of I_t

and I_{ss} by insulin, and restored the normal effect of insulin. Thus, mean I_t density (at +50 mV) in the presence of insulin, cytochalasin D and phalloidin was $22.8 \pm 1.7 \text{ pA pF}^{-1}$ ($n = 22$). The mean I_{ss} density under these conditions was $8.3 \pm 0.7 \text{ pA pF}^{-1}$. These values were significantly larger than those in the untreated cells ($P < 0.005$), and not significantly different from cells treated with insulin alone. These results, as well as a summarizing histograms, are shown in Fig. 4.

This last result is significant for two reasons. First, it demonstrates that the stabilization of the actin microfilament system with phalloidin prevents subsequent disruption by cytochalasin D and normalizes the augmenting effects of insulin on the K^+ currents. In addition, this result serves as a crucial control, indicating that cytochalasin D does not have non-specific actions on components other than the cytoskeleton. In other words, this result shows that

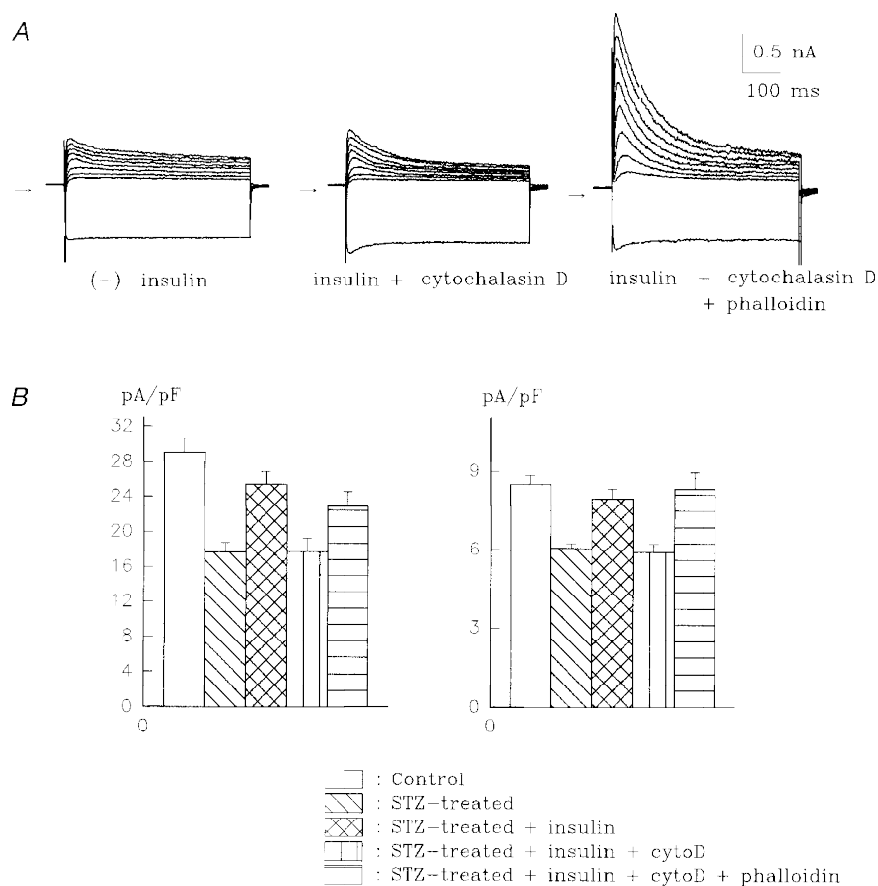


Figure 4. Phalloidin prevents the inhibition by cytochalasin D of the stimulatory insulin effect on K^+ currents

A, current traces (same voltages as in Fig. 1) from cells in the absence of insulin (left), 6 h after exposure to 10 nM insulin in the presence of $1 \mu\text{M}$ cytochalasin D (centre), and with insulin + cytochalasin D after a 4 h pre-incubation with $2.5 \mu\text{M}$ phalloidin (right). Note that cytochalasin D prevents insulin from augmenting the peak outward and steady-state currents (centre), but not if phalloidin is present (right). Cell capacitances are 51.6 (left), 57.4 (centre) and 48.8 pF (right). *B*, summary histogram showing current densities for I_t (left) and I_{ss} (right) in control myocytes ($n = 34$), in untreated STZ-diabetic myocytes ($n = 30$), and in diabetic myocytes treated with 10 nM insulin ($n = 16$), with insulin + cytochalasin D ($n = 18$), and with the combination of insulin, cytochalasin D and phalloidin ($n = 22$).

cytochalasin D does not interfere with any non-microfilament-associated actions of insulin (e.g. with K^+ channel assembly or processing) since the normal action of insulin on K^+ currents is obtained even with cytochalasin D present, as long as the cells are pretreated with phalloidin.

In another set of experiments (results not shown), we ruled out the unlikely possibility that the prolonged exposure to phalloidin somehow upregulates both K^+ currents, thereby producing the results shown in Fig. 4. In cells exposed to $2.5 \mu\text{M}$ phalloidin for 5–9 h, no changes were seen in any of the three K^+ currents. I_t density was $16.4 \pm 1.9 \text{ pA pF}^{-1}$ ($n = 16$) in the absence, and $17.1 \pm 1.8 \text{ pA pF}^{-1}$ ($n = 13$) in the presence of phalloidin. The corresponding values for I_{ss} were 4.9 ± 0.4 and $5.2 \pm 0.3 \text{ pA pF}^{-1}$; for I_{K1} they were -11.3 ± 0.7 and $-10.2 \pm 0.4 \text{ pA pF}^{-1}$.

An important issue in the context of our hypothesis regarding the mechanism of current augmentation by insulin (see Discussion) relates to the timing of the effects of cytochalasin D. In an additional series of experiments we examined whether the addition of cytochalasin D 5–9 h after insulin, when I_t and I_{ss} are already augmented, can reverse the effects of insulin. In 9 of 10 cells obtained from diabetic rats, addition of $1 \mu\text{M}$ cytochalasin D, between 5

and 9 h after insulin, did not diminish either current. In one cell there was a 10% decline, due to increased series resistance. Two examples are shown in Fig. 5. Figure 5A shows current amplitudes against time, after addition of cytochalasin D, while Fig. 5B shows superimposed current traces from another cell, before and 16 min after addition of cytochalasin D. Figure 5A and B illustrates the lack of a rapid change in current amplitude after it has already been augmented by insulin (see Discussion). The lack of effect of cytochalasin D was apparent for up to the longest exposure time of 50 min (1 cell). In the other cells, no effect was seen for up to 12–18 min (bath volume exchanged within 20 s).

Since longer exposure times to cytochalasin D were necessary to block the effects of insulin, we also tested whether long exposure times to cytochalasin D had any effect on the currents measured. In a subsequent set of experiments, current densities were compared in myocytes from control rats (no insulin treatment), in the absence or presence of $1 \mu\text{M}$ cytochalasin D (4–9 h). Figure 6 shows that prolonged exposure to this agent has no effect on any of the K^+ currents measured.

In the final set of experiments we investigated whether the integrity of the microtubular system is also required for the

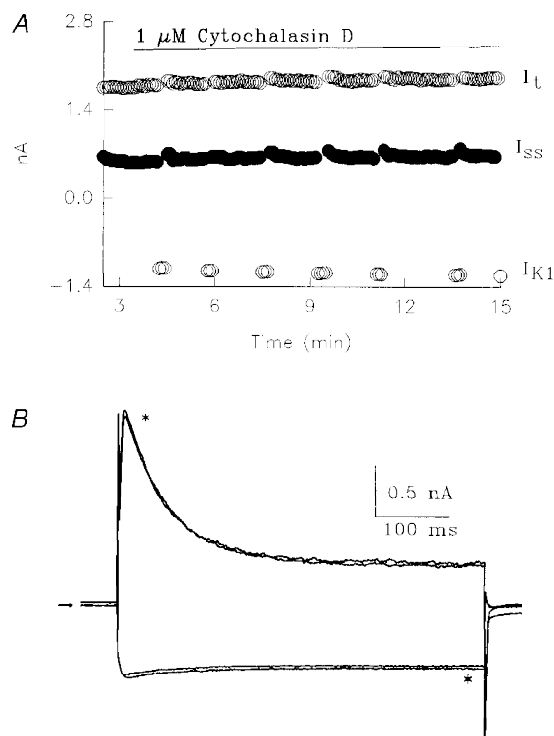


Figure 5. Lack of acute effect of cytochalasin D

Representative results obtained from two cells from diabetic rats, exposed for > 5 h to 100 nM insulin, and then to $1 \mu\text{M}$ cytochalasin D (CD). *A*, this cell was preincubated with 100 nM insulin for 6 h. Current amplitudes are plotted *vs.* time, showing no changes in I_t , I_{ss} or I_{K1} after adding CD to the bath. Bath volume was exchanged within 20 s. *B*, superimposed current traces (different cell, 8 h in insulin), in response to pulses from -80 mV to either $+50$ or -110 mV , before and 16 min after (*) addition of $1 \mu\text{M}$ CD. Arrow denotes zero current level.

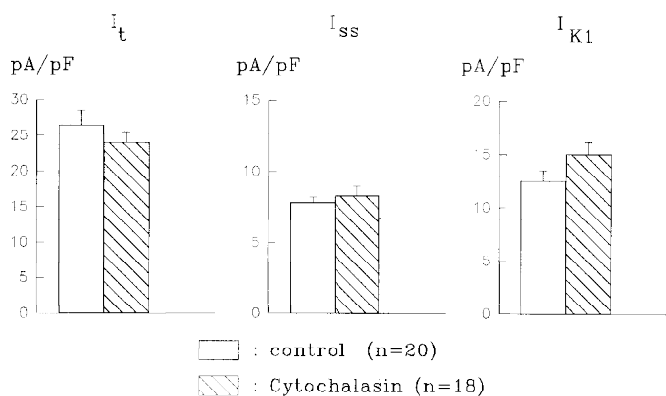


Figure 6. Lack of effect of prolonged exposure to cytochalasin D

Histograms show the mean current densities obtained from control myocytes or following 4–9 h in $1 \mu\text{M}$ CD. Densities for I_t (left), I_{ss} (middle) and I_{K1} (right) are not significantly ($P > 0.05$) altered by CD.

enhancement of I_t and I_{ss} by insulin. Cells from diabetic rats were incubated either with 100 nM insulin alone (for at least 5 h), or with insulin and colchicine ($5 \mu\text{M}$). Colchicine was added 1 h after insulin, and recordings made between 5 and 10 h after addition of insulin. The treatment with colchicine was also found to abolish the enhancement of both I_t and I_{ss} by insulin, as shown in Fig. 7. No deleterious effects of colchicine were observed, since the cells maintained a normal resting potential, and I_{K1} was unaffected. At -110 mV , the mean density of I_{K1} was 12.3 ± 0.7 ($n = 28$), 11.9 ± 0.6 ($n = 29$) and $11.0 \pm 0.8 \text{ pA pF}^{-1}$ ($n = 21$) in untreated, insulin-treated, and insulin + colchicine-treated cells, respectively.

DISCUSSION

Summary of findings

Our present results further characterize our previous findings by showing that depressed K^+ currents in ventricular myocytes from insulin-deficient rats can be restored by $1\text{--}10 \text{ nM}$ insulin (in contrast to 100 nM used previously). First, it is shown (Fig. 1) that although these effects can only be measured with a delay of at least 5–6 h, an insulin exposure time of only 1 h is sufficient to initiate the stimulatory effect on K^+ currents. This effect is sensitive to cycloheximide (Fig. 2), implying the involvement of protein synthesis, presumably of new K^+ channels. Our most significant result is that the stimulatory effect of insulin on

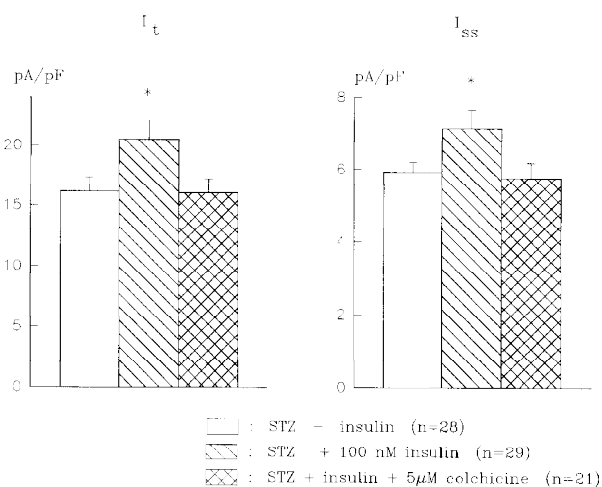


Figure 7. Block of insulin effects by colchicine

Summary histograms showing that the augmentation of both I_t (left) and I_{ss} (right) by 100 nM insulin is abolished by $5 \mu\text{M}$ colchicine. Cells were incubated for at least $5\frac{1}{2}$ h with insulin. Colchicine was added 1 h after insulin. The density of both currents in the presence of insulin is significantly ($*P < 0.05$) larger than the density in the absence of insulin, or the density in insulin + colchicine.

I_t and I_{ss} is sensitive to cytochalasin D (Fig. 3) and colchicine (Fig. 7), agents that disrupt the actin microfilament and microtubule networks, respectively. The inhibitory effect of cytochalasin D can be blocked by an actin microfilament stabilizer, phalloidin, so that the normal stimulatory effect of insulin can be restored (Fig. 4). Cytochalasin D has no effect once the currents have already been augmented by insulin, nor does it alter currents in control myocytes, even with prolonged (4–9 h) exposure.

Implications and significance

Our results suggest that K^+ currents are attenuated in the insulin-deficient diabetic state due to a reduction in the number of active channels. Addition of insulin stimulates the synthesis of new channels, which are presumably translocated to the cell membrane. This process depends on both the microtubule and the actin microfilament systems, based on the sensitivity of the insulin effects to cytochalasin D and colchicine.

However, this interpretation must be made with caution. Cytochalasin D disruption of the actin cytoskeleton has been reported to interfere with the initial events in insulin signalling, including activation of MAP kinase (Tsakiridis *et al.* 1997). Since in our experiments insulin addition to the cardiomyocytes preceded cytochalasin D by 60 min, it would appear that the subsequent cytochalasin D-sensitive steps involve more distal events, possibly the movement of newly synthesized K^+ channels to the plasma membrane. Other possible effects of cytochalasin D, unrelated to cytoskeletal disruption, were ruled out by the finding (Fig. 4) that insulin exerted its normal stimulating action in the presence of cytochalasin D when the cytoskeleton was stabilized with phalloidin. Nevertheless, it is still possible that cytochalasin D inhibits (channel) protein synthesis, if this process is itself dependent on an intact cytoskeleton. Cytochalasin D has been shown by Ornelles *et al.* (1986) to release mRNA from actin microfilaments, but these results were obtained at a concentration (10 μM) considerably higher than used here (their K_D values were 14 and 100 μM , whereas in the present experiments only 1 μM cytochalasin was used).

Earlier work on ion channels had shown that a polarized distribution of ion channels in *Xenopus* oocytes can also be disrupted by anticytoskeletal agents (Peter *et al.* 1991), although it was unclear from that work if the distribution of mRNA or of processed protein was affected by the disruption of the cytoskeleton. Earlier work showed that trafficking of transporting proteins depends on the integrity of the cytoskeleton (Bradbury & Bridges, 1994; Janmey, 1998). Our results show that this is also the case for voltage-dependent ion channels.

The present results differ from previous reports which showed that ion channel function can be affected by disruption in the microfilament or microtubule systems (Galli & Defelice, 1994; Undrovinas *et al.* 1995; Furukawa *et al.* 1996). Previous work dealt with cytoskeletal disruption affecting channels already in place. Our results suggest that

such disruption prevents the targeting of new channels to the cell membrane following their formation. When cytochalasin D is added acutely to cells from diabetic animals, after at least 5 h in insulin, when I_t and I_{ss} are already augmented, there is no effect on current amplitudes (Fig. 5). This suggests that once the new channels are inserted into the cell membrane, they are no longer sensitive to cytoskeletal disruption, during a short-term exposure to cytochalasin D (12–50 min). This strongly supports our hypothesis that it is the translocation of insulin-induced, newly synthesized channels that is disrupted by cytochalasin D and perhaps by colchicine. The lack of effect of prolonged exposure to cytochalasin D (Fig. 6) shows that the on-going activity of these channels does not depend on the integrity of the cytoskeleton. This further suggests that the turnover rate of these channels is slow, and unaffected by such disruption. It is possible that much longer periods of disruption would impair the renewal of channels in the membrane, and contribute to a faster run-down.

Our results have important implications for the normal and pathological control of the processing of ion channels and their targeting to the cell membrane. Actin microfilament disruption has also been found to affect channel activity in hippocampal neurons (Rosenmund & Westbrook, 1993), suggesting that our results may apply to other excitable tissues. It is now recognized that abnormalities in cytoskeletal proteins play a role in cardiomyopathies (Towbin, 1998). Our results suggest that insulin-deficient diabetes may induce functional abnormalities in cytoskeletal elements due to reduced levels of insulin, as has been suggested to occur in skeletal muscle (Chen-Zion *et al.* 1994). Tsakiridis *et al.* (1994) showed that the cytoskeletal microfilament system was essential for the rapid translocation of pre-formed glucose transporters to the cell membrane, in response to insulin. Following exposure to insulin, they observed a re-arrangement of the actin network. Future studies will have to address the issue of whether a deficiency in insulin affects the structure of the actin network in cardiomyocytes as well.

Limitations of this study

The major limitation of this study is that we are unable as yet to provide more direct evidence either for the increase in synthesis of K^+ channels by insulin, or for the translocation of newly synthesized K^+ channels to the cell membrane. Although we demonstrate that protein synthesis inhibition blocks the enhancing effect of insulin on I_t and I_{ss} , and suggest that new K^+ channels are formed, other interpretations are possible. For example, it is possible that in type I diabetes there is a deficiency not in K^+ channels *per se*, but in an accessory protein required for the activation of channels, or for transporting latent channels from an inactive pool to the membrane. In this scenario, addition of insulin would augment the synthesis of this accessory protein, so that more active channels are present in the membrane. This transfer of latent channels to the membrane may be the process requiring the cytoskeleton. Since the

channel isoforms underlying I_t in rat ventricle have been identified as Kv4.2 and Kv4.3 (Dixon *et al.* 1996; Fiset *et al.* 1997), it may be possible in the near future to use isoform-specific antibodies to examine specific K^+ channel content by immunoblotting. This may indicate whether new channels are synthesized in response to insulin. Tagging these channel isoforms with fluorescent probes may enable a visualization of their proposed translocation to the cell membrane.

Another limitation is the lack of direct visualization of actin microfilaments and associated proteins. This will be attempted in future studies, although separating the actin microfilament network elements from contractile elements will be extremely difficult in cardiac myocytes (Rothen-Rutishauser *et al.* 1998). Staining of these elements is usually reported for non-contractile cells or muscle cells maintained in culture (Tsakiridis *et al.* 1994; Fernandez-Borja *et al.* 1995), so that tagging of other cytoskeleton-associated proteins may be required.

Finally, our results depend on the selectivity of the pharmacological tools at our disposal. Nevertheless, the combination of the effects of cytochalasin D in the absence and presence of phalloidin (Figs 3 and 4) make a compelling case for the involvement of the cytoskeleton in the insulin-dependent augmentation of the K^+ currents. The results suggest that cytoskeletal elements are involved in the translocation of newly formed K^+ channels to the cell membrane.

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Acknowledgements

Some of this work was done in the laboratory of Dr W. Giles, whose support we gratefully acknowledge. Grant support from the Canadian Diabetes Association in honour of Mary Selina Jamieson (to Y.S.) and from the Medical Research Council of Canada (to D.S.) is also gratefully acknowledged.

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