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Effects of insulin and high glucose on mobilization of Slo1 BK_{Ca} channels in podocytes

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Abstract

Podocytes are dynamic polarized cells that lie on the surface of glomerular capillaries and comprise an essential component of the glomerular filtration barrier. Podocytes are affected in the earliest stages of diabetic nephropathy and insulin signaling to podocytes is essential for normal glomerular function. Large-conductance Ca²⁺-activated K⁺ channels (BK_{Ca} channels) encoded by the *Slo1* gene are expressed in podocytes in a complex with multiple glomerular slit diaphragm proteins including nephrin, TRPC6 channels, and several different actin-binding proteins. Here we show that insulin increases cell surface expression of podocyte BK_{Ca} channels, which is accompanied by a corresponding increase in the density of current flowing through these channels. Insulin stimulation of BK_{Ca} channels was detectable in 15 minutes and required activation of both Erk and Akt signaling cascades. Exposure to high glucose (36.1 mM) for 24 hr caused a marked reduction in the steady-state surface expression of BK_{Ca} channels as well as of the slit diaphragm signaling molecule nephrin. High glucose treatment also abolished the stimulatory effects of insulin on BK_{Ca} current density, although insulin continued to increase phosphorylation of Erk and Akt under those conditions. Therefore, in contrast to most other cell types, high glucose abrogates the effects of insulin in podocytes at relatively distal steps in its signaling pathway. Insulin stimulation of BK_{Ca} channels in podocytes may prepare podocytes to adapt to changes in pressure gradients that occur during postprandial hyperfiltration.

Keywords

podocytes; diabetes; K channel; trafficking; nephrin

INTRODUCTION

Polarized cells known as podocytes are essential components of the glomerular filtration barrier in the kidney. These cells extend primary processes that ramify and terminate in foot processes that attach to the glomerular basement membrane. The specialized contacts between adjacent podocyte foot process form porous structures known as slit diaphragms that are 30–40 nm in width (Rodewald and Karnovsky, 1974). The glomerular filtration barrier is permeable to small solutes, but normally inhibits passage of plasma proteins such as albumin into the ultrafiltrate. Type 1 and type 2 diabetes can lead to changes in the structure of podocyte foot processes, loss of podocytes, reduction in slit diaphragm proteins, and failure of the glomerular filtration barrier (Pagtalunan et al., 1997; Steffes et al., 2001; Doublier et al., 2003). This in turn triggers a cascade of events that can ultimately lead to loss of the entire nephron and to end-stage renal failure (Kriz and Lehir, 2005; Shankland, 2006).

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Podocytes are continually exposed to substantial mechanical forces owing to cyclical distension of the glomerular capillary wall and fluid shear stress (Endlich and Endlich, 2006; Peti-Peterdi and Sipos, 2010). The arrangement of F-actin, myosin, and α -actinin in podocyte foot processes has been proposed to facilitate adaptation of the glomerulus to changes in pressure gradients, or to modify the surface area for filtration (Drenckhahn and Franke, 1988; Kriz et al., 1994; Kriz et al., 1996). There is evidence that Ca^{2+} dynamics play a role in mediating adaptive responses in podocyte function (Peti-Peterdi, 2006; Saleem et al., 2008), although these cells are not excitable. Podocytes express large-conductance Ca^{2+} -activated K^{+} channels (BK_{Ca} channels) encoded by the *KCNMA1* gene (also known as *Slo1* and *KCa1.1*) which are likely to play a role in regulating these dynamics (Morton et al., 2004; Kim et al., 2008; Dryer and Reiser, 2010). BK_{Ca} channels contain binding sites for Ca^{2+} on a large cytosolic domain in the COOH-terminus of the pore-forming subunits. Binding of Ca^{2+} to these sites allows for voltage-dependent gating of BK_{Ca} channels at physiological membrane potentials. Within glomeruli, BK_{Ca} channels are expressed in podocytes and mesangial cells (Morton et al., 2004; Kim et al., 2010). It is notable that in addition to Ca^{2+} -dependent gating, the BK_{Ca} channels of cultured human podocytes also become active in response to membrane stretch (Morton et al., 2004). Moreover, they can be shown to interact directly with Ca^{2+} -permeable TRPC6 channels in podocytes (Kim et al., 2009a). BK_{Ca} channels in podocytes may play a role to facilitate Ca^{2+} influx through non-selective cation channels such as TRPC6 (Dryer and Reiser, 2010), which require a large driving force in order to efficiently transport Ca^{2+} into cells (Estacion et al., 2006). The very large conductance of BK_{Ca} channels—greater than 100 pS with physiological ionic gradients—ensures that activation of a small number of them can dominate the electrical behavior of an electrically compact cell by effectively clamping the membrane to the potassium equilibrium potential.

Podocytes are direct targets for insulin, which has been shown to cause an acute increase in glucose uptake into cultured podocyte cell lines and in human podocytes *ex vivo* owing to translocation of the glucose transporters GLUT1 and GLUT4 to the plasma membrane (Coward et al., 2005, 2007). In podocytes this effect of insulin requires nephrin, a protein that is not expressed in skeletal muscle or adipocytes (Coward et al., 2007). Nephrin is a transmembrane cell adhesion molecule whose ectodomains form an essential component of glomerular slit diaphragms (Kestilä et al., 1998; Putaala et al., 2001). The cytoplasmic domains of nephrin form a platform for assembly of signaling complexes, including the p85 subunit of phosphoinositide-3'-OH-kinase (PI3K) and nephrin signaling is functionally connected to cytoskeletal dynamics in podocytes (Benzing et al., 2004). Importantly, insulin signaling specifically in podocytes has been recently shown to be essential for normal glomerular filtration (Welsh et al., 2010).

In several recent studies we have shown that the steady-state surface expression of BK_{Ca} channels in podocytes requires nephrin (Kim et al., 2008) as well as other proteins that are normally expressed in the slit diaphragm domains of foot processes (Kim et al., 2009a, 2009b, 2010; Ridgway et al., 2009). We have also shown that growth factors can stimulate surface expression of neuronal BK_{Ca} channels (Dryer et al., 2003; Chae et al., 2005). We now report that insulin increases macroscopic currents through endogenously expressed podocyte BK_{Ca} channels owing at least in part to an increase in their steady-state expression on the cell surface. Culturing podocytes in the presence of very high glucose (36.1 mM) markedly suppressed basal surface expression of both BK_{Ca} channels and nephrin, and prevented insulin from increasing BK_{Ca} current density in podocytes. Surprisingly, high glucose did not abrogate more proximal steps in insulin signaling in podocytes, as insulin-evoked phosphorylation of Erk and Akt was similar to that observed in cells maintained in normal glucose. These results suggest that metabolic changes in diabetes could affect glomerular filtration by altering ion channel expression in podocyte foot processes.

MATERIALS AND METHODS

Cell culture protocols

Mouse podocyte cell lines (obtained from Dr. Peter Mundel of the University of Miami Miller School of Medicine) were maintained in RPMI-1640 medium containing 11.1 mM glucose supplemented with 10% fetal bovine serum and 100U/ml penicillin-streptomycin, with or without recombinant mouse γ -interferon, in humidified 5% CO₂ incubators. The cell line was allowed to propagate at 33°C. Removal of γ -interferon and temperature switch to 37°C induced podocyte differentiation and expression of podocyte markers in 14 days (Kim et al., 2009a, 2009b, 2010; Ridgway et al., 2009). Differentiated cells were transferred to serum-free media 24 hr before experiments with insulin. Cells were then treated with 100 nM recombinant human insulin for various lengths of time before analyses. High glucose media were prepared by adding 25 mM extra glucose to RPMI-1640 media (to make a total of 36.1 mM D-glucose). In some control experiments we used normal RPMI-1640, but in most of the experiments control cells were maintained in RPMI-1640 with 25 mM mannitol added to control for osmotic effects of high glucose.

Immunoblot analysis and cell-surface biotinylation assays

These were carried out as described in detail previously (Kim et al., 2008, 2010). Briefly, podocyte lysates were separated by SDS-PAGE on 10% gels and transferred to filters. Blots were blocked, incubated with a primary antibody overnight at 4 °C, washed again, and the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Proteins were visualized using a chemiluminescent substrate. The primary antibodies were anti-nephrin (Santa Cruz), anti-Slo1 (Chemicon), and anti- β -actin. In addition, immunoblot analyses of Akt and Erk1/2 phosphorylation were carried out using antibody pairs that recognize phosphorylated (active) and total forms of these enzymes (Cell Signaling Technology, Danvers, MA). For cell-surface biotinylation assays, intact podocytes were treated with a membrane impermeable biotinylation reagent, sulfo-*N*-hydroxy-succinimidobiotin (Pierce Biotechnology, Rockford, IL) (1 mg/ml) for 1 h. The reaction was stopped, cells were lysed, and biotinylated proteins from the cell surface were recovered by incubation with immobilized streptavidin-agarose beads. A sample of the initial cell lysate was retained for analysis of total proteins. These samples were quantified by immunoblot analysis using anti-Slo1 or anti-nephrin, followed by densitometric analysis using Image J software. All experiments were repeated at least three times.

Electrophysiology

Whole cell recordings were made as described in detail previously (Kim et al., 2008, 2009a, 2009b, 2010; Ridgway et al., 2009). Briefly, the bath solution contained 150 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 5.4 mM CaCl₂, 10 mM HEPES, pH 7.4. Pipette solutions contained 10 mM NaCl, 125 mM KCl, 6.2 mM MgCl₂, 10 mM HEPES, pH 7.2, and 5 μ M free Ca²⁺. Currents were evoked by a series of depolarizing voltage steps from a holding potential of -60 mV. The recording electrodes had resistances of 3–4 M Ω and it was possible to compensate up to 85% of this without introducing oscillations into the current output of the patch clamp amplifier. Whole cell currents were not observed when recording pipettes contained solutions with no added Ca²⁺ and 10 mM EGTA (data not shown). PD98059 and LY294002 were dissolved in DMSO before adding to culture media.

Statistics

All quantitative data are presented as mean $\pm$ s.e.m. Data were analyzed by Student's *t*-test, and by one-way ANOVA followed by *post hoc* analysis, when multiple comparisons were made. Throughout, *p* < 0.05 is regarded as significant.

RESULTS

In initial experiments we examined the effect of insulin (100 nM) on the steady-state surface expression of the pore-forming subunits of BK_{Ca} channels (Slo1 proteins) in differentiated cells of a mouse podocyte cell line. Cell-surface biotinylation assays showed that insulin exposure for 15 min could evoke an increase in the surface expression of Slo1 relative to the total amount of Slo1 expressed in podocytes (Fig. 1). Densitometric quantification of three repetitions of this experiment (Fig. 1B) showed a greater effect with longer insulin exposures of 3 hr and 24 hr. A stimulatory effect of insulin could also be seen in whole-cell recordings of BK_{Ca} channels from podocytes made using recording electrodes filled with a solution containing 5 μ M free Ca²⁺ (Fig. 2). Including micromolar concentrations of free Ca²⁺ in the recording pipettes allowed measurement of BK_{Ca} current density in response to depolarizing voltage steps from a holding potential of -60 mV. In previous studies we have shown that the outward currents evoked using these protocols are entirely attributable to BK_{Ca} channels (Kim et al., 2008,2010) and we observed here that the currents evoked in insulin-treated cells are completely blocked by the selective BK_{Ca} inhibitor paxilline (Fig. 2A, B). The results obtained by cell-surface biotinylation assays and whole cell recordings are not quantitatively identical. This may reflect differences intrinsic to the two methods, especially given the nonlinearity and high amplification of the chemiluminescent detection systems. However, we cannot exclude that insulin may also modulate activity of BK_{Ca} channels already in the plasma membrane as well as stimulating trafficking, especially given that insulin has been shown to regulate BK_{Ca} gating in other cell types (O'Malley and Harvey, 2004,2007). However, we did not observe any effect of insulin on the voltage-dependence (Fig. 2C) or kinetics (Fig. 2D) of BK_{Ca} activation in podocyte cell lines, and insulin increased mean currents at all membrane potentials where they could be detected.

The stimulatory effects of insulin on BK_{Ca} channels were associated with activation of mitogen-activated protein kinase (ERK1/2) and PI3K/Akt signaling cascades. Using immunoblot analysis we observed an increase in the phosphorylated (active) form of Erk1/2 (Fig. 3A) that could be seen as early as 1 min after insulin application and that was largely inhibited by pretreatment with the MEK1 inhibitor PD98059 (50 μ M) (Fig. 3B, C). Moreover, insulin-evoked stimulation of BK_{Ca} channels was blocked by PD98059 as assessed by cell-surface biotinylation assays (Fig. 3D) and by whole-cell recordings (Fig. 3E). Insulin also evoked a rapid increase in the phosphorylated (active) form of Akt (Fig. 4A) and this effect was reduced by pretreatment with the PI3K inhibitor LY294002 (50 μ M) (Fig. 4B, C). Moreover, insulin-evoked increases in Slo1 surface expression (Fig 4D) and macroscopic BK_{Ca} currents (Fig. 4E) were blocked by pretreatment of podocytes with LY294002 (Fig. 3F). These data indicate that both of these signaling pathways are necessary for insulin modulation of BK_{Ca}.

Elevated extracellular glucose is sufficient to cause insulin resistance in many different cells and tissues, and can act at several different steps along insulin signaling pathways (Pirola et al., 2004). We observed that culturing podocytes in high glucose (36.1 mM) for 24 hr or 72 hr caused a marked decrease in the steady-state expression of BK_{Ca} channels as assessed by cell-surface biotinylation assays (Fig. 5A, B). In these experiments, control cells were cultured in normal medium containing 11.1 mM glucose or in a medium containing 11.1 mM glucose and 25 mM mannitol in order to produce an osmotic effect similar to that of high glucose. A corresponding effect was also seen with whole-cell recordings, which showed a marked decrease in BK_{Ca} current density in cells cultured in high glucose (Fig. 5C). Given this, we were surprised to note that high glucose treatment did not completely abrogate insulin signaling in podocytes. Thus, treatment with insulin in the presence of 36.1 mM glucose evoked increases in Erk (Fig. 6A) and Akt (Fig. 6B) phosphorylation comparable to those observed in podocytes cultured in 11.1 mM glucose plus 25 mM

mannitol. Insulin treatment also increased surface expression of Slo1 channels in cells cultured in 36.1 mM glucose (Fig. 7A, B) although it should be noted that Slo1 surface expression always remains low compared to what is observed in insulin-treated cells maintained in 11.1 mM glucose.

Using more quantitative electrophysiological methods, we observed podocytes were resistant to insulin when the measured endpoint was BK_{Ca} current density assayed by whole-cell recordings (Fig. 7C, D). With those methods we observed that culturing podocytes in 36.1 mM glucose resulted in markedly reduced basal levels of macroscopic current density compared to cells cultured in 11.1 mM glucose plus 25 mM mannitol, as expected from the results of cell-surface biotinylation assays. Moreover, insulin did not evoke any discernible increase in BK_{Ca} current density from the already low baseline in cells cultured in high glucose. Insulin and glucose concentration did not affect the voltage-dependence of BK_{Ca} activation and uniformly affected the amplitudes of the currents at all step voltages tested (Fig. 7D, E).

The slit diaphragm protein nephrin is required for normal trafficking of BK_{Ca} channels and glucose transporters to the cell surface (Coward et al., 2007; Kim et al., 2008) as well as for the normal function of the glomerular filtration slit (Kestilä et al., 1998; Putaala et al., 2001). Therefore, we examined the effects of high glucose on the surface expression of nephrin using cell-surface biotinylation assays. We observed that culturing cells in 36.1 mM glucose markedly reduced surface expression of nephrin compared to controls maintained in 11.1 mM glucose. Although insulin treatment could partially restore surface expression of nephrin in the presence of 36.1 mM glucose, it remained substantially less than what is seen in control media (Fig. 8).

DISCUSSION

In this study we have shown that insulin stimulates the surface expression of the pore-forming subunits (Slo1 proteins) of BK_{Ca} channels in mouse podocytes. This is accompanied by an increase in BK_{Ca} channel activity that can be readily observed in whole-cell recordings. Insulin effects on BK_{Ca} channels appear to be the result of a complex transduction cascade that includes activation of Erk MAP kinase and PI3K/Akt signaling cascades. Exposing podocytes to elevated extracellular glucose for 24 hr caused a marked reduction in the expression of BK_{Ca} channels and nephrin on the cell surface. High glucose also blocked insulin-evoked increases in BK_{Ca} currents measured by whole-cell recordings. However high glucose did not completely abrogate insulin signaling in podocytes, since insulin continued to evoke robust stimulation of Erk and Akt phosphorylation and could restore some of the glucose-evoked loss of nephrin expression on the cell surface.

Previous studies have shown that podocytes are direct targets for insulin action (Coward et al., 2005, 2007), and a more recent study has shown that insulin signaling in podocytes is required for normal glomerular function (Welsh et al., 2010). In podocytes, as in other cells, insulin increases glucose uptake owing to translocation of GLUT1 and GLUT4 transporters to the cell surface (Coward et al., 2005, 2007). However, those initial studies already identified unique features of insulin signaling in podocytes compared to what is seen in many other cell types, since nephrin was required for insulin stimulation of GLUT4 translocation (Coward et al., 2007). Nephrin is a single-pass transmembrane adhesion protein of the immunoglobulin superfamily. It has a large ectodomain comprised of multiple IgG-like domains and a single fibronectin-like motif, and a cytoplasmic domain that can interact with several other proteins (Kestilä et al., 1998). It is not generally expressed in skeletal muscle or adipocytes. We have previously shown that nephrin can interact with Slo1 proteins and β 4-subunits of BK_{Ca} channels, and that nephrin and actin dynamics are

necessary for the normal expression of functional BK_{Ca} channels on the podocyte cell surface (Kim et al., 2008, 2010).

The mechanisms by which insulin stimulates of BK_{Ca} activity in podocytes appear to be complex, and may have similarities to growth factor effects on BK_{Ca} channels of neurons. For example, certain growth factors such as TGFβ1 increase translocation of BK_{Ca} to the neuronal cell surface through a process that depends on activation of PIK3 and Erk (Dryer et al., 2003; Chae et al., 2005) and remodeling of cortical actin (Chae and Dryer, 2005; Zou et al., 2008). Insulin can modulate the gating of neuronal BK_{Ca} channels through an Erk-dependent signaling cascade that is also associated with actin remodeling (O'Malley and Harvey, 2004, 2007). Insulin also stimulates BK_{Ca} channels of mesangial cells via an Erk-dependent process that may entail increased expression on the cell surface (Foutz et al., 2008). It seems likely that insulin regulation of BK_{Ca} channels in podocytes is as complex as it is in neurons. However, effects of insulin on podocyte BK_{Ca} channels that are already in the plasma membrane, if they occur, must be subtle, as we did not observe any effects on the kinetics or voltage-dependence of the activation of macroscopic currents, which are usually sensitive indicators of changes in gating properties.

Exposure to high glucose for 24 hr caused a very marked reduction in the steady-state surface expression of podocyte BK_{Ca} channel subunits accompanied by a quantitatively similar reduction in the surface expression of nephrin. The reduction in the surface expression of nephrin is sufficient to explain the reduction in basal BK_{Ca} channels (Kim et al., 2008). Based on previous studies, one would predict that high glucose would also result in a reduction in insulin-evoked glucose uptake into podocytes, which also requires nephrin (Coward et al., 2007), although to our knowledge this has not been examined directly. This effect of high glucose may be pathophysiologically significant, as reductions in glomerular nephrin expression are a feature of both type 1 and type 2 diabetes (Doublier et al., 2003; Benigni et al., 2004; Langham et al., 2002). They are also apparent in the earliest stages of animal models of diabetes (Aaltonen et al., 2001; Kim et al., 2007; Menne et al., 2006) and are mediated in part by increased endocytosis of nephrin (Tossidou et al., 2010). We should note that high glucose does not uniformly reduce surface expression of all membrane proteins, as we have observed that high glucose treatment causes a marked increase in the steady-state surface expression of TRPC6 cation channels (unpublished observations). Those observations will be the subject of a later report.

In adipocytes and fibroblasts high glucose can induce insulin resistance by acting at the initial steps in the transduction cascade, resulting in inhibition of insulin receptor autophosphorylation and phosphorylation of insulin receptor substrate (IRS) proteins (Müller et al., 1991; Berti et al., 1994; Ide et al., 1994). Given that, we were surprised to observe that insulin-evoked phosphorylation of Erk or Akt persisted in podocytes cultured in high glucose. On the other hand, high glucose completely blocked insulin-evoked increases in density of current flowing through functional BK_{Ca} channels. This indicates that high glucose can impinge on insulin responses in podocytes at relatively distal stages in the transduction pathway. In any case, the effects of high glucose are likely to be complex, cell type-dependent, and may impact multiple cell physiological processes in podocytes.

Insulin modulation of BK_{Ca} channels may proceed through several different pathways that are not mutually exclusive. One potential pathway arises from insulin stimulation of PI3K activity, which leads to production of phosphatidylinositol-3,4,5-tris-phosphate [InsP(3,4,5)P₃]. Phosphatidylinositol polyphosphates have been shown to stimulate activation of BK_{Ca} channels through a membrane delimited process that targets the channel or its proteolipid environment (Vaithianathan et al., 2008). Among the phosphoinositides, InsP(3,4,5)P₃ is by far the most effective at causing activation of BK_{Ca} (Vaithianathan et al.,

2008), and is therefore in a position to regulate both gating as well as cytoskeletal rearrangements that underlie trafficking to the cell surface. Disruption of nephrin expression could displace PI3K and therefore its catalytic product from the vicinity of BK_{Ca} channels (Benzing, 2004) thereby preventing their activation—even if total InsP(3,4,5)P₃ and Akt activation persist at normal levels. It is also possible that insulin affects actin dynamics via PI3k and Erk mediated pathways to regulate trafficking or gating of BK_{Ca} channels (O'Malley and Harvey, 2007; Zou et al., 2008).

How might this response to insulin impact glomerular physiology? It has long been known that feeding (Corman et al., 1988; Uemasu et al., 1991; Finco and Cooper, 2000) or intravenous glucose (Christiansen et al., 1981) or amino acid (Castellino et al., 1986) infusion evoke an acute increase in the glomerular filtration rate (GFR). Increases in GFR are generally associated with an increase in the net pressure gradient across the glomerular filtration barrier, which can occur through multiple mechanisms (Persson et al., 2010). Podocytes are thought to play an active role in maintaining the permselectivity of the glomerular filtration barrier in the face of distending forces (Kriz et al., 1994, 1996) and there is evidence that the failure of podocytes to generate sufficient force to counteract filtration barrier pressure gradients can lead to glomerulosclerosis (Kriz et al., 2005). Insulin-evoked mobilization of BK_{Ca} channels may therefore be part of a signaling mechanism that allows the glomerular filtration apparatus to adapt to mechanical challenges associated with increases in GFR. In this regard, an elegant recent study has shown that deletion of insulin receptors selectively in podocytes leads over time to failure of the glomerular filtration barrier, even though the animals are normoglycemic (Welsh et al., 2010). The precise role of BK_{Ca} channels in podocyte function has not been established, but they interact directly with Ca²⁺-permeable TRPC6 channels in these cells (Kim et al. 2009) and their coordinated activation would be expected to increase the driving force for Ca²⁺ influx into podocytes through TRPC6 and related channels (Estacion et al., 2006; Dryer and Reiser, 2010), thereby contributing to active responses in these cells. BK_{Ca} channels may also be involved in Ca²⁺ oscillations and Ca²⁺ waves observed in podocytes within intact glomeruli during tubuloglomerular feedback (Peti-Peterdi, 2006).

In summary, we have shown that insulin modulates BK_{Ca} channels in podocytes. We have also shown that cell surface expression of BK_{Ca} channels and nephrin are down-regulated by high glucose, and that exposure to high glucose for as little as 24 hr blocks insulin responses at a downstream step in the overall signal-transduction pathway.

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Figure 1.

Exposure to insulin (100 nM) stimulates increases the steady-state expression of the pore-forming subunits of BK_{Ca} channels (Slo1 proteins) on the cell surface of podocytes. *A*, Example of typical cell-surface biotinylation assay with duration of insulin treatments as indicated. *B*, Densitometric quantification of three repetitions of this experiment. In this and subsequent experiments, figures depict mean $\pm$ s.e.m.

Figure 2.

Insulin increases macroscopic currents carried by BK_{Ca} channels in cultured podocytes. **A**, Examples of families of currents evoked from a holding potential of -60 mV in a control cell and in a different cell exposed to insulin for 24 hr. The voltage-clamp protocol is denoted above traces. Recording pipettes contained 5 μ M free Ca^{2+} . Our previous studies (22) have shown that these protocols isolate macroscopic BK_{Ca} . Note complete inhibition of evoked outward currents in insulin-treated cells by bath application of the BK_{Ca} inhibitor paxilline (1 μ M). **B**, Quantification of current densities. Numbers in parentheses indicate number of cells tested in each group. Asterisk indicates $P < 0.05$ by one-way ANOVA followed by Scheffe's post hoc test. **C**, Mean currents (left) and conductance ratios (right) $\pm$ s.e.m. of control cells and cells treated for 24 hr with insulin. The ordinate G/G_{80} is the conductance at each voltage divided by the conductance evoked by a step depolarization to +80 mV. Note lack of effect of insulin on voltage-dependence of macroscopic BK_{Ca} . **D**, Typical currents from a control cell and from an insulin-treated cell (24 hr) with amplitudes normalized and overlaid so as to illustrate that activation kinetics are not altered by insulin.

Figure 3. Insulin-evoked Erk is necessary for stimulation of current through podocyte BK_{Ca} channels. *A*, Typical immunoblot analysis showing rapid increase in Erk phosphorylation evoked by 100 nM insulin. *B*, This increase is markedly attenuated by pretreating cells with 50 μ M PD98059, an inhibitor of MEK1. *C*, Densitometric analysis of three repetitions of the experiment shown in *B*. *D*, Cell-surface biotinylation assay showing that the effect insulin on BK_{Ca} trafficking is markedly attenuated by pretreatment with PD98059. *E*, Densitometric quantification of three repetitions of the experiment shown in *D*. *F*, Insulin stimulation of current through BK_{Ca} is blocked by pretreatment with PD98059. Asterisks indicate $P < 0.05$.

Figure 4.

Insulin-evoked activation of PI3K/Akt signaling cascade is necessary for stimulation of current through podocyte BK_{Ca} channels. *A*, Typical immunoblot analysis showing rapid increase in Akt phosphorylation evoked by 100 nM insulin. *B*, This increase is markedly attenuated by pretreating cells with 50 μ M LY294002, an inhibitor of PI3K. *C*, Densitometric analysis of three repetitions of the experiment shown in *B*. *D*, Cell-surface biotinylation assay showing that the effect insulin on BK_{Ca} trafficking is markedly attenuated by pretreatment with the PI3K inhibitor LY294002. *E*, Densitometric quantification of three repetitions of the experiment shown in *D*. *F*, Insulin stimulation of current through BK_{Ca} is blocked by pretreatment with LY294002. Asterisks indicate $P < 0.05$.

Figure 5.

Exposure to high glucose markedly reduces basal expression of functional cell surface BK_{Ca} channels. **A**, Typical cell-surface biotinylation assay showing that exposing cells to 36.1 mM glucose (HG) for 24 hr or 72 hr reduces surface expression of pore-forming subunits of BK_{Ca} channels compared to controls. LG refers to cells cultured in normal medium containing 11.1 mM glucose. LG + mannitol refers to a separate set of controls in which 25 mM mannitol was used to control for osmotic effects of high glucose. **B**, Densitometric quantification of three repetitions of the experiment shown in **A**. **C**, High glucose for 24 hr decreases BK_{Ca} current density measured by whole-cell recording. Asterisk indicates $P < 0.01$ by Student's unpaired t-test.

Figure 6.

Insulin increases phosphorylation of Erk and Akt in cells cultured in high glucose. Typical immunoblots phosphorylated and total Erk (A) and Akt (B) are shown above densitometric analyses of three repetitions of these experiments.

Figure 7.

Effects of high glucose on insulin modulation of BK_{Ca} channels. Insulin treatment for 24 hr continues to increase surface expression of pore-forming subunits of BK_{Ca} channels in high glucose (36.1 mM), although this occurs from a lower baseline (A and B). However, insulin treatment for 24 hr does not increase current density through BK_{Ca} channels in cells grown in high glucose (C, D) indicating a functional insulin resistance. Voltage-dependence of BK_{Ca} activation is not changed by any of these treatments (E).

Figure 8.

High glucose for 24 hr markedly reduces cell surface expression of nephrin, and insulin can partially restore normal expression. *A*, Typical cell-surface biotinylation assay. *B*, Densitometry of three repetitions of the experiment shown in *A*. Note that this experiment shows that some responses to insulin persist in podocytes cultured in high glucose.