Selective Insulin Signaling through A and B Insulin Receptors Regulates Transcription of Insulin and Glucokinase Genes in Pancreatic β Cells

Barbara Leibiger,*§ Ingo B. Leibiger,*§ Tilo Moede,* Sabine Kemper,* Rohit N. Kulkarni,† C. Ronald Kahn,‡
Lina Moitoso de Vargas,³ and Per-Olof Berggren*†
*The Rolf Luft Center for Diabetes Research
Department of Molecular Medicine
Karolinska Institutet
S-171 76 Stockholm
Sweden
†Research Division
Joslin Diabetes Center and
Department of Medicine
Harvard Medical School
Boston, Massachusetts 02215
‡Department of Medicine
New England Medical Center and
Tufts University School of Medicine
Boston, Massachusetts 02111

Summary
Insulin signaling is mediated by a complex network of diverging and converging pathways, with alternative proteins and isoforms at almost every step in the process. We show here that insulin activates the transcription of its own gene and that of the β cell glucokinase gene (βGK) by different mechanisms. Whereas insulin gene transcription is promoted by signaling through insulin receptor A type (Ex11–), PI3K class Ia, and p70s6k, insulin stimulates the βGK gene by signaling via insulin receptor B type (Ex11+), PI3K class II-like activity, and PKB (c-Akt). Our data provide evidence for selectivity in insulin action via the two isoforms of the insulin receptor, the molecular basis being preferential signaling through different PI3K and protein kinases.

Introduction
Understanding selectivity in signal transduction is one of the most challenging tasks in current cell biology. Over the years, insulin signaling has served as one of the model examples in hormone-induced signal transduction. Dysfunction of insulin signaling, referred to as insulin resistance, is one of the major causes of type 2 diabetes mellitus (non-insulin-dependent diabetes mellitus), the most common metabolic disorder in man.

Insulin has been shown to exhibit pleiotropic effects involving mitogenic and/or metabolic events. Moreover, the effect of insulin is tissue as well as development dependent. The fact that insulin may transduce its signal through a variety of pathways has been discussed in extensive detail (White and Kahn, 1994). The two major pathways described to date, which employ insulin recep-

tors as the primary target, include signaling via mitogen-activated protein (MAP) kinases and phosphoinositol-3 kinase (PI3K). The insulin receptor (IR), the first step in these cascades, exists in two isoforms as a result of alternative mRNA splicing of the 11th exon of the insulin receptor transcript (Seino et al., 1989). The A type (IR-A), or Ex11– (Ullrich et al., 1985), lacks whereas the B type (IR-B), or Ex11+ (Ebina et al., 1985), contains the respective sequence coding for 12 amino acids in the C terminus of the α chain of the receptor. To date, no insulin-induced effect has been reported that discriminates signaling via A- and B-type receptors. In fact, the functional significance of these IR isoforms remains unclear.

Recent studies have shown that the insulin-producing pancreatic β cell is a target for insulin action, with insulin effects on transcription, translation, Ca2+ flux, and exocytosis (Leibiger et al., 1998a, 2000; Xu and Rothenberg, 1998; Xu et al., 1998; Aspinwall et al., 1999; Kulkarni et al., 1999a). In an animal model with a β cell–specific knockout for IR, there is a decrease in glucose-stimulated insulin release and a decrease in the insulin content of the cell (Kulkarni et al., 1999a). In addition, disruption of insulin signaling in the β cell at the level of insulin receptor substrate (IRS)-1 (Kulkarni et al., 1999b) or IRS-2 (Withers et al., 1998) leads to altered growth and function of the β cell. Consequently, insulin resistance may not only affect the function of the “classical” insulin target tissues muscle, fat and liver, but also apply to the pancreatic β cell and thereby affect β cell function.

In the present study, we show selective insulin signaling via the two isoforms of the insulin receptor (i.e., IR-A and IR-B) in the pancreatic β cell. Insulin that is secreted by β cells upon glucose stimulation up-regulates transcription of its own gene as well as that of the β cell transcription unit of the glucokinase (βGK) gene in an autocrine feedback loop. More interestingly, while the insulin gene is activated by insulin signaling via IR-A involving PI3K class Ia, p70 s6 kinase (p70s6k), and Ca2+/calmodulin dependent kinases, insulin-stimulated βGK transcription occurs via IR-B, PI3K class II-like activity, and protein kinase B (PKB/c-Akt). These results provide evidence that signaling via either IR-A or IR-B and the subsequent activation of different classes of PI3K and protein kinases (i.e., p70s6k and PKB) represent a mechanism for selective insulin action. We furthermore show a preferential activation of p70s6k and PKB as a result of insulin signaling via IR-A and IR-B, respectively, in insulin-producing and non-insulin-producing cells.

Results and Discussion
Glucose Activates Glucokinase Gene Transcription via Secreted Insulin
Insulin, secreted upon glucose stimulation, is a key factor in the up-regulation of insulin gene transcription (Leibiger et al., 1998a). The promoters of both the insulin gene and the βGK gene contain many similar cis ele-
ments (Shelton et al., 1992; Leibiger et al., 1994a, 1994b; Watada et al., 1996). To test whether transcription of βGK is regulated by similar mechanisms as the insulin gene, we studied the role of glucose and insulin in regulation of βGK mRNA steady-state levels. Stimulation of cultured islets (Figure 1A) or insulin-producing HIT-T15 cells with 16.7 mM glucose led to an increase in βGK mRNA levels 60 min following start of stimulation. This is similar in time course to the effect of glucose to stimulate insulin mRNA levels (Leibiger et al., 1998a, 1998b).

To define in more detail the dynamics of βGK mRNA, we analyzed the half-life time, stability, and transcriptional rate of the βGK mRNA pool. As shown in Figure 1B, the half-life time of βGK mRNA was ~60 min and was not changed in the presence or absence of glucose. On the other hand, stimulation of HIT cells with 16.7 mM glucose led to an increase in βGK gene transcripts as early as 15 min and reached a maximum of transcriptional activity at 30 min in a nuclear run-off assay (Figure 1C). This effect of glucose on βGK transcription initiation was also observed in normal pancreatic islets (Figure 1D).

To further corroborate these data, we established a reporter gene assay using the βGK promoter coupled to the green fluorescent protein (GFP) (prβGK.GFP). We used the rat βGK promoter fragment up to nucleotide −278, since this has been shown to contain all cis elements responsible for both glucose-dependent and cell-type-specific transcriptional control (Jetton et al., 1994, 1998). Stimulation with 16.7 mM glucose led to an increase in βGK promoter–driven GFP fluorescence in HIT cells, isolated primary pancreatic β cells, and intact pancreatic islets (Figure 1E). As with the nuclear run-off assay, the dynamics of the activation of βGK promoter–driven GFP expression were similar, if not identical, to those of the glucose–stimulated insulin gene promoter (Leibiger et al., 1998a, 1998b).

To determine whether glucose metabolism per se or secreted insulin is a requirement for the up-regulation of βGK transcription, we investigated the effect of insulin secretagogues on βGK mRNA steady-state levels and βGK promoter–driven GFP expression. Insulin secretagogues, like KCl or the sulfonylurea compound glibenclamide, stimulate insulin secretion by depolarizing the β cell plasma membrane and provoking influx of extracellular Ca2+ through voltage-gated L-type Ca2+ channels (reviewed in Berggren and Larsson, 1994). As shown in Figure 2, stimulation with either 50 mM KCl or 1 μM glibenclamide for 5 min, at substimulatory glucose concentrations, led to an increase in βGK mRNA steady-state levels (Figure 2A) and to an elevation in βGK promoter–driven GFP expression (Figure 2C). Alternatively, preventing stimulus-induced insulin secretion by blocking L-type Ca2+ channels using nifedipine abolished up-regulation of βGK mRNA levels (Figure 2B).

We next studied the effect of exogenously administered insulin on βGK mRNA steady-state levels and βGK promoter–driven GFP expression at substimulatory glucose concentrations (Figures 2D and 2E). Addition of only 50 μU of insulin per ml to fully supplemented culture medium was sufficient to evoke βGK mRNA levels in pancreatic islets (Figure 2D). Interestingly, a more careful comparison of the necessary amounts of exogenous insulin to trigger promoter activities revealed that instead of 5–10 μU of insulin per ml, as is the case with the insulin gene, the addition of 20 μU per ml was required to gain an effect on βGK promoter activation (Figure 2E). Stimulation with 5 μU of insulin per ml of culture medium for 5 min led to an βGK promoter–driven increase in GFP fluorescence in isolated primary pancreatic β cells (Figure 2C), HIT cells, and intact pancreatic islets (data not shown).

Thus, our data support the view that the insulin gene and the βGK gene are both stimulated by insulin secreted in response to glucose. Interestingly, a higher concentration of insulin is needed to activate βGK transcription when compared with the insulin gene.

Insulin-Stimulated Glucokinase Gene Transcription Utilizes Signal Transduction, which Is Different from that of the Insulin Gene

Our studies on insulin-stimulated insulin gene transcription have shown the involvement of PI3K, p70s6k, and Ca2+/calmodulin-dependent kinase(s) in the signaling cascade (Leibiger et al., 1998a). Because previous data from others and our laboratory suggest that insulin-and βGK-promoters can bind the same transcription factors (Shelton et al., 1992; Leibiger et al., 1994a, 1994b; Watada et al., 1996) and both genes respond positively to many of the same stimuli (glucose, insulin, secretagogues) at the level of transcription, we questioned whether both genes might be regulated by the same signaling pathway.

To test whether the same protein kinases that are involved in insulin-triggered insulin gene transcription contribute to insulin-triggered transcription of βGK, we studied the effect of pharmacological inhibitors on insulin-stimulated βGK promoter activity (Figure 3). We combined insulin stimulation (5 μU/ml for 5 min at substimulatory glucose concentrations) with the cotreatment of islet cells and HIT cells with inhibitors of protein kinase C (PKC; 150 nM bisindolylmaleimide I [BIM]), PI3K (25 μM LY294002 [LY]), p70s6k (10 nM rapamycin [rap]), MAP kinases Erk1/2 (20 μM PD98059 [PD9]) and p38/RK/SAPK2a + SAPK1/JNK (10 μM PD169316 [PD1]), IR tyrosine kinase (100 μM HNMPA-AM), [HNMPA], and Ca2+/calmodulin-dependent kinase II (CaMKII; 400 nM autacamide-2 related inhibited peptide [AC]). The efficiency of these inhibitors was verified by the respective protein kinase assays in cell lysates of inhibitor-treated and untreated cells following glucose/insulin stimulation (data not shown). In agreement with the data on insulin-stimulated insulin gene transcription, insulin-stimulated βGK transcription was not sensitive to inhibition of PKC or MAP kinases Erk1/2 and p38 but was sensitive to inhibition of IR tyrosine kinase by HNMPA-AM (Figure 3A). However, to our surprise, insulin-stimulated βGK transcription was not inhibited by LY294002, rapamycin, or autacamide-2 related inhibited peptide, suggesting that signaling via PI3K/p70s6k and via CaMKII, respectively, is not involved (Figures 3A and 3B). To further confirm that insulin stimulates insulin gene transcription and βGK transcription using different signaling pathways, we established a technique that allowed monitoring of insulin and βGK promoter activities simultaneously in the same cell. In addition to prβGK.GFP, we generated an expression construct where the rat insulin I promoter (~410/−1 bp) controlled the expres-
Figure 1. Effect of Glucose on βGK mRNA Steady-State Levels, Transcription Initiation, and mRNA Stability

(A) Elevation of βGK mRNA steady-state levels in isolated islets after stimulation with 16.7 mM glucose (15 min).

(B) Dynamics of βGK mRNA stability in islet cells at 3 mM glucose (closed squares) and after stimulation with 16.7 mM glucose for 15 min (open squares). Actinomycin D (5 μg/ml) was present all the time under nonstimulatory conditions (closed squares), whereas in the case of stimulation (open squares) the inhibitor was added 45 min after start of stimulation. In (A) and (B), βGK mRNA values are presented as percentages of mRNA levels of the nonstimulated control at minute 0 (given as 100%).

(C and D) Dynamics of βGK transcription initiation in response to glucose stimulation in HIT cells (C) and isolated islets (D). Transcription initiation was studied by nuclear run-off analysis. Elevation of RNA levels in stimulated cells is shown as the percentage of RNA levels of the nonstimulated control at minute 0 (given as 100%). In (A)–(D), all data are shown as mean values ± S.E. (n = 3).

(E) On-line monitoring of glucose-stimulated βGK promoter–driven GFP expression in transfected HIT-T15 cells, islet cells, and whole islets. Representative images of HIT cells (n = 40), islet cells (n = 40), and islets (n = 3) are shown 60 and 240 min after start of glucose stimulation. The pseudo-color images were created by converting the original “gray-scale” data using Isee software; the fluorescence increases from blue to red. Scale bars, 10 μm.
Figure 2. Effect of Secretagogues, Voltage-Dependent L-Type Ca\textsuperscript{2+} Channel Blockers, and Exogenous Insulin on Endogenous \( \beta \)GK mRNA Levels and \( \beta \)GK Promoter–Driven GFP Expression

(A) Elevation of endogenous \( \beta \)GK mRNA levels in cultured pancreatic islets in response to stimulation for 5 min with either 50 mM KCl (KCl) or 1 \( \mu \)M glibenclamide (glib) at 3 mM glucose.

(B) Elevation of endogenous \( \beta \)GK mRNA levels in islet cells in response to stimulation for 15 min with 16.7 mM glucose with or without 10 \( \mu \)M nifedipine (nif). In (A) and (B), data are shown as mean values ± S.E. (n = 3), and amounts of \( \beta \)GK mRNA are presented as the percentage of mRNA levels of the nonstimulated control (given as 100%).

(C) On-line monitoring of \( \beta \)GK promoter–driven GFP expression in islet cells. Islet cells were transfected with pr\( \beta \)GK.GFP (\( \beta \)GK) or with pRC.CMV.GFP (CMV) as control and incubated with 16.7 mM glucose, 50 mM KCl, 1 \( \mu \)M glibenclamide (glib) or 5 mU/ml insulin (ins). Data are shown as mean values ± S.E. (n = 8).

(D and E) Effect of increasing concentrations of insulin added to the culture medium for 5 min, on (D) endogenous \( \beta \)GK mRNA levels in isolated pancreatic islets and on (E) \( \beta \)GK promoter–driven GFP expression and insulin promoter–driven DsRed expression in transfected HIT cells. In (D), amounts of \( \beta \)GK mRNA are presented as the percentage of mRNA levels of nonstimulated control (given as 100%), and data are shown as mean values ± S.E. (n = 3). In (E), HIT cells were cotransfected with pr\( \beta \)GK.GFP (open bars) and pr\( \alpha \)s1.DsRed (closed bars) and stimulated for 5 min with the indicated amounts of exogenous insulin. On-line monitoring data are presented as the ratio of fluorescence obtained at minutes 240 and 60 and represent mean values ± S.E. (n = 7).

Thus, insulin activates the \( \beta \)GK promoter by employing a signaling pathway that is different from that utilized by the insulin promoter.

Besides signaling via the MAP kinase and the PI3K/mTOR/p70s6k pathways, insulin has been shown to exert its effect via the activation of PKB (c-Akt) (Coffer et al., 1998). To test whether stimulation with either glucose or insulin leads to the activation of PKB in pancreatic \( \beta \) cells, we studied PKB activity following stimulation with either 16.7 mM glucose or 5 mU of insulin/ml. As shown in Figure 4, PKB activation was observed 5 min following stimulation with 16.7 mM glucose (Figure 4A) and 2 min following stimulation with 5 mU of insulin/ml, at substimulatory glucose concentrations (Figure 4B). Prevention of glucose-induced insulin secretion by treatment of insulin-producing cells with the L-type Ca\textsuperscript{2+} channel blocker nifedipine abolished glucose-induced activation of PKB, as did inhibition of insulin signaling by HNMPA-(AM)\textsubscript{3} (data not shown). These data suggest that PKB is activated in response to glucose-stimulated insulin secretion. Because of the lack of a selective pharmacological inhibitor of PKB, we tested its involvement in insulin-stimulated \( \beta \)GK gene transcription by transiently
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Figure 3. Effect of Various Protein Kinase Inhibitors on Insulin-Stimulated Insulin and βGK Promoter Activity and Endogenous βGK mRNA Levels

(A) On-line monitoring of insulin promoter–driven (closed bars) and βGK promoter–driven (open bars) GFP expression in transfected islet cells. Data are presented as the ratio of fluorescence obtained at minutes 240 and 60 and represent mean values ± S.E. (n = 10).

(B) Amounts of βGK mRNA are presented as the percentage of mRNA levels of nonstimulated control (given as 100%). Data are shown as mean values ± S.E. (n = 3).

(C) HIT cells were cotransfected with prβGK.GFP (open bars) and prIns1.DsRed (closed bars). On-line monitoring data are presented as the ratio of fluorescence obtained at minutes 240 and 60 and represent mean values ± S.E. (n = 13).

overexpressing PKBα/c-Akt1. Whereas overexpression of PKBα had no effect on insulin-stimulated insulin gene transcription, it led to a more pronounced effect on insulin-stimulated βGK promoter–driven GFP expression (Figure 4C). According to the current view, insulin-stimulated PKB activation involves the phosphorylation of PKB by the phosphoinositol-dependent kinase 1, PDK1 (Vanhaesebroeck and Alessi, 2000). Indeed, transient overexpression of PDK1 led to a pronounced stimulation of insulin-triggered βGK promoter activity, whereas overexpression of the antisense transcript of PDK1 abolished the stimulatory effect of insulin on insulin-triggered βGK promoter activity (Figure 4C). Noteworthily, transient overexpression of either PKBα or PDK1 did not lead per se to an increased basal insulin or βGK promoter activity (data not shown).

Interestingly, the activation of PKB has so far been shown to be dependent on the activity of PI3K (Vanhaesebroeck and Alessi, 2000) and therefore to be sensitive to the independent pharmacological inhibitors wortmannin and LY294002. Whereas treatment of insulin-producing cells with 25 μM LY294002 clearly abolished insulin-stimulated rat insulin I gene promoter activity, it did not block insulin-stimulated rat βGK promoter activity (Figure 3). When analyzing the effect of LY294002 on insulin-stimulated insulin and βGK promoter activity in a dose-dependent manner in cells cotransfected with prβGK.GFP and prIns1.DsRed, we observed that LY294002 inhibited the two promoters at different concentrations. Whereas 25 μM LY294002 blocked insulin-stimulated insulin promoter activity, 100 μM LY294002 was needed to completely abolish insulin-stimulated βGK promoter activity (Figure 4D). The effect of wortmannin was similarly concentration dependent. Treatment of cells with 50 nM wortmannin was sufficient to inhibit insulin-stimulated insulin promoter activity, whereas 150 nM wortmannin was necessary to block insulin-stimulated βGK promoter activity (Figure 4E).

These data indicate that insulin-stimulated βGK gene transcription occurs by signaling via PDK1/PKB, whereas insulin-stimulated insulin gene transcription is mediated via PI3K/p70s6k and CaMKII.

Insulin Signaling via IR-A Activates Insulin Gene Promoter Whereas Signaling via IR-B Activates βGK Promoter

Previous data on insulin-stimulated insulin gene transcription (Leibiger et al., 1998a) favored signaling via IR but did not exclude the signaling via IGF-I receptors or possible hybrids of insulin- and IGF-I receptors. The loss of insulin effect when treating cells with HNMPA-AMβ, an inhibitor of the IR tyrosine kinase (Saperstein et al., 1989), supported the idea that signaling via IR is crucial. Consequently, we examined whether the expression of IR per se is an absolute requirement for insulin-stimulated insulin and βGK gene expression. Therefore, we analyzed insulin and βGK mRNA levels in response to glucose/insulin stimulation in isolated islets from βIRKO mice, a knockout model that lacks the expression of IR specifically in the pancreatic β cell (Kulkarni et al., 1999a). Stimulation with either 16.7 mM glucose or 5 mU of insulin/ml led to an increase in both endogenous insulin and βGK mRNA levels in islets of wild-type mice, whereas no increase in insulin and βGK mRNA levels was observed in islets prepared from βIRKO mice (Figure 5A). These data suggest that the expression of the IR in pancreatic β cells is an absolute requirement to gain the stimulatory effect by insulin on both insulin and βGK gene expression and that signaling via IGF-I receptors is unlikely to be involved. This is consistent with the finding that activation of IGF-I receptors by stimulation with 2.6 nM IGF-I did not activate
insulin promoter– or βGK promoter–driven reporter gene expression in insulin-producing cells (data not shown).

Employing RT–PCR with subsequent DNA sequence analysis, we have previously observed that insulin-producing cells express both IR-A and IR-B (Leibiger et al., 1998a). Transient overexpression of IR-A led to a pronounced effect of insulin stimulation on insulin promoter activity, whereas overexpression of IR-B had no effect. To test a similar effect for insulin-stimulated βGK transcription, we cotransfected islet cells and HIT cells with prβGK.GFP and prins1.DsRed in combination with either IR-A or IR-B. To our surprise, we found that overexpression of IR-B led to a pronounced activation of the βGK promoter while overexpression of IR-A had no effect (Figure 5B). Moreover, overexpression of an inactive IR-B mutant (IR-Bm), described as M1153I (Levy-Toledano et al., 1994), did not lead to a further effect on βGK promoter–driven GFP expression (Figure 6B). To test the involvement of IR-B in insulin-stimulated βGK promoter activation in more detail, we treated islet cells and HIT cells, prior to glucose/insulin stimulation, with an anti-IR-B antibody. This antibody selectively binds to the α chain of IR-B and therefore selectively blocks insulin binding to IR-B and signaling via IR-B.

Employing coexpression of prβGK.GFP and prins1.DsRed in the same cell, we observed that treatment with the B-type receptor-specific antibody (αIR-B) abolished insulin-stimulated prβGK.GFP expression, whereas it did not affect insulin-stimulated prins1.DsRed expression (Figure 5C). In addition, treatment of insulin-producing cells with αIR-B abolished elevation of βGK mRNA levels following stimulation with either 16.7 mM glucose or 5 μU of insulin/ml at substimulatory glucose concentrations (data not shown). As expected, treatment of transfected cells with an antibody that blocks insulin signaling via both receptor isoforms (αIR-AB) suppressed insulin-stimulated activation of βGK– and insulin-promoters (Figure 5C). Accordingly, treatment of insulin-producing cells with this antibody also abolished insulin-stimulated elevation of βGK mRNA steady-state levels (data not shown). By contrast, treatment of transfected cells with an antibody that blocks signaling via IGF-1 receptors (αIGF-1R) did not affect insulin-stimulated activation of βGK promoter– and insulin promoter–driven reporter gene expression (Figure 5C).

These data indicate that insulin stimulates the insulin gene promoter through IR-A, whereas it stimulates the βGK gene promoter via IR-B.
To start to understand the molecular mechanisms that underlie the selectivity in insulin signaling via the two IR isoforms, we aimed to explain the different sensitivity for PI3K inhibitors we observed between insulin-stimulated insulin- and βGK-promoter activation (Figures 4D and 4E). One possible interpretation would be that the same PI3K is involved in the transcription of both genes but that a lower PI3K activity is sufficient to trigger the cascade that activates βGK gene transcription via PKB. If this is the case, inhibition of PI3K-mediated βGK transcription should require higher concentrations of wortmannin or LY294002 to be fully effectuated. This should also be reflected upon by an inverse relationship between required inhibitor concentration and sensitivity of the respective promoter activity to insulin. If the same PI3K is sufficient to lead to the activation of both insulin- and βGK-promoters, then the promoter activity that is least sensitive to the inhibitors (i.e., βGK) would be expected to require less insulin to become stimulated. As demonstrated in Figure 2E, this is not the case. On the contrary, more insulin is needed to stimulate βGK promoter–driven GFP expression. Another interpretation is that the different sensitivity in vivo could be due to a different accessibility of the inhibitor for the same type of PI3K as a result of a different distribution/localization of the two IR isoforms, or it could be due to the involvement of different classes of PI3K, exhibiting a different sensitivity to wortmannin and LY294002 as described for PI3K classes I and III versus class II (reviewed in Fruman et al., 1998). To test whether IR-A and IR-B exhibit a distinct distribution in vivo, we tagged both receptor isoforms with GFP and DsRed at the C terminus of the β subunit. Tagging both IR isoforms did not interfere with their physiological function (e.g., overexpression of the tagged IR isoform led to a pronounced insulin effect on the respective promoter activity to the same extent as the untagged IR) (data not shown). Whereas transient coexpression of the differently tagged IR isoform led to a complete colocalization (data not shown), coexpression of the differently tagged IR-A and IR-B in either combination (IR-A–DSRed/IR-B–GFP and IR-A–GFP/IR-B–DSRed) clearly showed IR isoforms that are not colocalized. This pattern of distinct IR isoform distribution was observed in insulin-producing cells HIT (Figure 6A), INS1, and MIN6, as well as in non-insulin-producing cells HEK293 and COS7 (data not shown). To test whether the two IR isoforms do utilize different classes of PI3K, we overexpressed either IR-A–GFP or IR-B–GFP in HIT cells and studied the sensitivity of PI3K activity to wortmannin in vitro following immunoprecipitation with GFP antibodies. Whereas the PI3K activity in the IR-A immunoprecipitate was inhibited by wortmannin in the lower nanomolar range, as typical for PI3K class I and III, the PI3K activity in the IR-B immunoprecipitate was only inhibited at higher concentrations (Figure 6B), as described for PI3K class II (see Fruman et al., 1998). To test whether insulin-stimulated insulin gene transcription involves IR-A–mediated insulin signaling via PI3K class Ia, we combined insulin stimulation with the transient overexpression of the dominant-negative form of the PI3K
Figure 6. Molecular Mechanisms Involved in the Selective Insulin Signaling via IR-A and IR-B

(A) Distribution of IR-A–GFP (green) and IR-B–DsRed (red) in HIT cells obtained by laser scanning confocal microscopy. Areas in yellow indicate colocalization of the two IR isoforms. This is a representative image out of a total of 25.

(B) PI3K activity in GFP immunoprecipitates obtained from insulin-stimulated (150 nM insulin for 5 min) HIT cells overexpressing either IR-A–GFP (closed bars) or IR-B–GFP (open bars). The amount of wortmannin included in the in vitro assay is indicated. Data are presented as mean values ± S.E. (n = 3).

(C) Effect of overexpression of dominant-negative p85 PI3K subunit, Δp85, on insulin-stimulated βGK promoter–driven GFP expression (open bars) and insulin promoter–driven DsRed expression (closed bars) in cotransfected HIT cells. Data are presented as the ratio of fluorescence obtained at minutes 240 and 60 and represent mean values ± S.E. (n = 10).

(D and E) Analysis of insulin-stimulated p70s6k and PKB activities in HIT (D) and HEK293 (E) cells following transfection with IR-A or IR-B. Cells were stimulated with insulin for 10 min and lysed after a further 10 min. Data are represented as percentages of the nonstimulated, mock-transfected control, set as 100%, and presented as mean values ± S.E. (n = 3).

Taken together, these data suggest a selectivity in insulin signaling in insulin-producing cells via IR-A through PI3K class Ia and p70s6k on the one hand, and via IR-B through a different PI3K activity, very similar to that of class II, and PKB on the other.

When separately overexpressing IR isoforms in HIT cells, we observed a more pronounced activation of p70s6k in cells overexpressing IR-A in response to insulin stimulation, while cells overexpressing IR-B showed...
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Figure 7. Selective Activation of Insulin and Glucokinase Gene Transcription by Selective Insulin Signaling via A- and B-Type Insulin Receptors

The scheme illustrates the coupling between insulin exocytosis and the activation of transcription of insulin and glucokinase genes.

Conclusion

Selectivity in insulin signaling is currently discussed as the result of the activation of specific signal transduction pathways. This selectivity may be gained by activating specific adaptor proteins (i.e., IRS and Shc proteins) that "channel" the insulin signal in a more defined way by specifically interacting with downstream located effector proteins (Myers and White, 1996; Virkamäki et al., 1999). Whereas the importance of IRS proteins in achieving insulin effects in different tissues is currently under extensive investigation, the possibility of selective insulin signaling via the two isoforms of the IR has been neglected. Studies on general and tissue-specific IR knockout models have demonstrated that a defect IR-mediated insulin signaling leads to a type 2 diabetes-like phenotype (reviewed in Taylor, 1999). However, these knockout models do not discriminate between the two IR isoforms. This is of importance, since earlier studies clearly established differences in tissue-specific IR isoform expression as well as in their activation profile. Whereas IR-B, which shows a 2-fold lesser affinity for insulin in comparison to IR-A (Mosthaf et al., 1990; Yamaguchi et al., 1991; McClain, 1991), is predominantly expressed in liver and muscle, IR-A is mainly expressed in brain (Moller et al., 1989; Seino and Bell, 1989; Mosthaf et al., 1990). Although it is tempting to link the phenotypes of the liver- and brain-specific IR knockouts (Brüning et al., 2000; Michael et al., 2000) to the failure in IR-B or IR-A function, respectively, a direct proof of the involvement of IR isoforms remains to be shown in IR isoform-specific knockout models. Attempts to correlate tissue-specific expression of IR isoforms with diabetes mellitus have generated conflicting results (Mosthaf et al., 1991; Benecke et al., 1992; Norgren et al., 1993) that do not clarify the functional role of either isoform.

Little is known about selective insulin signaling via A- and B-type IR. Besides the affinity for insulin, differences in their kinase activity (Kellerer et al., 1992) as well as internalization and recycling (Vogt et al., 1991; Yamaguchi et al., 1991) have been described. These data have implied differences in the function of either IR isoform, but no isoform-specific insulin-induced effect has been reported so far. In the present paper, we provide a "read-out" system for discriminating selective signaling via the two IR isoforms. We have demonstrated that the molecular basis for this selectivity could be provided by the different localization of the two IR isoforms in the plasma membrane and their different sensitivity for insulin. Mechanistically, this enables preferential activation of IR-A/PI3K I/II/p70s6k in pancreatic β cell glucose/insulin-stimulated insulin gene transcription and IR-B/PI3K class II-like/PKB in glucose/insulin-stimulated βGK transcription (Figure 7). That this specificity in signaling has a wider implication than to the pancreatic β cell is demonstrated by the data obtained in non-insulin-producing HEK293 cells.

Thus, our data clearly demonstrate that selectivity of insulin signaling can be gained by signaling through the two IR isoforms and reinforce the concept of the pancreatic β cell as a target for positive insulin action.

Experimental Procedures

Materials

Bisindolylsmaleimide I, PD98059, wortmannin, LY294002, rapamycin, HNMPA-(AM), autocamtide-2 related inhibitory peptide, and nifedi-
pine were purchased from Calbiochem, and Actinomycin D was from Sigma. pRcCMVIL and pCMV5.PKB were obtained as described previously (Leibiger et al., 1998a, 1998b). pDsRed was a gift from Dr. Isao Takeichi (Department of Developmental Biology, University of Tokyo). pCMV5.PDK1 antisense was kindly provided by D. Alessi (MRCagini/Lipid Research Unit, University of Dundee, UK), and pcDNA3- pRcCMVi.hIR(B) were kindly provided by D.R. Alessi (MRC HIT cells were transfected with either pRcCMVi.hIR(A) and pcDNA3- pRcCMVi.hIR(B), or pRcCMVIL and pcDNA3- pRcCMVIL, respectively. Nucleotide subcloning was performed by employing the QuikChange Mutagenesis Kit (Stratagene). Adenovirus-based vector Ad.\( \gamma\)iGK.GFP was constructed by subcloning the \( \gamma\)iGK.GFP cassette into pAC.CMV.pLpA and performing homologous recombination as described in Moitoso de Vargas et al. (1997). All vector constructions were verified by DNA sequence analysis. Plasmids pCMVS.PKB+, pCMV5.PKB+, pCMV5.PDK1, and pCMV5.PDK1 antisense were kindly provided by D.R. Alessi (MRC Phosphorylation Unit, University of Dundee, UK), and pcDNA3- Zeo.\( \gamma\)p85 was a gift from C.P. Downes (Department of Biochemistry, University of Dundee, UK).

Cell Culture and Transfection
Isolation of pancreatic islets, the culture of islets, and HIT-T15 cells, and their transfection have been described in Leibiger et al. (1998a; 1998b).

Analysis of PKB Activity
Analysis of PKB activity was performed employing the Akt1/PKB Immunoprecipitation Kinase Assay Kit (Upstate Biotech) according to the manufacturer's instructions.

Analysis of \( \gamma\)t06k Activity
p\( \gamma\)t06 kinase from cell lysates was immunoprecipitated using a \( \gamma\)t06k antibody (Upstate Biotech). Analysis of \( \gamma\)t06k activity was performed employing the S6 Kinase Assay Kit (Upstate Biotech) according to the manufacturer's instructions.

Analysis of P63K Activity
HIT cells were transfected with either pRcCMVi.hIR(A)–GFP or pRcCMVi.hIR(B)–GFP. Cell lysates containing 2 mg of protein were subjected to immunoprecipitation using anti-GFP antibody A-11122 (Molecular Probes). P63K activity was analyzed in the GFP immunoprecipitates as described in Krook et al. (1997) using L-\( \alpha\)-phosphatidylinositol from Avanti Polar-Lipids, Inc.

On-Line Monitoring of GFP and DsRed Expression and Detection of Fluorescence
Detection of fluorescence by digital imaging fluorescence microscopy was performed as described previously (Leibiger et al., 1998a; 1998b).

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