Fluoreszenzmikroskopische Untersuchungen zur intrazellulären Glucosehomöostase in Beta-Zellen und in der Leber

Von der Naturwissenschaftlichen Fakultät der

Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades

DOKTOR DER NATURWISSENSCHAFTEN

Dr. rer. nat.

Genehmigte Dissertation

von

Dipl.-Biol. Martin Tobias Kaminski

geboren am 6. Mai 1980 in Hannover

2012

Referent: Prof. Dr. Sigurd Lenzen

Korreferentin: Prof. Dr. Simone Baltrusch

Tag der Promotion: 15. Februar 2012

Kurzzusammenfassung

Die Beta-Zellen des Pankreas und die Leber sind wesentlich an der Blutglucoseregulation beteiligt. In diesen Organen werden Glucosetransport und -phosphorylierung über den GLUT2 und die Glucokinase vermittelt. In den Beta-Zellen des Pankreas stimuliert der Glucosemetabolismus Signalwege, die zur Insulinsekretion führen. Man unterscheidet dabei zwischen dem auslösenden Weg (*triggering pathway*), der abhängig von K_{ATP}-Kanälen abläuft und einem verstärkenden Weg (*amplifying pathway*). Diese Signalwege führen zu einem Anstieg der intrazellulären Calciumkonzentration, wodurch es zur Exozytose der Insulingranula kommt. Echtzeitanalysen der intrazellulären Glucosekonzentration können dazu beitragen, das Zusammenspiel dieser Signalwege in den Beta-Zellen besser zu verstehen. Solche Messungen wurden in dieser Arbeit mithilfe des Glucosesensors FLII^{12}Pglu-700µ-δ6 fluoreszenzmikroskopisch durchgeführt. Damit konnte bestätigt werden, dass in Beta-Zellen die Phosphorylierung der Glucose durch die Glucokinase die Geschwindigkeit des Glucosefluxes bestimmt. Weiterführend wurde erstmals eine simultane intrazelluläre Analyse von Glucose und Calcium in Beta-Zellen durchgeführt. Diese Studien zeigten, dass der initiale Calciumanstieg durch eine Erhöhung der intrazellulären Glucosekonzentration auf 4 mmol/l erreicht wird. Oszillationen der intrazellulären Calciumkonzentration waren dagegen erst bei einer Glucosekonzentration von 6-7 mmol/l sichtbar. Der Calciumanstieg konnte auch durch den Verschluss von K_{ATP}-Kanälen mittels Tolbutamid erzwungen werden. Da Calciumoszillationen durch Tolbutamid nicht ausgelöst werden konnten, lässt sich schlussfolgern, dass sie glucoseabhängig über einen *amplifying pathway* generiert werden. Auch in der Leber wird der Glucoseflux maßgeblich durch die Glucokinase reguliert. Hier unterliegt das Enzym einer spezifischen posttranslationalen Modulation. Das Glucokinase Regulatorprotein (GRP) inaktiviert die Glucokinase bei niedriger Glucose und translozíert sie in den Zellkern. Durch Generierung eines nukleären Glucosesensors konnte in dieser Arbeit nachgewiesen werden, dass sich bei einem Anstieg der extrazellulären Glucosekonzentration Glucose schnell im Zellkern anreichert. Dadurch wird die Glucokinase aus dem Komplex mit dem GRP freigesetzt. Es lässt sich schlussfolgern, dass der nukleäre Export der Glucokinase im Gegensatz zum Import von der Glucosekonzentration, nicht aber vom GRP abhängig ist. Durch die Etablierung der intrazellulären Glucosemessung konnten physiologische Prozesse in den Beta-Zellen des Pankreas und der Leber weiter aufgeklärt werden. Zukünftig kann diese neue Methodik zum besseren Verständnis der Pathogenese des Typ 2 Diabetes mellitus beitragen.

Schlagwörter: Diabetes mellitus, Glucokinase, Glucokinase Regulatorprotein

Abstract

Blood glucose regulation is mediated by the interplay of pancreatic beta cells and liver. In both organs, glucose transport and phosphorylation are controlled by the glucose transporter GLUT2 and glucokinase. In beta cells, glucose stimulates metabolic pathways that result in insulin secretion. The mechanism of glucose-stimulated insulin secretion can be divided into a triggering pathway that is dependent on K_{ATP}-channel closure, and an amplifying pathway. Both pathways eventually lead to calcium influx into the cell triggering insulin granule exocytosis. Real time analysis of intracellular glucose flux can provide further insights into the interplay between these signaling pathways in beta cells. In the present study, glucose measurements were performed by means of the fluorescence-based glucose sensor FLII^{12}Pglu-700µ-δ6. Using this method, glucose phosphorylation by glucokinase could be confirmed as the rate-limiting step of glucose flux. Furthermore, a concomitant analysis of intracellular glucose and calcium was performed for the first time in beta cells. Our study suggests that the intracellular calcium influx is initiated at a glucose threshold of 4 mmol/l. In contrast, large amplitude calcium oscillations were detected at an intracellular glucose concentration of 6-7 mmol/l. Calcium influx could be enforced by tolbutamide-mediated closure of K_{ATP}-channels. However, since tolbutamide did not evoke calcium oscillations, it can be concluded that oscillations are generated in a glucose-dependent manner through the amplifying pathway. In the liver, glucose metabolism is also regulated by glucokinase. Glucokinase activity is inhibited on the posttranslational level by binding to a liver specific “Glucokinase Regulatory Protein” (GRP). This protein translocates glucokinase into the nucleus at low glucose concentrations. Using a nuclear targeted version of the glucose sensor, our results showed that glucose accumulates very rapidly in the nucleus upon an increase of extracellular glucose. High glucose in the nucleus mediates dissociation of glucokinase from the GRP. Thus, we could show that the nuclear export of glucokinase is glucose-dependent, but independent from the GRP. In conclusion, the developed fluorescence based technique provided new insights into glucose-dependent processes in pancreatic beta cells and liver. This innovative method enables future studies towards understanding the pathogenesis of type 2 diabetes mellitus.

Keywords: Diabetes mellitus, Glucokinase, Glucokinase Regulatory Protein

Inhaltsverzeichnis

1 Einleitung

1.1 Glucosehomöostase

Die Glucose ist die Hauptenergiequelle aller Stoffwechselvorgänge des menschlichen Körpers. Die Glucosekonzentration im Blut ist unter physiologischen Bedingungen stets in relativ engen Grenzen von 4-8 mmol/l unter hormonellem Einfluss reguliert. In diesem Zusammenhang ist das wichtigste Hormon, das für die Senkung der Blutglucosekonzentration verantwortlich ist, das Insulin, welches in den Beta-Zellen der Langerhans'schen Inseln des Pankreas gebildet wird (Waldhäusl & Lenzen 2007). Die Ausschüttung des Insulins hat zum einen eine Erhöhung der Glykogensynthese in Leber und Muskelgewebe, und zum anderen eine vermehrte Glykolyse und Fettsäuresynthese in der Leber zur Folge. Weiterhin wird die Aufnahme der Glucose in der Muskulatur und im Fettgewebe über eine verstärkte Translokation des Glucosetransporters GLUT4 in die Plasmamembran dieser Zielgewebe erhöht. Das Insulin ist dabei der Auslöser einer rezeptorvermittelten Signalkaskade, die diese Translokation zur Folge hat. Zusätzlich wird eine Reihe von anabolen Stoffwechselwegen durch das Insulin positiv beeinflusst. Dies führt zu einer allgemeinen Erhöhung der Proteinbiosynthese bei gleichzeitiger Hemmung des Proteinabbaus, sowie der vermehrten Speicherung von Triacylglyceriden im Fettgewebe. Als hormoneller Gegenspieler zum Insulin bei niedrigen Blutglucosekonzentrationen tritt das in den Alpha-Zellen des Pankreas gebildete Peptidhormon Glucagon auf, dessen primäres Zielorgan die Leber ist. Dort vermittelt es gegenläufig zur Insulinwirkung die Bereitstellung und Abgabe von Glucose ins Blut durch Gluconeogenese und den Abbau von Glykogen. Muskel- und Fettgewebe tragen in der postprandialen Phase ebenfalls durch eine verminderte Glucoseverwertung zur Blutglucosehomöostase bei.

1.2 Diabetes mellitus

Der Diabetes mellitus ist eine Stoffwechselerkrankung, bei der eine Störung der Regulation der Blutglucosehomöostase vorliegt. Als Folge eines Insulinmangels tritt als Leitbefund eine chronische Hyperglykämie auf. Zu den klassischen Symptomen gehören Gewichtsverlust, Polyurie, Polydipsie und Ketoazidose.

Im Wesentlichen wird der Diabetes mellitus in zwei Typen eingeteilt, den Typ 1 und den Typ 2. Der Typ 1 betrifft ungefähr 5-10% aller Diabetesfälle in Deutschland und ist durch

einen absoluten Insulinmangel als Folge einer T-Zell vermittelten Autoimmunreaktion auf die Beta-Zellen des Pankreas charakterisiert (Waldhäusl & Lenzen 2007). Er erfordert eine obligate Substitution mit Insulin. Der Typ 2 Diabetes mellitus ist durch einen relativen Insulinmangel gekennzeichnet, welcher entweder die Folge einer peripheren Insulinresistenz und / oder einer verminderten Insulinsekretion der Beta-Zellen des Pankreas ist. Zu diesem Typ zählen in Deutschland ca. 90% der Diabetesfälle. Die Prävalanz des Typ 2 Diabetes mellitus beträgt in den westlichen Industrienationen ca. 4-5 % (Kerner 2009; Matthaei & Kloos 2009). Als Ursache für die Enstehung des Typ 2 Diabetes mellitus sind zum einen genetische Prädisposition und zum anderen Umweltfaktoren und Fehlernährung, bzw. eine Kombination dieser Parameter.

1.3 Glucosesensorisches Netzwerk

Die Aufrechterhaltung der Glucosehomöostase erfordert das Zusammenspiel mehrerer Organe, welche als glucosesensorisches Netzwerk beschrieben wurden. Dazu gehören Hepatozyten, pankreatische Alpha- und Beta-Zellen, endokrine Zellen der Darmmukosa (K- und L-Zellen), sowie neuronale Zellen der Hypophyse und des Hypothalamus. Hormonelle und neuronale Signalkaskaden zwischen diesen Organen ermöglichen eine schnelle Anpassung der Glucosehomöostase an die aktuelle Stoffwechsellage. Im Zentrum dieser Regulation stehen die Beta-Zellen des Pankreas (Kap. 1.4). In diesen Zellen, wie auch in den anderen Geweben des glucosesensorischen Netzwerks, wird das Enzym Glucokinase exprimiert (Kap. 1.5). Es nimmt eine Schlüsselfunktion in der Erhaltung der Glucosehomöostase ein.

Der durch die Glucokinase vermittelte Glucosestoffwechsel in den Beta-Zellen stellt den primären Stimulus der Insulinsekretion dar. Signale aus dem glucosesensorischen Netzwerk haben zudem einen nachweisbaren Einfluss. Die Absorption der Glucose durch die Zellen der Darmmukosa führt zunächst zu einer Ausschüttung der Inkretinhormone GLP-1 und GIP. Die Beta-Zellen exprimieren eine Reihe von spezifischen Zelloberflächenrezeptoren, wie z.B. den GLP-1 und GIP Rezeptor, sowie weitere G-Protein gekoppelte Rezeptoren, welche intrazelluläre Signalwege beeinflussen (Thorens 2008), die final zur Insulinsekretion führen. Studien heben überdies eine Verbindung zwischen dem Hypothalamus und dem endokrinen Pankreas hervor, welche ebenfalls die Insulinsekretion beeinflusst (Parton *et al.* 2007; Thorens 2010).

Neben den Beta-Zellen des Pankreas ist die Leber mit ihrer Fähigkeit des schnellen Wechsels zwischen Glucoseaufnahme und Glucosebereitstellung wesentlich an der Regulation der Blutglucosehomöostase beteiligt. Beiden Geweben ist gemeinsam, dass sie neben der Glucokinase den für die Glucoseerkennung wichtigen Glucosetransporter GLUT2 exprimieren. Dieser niedrigaffine Glucosetransporter sorgt für die Kopplung extrazellulärer millimolarer Glucosekonzentrationen an die intrazelluläre Glucosekonzentration (Bell *et al.* 1990; Kayano *et al.* 1990; Unger 1991; Thorens 1992; Lenzen & Tiedge 1994; Thorens 2008). Das glucosephosphorylierende Enzym Glucokinase übernimmt nachfolgend die Rolle

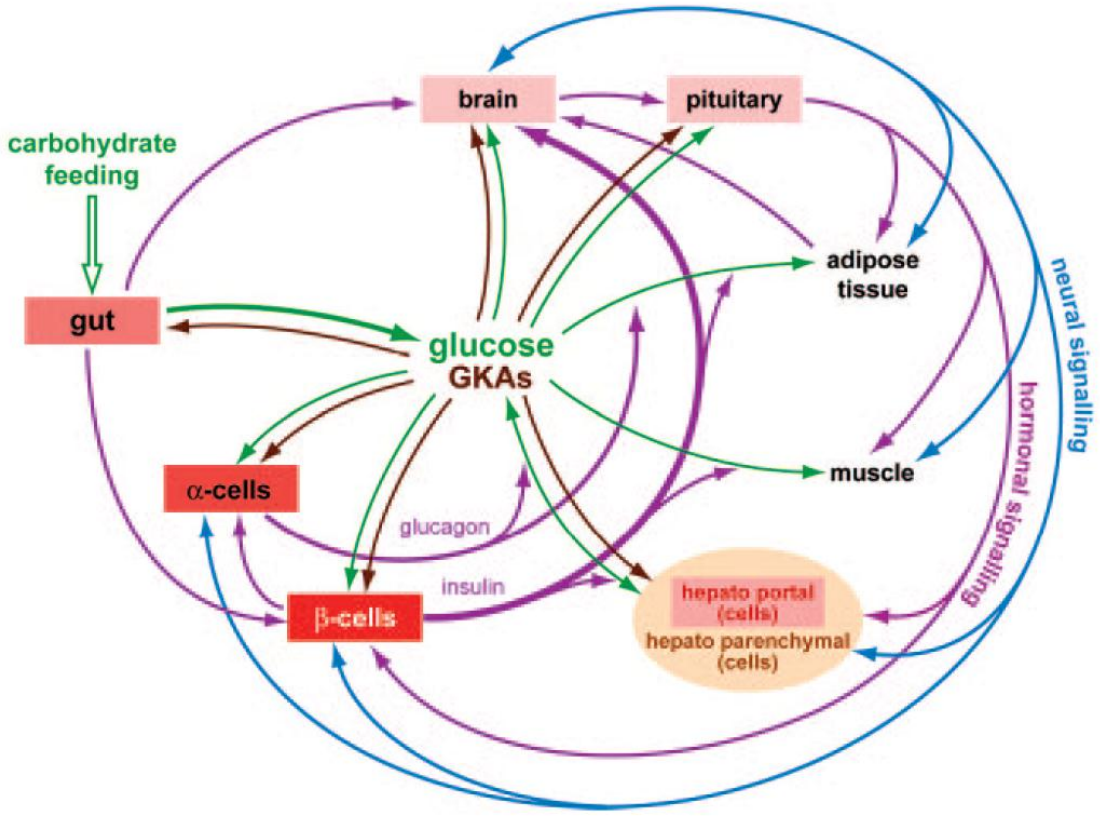


Abb. 1.1 Einfluss der Glucose auf glucosesensorische Gewebe. Der Anstieg der Blutglucose nach Nahrungsaufnahme führt zur Aufnahme des Substrates in das Gehirn, die Muskulatur und das Fettgewebe und zur Speicherung der Glucose in der Leber. Die Beta-Zellen des Pankreas sezernieren das Insulin, welches in der Leber anabole Stoffwechselwege verstärkt und gleichzeitig die Gluconeogenese und den Glucoseausstrom verhindert. Überdies bewirkt das Insulin eine rezeptorvermittelte Translokation des GLUT4 Glucosetransporters in die Zielgewebe Muskel- und Fettgewebe, welche die Aufnahmerate für die Glucose erhöht. Niedrige Glucosespiegel verursachen die Ausschüttung von Glucagon, dem Gegenspieler des Insulins, aus den Alpha-Zellen des Pankreas. Neuronale und hormonelle Signale beeinflussen überdies die Glucoseverwertung und –wirkung in ihren Zielgeweben (Matschinsky *et al.* 2006).

des eigentlichen „Glucosesensors“ (Meglasson & Matschinsky 1984; Meglasson & Matschinsky 1986; Lenzen & Panten 1988b; Matschinsky *et al.* 1993).

Im Hinblick auf die Regulation der Blutglucosehomöostase sind die intrazellulären Regulationsprinzipien von Leber und Beta-Zellen des Pankreas somit von besonderem Interesse. Ihre weitere Aufklärung steht daher im Fokus der vorliegenden Arbeit.

1.4 Glucosestimulierte Insulinsekretion in Beta-Zellen

Die Beta-Zellen des Pankreas erfüllen eine wichtige Rolle in der Kontrolle der Blutglucosehomöostase, indem sie das Hormon Insulin synthetisieren und sezernieren. Insulin ist ein Peptidhormon, welches in seiner reifen Form aus zwei Peptidketten besteht, die über zwei Disulfidbrücken miteinander verbunden sind. Die Proteinbiosynthese des Insulins erfolgt zunächst durch Translation der codierenden mRNA, durch die eine Vorstufe des Proteins im ER synthetisiert wird, das Präproinsulin. Durch weitere Prozessierung, Abspaltung der Signalsequenz sowie der Ausbildung von Disulfidbrücken reift das Peptid weiter zum Proinsulin, welches im Golgi-Apparat in Sekretgranula verpackt wird. Hier kommt es unter Abspaltung des zwischen der A- und B-Kette gelegenen C-Peptids zur Entstehung des reifen Insulins.

Die Glucose ist der wichtigste Stimulus der Insulinsekretion. Dieser Prozess unterliegt einer stringenten Kontrolle, die die Sezernierung des Hormons in Abhängigkeit der Blutglucosekonzentration steuert und als glucosestimulierte Insulinsekretion bezeichnet wird. Sie beschreibt eine Signalkaskade, die im Gegensatz zu anderen endokrinen Zelltypen aus dem Metabolismus der Glucose generiert wird (Lenzen & Panten 1988b; Lenzen 1990). Diese Stimulus-Sekretionskopplung kann im Wesentlichen in zwei Vorgänge geteilt werden, den sogenannten *triggering pathway*, welcher primär die glucoseabhängige Insulinsekretion auslöst, sowie den *amplifying pathway*, welcher durch Stoffwechselintermediate die Sezernierung des Insulins verstärkt.

1.4.1 Triggering Pathway

Die Glucose, welche extrazellulär in millimolaren Konzentrationen vorliegt, wird über den Glucosetransporter 2 (GLUT2) mittels erleichterter Diffusion entlang des Konzentrationsgradienten aus dem Blut in die Beta-Zellen aufgenommen. Hier erfolgt der erste Schritt der Glykolyse durch die Phosphorylierung mit dem Schrittmacherenzym der Glykolyse, der Glucokinase (Hexokinase Typ IV). Die Glucosephosphorylierung ist hierbei im Gegensatz zur Glucoseaufnahme der geschwindigkeitsbestimmende Schritt (Lenzen &

Panten 1988b). Durch den anschließenden Stoffwechselfluss über die Glykolyse, den Citratzyklus und die Atmungskette wird in den Mitochondrien ATP generiert, welches den ATP/ADP Quotienten in der Zelle ansteigen lässt. Hierdurch kommt es zum Schließen von ATP-abhängigen Kaliumkanälen (K_{ATP}-Kanäle), welche aus 2 Untereinheiten zusammengesetzt sind, dem Sulfonylharnstoffrezeptor SUR1 und dem Porenprotein KIR6.2 (Ashcroft *et al.* 1984; Cook & Hales 1984; Rorsman & Trube 1985; Aguilar-Bryan *et al.* 1995; Inagaki *et al.* 1995). Das Schließen dieses Kanals durch Einwirkung von ATP hat eine Depolarisation der Zellmembran zur Folge. Dies bewirkt einen Einstrom von extrazellulärem Calcium durch spannungsabhängige Ca^{2+}-Kanäle und damit schlussendlich die Verschmelzung von Insulinsekretgranula mit der Zellmembran und die Exozytose des Insulins (Abb. 1.2).

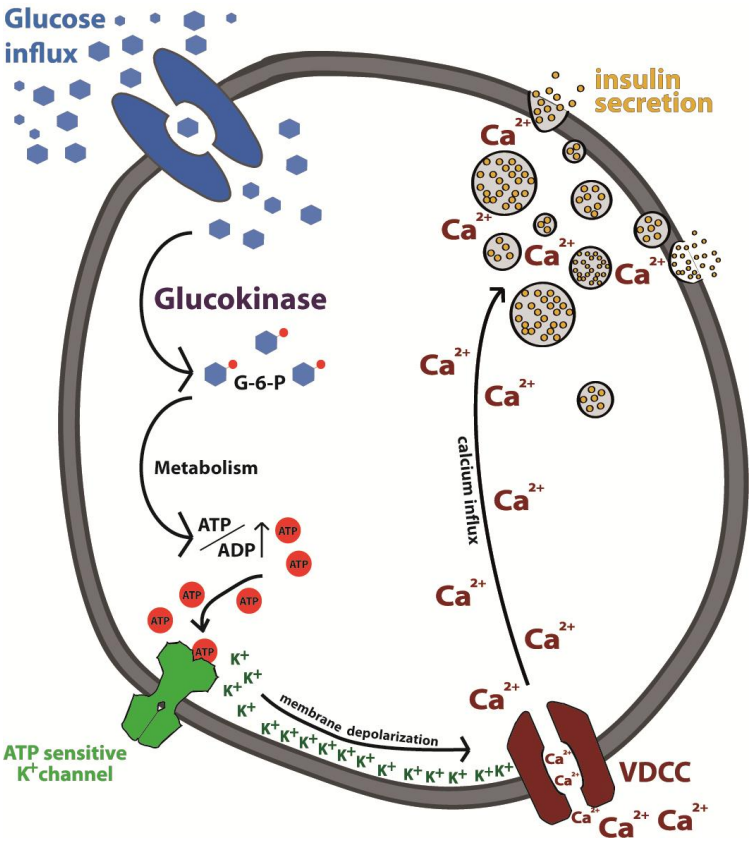


Abb. 1.2 Konsensusmodell der glucosestimulierten Insulinsekretion.

1.4.2 Amplifying pathway

Die Insulinsekretion ist von dem primären Stimulus des Glucoseeinstroms und der nachfolgenden Phosphorylierung der Hexose abhängig. Jedoch können durch externe

hormonelle Einflüsse über rezeptorgekoppelte Kaskaden eine Reihe von zusätzlichen sekretionsverstärkenden Signalen gebildet werden. Überdies können weitere metabolische Signale im Kohlenhydratmetabolismus gebildet werden, die eine verstärkende Wirkung auf die Insulinsekretion ausüben. Im Unterschied zum auslösenden *triggering pathway* ist der *amplifying pathway* jedoch von dem Vorhandensein eines primären Auslösers abhängig. Neben der Glucose, welche in ihrer α-anomeren Form effektiv die Insulinsekretion stimuliert, können auch die Hexose Mannose, sowie α-Ketoisocapronsäure (KIC), die Aminosäure L-Leucin und das Leucin-Analog BCH die Insulinfreisetzung bewirken (Gylfe 1976; Ashcroft 1981; Lenzen *et al.* 1986; Lenzen 1990). Die die Insulinsekretion verstärkende Wirkung der aus dem mitochondrialen Stoffwechsel generierten Metaboliten wird in zahlreichen Studien diskutiert (MacDonald *et al.* 2005). Der mögliche Einfluss der Generierung von NADPH wird dabei kontrovers diskutiert (Ivarsson *et al.* 2005; Urban & Panten 2005; Panten & Rustenbeck 2008). Insbesondere der Export von Citrat aus dem Mitochondrium ins Zytoplasma sowie ein direkter Einfluss des Citrats auf die Insulinsekretion wurde postuliert (Panten & Rustenbeck 2008).

Die Abgabe des Insulins durch Exozytose erfolgt wie auch der Calciumeinstrom und Veränderungen des cAMP Spiegels in einer pulsatilen Form (Gylfe *et al.* 2000; Dyachok *et al.* 2008a). Das bedeutet, dass der konstante stimulatorische Glucoseflux im nachfolgenden Metabolismus zyklische Schwankungen generiert, die sich auf die Insulinsekretion auswirken. Es stellt sich jedoch die Frage, an welcher Stelle im Metabolismus diese Oszillationen generiert werden. In diesem Zusammenhang wurde postuliert, dass bereits der Glucoseflux einen oszillierenden Charakter besitzt und damit nachfolgende metabolische Signalwege maßgeblich mitbeeinflusst (Jung *et al.* 2000a; Kennedy *et al.* 2002). Charakteristisch für die Insulinsekretion aus isolierten pankreatischen Inselzellen ist überdies die Aufteilung in eine erste und zweite Phase. Die erste Phase ist durch einen direkten Anstieg der Insulinsekretion nach Erreichen von korrespondierenden Ca^{2+}-Oszillationen gekennzeichnet, während die zweite Phase bei gleichbleibendem Glucosestimulus durch eine konstante, aber im Vergleich zur ersten Phase verminderte Insulinabgabe charakterisiert wird. Der genaue Einfluss von amplifizierenden Signalen auf jede dieser beiden Phasen ist bislang noch ungeklärt (Henquin 2009).

1.5 Glucokinase

Das Enzym Glucokinase (EC 2.7.1.1) wird in der Leber und den pankreatischen Beta-Zellen (Lenzen & Panten 1988a), sowie in geringerem Maße auch in einigen endokrinen Zellen des

Darms und des Gehirns exprimiert (Jetton *et al.* 1994). Es gehört zur Gruppe der Hexokinasen und katalysiert den ersten Schritt des Glucosemetabolismus durch den Transfer einer Phosphatgruppe des ATPs auf das Glucosemolekül unter Generierung von Glucose-6-Phosphat. Die Hexose-Phosphotransferasen können generell in zwei Gruppen unterteilt werden. Die Hexokinasen der ersten Gruppe umfassen die Isoenzyme I-III und zeichnen sich durch ein Molekulargewicht von ca. 100 kDa und eine hohe Substrataffinität für Glucose mit K_m-Werten von 20-130 µM aus. Ihre Aktivität wird durch Produkthemmung in physiologischen Konzentrationen durch Glucose-6-Phosphat reguliert (Middleton 1990).

Die Glucokinase, auch als Hexokinase Typ IV bezeichnet, weist ein Molekulargewicht von ca. 52 kDa auf und ist damit etwa halb so groß wie die übrigen Hexokinasen. Sie besitzt eine kooperative Reaktionskinetik für das Substrat Glucose mit einem sigmoidalen Reaktionsverlauf und einem Hill-Koeffizienten von 1,5 – 1,7. Der $S_{0.5}$-Wert für die Glucose liegt bei 7 – 10 mmol/l. Dies bedeutet eine deutlich geringere Substrataffinität im Vergleich zu den übrigen Hexokinasen (Lenzen & Panten 1988b; Lenzen 1990; Matschinsky 1990; Lenzen & Mirzaie-Petri 1992; Iynedjian 1993; Printz *et al.* 1993). Das Enzym unterliegt überdies keiner Produkthemmung durch Glucose-6-Phosphat. Der Umkehrpunkt der sigmoidalen Reaktionskinetik liegt im Bereich von etwa 5 mmol/l Glucose und damit nahe des Schwellenwertes zur Auslösung der glucoseinduzierten Insulinsekretion in Beta-Zellen. Diese Eigenschaften machen das Enzym zu einem idealen Kopplungsglied zwischen postprandial erhöhter extrazellulärer Blutglucose und metabolischen intrazellulären Glucosefluxänderungen, die in der Beta-Zelle zur bedarfsgerechten Insulinsekretion führen und in der Leber anabole Stoffwechselwege zur Speicherung der Glucose beeinflussen. Neben der Glucose, welche durch die Glucokinase bevorzugt in ihrer Alpha-anomeren Form phosphoryliert wird, können auch die Hexosen D-Mannose (80%), D-Fructose (20%) und D-2-Deoxyglucose (40% des V_{max}-Wertes der D-Glucose) durch die Glucokinase phosphoryliert werden (Printz *et al.* 1993; Tiedge *et al.* 1997).

Die Aminosäuresequenz der beiden Glucokinaseisoformen in Leber und Pankreas ist weitgehend identisch und unterscheidet sich nur in den ersten 11 N-terminalen Aminosäuren des insgesamt 465 Aminosäuren umfassenden Proteins (Magnuson & Shelton 1989). Die Transkription der mRNA steht in beiden Geweben unter der Kontrolle eines gewebespezifischen Promotors. Sie besteht aus insgesamt 10 Exons, von denen die Exons 2-10 in beiden Zelltypen identisch sind. Die gewebespezifischen Kontrollregionen sind 27 kb

voneinander entfernt und beinhalten neben den Promotoren auch das erste Exon (Magnuson *et al.* 1989; Magnuson & Shelton 1989).

Die Proteinstruktur konnte erstmals 1994 anhand von Kristallstrukturanalysen der Hefe-Glucokinase beschrieben werden, welche eine 33%ige Homologie zur humanen Isoform aufweist (St Charles *et al.* 1994). Eine Röntgenkristallstrukturanalyse an der humanen Form gelang 2004 (Kamata *et al.* 2004). Diese Analysen geben Auskunft über die Substratbindung und eine mit dieser assoziierten Konformationsänderung, welche die Aktivität des Enzyms beeinflussen. Demnach besteht die Glucokinase aus zwei Domänen, die durch eine kurze Region miteinander verbunden sind, in dem sich auch das aktive Zentrum des Enzyms befindet und die ein Aufklappen des Enzyms ermöglicht. Hieraus ergeben sich mindestens 2 Konformationen der Glucokinase, die in Abhängigkeit vom Öffnungswinkel der beiden Domänen zueinander variieren. Die aktive Konformation des Enzyms korrespondiert mit einer geschlossenen Form (*closed form*), bei der das Substrat eng von den beiden Domänen umschlossen wird. Ein Glucosemolekül ist dabei über 6 Aminosäuren an die Glucokinase gebunden. Die zweite weit offene Konformation (*super-opened form*) ermöglicht keine Bindung der Glucose an das aktive Zentrum und inaktiviert dadurch das Enzym (Abb. 1.3). Im Gegensatz zu den anderen Enzymen der Hexokinase-Familie unterscheidet sich die Glucokinase durch diese Flexibilität, die auch als eine Erklärung für die positive Kooperationskinetik dient (Cornish-Bowden & Storer 1986; Cardenas 1997). Darüber hinaus werden weitere Übergangsformen zwischen der weit offenen und der geschlossenen Konformation postuliert, die in einem durch die Glucosekonzentration definierten Gleichgewicht vorliegen (Kamata *et al.* 2004; Zhang *et al.* 2006; Antoine *et al.* 2009).

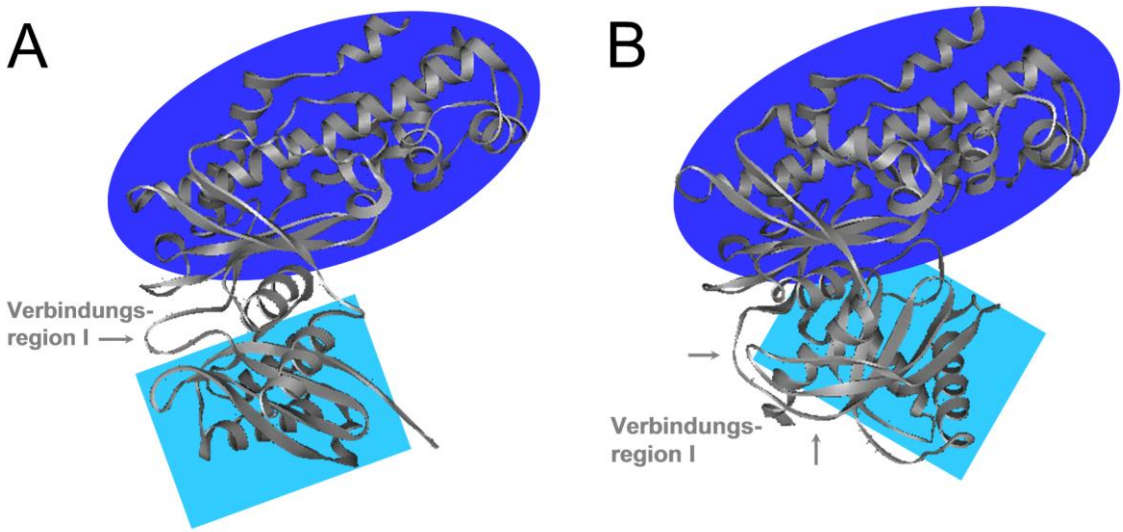


Abb. 1.3 Dreidimensionale Modellstruktur der Glucokinase in der weit offenen und der geschlossenen Form. Strukturmodell der Glucokinase in der weit offenen (A) (*Protein Databank Identification* 1V4T) und der geschlossenen (B) Konformation (*Protein Databank Identification* 1V4S). Die Darstellung erfolgte mit Hilfe des *3d molecule viewer* (Invitrogen) (Baltrusch & Tiedge 2006a).

1.6 Prinzipien der Regulation der Glucokinase in Leber und Beta-Zellen des Pankreas

Die Glucokinase übernimmt sowohl in den Beta-Zellen als auch in der Leber die entscheidende Rolle des Glucosesensorenzyms. Obwohl die Genstruktur der Glucokinase, wie bereits erwähnt, in beiden Geweben weitgehend identisch ist, erfolgt die Regulation dieses Enzyms jedoch auf unterschiedliche Art und Weise.

Auf der Ebene der Genregulation unterliegt die Transkription des Glucokinasegens in der Leber dem hormonellen Einfluss von Insulin (stimulierend) und Glucagon (antagonistisch) (Iynedjian 1993). Längeres Fasten bewirkt dabei eine Senkung der Transkriptionsrate (Tiedge & Lenzen 1995). Im Gegensatz hierzu erfolgt die Transkription des Glucokinasegens in der Beta-Zelle nicht unter Einfluss des Insulins, sondern unter Einfluss der Glucose (Tiedge & Lenzen 1991; Tiedge & Lenzen 1995). Wie in der glucosestimulierten Insulinsekretion zeigt sich hierbei erneut die direkte Abhängigkeit der Beta-Zellen von diesem Substrat, welches nicht nur als Signalmolekül für die Insulinsekretion fungiert, sondern überdies auch die transkriptionelle Regulation beeinflusst.

Um jedoch einen schnellen Wechsel der Enzymaktivität nach Blutglucoseanstieg gewährleisten zu können, existieren in beiden Geweben weitere regulatorische Mechanismen, die auf posttranslationaler Ebene die Aktivität der Glucokinase beeinflussen. Sowohl die Beta-Zellen des Pankreas, als auch die Leber exprimieren physiologische Aktivatoren, deren Wirkung durch eine direkte Bindung an das Enzym ermöglicht wird und die die Enzymaktivität der Glucokinase steigern.

1.6.1 PFK-2/FBPase-2 als endogener Aktivator der Glucokinase in Leber und Beta-Zellen

Das bifunktionelle Enzym 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase (PFK-2/FBPase-2) wurde als Bindungspartner für die Glucokinase mit Hilfe von *Random Peptide Phage Display Library Screening* identifiziert (Baltrusch *et al.* 2001). Dieses Enzym tritt als Homodimer auf und katalysiert den Auf- und Abbau von Fructose-2,6-bisphosphat. Die Dimerisierung des Enzyms wird dabei über Bindung der beiden Kinasedomänen des Enzymkomplexes verursacht, nicht jedoch über die Bisphosphatasedomänen (Baltrusch *et al.* 2001; Okar *et al.* 2001). Die Interaktion der Glucokinase mit der PFK-2/FBPase-2 erfolgt über das Konsensusmotiv (SL)KVWT an den beiden gegenüberliegenden Bisphosphatasedomänen und führt sowohl in Hepatozyten als auch in pankreatischen Beta-Zellen zu einer Steigerung der intrinsischen Enzymaktivität (Massa *et al.* 2004). Da in den Beta-Zellen des Pankreas die allgemeine Nährstoffverfügbarkeit keinen unmittelbaren Einfluss auf die Expression der Glucokinase hat, nimmt die PFK-2/FBPase-2 in diesen Zellen die besondere Rolle eines endogenen Aktivators ein.

1.6.2 Weitere potentielle Regulationsmechanismen

Für die Glucokinase wurde zudem eine Interaktion mit dem alpha-Tubulin nachgewiesen, die insbesondere in den Beta-Zellen des Pankreas in den Zusammenhang mit der tubulinassoziierten Bewegung der Insulinsekretgranula (Rizzo *et al.* 2002; Arden *et al.* 2004) gebracht wurde (Baltrusch & Lenzen 2007a). Die Glucokinase tritt hierbei als Bindeglied zwischen dem Motorprotein Kinesin und den Insulingranula auf. Darüber hinaus wurden in Beta-Zellen eine Assoziation der Glucokinase mit den Mitochondrien (Danial *et al.* 2003; Kim *et al.* 2005) sowie Polyubiquitin bzw. Ubiquitin selbst (Bjorkhaug *et al.* 2007) beschrieben. Die genaue physiologische Rolle der Glucokinase für diese Protein-Protein-Interaktionen ist noch ungeklärt. Die Bindung der Glucokinase an subzelluläre Kompartimente oder Matrixproteine in den Beta-Zellen des Pankreas sowie die direkte Interaktion mit der PFK-2/FBPase-2 in Hepatozyten stellen für beide Gewebe spezifische

Möglichkeiten zur posttranslationellen Modifikation der Enzymaktivität dar (Baltrusch & Tiedge 2006a; Baltrusch & Lenzen 2007a). Abbildung 1.4 veranschaulicht die in den Beta-Zellen des Pankreas bekannten Regulationsmechanismen für die Glucokinaseaktivität.

Neben den physiologischen Aktivatoren werden seit fast einem Jahrzehnt chemische Aktivatoren der Glucokinase und ihre Möglichkeiten für die Pharmakotherapie des Diabetes mellitus Typ 2 untersucht (Grimsby *et al.* 2003; Matschinsky *et al.* 2006; Rustenbeck *et al.* 2010; Massa *et al.* 2011).

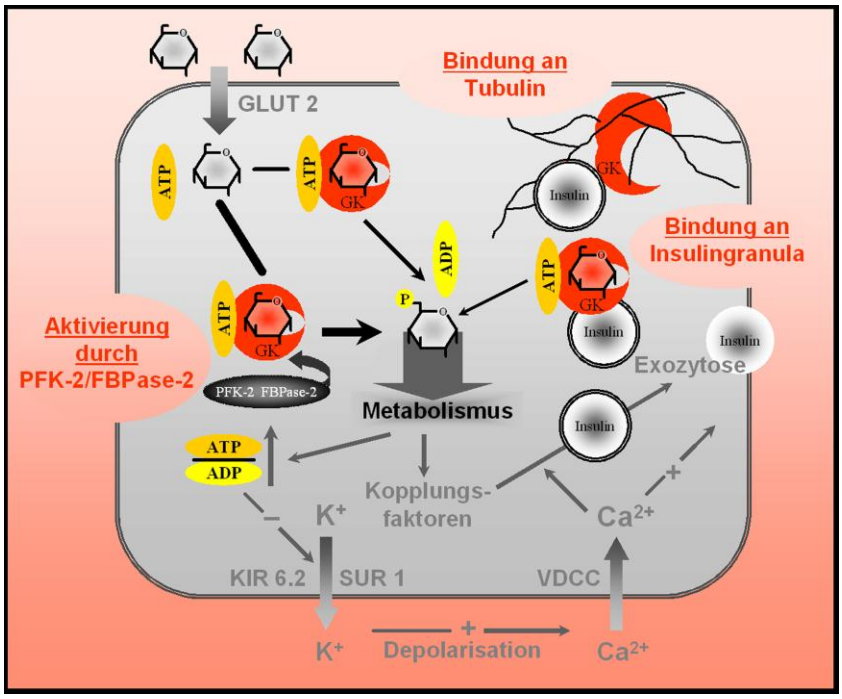


Abb. 1.4 Regulation der Glucokinase. Schematische Darstellung der posttranslationellen Aktivitätsregulation in pankreatischen Beta-Zellen. In den Beta-Zellen des Pankreas wird die GK Aktivität durch Bindung an PFK2/FBPase-2 sowie GK-Aktivatoren bestimmt. Zudem ist die GK mit einer Reihe von zellulären Strukturen wie Tubulin und Insulinsekretgranula assoziiert, deren möglicher Einfluss auf die Enzymaktivität jedoch nicht nachgewiesen ist (Baltrusch & Tiedge 2006a).

1.6.3 Glucokinase Regulatorprotein in der Leber

Im Gegensatz zu den Beta-Zellen des Pankreas wird die Regulation der hepatischen Glucokinaseaktivität entscheidend durch das Vorhandensein eines leberspezifischen Glucokinase-Regulatorproteins (GRP) als allosterischen Bindungspartner beeinflusst (s. auch Abb. 1.5). Dieses 68 kDa große und 626 Aminosäuren umfassende Protein wurde

erstmals 1989 in der Rattenleber nachgewiesen (Van Schaftingen 1989a). Das GRP fungiert als kompetitiver Inhibitor der Glucokinase. Diese Bindung wird durch den Glucosestoffwechsel der Leberzelle und durch Fructosephosphate beeinflusst. Die Anwesenheit von Fructose-1-Phosphat mindert die Interaktion des GRP mit der Glucokinase, während Fructose-6-Phosphat eine Verstärkung dieser Bindung verursacht (Van Schaftingen 1994; Van Schaftingen *et al.* 1994). Dies reflektiert den prä- und postabsorptiven Status der Leber, in dem neben der Blutglucose auch die Fructose erhöht ist und in der Leber Fructose-1-Phosphat gebildet wird. Neben der direkten Bindung und Inaktivierung der Glucokinase durch das GRP, die nur in der weit offenen Konformation der Glucokinase möglich ist (Futamura *et al.* 2006), vermittelt das GRP eine Translokation der Glucokinase vom Zytoplasma in den Nukleus von Hepatozyten (Toyoda *et al.* 1995a; Brown *et al.* 1997; Niculescu *et al.* 1997; Shiota *et al.* 1999b). Unter basalen Glucosekonzentrationen wird dadurch ein Anteil der exprimierten Glucokinase in den Nukleus transloziert, während im postprandialen Zustand nach Erhöhung der Glucosekonzentration und von Fructose-1-Phosphat eine rückwärtige Verteilung der Glucokinase in das Zytoplasma beobachtet wurde. Diese GRP – abhängige Translokation der Glucokinase in den Nukleus konnte auch in einem artifiziellen Zellsystem gezeigt werden. Die Coexpression der Glucokinase und des GRP als Fusionskonstrukte mit EYFP bzw. ECFP wurde in COS-1 Zellen untersucht, in denen weder die Glucokinase noch das GRP endogen vorkommen. Eine solche Überexpression von fluoreszenzmarkierten Fusionskonstrukten aus ECFP-GK und EYFP-GRP zeigte eine deutliche Verlagerung der Glucokinase in den Nukleus bei niedriger Glucosekonzentration (5,5 mmol/l). Nach Erhöhung der Glucosekonzentration auf 25 mmol/l zeigte sich in den COS-1 Zellen eine deutliche Erhöhung des ECFP-GK Proteins im Zytoplasma (Baltrusch *et al.* 2005). Im Gegensatz dazu ist das GRP sowohl in Hepatozyten, als auch nach Überexpression in COS-Zellen vorwiegend im Nukleus lokalisiert und nur zu einem geringeren Teil im Zytoplasma (Baltrusch & Tiedge 2006a). Der Import der Glucokinase in den Nukleus bei niedriger Glucose ist somit an die Bindung zum GRP gekoppelt. Glucokinasemutanten, denen eine Interaktion mit dem GRP nicht möglich ist, zeigten eine von der Anwesenheit des GRP und der Glucosekonzentration unbeeinflusste subzelluläre Verteilung. Die durch eine korrekte Konformation der Glucokinase ermöglichte Interaktion mit dem GRP ist daher ausschlaggebend für einen erfolgreichen Import in den Zellkern (Baltrusch *et al.* 2005). Im Gegensatz dazu ist der nukleäre Export der Glucokinase nach Glucoseanstieg bislang nicht schlüssig aufgeklärt. Die Glucokinase verfügt zwar über ein nukleäres Exportsignal, jedoch

ist der GK-Export nicht durch den Einsatz von Leptomycin B, einem Inhibitor des nukleären Exports beeinträchtigt (Shiota *et al.* 1999b). Andererseits könnte die GK mit ca. 52 kDa den Kern möglicherweise noch ohne Hilfe des Exportins verlassen. Dabei stellt sich insbesondere die Frage, ob der Export der Glucokinase unabhängig vom GRP erfolgt. In diesem Fall müsste es zu einer Dissoziation der beiden Interaktionspartner nach Glucoseanstieg im Nukleus kommen. Weiterhin gilt es zu klären, ob die Glucokinase im Nukleus eine spezifische Funktion übernimmt (Van Schaftingen & Veiga da Cunha 2004). Die Rolle des GRP als Interaktionspartner der Glucokinase innerhalb des Zytoplasmas ist ebenso weitgehend ungeklärt. Das GRP konnte zusammen mit der Glucokinase und dem pro-apoptotischen Protein BAD in Assoziation mit Mitochondrien in Hepatozyten nachgewiesen werden (Danial *et al.* 2003; Arden *et al.* 2006). Jedoch bleiben auch hier die exakten physiologischen Zusammenhänge eines solchen Multienzymkomplexes Gegenstand weiterer Untersuchungen. Studien an GRP-*knockout* Mäusen ergaben eine verminderte Expression der Glucokinase in der Leber dieser Tiere. Die Abwesenheit eines Glucokinasereservoirs im Nukleus führte hierbei zu einer verminderten Glucosetoleranz nach Fütterung der Tiere. Dies hebt die stabilisierende Rolle des GRP für die Glucokinase hervor, in der überdies durch die Bereitstellung ausreichender Mengen der Glucokinase im Nukleus eine direkte und schnelle Verfügbarkeit des Enzyms für die postprandiale Verstoffwechselung der Glucose gewährleistet wird (Farrelly *et al.* 1999; Grimsby *et al.* 2000).

Die Interaktion des GRP mit der Glucokinase erfolgt bei beiden Bindungspartnern über spezifische Epitope mit konservierter Aminosäuresequenz. Durch Einsatz eines systematisch *phage display screenings* konnte die Aminosäuresequenz –LSA-XX-VAG- als ein Konsensusmotiv für die Bindung an die Glucokinase identifiziert werden (Baltrusch *et al.* 2001). Diese Sequenz ist homolog zu einem Motiv des humanen GRP mit der Sequenz 185-SVGLSAPVAGQMD-197. Weiterhin wurde die Aminosäure S179 in der Umgebung dieser Konsensussequenz als kritischer Faktor für die Affinität zur Glucokinase identifiziert (Veiga-da-Cunha & Van Schaftingen 2002). Die Glucokinase hingegen besitzt eine Reihe von potentiellen Konsensusmotiven, die eine Bindung an das GRP ermöglichen können. Darunter treten besonders die beiden Leucin-Asparagin Bindungsstellen L58/N204 und L355/N350 hervor, die direkte Auswirkungen auf die GRP vermittelte Translokation der Glucokinase haben. Die Konformation der GK ist dabei für eine Bindung an das GRP von entscheidender Bedeutung und scheint in der bereits erwähnten weit offenen Konformation bevorzugt zu sein. Eine spezifische Aktivierung der Glucokinase in der Leber durch

pharmakologische GK-Aktivatoren könnte daher im Gegensatz zu den Beta-Zellen des Pankreas durch Interferenz mit dem GK-GRP Komplex erreicht werden (Baltrusch & Tiedge 2006a).

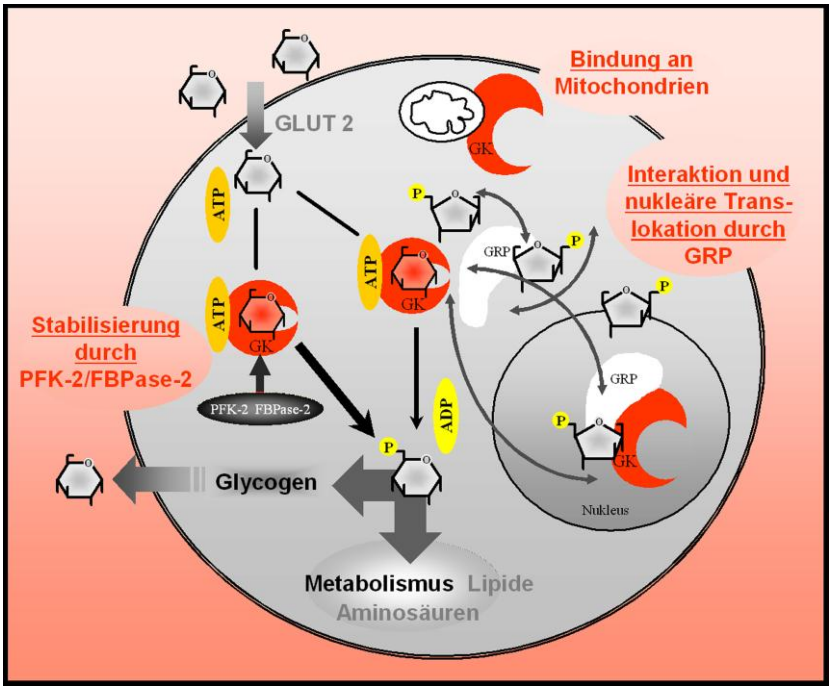


Abb. 1.5 Regulation der Glucokinase in der Leber. In Hepatozyten erfolgt die Aktivitätsregulation der Glucokinase hauptsächlich durch das leberspezifische Glucokinase Regulatorprotein (GRP). Diese Bindung wird durch Fructose-1-Phosphat inhibiert und durch Fructose-6-Phosphat verstärkt. Des Weiteren wirken synthetische GK-Aktivatoren sowie der endogene Aktivator PFK2/FBPase-2 aktivierend.

1.7 FRET in der Aufklärung zellulärer Metabolitenströme

Die Entwicklung und Verbesserung moderner fluoreszenzbasierter Farbstoffe und Proteine innerhalb der letzten Jahrzehnte ist eine wichtige Voraussetzung für das Verständnis von physiologischen Vorgängen innerhalb einzelner Zellen. Die Entdeckung und Klonierung des *green fluorescent protein* (GFP) aus *Aequorea victoria* und die Generierung einer Reihe von Derivaten mit unterschiedlichen spektralen Eigenschaften (Shimomura *et al.* 1962; Prasher *et al.* 1992; Chalfie *et al.* 1994; Inouye & Tsuji 1994; Tsien 1998) ist ein Meilenstein in der Fluoreszenzmikroskopie. Diese fluoreszierenden Proteine können als noninvasive Marker und Fusionskonstrukte mit einem zu untersuchenden Protein zur Expression gebracht werden.

Das Auflösungsvermögen eines konventionellen Fluoreszenzmikroskops liegt bei etwa 200 nm. Der Nachweis einer direkten Interaktion zweier unterschiedlich fluoreszenzmarkierter Proteine ist dadurch nicht direkt möglich. Eine Protein-Protein-Interaktion kann jedoch unter Einbindung des *fluorescence resonance energy transfer* (FRET)–Prinzips mit Hilfe der Fluoreszenzmikroskopie nachgewiesen werden. Das FRET-Prinzip wurde erstmals 1932 von Perrin (Perrin 1932) theoretisch beschrieben, wurde jedoch entscheidend von Förster als ein quantenmechanischer Effekt beschrieben (Förster 1948). Es beruht auf der Tatsache, dass ein Fluorophor (sogenannter Donor) nach Anregung einen charakteristischen elektrischen Dipol erzeugt, dessen Energie auf direktem Weg auf ein benachbartes Fluorophor (sogenannter Akzeptor) übertragen werden kann und dieses wiederum zur Emission anregt. Der Energietransfer benötigt dabei im Wesentlichen zwei Voraussetzungen. Zum einen hängt der Energietransfer vom Abstand der beiden potentiellen FRET-Partner ab. Die Effizienz des Energieübertrages nimmt dabei mit der sechsten Potenz des Abstandes zueinander ab. Der sogenannte Förster Radius R_0 beschreibt dabei den Abstand in dem die FRET-Effizienz bei 50% liegt. Dies resultiert in einem typischen Abstand von ca. 3 nm, maximal jedoch 10 nm, zwischen den beiden Fluorophoren. Als zweite Voraussetzung müssen das Emissionsspektrum des Donors und das Absorptionsspektrum des Akzeptors überlappen (Abb. 1.5). Die räumliche Orientierung der beiden Fluorophore zueinander kann als dritte Voraussetzung für einen effektiven Energietransfer angesehen werden (Schaufele *et al.* 2005).

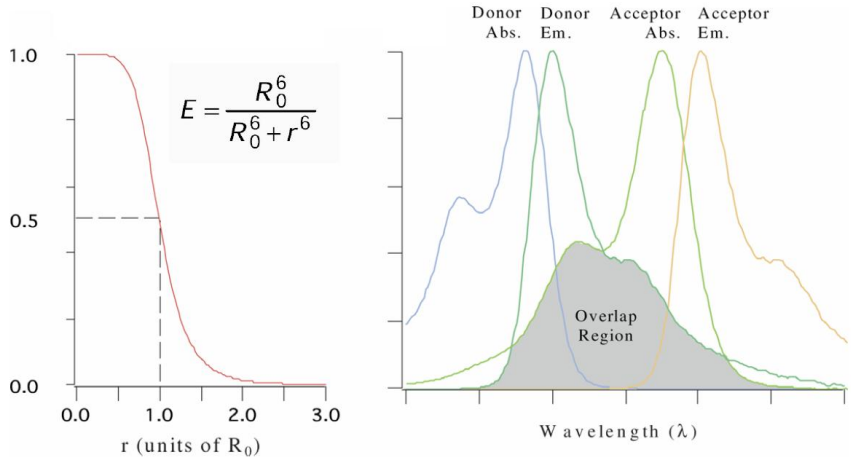


Abb. 1.6 Bedingungen für FRET.

Neben der ursprünglichen Anwendung des FRET-Prinzips zur Distanzmessung zweier Moleküle und dem Nachweis von Protein-Protein-Interaktionen kann die räumliche Annäherung von Donor und Akzeptormolekülen auch für die Konzentrationsbestimmung von Metaboliten genutzt werden. Das Prinzip solcher Biosensoren beruht dabei auf der Nutzung eines spezifischen Bindungsmoleküls für einen Metaboliten. Man kann zwei verschiedene Prinzipien unterscheiden. Entweder kommt es durch Anwesenheit des Metaboliten zu einer Konformationsänderung im Bindungsmolekül und damit zu einer Annährung von Donor und Akzeptor, die jeweils endständig an das Bindungsmolekül fusioniert sind, oder zwei Bindungspartner, die jeweils mit Donor oder Akzeptor fusioniert sind, assoziieren oder dissoziieren in Anwesenheit des Metaboliten (Tsien *et al.* 1993). Diese Art von Biosensor konnte erstmals 1991 zur Detektion des intrazellulären cAMP-Spiegels angewendet werden und basierte auf einer intermolekularen Energieübertragung zwischen der Rhodamin-markierten regulatorischen Untereinheit der Proteinkinase A (PKA) und der Fluorescein-markierten katalytischen Untereinheit (Adams *et al.* 1991). Intramolekulare FRET-basierte Biosensoren wurden für eine Reihe weiterer Metaboliten entwickelt, darunter das cAMP (Ponsioen *et al.* 2004). Das *cameleon*, ein Ca^{2+}-Sensor für die Detektion intrazellulärer Calciumveränderungen (Miyawaki *et al.* 1997; Nagai *et al.* 2001; Zhang *et al.* 2002), hat bereits besonders breite Anwendung gefunden. Im Vergleich zum etablierten Calciumfarbstoff FURA kann das *cameleon* als Expressionskonstrukt durch spezifische *targeting*-Sequenzen auch gezielt in subzellulären Kompartimenten zur Expression gebracht werden und ermöglicht damit kompartimentspezifische Analysen des Calciumfluxes. FRET-basierte Biosensoren kamen weiterhin bereits zum Nachweis von cGMP (Honda *et al.* 2001), Proteinkinasen (Sato *et al.* 2002), des Redoxpotentials (Dooley *et al.* 2004) sowie der Aktivität von Ras und Rap1 (Mochizuki *et al.* 2001) zum Einsatz.

Die Entwicklung von FRET-basierten Biosensoren für die Detektion von intrazellulärer Glucose und Galaktose basiert auf der Modifikation von bakteriellen periplasmatischen Bindeproteinen, die spezifisch für Monosaccharide sind. Als Donor-Akzeptor FRET-Paar wurden ECFP und Citrin (ein Derivat des EYFP) an solche Bindeproteine fusioniert (Deuschle *et al.* 2005a). Glucose verursacht durch die Bindung an die Erkennungsdomäne des Bindeproteins eine Konformationsänderung, welche die beiden FRET-Partner in ihrer räumlichen Zuordnung beeinflusst. Dies hat eine Veränderung der Energieübertragung zwischen Donor und Akzeptor zur Folge. Messbar ist dies durch Aufnahme der Emission von Donor und Akzeptor nach Anregung des Donors. Die gemessene Veränderung der FRET-Effizienz steht damit in direkter Abhängigkeit zur Konzentration des analysierten

zellulären Substrates. Ein linearer Anstieg der FRET-Effizienz in Abhängigkeit der Substratkonzentration kennzeichnet hierbei das Auflösungsvermögen eines solchen Sensors (Fehr *et al.* 2003a; Fehr *et al.* 2004b). Neben den glucosespezifischen Nanosensoren wurden weitere Konstrukte generiert, die z.B. für Ribose (Lager *et al.* 2003) und Maltose (Fehr *et al.* 2002) spezifisch sind. Durch gezielte Mutation war es zudem möglich, die Affinität sowie gleichzeitig das Signal/Rausch-Verhältnis solcher Sensoren zu optimieren (Deuschle *et al.* 2005b). Der in der vorliegenden Arbeit verwendete Glucosesensor FLII^{12}Pglu-700µ-δ6 (Takanaga *et al.* 2008b) besteht aus dem periplasmatischen Bindeprotein mit besonderer Spezifität für Glucose und dem FRET-Paar ECFP/Citrin. Er erlaubt die ratiometrische Bestimmung intrazellulärer Glucose, durch Bestimmung des Emmissionsquotienten aus Citrin/ECFP nach Anregung des ECFP (Abb. 1.6).

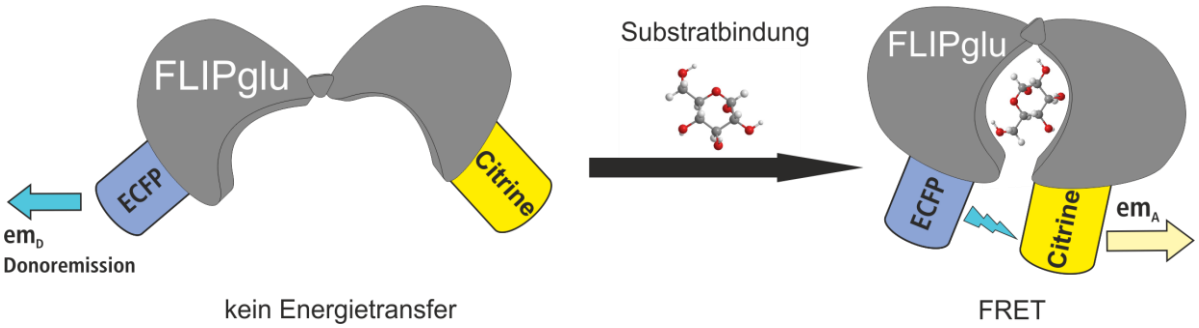


Abb. 1.7 Schematische Darstellung des FLIPglu Glucosesensors. Durch Fusionierung des FRET-Paares ECFP/Citrin an einen bakteriellen periplasmatischen Glucosetransporter (grau) entstand der FRET-Glucosesensor FLIPglu. Die Bindungsdomäne bringt dabei durch eine Konformationsänderung nach Substratbindung die beiden FRET-Partner in räumliche Nähe und ermöglicht somit einen Energietransfer von Donor zu Akzeptor.

1.8 Fragestellung

Die Beta-Zellen des Pankreas und die Leber übernehmen essentielle Funktionen in der Regulation der Blutglucosehomöostase. In beiden Geweben werden der Glucosetransporter GLUT2 und das glucosephosphorylierende Enzym Glucokinase exprimiert. Diese sind in der Lage, den intrazellulären Glucoseflux an nachgeschaltete metabolische Signalkaskaden zu koppeln.
Die vorliegende Promotionsarbeit hatte daher die folgenden Ziele:

1. Die Entwicklung von Fluoreszenz Resonanz Energie Transfer (FRET) – basierten intrazellulären Glucosesensoren ermöglicht im Gegensatz zu bereits etablierten Methoden der Glucosemessung eine direkte Erfassung des Glucosefluxes in Echtzeit. Der Glucosesensor FLIPglu sollte daher in klonalen und primären Beta-Zellen, sowie primären Hepatozyten zur Expression gebracht werden, um damit eine Glucosemessmethode in diesen Zellen zu etablieren. Zu diesem Zweck sollten sowohl eine stabil überexprimierende MIN6 Beta-Zelllinie, als auch adenovirale Vektoren mit der cDNA für das Sensorkonstrukt generiert werden.

2. Das Zusammenspiel von Glucoseaufnahme und -phosphorylierung reguliert die Glucosekonzentration in der Zelle. Durch den Einsatz des Glucosesensors FLIPglu sollte daher eine Echtzeitanalyse und nachfolgende Quantifizierung von Glucoseinflux, -efflux und -stoffwechselkapazität durchgeführt werden. Dabei sollten insulinproduzierende mit nicht insulinproduzierenden Zellen verglichen werden.

3. Störungen in der glucosestimulierten Insulinsekretion der Beta-Zellen des Pankreas sind eine Ursache des Typ 2 Diabetes mellitus. Daher sollte eine simultane fluoreszenzmikroskopische Analyse der intrazellulären Glucose- und Calciumkonzentration durchgeführt werden. Diese Messungen sollten eine zeitliche Korrelation des initialen Stimulus Glucose mit nachfolgenden Signalkaskaden der Insulinsekretion ermöglichen.

4. Nur in der Leber vermittelt das GRP eine Translokation der Glucokinase zwischen Zytoplasma und Zellkern in Abhängigkeit von der Glucosekonzentration. Der Mechanismus des Glucokinaseexportes nach dem Anstieg der Blutglucose ist bislang weitgehend ungeklärt. Es wurde jedoch eine Dissoziation der Glucokinase vom GRP im Zellkern von Hepatozyten postuliert. Daher sollte in der vorliegenden Arbeit anhand des Glucosemetabolismus untersucht werden, ob im Zellkern eine Dissoziation der

Glucokinase von ihrem regulatorischen Protein erfolgt und die Glucokinase dort bereits eine katalytische Aktivität aufweist. Hierzu sollte der Glucosesensor FLIPglu mit Hilfe einer NLS-Zielsequenz im Zellkern von Hepatozyten zur Expression gebracht werden.

2 Publikationen

Titel:

Endogenous activation of glucokinase by
6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase is glucose dependent

Zeitschrift:

Molecular Endocrinology 2010 Oct 1; 24(10):1988-1997 Epub 2010 Aug 11

Titel:

Real-time analysis of intracellular glucose and calcium in pancreatic beta cells by fluorescence microscopy

Zeitschrift:

The Journal of Biological Chemistry

Zur Publikation eingereicht.

Titel:

Glucose causes dissociation of the glucokinase-glucokinase regulatory protein complex already in the nucleus of hepatocytes

Zeitschrift:

Molecular Endocrinology

In Vorbereitung

ORIGINAL RESEARCH

Endogenous Activation of Glucokinase by 6-Phosphofructo-2-Kinase/Fructose-2,6-Bisphosphatase Is Glucose Dependent

Sara Langer, Martin T. Kaminski, Sigurd Lenzen, and Simone Baltrusch

Institute of Clinical Biochemistry (S.L., M.T.K., S.L., S.B.), Hannover Medical School, 30623 Hannover, Germany; and Institute of Medical Biochemistry and Molecular Biology (S.B.), University of Rostock, 18057 Rostock, Germany

Glucokinase (GK) plays a crucial role as glucose sensor in glucose-induced insulin secretion in pancreatic β-cells. The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) acts as an endogenous GK activator. Therefore, the goal of this study was the analysis of GK-PFK-2/FBPase-2 complex formation and its effect on metabolic stimulus-secretion coupling in β-cells in dependence upon glucose. The interaction between GK and PFK-2/FBPase-2 was analyzed in insulin-secreting MIN6 cells with a new fluorescence-based mammalian two-hybrid system. In contrast to the commonly used mammalian two-hybrid systems that require sampling before detection, the system used allows monitoring of the effects of environmental changes on protein-protein interactions on the single-cell level. Increasing the glucose concentration in the cell culture medium from 3 to 10 and 25 mmol/liter amplified the interaction between the enzymes stepwise. Importantly, in line with these results, overexpression of PFK-2/FBPase-2 in MIN6 cells evoked only at 10 and 25 mmol/liter, an increase in insulin secretion. Furthermore, a PFK-2/FBPase-2 mutant with an abolished GK-binding motif neither showed a glucose-dependent GK binding nor was able to increase insulin secretion. The results obtained with the mammalian two-hybrid system could be confirmed by fluorescence resonance energy transfer experiments in COS cells. Furthermore, the established interaction between GK and the liver GRP served in all experiments as a control. Thus, this study clearly showed that binding and activation of GK by PFK-2/FBPase-2 in β-cells is promoted by glucose, resulting in an enhancement of insulin secretion at stimulatory glucose concentrations, without affecting basal insulin secretion. (***Molecular Endocrinology*** **24: 1988–1997, 2010)**

The glucose sensor enzyme glucokinase (GK) plays a pivotal role in coupling millimolar changes of the extracellular glucose concentration to metabolism both in pancreatic β-cells and liver (1–4). In β-cells glucose phosphorylation by GK is the rate-limiting step of glucose metabolism and thus of glucose-induced insulin secretion (5–7). Glucose is the most potent activator of GK, inducing a comprehensive conformational change. Furthermore, for short-term regulation of GK activity, posttranslational mechanisms including protein-protein interactions proved to be of particular importance. The liver-specific GK-regulatory protein (GRP), which competitively inhibits GK activity, was the first GK-binding protein to be identified (8). The interaction between GK and GRP takes place at low glucose concentrations and is stimulated in the presence of fructose-6-phosphate (8, 9). Furthermore the GK-GRP complex is translocated to the nucleus (10–13). In previous experiments binding between GK and the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) has been documented (14–17). The GK-binding motif is localized in the FBPase-2 domain and consists of the amino acids LKVWT for the β-cell isoform (15). Yeast two-hybrid studies have shown that both the liver and β-cell isoform of PFK-2/FBPase-2 are able to bind to the GK

ISSN Print 0888-8809 ISSN Online 1944-9917
Printed in U.S.A.

doi: 10.1210/me.2010-0115 Received March 25, 2010. Accepted July 9, 2010.
First Published Online August 11, 2010

Abbreviations: AD, Activation domain; BD, binding domain; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; FRET, fluorescence resonance energy transfer; FRETN, sensitized emission-based FRET efficiency; GK, glucokinase; GRP, glucokinase regulatory protein; PFK-2/FBPase-2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase.

protein (15). PFK-2/FBPase-2 determines intracellular levels of fructose-2,6-bisphosphate by synthesizing and degrading this important regulator of carbohydrate metabolism (18–20). Overexpression of PFK-2/FBPase-2 results in a significant activation of GK enzyme activity in insulin-producing cells (16). In pancreatic β-cells the PFK-2/FBPase-2 acts not only as an endogenous activator of GK enzyme activity but also significantly improves the coupling between glycolysis and oxidative flux (14). Importantly, the activation of GK by PFK-2/FBPase-2 affects only the V_{max} value of the enzyme, but not the $S_{0.5}$ for glucose (16). Recently discovered small chemical GK activators allosterically activate the enzyme by lowering the glucose $S_{0.5}$ (21–29). Some of the compounds were able to additionally increase the V_{max} (21, 22, 25). GK activators enhance glucose-stimulated insulin release from pancreatic islets as well as glucose disposal by the liver (26, 28, 30). Further elucidation of the physiological GK activation by PFK-2/FBPase-2 will help to advance the concept of GK activation based on physiological needs for treatment of type 2 diabetes.

Because detection of protein-protein interactions is a major challenge in proteome research, two-hybrid assays have been established and modified in various ways in the past decade (31–37). Thus, a two-hybrid system was further adapted in this study to analyze interactions in insulin-producing cells. Both in mammalian cells and in yeast these assays make use of the fact that many eukaryotic transcriptional activators consist of two separable domains, which only initiate reporter gene transcription when they are closely approximated (38–40). The required proximity is established through direct binding between the proteins the interaction of which is studied. Solubility, folding, and posttranslational modifications required for specific protein interactions of mammalian proteins are partially insufficient in yeast. Thus, only the mammalian system provides a natural environment. In the systems used in this study the transcriptional activator domains are a herpes simplex virus VP16 transcriptional activation domain (AD) and the yeast GAL4 DNA-binding domain (BD), each of which is fused to one of the proteins of interest. Association of GAL4-DNA-BD and VP16-AD, driven by the interaction of the fused proteins of interest, results in binding to the promoter region and transcriptional activation of the reporter gene. In most mammalian two-hybrid experiments analyzing strong interactions of viral proteins, luciferase or chloramphenicol acetyltransferase activity was determined as a reporter signal (34, 35). However, in some studies fluorescent proteins, namely green fluorescent protein and enhanced yellow fluorescent protein (EYFP), have already been applied successfully as a reporter providing a higher resolution of signal detection (36, 41). The use of EYFP as a reporter gene, together with enhanced cyan fluorescent protein (ECFP) expression under control of a constitutive promoter, allows normalization for differences in transfection efficiency and enables quantitative analysis of protein-protein interactions on the single-cell level (41).

The present study applied such a fluorescence-based mammalian two-hybrid system in combination with a semiautomated microscopy approach to elucidate the regulatory principle of the interaction between GK and its activator PFK-2/FBPase-2 in insulin-secreting MIN6 cells. Moreover, this system was compared with the analysis of protein-protein interactions by the use of fluorescent fusion proteins and intracellular fluorescence resonance energy transfer (FRET). Finally, the effect of the GK-PFK-2/FBPase-2 interaction on the threshold of glucose-induced insulin secretion was determined.

Results

Glucose-dependent interaction between GK and the GRP in the mammalian two-hybrid system

The well-characterized interaction between GK and the GRP (42) could be assayed with the fluorescence-based mammalian two-hybrid system. The system was initially performed in COS cells, a previously established cellular model for the GK-GRP interaction (10). Transient transfection of COS cells with the GK and GRP two-hybrid plasmids whereas culturing the cells in medium containing 25 mmol/liter glucose resulted in a significant interaction signal, given as the ratio of EYFP over ECFP that was 70% higher than in negative controls. Because a two-hybrid signal is only produced by direct binding of the fusion proteins to each other, this indicates complex formation of GK and GRP. Incubation of the transfected cells with medium containing 5 mmol/liter glucose resulted in a 50% higher EYFP/ECFP ratio than with 25 mmol/liter glucose (Fig. 1). This is consistent with the role of GRP to bind GK in hepatocytes at low glucose concentrations. Thus, this mammalian two-hybrid system, which allows the calculation of the interaction strength on the single-cell level, is a suitable tool for analyzing protein-protein interactions in a quantitative manner while different extracellular parameters can be set.

Glucose-dependent binding of islet PFK-2/FBPase-2 to GK

The two-hybrid system was established in MIN6 cells to study the interaction between GK and PFK-2/FBPase-2 in insulin-secreting cells. Furthermore, a triple mutation was inserted in the GK-binding motif of PFK-2/FBPase-2, and the GK-binding behavior of this mutant (PFK-2/FBPase-2-Mut) was compared with the wild type. The

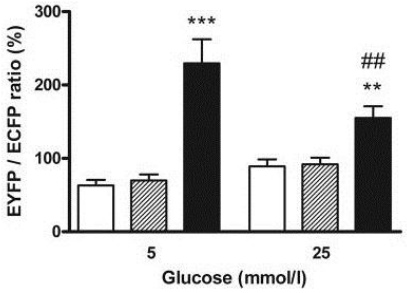


FIG. 1. Glucose-dependent modulation of GK binding to the GRP. The interaction strength of the fusion proteins DNA-BD-GRP and AD-GK was measured in a fluorescence-based mammalian two-hybrid assay. DNA-BD and AD-GK (*white bars*) or DNA-BD-GRP and AD (*dashed bars*) or DNA-BD-GRP and AD-GK (*black bars*) were coexpressed in COS cells. The cells were preincubated in the presence of 5 mmol/liter or 25 mmol/liter glucose. Thereafter, the fluorescence intensities of ECFP and EYFP were determined. Data are expressed as means ± SEM of three to five individual experiments with a total of 142–266 nuclei analyzed. **, $P < 0.01$; ***, $P < 0.001$ compared with negative controls; ##, $P < 0.01$ compared with cells incubated with 5 mmol/liter glucose (ANOVA/Bonferroni's test).

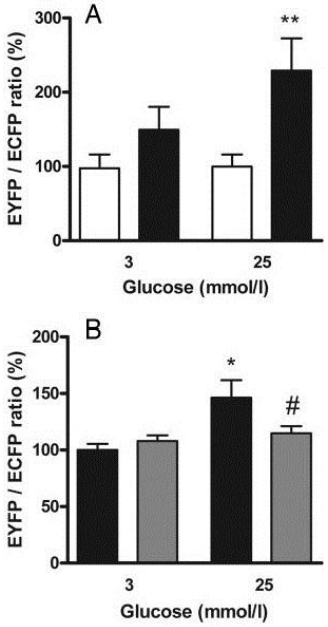


FIG. 2. Glucose promoted binding of islet PFK-2/FBPase-2 to GK. The interaction strength of the fusion proteins DNA-BD-PFK-2/FBPase-2 or DNA-BD-PFK-2/FBPase-2-Mut and AD-GK was measured in a fluorescence-based mammalian two-hybrid assay in insulin-secreting MIN6 cells. A, DNA-BD-PFK-2/FBPase-2 and AD (*white bars*) or DNA-BD-PFK-2/FBPase-2 and AD-GK (*black bars*) were coexpressed in MIN6 cells and preincubated with 3 mmol/liter or 25 mmol/liter glucose. Thereafter, the fluorescence intensities for ECFP and EYFP were determined. Data are expressed as means ± SEM of three individual experiments with a total of 60–70 nuclei analyzed. **, $P < 0.01$ compared with negative control (ANOVA/Bonferroni's test). B, DNA-BD-PFK-2/FBPase-2 and AD-GK (*black bars*) or DNA-BD-PFK-2/FBPase-2-Mut and AD-GK (*gray bars*) were coexpressed in MIN6 cells and preincubated with 3 mmol/liter or 25 mmol/liter glucose. Thereafter, the fluorescence intensities for ECFP and EYFP were determined. Data are expressed as means ± SEM of three individual experiments with a total of 44–152 nuclei analyzed. *, $P < 0.05$ compared with cells incubated with 3 mmol/liter glucose; #, $P < 0.05$ compared with DNA-BD-PFK-2/FBPase-2 wild type (ANOVA/Bonferroni's test).

islet PFK-2/FBPase-2 isoform fused to the GAL4-DNA-binding domain showed a significant interaction with GK fused to the VP16 AD when the cells were incubated with standard medium containing 25 mmol/liter glucose. The EYFP/ECFP ratio was 2.3 times higher than the ratio of the negative controls. Thus, the interaction of GK with PFK-2/FBPase-2 can be regarded as a weak interaction. At a low glucose concentration of 3 mmol/liter the EYFP reporter signal expression in relation to constitutively expressed ECFP was diminished to a value only slightly above the negative control (Fig. 2A). This suggests that an even smaller fraction of GK is bound in the complex with PFK-2/FBPase-2 when the glucose concentration is decreased. The PFK-2/FBPase-2-Mut showed at 3 mmol/liter glucose with 108% a minor GK interaction comparable to the wild-type PFK-2/FBPase-2 (Fig. 2B). The significant increase in GK binding of the PFK-2/FBPase-2 wild type to 146% at 25 mmol/liter glucose was not detectable for the mutant. Interaction between GK and PFK-2/FBPase-2-Mut at 25 mmol/liter glucose was with 114% significantly lower compared with wild-type PFK-2/FBPase-2 (Fig. 2B).

A semiautomated approach to analyze protein-protein interactions with the mammalian two-hybrid system

The use of a semiautomated microscopy approach made it possible to simultaneously monitor the interaction strength of GK binding to PFK-2/FBPase-2 at three different glucose concentrations over a time period of 40 h. The signals were highly variable for 10 h after adjusting the different glucose concentrations. The number of cells expressing fluorescent protein was low during this 10-h time period. Then the values ranged around a plateau before the negative controls started to increase slightly from 50 h after transfection. Generally, the negative controls showed less variability than the samples in which GK and islet PFK-2/FBPase-2 were coexpressed. It is noteworthy that the EYFP/ECFP ratio exhibited an oscillating profile particularly in the coexpressing cells and slightly in the negative controls, possibly due to batch expression of the proteins (Fig. 3A). Therefore, the values were averaged over the period from 36–48 h post transfection for final analysis. GK and islet PFK-2/FBPase-2 showed a significant interaction signal when the cells were preincubated at 25 mmol/liter glucose. Also at 10 mmol/liter glucose, the proteins showed a distinct inter-

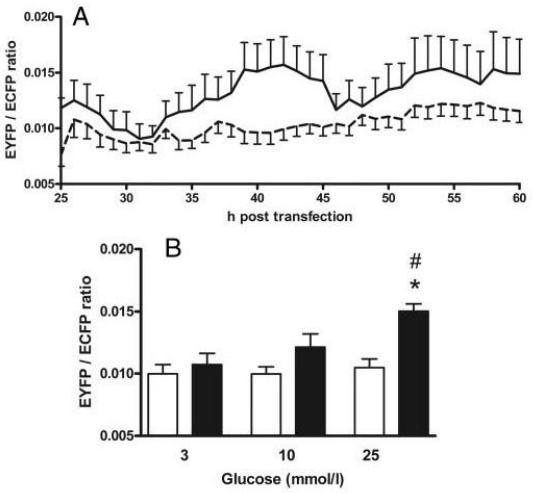


FIG. 3. The interaction strength between GK and PFK-2/FBPase-2 rises with increasing glucose concentrations. The interaction of the fusion proteins DNA-BD-PFK-2/FBPase-2 and AD-GK was measured in a semiautomated fluorescence-based mammalian two-hybrid assay. DNA-BD-PFK-2/FBPase-2 and AD (*dashed line* in A and *white bars* in B) or DNA-BD-PFK-2/FBPase-2 and AD-GK (*solid line* in A and *black bars* in B) were coexpressed in insulin-secreting MIN6 cells. In cells cultured at 3, 10, or 25 mmol/liter glucose, respectively, the degree of interaction between DNA-BD and AD proteins was analyzed every hour as the mean value for the nuclear EYFP/ECFP ratio. A, Images were taken hourly over a period of 40 h. The time lapse of the calculated EYFP/ECFP ratio is exemplary, shown for a representative single experiment for the interaction of GK with islet PFK-2/FBPase-2 at 25 mmol/liter glucose (*solid line*) together with the respective negative control at 25 mmol/liter glucose (*dashed line*). Data are expressed as means ± SEM of 2–120 nuclei analyzed per time point. B, The mean nuclear EYFP/ECFP ratios obtained between 36 and 48 h after transfection were determined together with the respective SEM for 341–1369 cells per sample. The means ± SEM of the EYFP/ECFP ratio are shown for three to five independent experiments with a total of 1331–5905 nuclei analyzed. *, $P < 0.05$ compared with negative control, #, $P < 0.05$ compared with cells incubated with 3 mmol/liter glucose (ANOVA/Bonferroni's test).

action. At 3 mmol/liter glucose only a slightly higher EYFP/ECFP ratio than in the negative controls was detected. The interaction strength at 3 mmol/liter glucose was significantly lower than at 25 mmol/liter (Fig. 3B).

Effect of low *vs.* high glucose on colocalization between GK and PFK-2/FBPase-2

The morphology and size of COS cells allow a standardized cytoplasmic region analysis necessary for colocalization as well as FRET studies. Thus, COS cells were cotransfected with expression vectors encoding the fluorescent fusion proteins ECFP-GK and wild-type or mutant EYFP-PFK-2/FBPase-2 and preincubated in medium containing 3 mmol/liter or 25 mmol/liter glucose. After both preincubation conditions, cells showed comparable cell viability and cytoplasmic localization of ECFP-GK and wild-type or mutant EYFP-PFK-2/FBPase-2. GK and wild-type PFK-2/FBPase-2 as well as GK and PFK-2/FBPase-2-Mut exhibited distinct colocalization at both glucose concentrations indicating cytoplasmic cocompartmentalization (Fig. 4). The degree of colocalization between ECFP-GK and EYFP-PFK-2/FBPase-2 wild type was 7.8% at 3 mmol/liter glucose and 11.0% at 25 mmol/liter glucose (Fig. 4C). Mutation of the GK-binding motif of PFK-2/FBPase-2 resulted in degrees of colocalization of

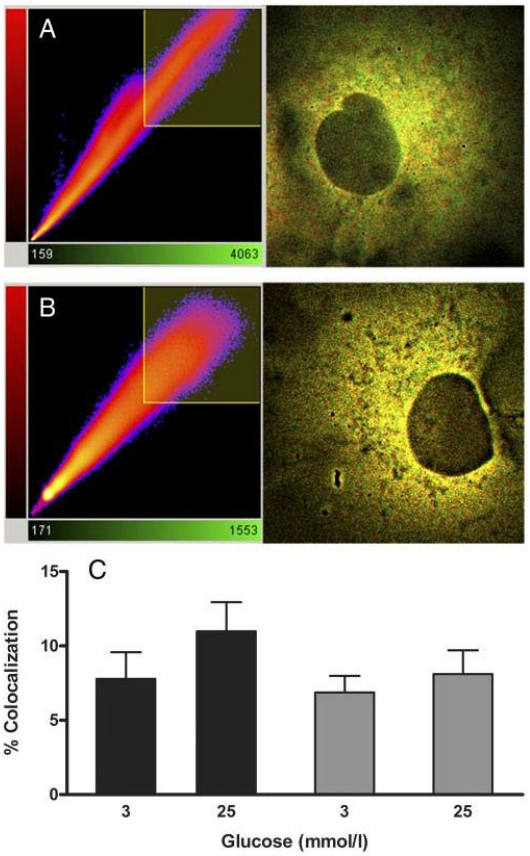


FIG. 4. Effect of low *vs.* high glucose on colocalization between GK and islet PFK-2/FBPase-2. ECFP-GK and EYFP-PFK-2/FBPase-2 or EYFP-PFK-2/FBPase-2-Mut fusion proteins were co-overexpressed in COS cells and precultured in medium containing 3 mmol/liter or 25 mmol/liter glucose, respectively. Fluorescence images were taken, and the degree of colocalization was calculated with the Imaris software (Bitplane). A typical image overlay of one cell co-overexpressing ECFP-GK and EYFP-PFK-2/FBPase-2 incubated at 3 mmol/liter glucose (A) and one at 25 mmol/liter glucose (B) is shown with the associated correlation diagram. ECFP-GK is depicted in *green* and EYFP-PFK-2/FBPase-2 is shown in *red*. C, Colocalization of ECFP-GK and EYFP-PFK-2/FBPase-2 (*black bars*), and of ECFP-GK and EYFP-PFK-2/FBPase-2-Mut (*gray bars*) is expressed as means ± SEM of three individual experiments with a total of five to eight cells analyzed.

6.9% at 3 mmol/liter glucose and 8.1% at 25 mmol/liter glucose (Fig. 4C).

Effect of low *vs.* high glucose on FRET between GK and GRP or PFK-2/FBPase-2

Sensitized emission-based FRET efficiency (FRETN) was measured between ECFP and EYFP fused to each other to determine the FRETN detection range of the microscopy setup. FRETN was 9.1 and therefore by the factor of 31 higher than the negative control in which ECFP and EYFP were expressed separately. A low FRET rate was also detectable in the negative control because of a residual dimerization tendency of ECFP and EYFP as well as bleed-through that happens when the donor ECFP is excited by the acceptor EYFP excitation wavelength or *vice versa* (Fig. 5A). ECFP-GK and EYFP-GRP showed a significant FRET signal both at 5 mmol/liter and 25 mmol/liter glucose. At low glucose concentration, FRETN was 2.3 times higher than the negative control and 50% higher in comparison to cells incubated at 25 mmol/liter glucose.

Although the fusion proteins ECFP-GK and EYFP-PFK-2/FBPase-2 wild type showed only a slightly higher colocalization at 25 mmol/liter glucose than at 3 mmol/liter glucose when they were co-overexpressed in COS cells, the enzymes exhibited FRET exclusively at 25 mmol/liter glucose. At 25 mmol/liter glucose FRETN was, with 0.82, 2.5 times higher than the negative control. In contrast, no significant FRETN efficiency was detectable between ECFP-GK and EYFP-PFK-2/FBPase-2-Mut at 25 mmol/liter glucose. When the cells were preincubated with medium containing 3 mmol/liter glucose, no FRET was observed between ECFP-GK and EYFP-PFK-2/FBPase-2, neither for the wild type nor for the mutant PFK-2/FBPase-2 (Fig. 5C).

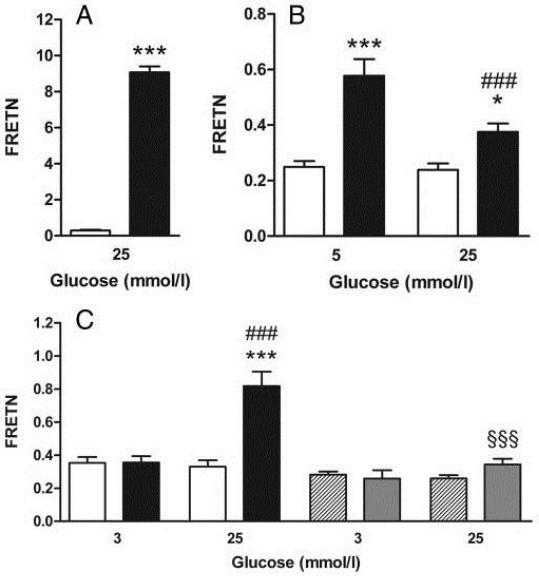


FIG. 5. Effect of low *vs.* high glucose on FRET between GK and its binding partners GRP and islet PFK-2/FBPase-2. Fluorescent proteins were coexpressed in COS cells and precultured with the indicated glucose concentrations. Thereafter, fluorescence images were taken in living cells, and the sensitized emission-based FRET efficiency (FRETN) was calculated from the ECFP and EYFP emission intensities. A, FRETN between separately expressed ECFP and EYFP (*white bars*) or the fluorescent fusion protein ECFP-EYFP (*black bars*). B, FRETN between EYFP-GRP and ECFP (*white bars*) or ECFP-GK (*black bars*). C, FRETN between EYFP-PFK-2/FBPase-2 and ECFP (*white bars*) or ECFP-GK (*black bars*), and between EYFP-PFK-2/FBPase-2-Mut and ECFP (*dashed bars*) or ECFP-GK (*gray bars*). Data are expressed as means ± SEM of three individual experiments with a total of 8–22 cells analyzed. *, $P < 0.05$; ***, $P < 0.001$ compared with negative control; ###, $P < 0.001$ compared with cells incubated with 3 mmol/liter or 5 mmol/liter glucose; §§§, $P < 0.001$ compared with EYFP-PFK-2/FBPase-2 wild type, respectively (panel A, Student's *t* test; panels B and C, ANOVA/Bonferroni's test).

Islet PFK-2/FBPase-2 expression evoked a glucose-dependent increase in insulin secretion

MIN6 cells were transfected with EYFP as a control or EYFP-PFK-2/FBPase-2 or EYFP-PFK-2/FBPase-2-Mut and insulin secretion were determined after 1-h starvation and subsequent stimulation with 3, 10, or 25 mmol/liter glucose. Comparable transfection efficiency and protein expression level of EYFP, EYFP-PFK-2/FBPase-2, and EYFP-PFK-2/FBPase-2-Mut were verified by fluorescence microscopy. Control cells showed a glucose-induced insulin secretion with a threshold above 3 mmol/liter glucose (Fig. 6). Whereas at 3 mmol/liter glucose insulin secretion in EYFP-PFK-2/FBPase-2 transfected cells was

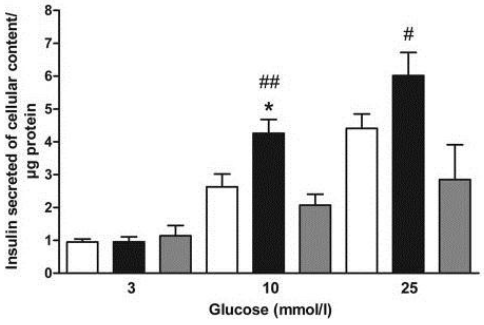


FIG. 6. Effect of islet PFK-2/FBPase-2 on glucose-induced insulin secretion. Insulin-secreting MIN6 cells were transfected with EYFP (*white bars*), EYFP-PFK-2/FBPase-2 (*black bars*), or EYFP-PFK-2/FBPase-2-Mut (*gray bars*) and cultured for 48 h. Cells were starved for 1 h and thereafter stimulated for 1 h with 3, 10, or 25 mmol/liter glucose, respectively. Insulin secretion is shown as insulin secreted per cellular insulin content and protein. Data are expressed as means ± SEM of four individual experiments. *, $P < 0.05$ compared with negative control; #, $P < 0.05$; ##, $P < 0.01$ compared with EYFP-PFK-2/FBPase-2-Mut stimulated with the same glucose concentration (ANOVA/Bonferroni's test).

comparable to control cells, at 10 and 25 mmol/liter EYFP-PFK-2/FBPase-2 expression induced a higher insulin secretion. At 10 mmol/liter glucose insulin secretion of EYFP-PFK-2/FBPase-2-transfected cells was significantly higher in comparison to control cells (Fig. 6). In contrast, transfection with EYFP-PFK-2/FBPase-2-Mut did not evoke an increase in insulin secretion in MIN6 cells. Thus, insulin secretion of wild-type PFK-2/FBPase-2-expressing cells was at 10 and 25 mmol/liter glucose significantly higher compared with mutant PFK-2/FBPase-2-expressing cells (Fig. 6).

Discussion

The glucose sensor enzyme GK plays a crucial role during stimulus-secretion coupling in pancreatic β-cells. In contrast to the transcriptional regulation of liver GK by insulin and glucagons, the β-cell isoenzyme protein levels are only marginally affected by these hormones (43, 44). Posttranslational regulation mechanisms of GK, including protein-protein interaction and compartmentalization processes, therefore make an important contribution to the complex network maintaining glucose homeostasis (44–46). In β-cells the GK protein is exclusively localized in the cytoplasm. The cytoplasmic bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2), a key regulator of glucose metabolism, proved to be a binding partner of GK (15, 17). Unlike the GRP, which competitively inhibits GK activity only in liver (10–13), PFK-2/FBPase-2 proved to be an activating GK-binding partner in both liver and pancreatic β-cells (14, 16, 17). Furthermore, PFK-2/FBPase-2 amplified the increase of intrinsic GK activity by glucose, whereas both the affinity of GK for glucose and the positive cooperativity were unaffected (16). Thus, it becomes apparent, that the endogenous GK activator PFK-2/FBPase-2 plays an important role in glucose-induced insulin secretion in pancreatic β-cells.

The objective of this study was to analyze the interaction of GK with the β-cell isoform of PFK-2/FBPase-2 with special emphasis on glucose dependency. For the purpose of detecting differences in the interaction of the enzymes in a physiological environment, fusion proteins of the wild type and a mutant PFK-2/FBPase-2 were generated to perform mammalian two-hybrid and FRET experiments. The GK-binding motif is located within the FBPase-2 domain of PFK-2/FBPase-2 (15). Three of the five amino acids forming this motif were mutated to abolish the GK-binding characteristics in this mutant PFK-2/FBPase-2.

Mammalian two-hybrid and FRET experiments were similarly applied to the interaction of GK with its regulatory protein (GRP) the modulation of which by glucose has been described earlier (10–13). Thus, the action of both GK interaction partners can be compared with respect to the liver metabolism. FRET efficiency strongly depends on the distance of the donor-acceptor pair of fluorophores but also on the relative orientation of the dipole moments of donor and acceptor. Although the performed sensitized emission-based FRET measurements must be considered as semiquantitative, the obtained values for the interaction of GK both with PFK-2/FBPase-2 and the GRP in the cytoplasm of COS cells in relation to the internal positive control support the classification of both interactions as weak. However, it must be taken into account that in the positive control ECFP and EYFP are directly fused to each other, providing a 1:1 ratio of donor and acceptor; an exact 1:1 stoichiometry, however, cannot be achieved by expression of proteins from different vectors, in this case ECFP-GK and EYFP-GRP or EYFP-PFK-2/FBPase-2. Although there is a significant interaction between GK and wild-type PFK-2/FBPase-2 at high glucose, the binding to the GRP was minor. At low glucose the GK-PFK-2/FBPase-2 interaction was diminished, but a significant binding between GK and GRP was detectable. Comparable results were obtained with the mammalian two-hybrid system.

Crystal structure analysis of GK protein revealed a closed conformation of catalytically active GK and a superopened conformation in which GK is inactive (27). From kinetic modeling there is strong evidence that up to three additional conformational intermediates exist between the two already established ones and that the percentage of each conformation depends upon the glucose concentration (27, 47, 48). Transferring this multiple conformational state model to the situation in the liver, this study provides evidence for an increase of the closed conformation at high glucose through interaction of GK with PFK-2/FBPase-2. In contrast, at low glucose PFK-2/FBPase-2 did not interact with GK, and the enzyme switches to the superopened conformation. In this conformation GRP is able to bind GK and to translocate the enzyme to the nucleus (10, 13). Thus, both GK binding partners seem to be important to adjust liver metabolism to the needs of fasting and refeeding. As posttranslational modifications of PFK-2/FBPase-2 (17–20) are not taken into account here, the hypothesis must be further tested in future experiments with special emphasis on the liver metabolism.

Colocalization in COS cells was highest at 25 mmol/liter between GK and wild-type PFK-2/FBPase-2 but not significantly lower at 3 mmol/liter glucose or between GK and the mutant PFK-2/FBPase-2. Assuming a compartmentalization of the cytoplasm for macromolecular chan-

nelling of glycolytic intermediates, this indicates that both proteins are located in close proximity, which is a prerequisite for a direct interaction. The direct interaction, determined by FRET, was detectable only between GK and wild-type PFK-2/FBPase-2 at 25 mmol/liter. Thus, GK-PFK-2/FBPase-2 complex formation seems to be directly dependent upon the glucose concentration and feasible only with an intact binding motif in the FBPase-2 domain. Interestingly, mammalian two-hybrid experiments in insulin-secreting MIN6 cells provided further evidence for an only very weak, and thus not significant, GK interaction at 3 mmol/liter glucose with both the wild-type and the mutant PFK-2/FBPase-2. At 25 mmol/liter glucose, a significant interaction was detectable exclusively between GK and wild-type PFK-2/FBPase-2.

Two-hybrid systems offer a great advantage for measurement of direct binary interactions. Furthermore, implementation of the fluorescent protein EYFP as a reporter in the mammalian two-hybrid system allows detection of weak protein-protein interactions in living mammalian cells in a quantitative manner, not achievable through analysis of the reporter gene alkaline phosphatase in the supernatant medium of cultured cells. Outlier cells that would strongly influence the mean value of a data set can be easily identified by single-cell analysis. However, fluorescence microscopy-based data acquisition, as well as analysis, is time consuming. Thus, the number of analyzed cells within a manually performed two-hybrid experiment is limited. Furthermore protein-protein interactions could be monitored only during a narrow time period. To increase both the number of analyzed cells and the recorded time points, an open fluorescence microscope scanning system was used in a semi-automated approach. In addition, high content data analysis was performed including background correction, automated cell detection, and cell tracking. Finally the EYFP/ECFP ratio was directly available on the single-cell level. Cells with a low or high constitutive ECFP expression were excluded from the final analysis using gates in the scan^R analysis software. This procedure has significantly reduced the SE of the measurement. Thus, this approach made it possible to determine the interaction between GK and PFK-2/FBPase-2 at three different glucose concentrations in parallel for a time period of 40 h in insulin-secreting MIN6 cells. The data obtained with this approach, in fact, suggest a stepwise amplification of interaction strength between the proteins with increasing glucose concentrations. Thus, it could be shown for the first time that the GK-PFK-2/FBPase-2 binding process itself is glucose dependent.

Together with the previous observation that overexpression of β-cell PFK-2/FBPase-2 resulted in a greater stimulatory effect of glucose on GK enzyme activity when the glucose concentration was increased (14), the present study provides direct evidence for a shift of the GK conformational equilibrium to its enzymatically active closed conformation by PFK-2/FBPase-2 binding. Thus, complex formation prevents the slow conformational change of GK into the superopened conformation. Finally, the interaction leads to an increase in GK enzyme activity, thereby facilitating the role of GK as a sensor for glucose-induced insulin secretion. Actually, in line with the increase in GK-PFK-2/FBPase-2 binding, an increase in insulin secretion was obtained at 10 and 25 mmol/liter glucose through overexpression of PFK-2/FBPase-2 in MIN6 cells. Furthermore, an increase in insulin secretion was not detectable after overexpression of the mutant PFK-2/FBPase-2 not able to interact with GK. The results fit with previous observations in β-cells with respect to the threshold of glucose-induced insulin secretion (2, 4–6), because at 3 mmol/liter glucose, overexpression of PFK-2/FBPase-2 did not evoke an increase in insulin secretion. In contrast, treatment of β-cells with small synthetic GK activators (21, 22, 24, 25) caused an increase in insulin secretion already at such glucose concentrations, thus changing the threshold of glucose-induced insulin secretion.

The present study underlines the physiological importance of posttranslational GK regulation by protein-protein interactions. Because GK activation has been described as a promising therapeutic strategy in the treatment of type 2 diabetes, further elucidation of the endogenous GK activation mechanisms will contribute to a better understanding of the metabolic coupling between GK activation and glucose-stimulated insulin secretion in β-cells. Furthermore, a viable method for the intracellular detection of a weak protein-protein interaction and its posttranslational modification was established.

Materials and Methods

Materials

Restriction enzymes and modifying enzymes for the cloning procedures were from New England Biolabs (Beverly, MA) or Fermentas (St. Leo-Rot, Germany). Custom oligonucleotides were synthesized by MWG Biotech (Ebersberg, Germany). The reporter vector pHASH-3 was a kind gift of Dr. S. Herlitze (Department of Neurosciences, Case Western Reserve University, Cleveland, OH). All reagents of analytical grade were from Merck (Darmstadt, Germany). All tissue culture equipment was from Invitrogen (Karlsruhe, Germany) and Greiner-Bio One (Frickenhausen, Germany).

Cell culture and transient transfection

COS cells and MIN6 cells were grown in DMEM supplemented with 25 mmol/liter glucose, 10% (vol/vol) fetal calf serum, 10 U/ml penicillin, 10 μg/ml streptomycin, and 2 mmol/

liter glutamine in a humidified atmosphere at 37 C and 5% CO_2. For the mammalian two-hybrid experiments, COS cells (passages 13–35) were seeded in six-well microplates at a density of 3×10^4 cells and grown for 1 d. MIN6 cells (passages 37–44) were seeded in six-well microplates at a density of 8×10^4 cells and grown for 5 d. Thereafter, cells were transfected with the mammalian two-hybrid vectors with jetPEI (Qbiogene, Montreal, Quebec, Canada) according to the manufacturer's instructions. For each transfection 0.4 pmol of the reporter vector pHASH-3 was used in combination with 0.1 pmol of the expression vector pM (negative control), pM-GRP, pM-PFK-2/FBPase-2 or pM-PFK-2/FBPase-2-Mut, and 0.1 pmol of the expression vector pVP16 (negative control) or pVP16-GK. COS cells were incubated for 21 h in the presence of 5 mmol/liter or 25 mmol/liter glucose, respectively, before image acquisition. MIN6 cells were incubated for 20 h after transfection with standard DMEM containing 25 mmol/liter glucose. Thereafter, cells were incubated in the presence of 3 mmol/liter or 25 mmol/liter glucose, respectively, for another 24 h before image acquisition. For the semiautomated scan^R approach MIN6 cells (passages 39–40) were seeded at a density of 1.5×10^5 in 12-well microplates and grown for 3 d. Then the cells were transfected with equimolar amounts of the vectors pGL4.EYFP, pBIND.ECFP-PFK-2/FBPase-2, and pACT or pACT-GK with a total amount of 1 μg DNA and grown for 24 h in standard DMEM. Thereafter, the medium was replaced, and cells were incubated in DMEM containing 3, 10, or 25 mmol/liter glucose. Image acquisition was started 1 h after medium replacement. For colocalization and FRET studies, COS cells (passages 5–20) were seeded at a density of 2×10^4 cells on 35-mm glass-bottom dishes (MatTek, Ashland, MA) and grown for 1 d. Thereafter, cells were transfected as described above with either 0.5 μg of pECFP or pECFP-GK plasmid and 0.5 μg pEYFP-GRP or 0.5 μg of pEYFP-PFK-2/FBPase-2 or 0.5 μg of pEYFP-PFK-2/FBPase-2-Mut, or with 0.5 μg pECFP and 0.5 μg pEYFP, or with 1 μg pECFP-EYFP alone. The culture medium was replaced 1 d after transfection, and the cells were incubated for another 24 h with medium containing 3, 5, or 25 mmol/liter glucose, respectively, before image acquisition.

Mammalian two-hybrid analysis

Mammalian two-hybrid analysis was performed using the cloning vectors pM and pVP16 from the BD Matchmaker Mammalian Assay Kit 2 (CLONTECH Laboratories, Inc., Palo Alto, CA) to create the GAL4 DNA-BD and VP16 AD fusion constructs, respectively. The cDNA sequences of rat β-cell PFK-2/FBPase-2 and rat liver GRP were amplified by PCR and subcloned in frame (*Sal*I and *Hin*dIII restriction sites) to the GAL4-DNA-BD into pM. Human β-cell GK coding cDNA was amplified by PCR and subcloned in frame (*Sal*I and *Hin*dIII restriction sites) to the VP16-AD into pVP16 (CLONTECH). The fluorescence-based reporter plasmid pHASH-3 (41) was used for transfection in combination with the pM and pVP16 constructs. pHASH-3 contains the gene for the yellow fluorescent protein (EYFP) under control of an inducible promoter containing GAL4-binding sites. Furthermore, pHASH-3 carries the cyan fluorescent protein (ECFP) gene, which is constitutively expressed. Both EYFP and ECFP carry a nuclear localization signal so that fluorescence is detectable in the nucleus of transfected cells. COS cells or MIN6 cells were transfected with the two-hybrid vectors for expression of the fusion proteins and the reporter vector.

For a semiautomated microscope approach of mammalian two-hybrid analysis, vectors of the CheckMate/Flexi Vector Mammalian Two-Hybrid System (Promega Corp., Madison, WI) were modified as follows. Human β-cell GK coding cDNA was amplified by PCR and subcloned in frame (*Sgf*I and *Pme*I restriction sites) to the VP16-AD into pFN10A (ACT) Flexi Vector to generate pACT-GK. The cDNA sequence of rat β-cell PFK-2/FBPase-2 was amplified by PCR and subcloned in frame (*Sgf*I and *Pme*I restriction sites) to the GAL4-DNA-BD into pFN11A (BIND) Flexi Vector to generate pBIND-PFK-2/FBPase-2. To generate pECFP-Nuc, the vectors pECFP-N1 and pEYFP-Nuc (CLONTECH) were digested with the restriction enzymes *Age*I and *Bsr*GI, and the EYFP was replaced by ECFP. The cDNA of ECFP-Nuc, including the cytomeglovirus promoter and simian virus 40 early polyadenylation signal, was amplified by PCR using composite primers to introduce *Pvu*II (5′) and *Cla*I (3′) restriction sites. *Renilla Luciferase* coding cDNA was removed from pBIND-PFK-2/FBPase-2 by digestion with *Pvu*II and *Cla*I, and ECFP-Nuc was subcloned into the vector frame. The resulting vector was called pBIND.ECFP-PFK-2/FBPase-2. The cDNA sequence of EYFP-Nuc was amplified by PCR using composite primers to introduce *Hin*dIII (5′) and *Fse*I (3′) restriction sites. The coding *luc2P* firefly luciferase cDNA was removed from the reporter vector pGL4.31 (Promega) by digestion with *Hin*dIII and *Fse*I, and EYFP-Nuc was subcloned into the vector frame. The resulting reporter vector was called pGL4.EYFP. MIN6 cells were transfected with pGL4.EYFP, pBIND.ECFP-PFK-2/FBPase-2 and pACT (Promega) as negative control or with pACT-GK. For analysis of an interaction of the fusion proteins fluorescence intensities of EYFP and ECFP were determined in the nuclei of transfected cells. To quantify the interaction strength the ratio EYFP/ECFP was calculated for single cells from the background corrected average gray values of the EYFP and ECFP fluorescence intensities.

Colocalization and FRET analyses

The expression vectors pECFP-GK and pEYFP-GRP, as described previously (10), were used for overexpression of fluorescent fusion proteins. Rat β-cell PFK-2/FBPase-2 coding cDNA was amplified by PCR and subcloned in frame (*Kpn*I and *Bam*HI restriction sites) to the EYFP into pEYFP-C1 (CLONTECH). A plasmid encoding an ECFP-EYFP fusion protein with a two-amino acid spacer between ECFP and EYFP was generated as described elsewhere (49) and used as a positive control for FRET. COS cells were transfected with combinations of ECFP and EYFP plasmids. ECFP and EYFP fluorescence intensities were recorded in the cytoplasm of living cells by fluorescence microscopy. Colocalization of ECFP and EYFP was determined with the Imaris software (Bitplane, Zurich, Switzerland). The sensitized emission-based FRET efficiency (FRETN) was calculated from the ECFP emission with excitation at 436 nm, EYFP emission with excitation at 436 nm, and EYFP emission with excitation at 500 nm, based on the calculation of Vanderklish *et al.* (50).

Mutagenesis

The LKVWT GK-binding motif in the FBPase-2 domain of rat islet PFK-2/FBPase-2 (15) was changed to **HKEWR** (PFK-2/FBPase-2-Mut). Site-directed mutagenesis was performed in pM-PFK-2/FBPase-2 and pEYFP-PFK-2/FBPase-2 using the QuikChange II Site-Directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). The sequences of the specific mismatch oligonucleotides for generation of the triple mutant were g gag atc cag gac cac aaa gag tgg aga agc cag ttg aag ag (forward)

and ct ctt caa ctg gct tct cca ctc ttt gtg gtc ctg gat ctc c (reverse). Mutagenesis was verified by sequence analysis.

Fluorescence microscopy

A cell^R/Olympus IX81 inverted microscope system equipped with a Cellcubator, as described previously (45), was used. D436/10-455DCLP-D480/40 and HQ500/20-530DCLP-D560/40 single-band filter sets were used for ECFP and EYFP, respectively (AHF Analysentechnik, Tübingen, Germany). For the mammalian two-hybrid analyses the cell culture plates with transfected, living cells were fixed on the microscope stage, and images were obtained with an UPLSAPO 20 × 0.75 numerical aperture objective (Olympus, Hamburg, Germany). For colocalization and FRET studies glass-bottom dishes were fixed on the microscope stage, and ECFP and EYFP images were obtained with an UPLSAPO 60 × 1.35 numerical aperture oil-immersion objective (Olympus). For FRET measurements, ECFP and EYFP emission were detected simultaneously using a DV-CC Dual View Simultaneous Imaging System (Optical Insights, LLC, Tucson, AZ) equipped with a 505 dcxr beam splitter and D465/30 and HQ535/30 emission filters (AHF Analysentechnik). For multiple image analysis of 12-well microplates, all motorized devices of the microscope system were synchronized by the scan^R acquisition software (Olympus Soft Imaging Solutions, Munich, Germany). The image focus was automatically determined with a gradient method from transmitted light images. In a 40-h time-lapse experiment 20 fluorescence images of each well were taken hourly with an UPLSAPO 20 × 0.75 numerical aperture objective (Olympus, Hamburg, Germany). Analysis parameters were specified and image processing was carried out with the scan^R analysis software. After background correction, individual cells were detected using an intensity threshold method and tracked over time. By setting gates, only cells with a mean ECFP fluorescence were finally considered to exclude artifacts. Thereafter the ECFP/EYFP ratio of each single cell was available over time.

Measurement of insulin secretion

MIN6 cells (passages 37–40) were seeded in six-well microplates at a density of 3.5 x 10^5 cells and grown for 3 d. Thereafter, cells were transfected with jetPEI (Qbiogene, Montreal, Quebec, Canada) according to the manufacturer's instructions and 2 μg EYFP, EYFP-PFK-2/FBPase-2 or EYFP-PFK-2/FBPase-2-Mut and grown for 24 h. Thereafter, the medium was replaced by fresh standard DMEM. Transfection efficiency and protein expression were quantified using automated scan^R fluorescence microscopy. Cells were incubated for an additional 24 h in DMEM. Finally, cells were incubated for 1 h in bicarbonate-buffered Krebs-Ringer solution without glucose supplemented with 0.1% albumin and thereafter stimulated for 1 h with 3, 10, or 25 mmol/liter glucose, respectively. Thereafter, 1 ml of the incubation buffer from each well was carefully harvested and gently centrifuged to remove detached cells. In the final supernatants the secreted insulin was measured. Cells were homogenized by sonication in PBS (pH 7.4), and insulin content was measured in soluble fractions. Insulin was measured by ELISA, and the protein concentration was quantified by a Bradford protein assay.

Statistical analyses

Data are expressed as means ± SEM. Statistical analyses were performed by ANOVA followed by Bonferroni's test for multiple comparison or Student's *t* test using the Prism analysis program (Graphpad, San Diego, CA).

Acknowledgments

We thank Dr. S. Herlitze (Department of Neurosciences, Case Western Reserve University, Cleveland, OH) for providing the reporter vector pHASH-3 and Dr. D. Krüger (Olympus Soft Imaging Solutions, Munich, Germany) for excellent technical support regarding the scan^R system. We also thank J. Kresse and R. Waterstradt for skillful technical assistance.

Address all correspondence and requests for reprints to: Professor Dr. Simone Baltrusch, Institute of Medical Biochemistry and Molecular Biology, University of Rostock, 18057 Rostock, Germany. E-mail: simone.baltrusch@med.uni-rostock.de.

This work was supported by the European Union (Integrated Project EuroDia LSHM-CT-2006-518153 in the Framework Program 6 of the European-Community) and grants from the Dr. Buding Foundation and the German Diabetes Association (to S.B.).

Disclosure summary: The authors have nothing to disclose.

References

1. Baltrusch S, Tiedge M 2006 Glucokinase regulatory network in pancreatic β-cells and liver. Diabetes 55(Suppl 2):S55–S64
2. Lenzen S, Panten U 1988 Signal recognition by pancreatic B-cells. Biochem Pharmacol 37:371–378
3. Matschinsky F, Liang Y, Kesavan P, Wang L, Froguel P, Velho G, Cohen D, Permutt MA, Tanizawa Y, Jetton TL, Niswender K, Magnuson MA 1993 Glucokinase as pancreatic β cell glucose sensor and diabetes gene. J Clin Invest 92:2092–2098
4. Meglasson MD, Matschinsky FM 1984 New perspectives on pancreatic islet glucokinase. Am J Physiol 246:E1–E13
5. Matschinsky FM 1996 Banting Lecture 1995. A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. Diabetes 45:223–241
6. Matschinsky FM 2002 Regulation of pancreatic β-cell glucokinase: from basics to therapeutics. Diabetes 51(Suppl 3):S394–S404
7. Matschinsky FM, Glaser B, Magnuson MA 1998 Pancreatic β-cell glucokinase: closing the gap between theoretical concepts and experimental realities. Diabetes 47:307–315
8. Van Schaftingen E 1989 A protein from rat liver confers to glucokinase the property of being antagonistically regulated by fructose 6-phosphate and fructose 1-phosphate. Eur J Biochem 179:179–184
9. Vandercammen A, Detheux M, Van Schaftingen E 1992 Binding of sorbitol 6-phosphate and of fructose 1-phosphate to the regulatory protein of liver glucokinase. Biochem J 286:253–256
10. Baltrusch S, Francini F, Lenzen S, Tiedge M 2005 Interaction of glucokinase with the liver regulatory protein is conferred by leucine-asparagine motifs of the enzyme. Diabetes 54:2829–2837
11. Bosco D, Meda P, Iynedjian PB 2000 Glucokinase and glucokinase regulatory protein: mutual dependence for nuclear localization. Biochem J 348:215–222
12. de la Iglesia N, Veiga-da-Cunha M, Van Schaftingen E, Guinovart JJ, Ferrer JC 1999 Glucokinase regulatory protein is essential for the proper subcellular localisation of liver glucokinase. FEBS Lett 456:332–338
13. Shiota C, Coffey J, Grimsby J, Grippo JF, Magnuson MA 1999 Nuclear import of hepatic glucokinase depends upon glucokinase regulatory protein, whereas export is due to a nuclear export signal sequence in glucokinase. J Biol Chem 274:37125–37130

14. Baltrusch S, Langer S, Massa L, Tiedge M, Lenzen S 2006 Improved metabolic stimulus for glucose-induced insulin secretion through GK and PFK-2/FBPase-2 coexpression in insulin-producing RINm5F cells. Endocrinology 147:5768–5776
15. Baltrusch S, Lenzen S, Okar DA, Lange AJ, Tiedge M 2001 Characterization of glucokinase-binding protein epitopes by a phage-displayed peptide library. Identification of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase as a novel interaction partner. J Biol Chem 276:43915–43923
16. Massa L, Baltrusch S, Okar DA, Lange AJ, Lenzen S, Tiedge M 2004 Interaction of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) with glucokinase activates glucose phosphorylation and glucose metabolism in insulin-producing cells. Diabetes 53:1020–1029
17. Smith WE, Langer S, Wu C, Baltrusch S, Okar DA 2007 Molecular coordination of hepatic glucose metabolism by the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase:glucokinase complex. Mol Endocrinol 21:1478–1487
18. Claus TH, El-Maghrabi MR, Regen DM, Stewart HB, McGrane M, Kountz PD, Nyfeler F, Pilkis J, Pilkis SJ 1984 The role of fructose 2,6-bisphosphate in the regulation of carbohydrate metabolism. Curr Top Cell Regul 23:57–86
19. Okar DA, Manzano A, Navarro-Sabatè A, Riera L, Bartrons R, Lange AJ 2001 PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2,6-bisphosphate. Trends Biochem Sci 26:30–35
20. Pilkis SJ, Claus TH, Kurland IJ, Lange AJ 1995 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase: a metabolic signaling enzyme. Annu Rev Biochem 64:799–835
21. Brocklehurst KJ, Payne VA, Davies RA, Carroll D, Vertigan HL, Wightman HJ, Aiston S, Waddell ID, Leighton B, Coghlan MP, Agius L 2004 Stimulation of hepatocyte glucose metabolism by novel small molecule glucokinase activators. Diabetes 53:535–541
22. Efanov AM, Barrett DG, Brenner MB, Briggs SL, Delaunois A, Durbin JD, Giese U, Guo H, Radloff M, Gil GS, Sewing S, Wang Y, Weichert A, Zaliani A, Gromada J 2005 A novel glucokinase activator modulates pancreatic islet and hepatocyte function. Endocrinology 146:3696–3701
23. Futamura M, Hosaka H, Kadotani A, Shimazaki H, Sasaki K, Ohyama S, Nishimura T, Eiki J, Nagata Y 2006 An allosteric activator of glucokinase impairs the interaction of glucokinase and glucokinase regulatory protein and regulates glucose metabolism. J Biol Chem 281:37668–37674
24. Fyfe MC, White JR, Taylor A, Chatfield R, Wargent E, Printz RL, Sulpice T, McCormack JG, Procter MJ, Reynet C, Widdowson PS, Wong-Kai-In P 2007 Glucokinase activator PSN-GK1 displays enhanced antihyperglycaemic and insulinotropic actions. Diabetologia 50:1277–1287
25. Grimsby J, Sarabu R, Corbett WL, Haynes NE, Bizzarro FT, Coffey JW, Guertin KR, Hilliard DW, Kester RF, Mahaney PE, Marcus L, Qi L, Spence CL, Tengi J, Magnuson MA, Chu CA, Dvorozniak MT, Matschinsky FM, Grippo JF 2003 Allosteric activators of glucokinase: potential role in diabetes therapy. Science 301:370–373
26. Guertin KR, Grimsby J 2006 Small molecule glucokinase activators as glucose lowering agents: a new paradigm for diabetes therapy. Curr Med Chem 13:1839–1843
27. Kamata K, Mitsuya M, Nishimura T, Eiki J, Nagata Y 2004 Structural basis for allosteric regulation of the monomeric allosteric enzyme human glucokinase. Structure 12:429–438
28. Leighton B, Atkinson A, Coghlan MP 2005 Small molecule glucokinase activators as novel anti-diabetic agents. Biochem Soc Trans 33:371–374
29. Matschinsky FM 2009 Assessing the potential of glucokinase activators in diabetes therapy. Nat Rev Drug Discov 8:399–416
30. Printz RL, Granner DK 2005 Tweaking the glucose sensor: adjusting glucokinase activity with activator compounds. Endocrinology 146:3693–3695
31. Hoat TX, Bertin N, Ninomiya N, Fukuda S, Usui K, Kawai J, Hayashizaki Y, Suzuki H 2009 Development of a high-throughput method for the systematic identification of human proteins nuclear translocation potential. BMC Cell Biol 10:69
32. Ikeda M, Inoue S, Muramatsu M, Minatogawa Y 2007 Characterization and identification of a steroid receptor-binding protein, SRB-RGS. Biol Pharm Bull 30:1056–1064
33. Massoud TF, Paulmurugan R, De A, Ray P, Gambhir SS 2007 Reporter gene imaging of protein-protein interactions in living subjects. Curr Opin Biotechnol 18:31–37
34. Murakami Y, Fukazawa H, Kobatake T, Yamagoe S, Takebe Y, Tobiume M, Matsuda M, Uehara Y 2002 A mammalian two-hybrid screening system for inhibitors of interaction between HIV Nef and the cellular tyrosine kinase Hck. Antiviral Res 55:161–168
35. Pan J, Peng X, Gao Y, Li Z, Lu X, Chen Y, Ishaq M, Liu D, Dediego ML, Enjuanes L, Guo D 2008 Genome-wide analysis of protein-protein interactions and involvement of viral proteins in SARS-CoV replication. PLoS One 3:e3299
36. Shioda T, Andriole S, Yahata T, Isselbacher KJ 2000 A green fluorescent protein-reporter mammalian two-hybrid system with extrachromosomal maintenance of a prey expression plasmid: application to interaction screening. Proc Natl Acad Sci USA 97:5220–5224
37. Zhao HF, Kiyota T, Chowdhury S, Purisima E, Banville D, Konishi Y, Shen SH 2004 A mammalian genetic system to screen for small molecules capable of disrupting protein-protein interactions. Anal Chem 76:2922–2927
38. Bartel P, Chien CT, Sternglanz R, Fields S 1993 Elimination of false positives that arise in using the two-hybrid system. Biotechniques 14:920–924
39. Chien CT, Bartel PL, Sternglanz R, Fields S 1991 The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc Natl Acad Sci USA 88:9578–9582
40. Fields S, Song O 1989 A novel genetic system to detect protein-protein interactions. Nature 340:245–246
41. Hümmer A, Delzeith O, Gomez SR, Moreno RL, Mark MD, Herlitze S 2003 Competitive and synergistic interactions of G protein $\beta(2)$ and Ca(2+) channel $\beta(1b)$ subunits with Ca (v) 2.1 channels, revealed by mammalian two-hybrid and fluorescence resonance energy transfer measurements. J Biol Chem 278:49386–49400.
42. van Schaftingen E, Veiga-da-Cunha M, Niculescu L 1997 The regulatory protein of glucokinase. Biochem Soc Trans 25:136–140
43. Iynedjian PB, Pilot PR, Nouspikel T, Milburn JL, Quaade C, Hughes S, Ucla C, Newgard CB 1989 Differential expression and regulation of the glucokinase gene in liver and islets of Langerhans. Proc Natl Acad Sci USA 86:7838–7842
44. Tiedge M, Steffeck H, Elsner M, Lenzen S 1999 Metabolic regulation, activity state, and intracellular binding of glucokinase in insulin-secreting cells. Diabetes 48:514–523
45. Baltrusch S, Lenzen S 2007 Novel insights into the regulation of the bound and diffusible glucokinase in MIN6 β-cells. Diabetes 56: 1305–1315
46. Rizzo MA, Magnuson MA, Drain PF, Piston DW 2002 A functional link between glucokinase binding to insulin granules and conformational alterations in response to glucose and insulin. J Biol Chem 277:34168–34175
47. Antoine M, Boutin JA, Ferry G 2009 Binding kinetics of glucose and allosteric activators to human glucokinase reveal multiple conformational states. Biochemistry 48:5466–5482
48. Zhang J, Li C, Chen K, Zhu W, Shen X, Jiang H 2006 Conformational transition pathway in the allosteric process of human glucokinase. Proc Natl Acad Sci USA 103:13368–13373
49. He L, Bradrick TD, Karpova TS, Wu X, Fox MH, Fischer R, McNally JG, Knutson JR, Grammer AC, Lipsky PE 2003 Flow cytometric measurement of fluorescence (Forster) resonance energy transfer from cyan fluorescent protein to yellow fluorescent protein using single-laser excitation at 458 nm. Cytometry A 53:39–54
50. Vanderklish PW, Krushel LA, Holst BH, Gally JA, Crossin KL, Edelman GM 2000 Marking synaptic activity in dendritic spines with a calpain substrate exhibiting fluorescence resonance energy transfer. Proc Natl Acad Sci USA 97:2253–2258

REAL-TIME ANALYSIS OF INTRACELLULAR GLUCOSE AND CALCIUM IN PANCREATIC BETA CELLS BY FLUORESCENCE MICROSCOPY

Martin Tobias Kaminski[1], Sigurd Lenzen[1], and Simone Baltrusch[1,2,*]

[1]Institute of Clinical Biochemistry, Hannover Medical School, 30623 Hannover, Germany

[2]Institute of Medical Biochemistry and Molecular Biology, University of Rostock, 18057 Rostock, Germany

Running title: Intracellular glucose measurements

[*]Corresponding author: Prof. Simone Baltrusch, Institute of Medical Biochemistry and Molecular Biology, University of Rostock, D-18057 Rostock, Germany, Tel. 0049-381-494-5760, Fax 0049-381-494-5752, e-mail: simone.baltrusch@med.uni-rostock.de

Keywords: glucokinase, GLUT2, glucose metabolism, glucose transport, fluorescence-based nanosensor, fluorescence resonance energy transfer (FRET), FLII^{12}Pglu-700µ-δ6, Calcium imaging, insulin secretion

Abbreviations: GK, glucokinase; ECFP, enhanced cyan fluorescent protein; FRET, fluorescence resonance energy transfer; HK, hexokinase; GLUT, glucose transporter; MH, mannoheptulose; 3-OMG, 3-O-methylglucose; FLIPglu, FLII^{12}Pglu-700µ-δ6; $[Ca^{2+}]_i$, intracellular calcium

Background: Glucose stimulates insulin secretion in beta cells.

Results: The FLII^{12}Pglu-700µ-δ6 nanosensor is useful for real-time measurements of the intracellular glucose concentration in beta-cells.

Conclusion: Concomitant analysis of glucose and calcium determined a glucose threshold of 4 mM for the $[Ca^{2+}]_i$ increase and of 7 mM for $[Ca^{2+}]_i$ oscillations.

Significance: Our findings provide new insights into the role of the amplifying insulin secretion pathway.

SUMMARY

Glucose is the physiological stimulus for insulin secretion in pancreatic beta cells. The uptake and phosphorylation of glucose initiates and controls downstream pathways, resulting in insulin secretion. However, the temporal coordination of these events in beta cells is not fully understood. The recent development of the FLII^{12}Pglu-700µ-δ6 glucose nanosensor facilitates real-time analysis of intracellular glucose within a broad concentration range. Using this fluorescence-based technique, we show the shift in intracellular glucose concentration upon external supply and removal in primary mouse beta cells with high resolution. Glucose

influx, efflux, and metabolism rates were calculated from the time-dependent plots. The glucose influx rate of beta cells expressing GLUT2 was ten-fold higher than that of non-insulin-producing COS cells solely expressing GLUT1. Glucose metabolism was four-fold faster in beta cells than COS cells due to expression of the glucose sensor enzyme glucokinase. We found no evidence of oscillations of the intracellular glucose concentration in beta cells. Concomitant real-time analysis of glucose and calcium dynamics using FLII^{12}Pglu-700µ-δ6 and fura-2-acetoxymethyl-ester determined a glucose threshold of 4 mM for the $[Ca^{2+}]_i$ increase in beta cells. Indeed, a glucose concentration of 7 mM had to be reached to evoke large amplitude $[Ca^{2+}]_i$ oscillations. The K_{ATP} channel closing agent tolbutamide was not able to induce large amplitude $[Ca^{2+}]_i$ oscillations in the absence of glucose. Our findings suggest that glucose has to reach a threshold to evoke the $[Ca^{2+}]_i$ increase and subsequently initiate $[Ca^{2+}]_i$ oscillations in a K_{ATP} channel independent manner.

Glucose-stimulated insulin secretion from pancreatic beta cells determines the blood glucose concentration. Upon facilitative uptake via the glucose transporter 2 (GLUT 2) (Thorens 1992; Schuit 1997; Thorens & Mueckler 2010), glucose is phosphorylated by glucokinase and subsequently metabolized, leading to an increase in the ATP/ADP ratio (Lenzen & Panten 1988b; Lenzen 1992; Matschinsky 1996; Baltrusch & Tiedge 2006b). This increase mediates the closure of ATP-sensitive potassium (K_{ATP}) channels, followed by depolarization of the plasma membrane, causing voltage-gated calcium channels to open (Henquin 2009). Ultimately, insulin is released by an increase in intracellular free calcium ($[Ca^{2+}]_i$) (Lenzen *et al.* 2000; Rorsman & Renstrom 2003; Baltrusch & Lenzen 2007b; Henquin 2009). However, this triggering pathway requires additional amplification signals generated through metabolic and neuronal mechanisms (Henquin 2000; Henquin 2009). Exploring the interplay of these pathways is a significant challenge and requires sophisticated methodologies (Henquin 2009).

A rapid expansion in fluorescence-based sensors has facilitated real-time analysis of ions and metabolites, such as calcium (Miyawaki *et al.* 1997), cAMP (Adams *et al.* 1991; Dyachok *et al.* 2006), and ATP (Kennedy *et al.* 1999a; Heart *et al.* 2006a; Berg *et al.* 2009) in intact cells. Such measurements display changes in metabolites with much higher resolution than static analyses requiring cell disruption (Fehr *et al.* 2005a; Dyachok *et al.* 2006). Thus, live cell fluorescence microscopy approaches substantially contribute to our knowledge of regulatory networks controlling stimulus-secretion coupling in beta cells (Lalonde *et al.* 2005; Tiedge & Baltrusch 2011).

Glucose uptake and metabolism have been quantified in beta cells and islets using enzymatic assays and radiolabeled glucose

analogues (Sener *et al.* 1993; Heimberg *et al.* 1995; Sener *et al.* 1999b; Rasschaert *et al.* 2001; Baltrusch *et al.* 2006). Fluorescence labeled glucose has also been used successfully to study glucose uptake (Yamada *et al.* 2000; Yamada *et al.* 2007). In the past few years, so-called glucose nanosensors became available for intracellular glucose imaging in mammalian cells (Fehr *et al.* 2003b; Fehr *et al.* 2004c; Fehr *et al.* 2005a; Lalonde *et al.* 2005). These nanosensors are based on the intramolecular fluorescence resonance energy transfer (FRET) response of an enhanced cyan fluorescent protein (ECFP)/citrine pair fused to a glucose-specific bacterial periplasmic binding protein (Fehr *et al.* 2005a; Lalonde *et al.* 2005). A conformational change induced by glucose binding results in a concentration-dependent shift of the emission intensity ratios (Fehr *et al.* 2003b; Fehr *et al.* 2004c).

Another advance in this development is FLII^{12}Pglu-700µ-δ6, a glucose nanosensor with a broad detection range of millimolar glucose concentrations (Takanaga *et al.* 2008a; Hou *et al.* 2009; Takanaga & Frommer 2010). The aim of the present study was to investigate changes in the intracellular glucose concentration in beta cells in real-time using this new glucose nanosensor in combination with simultaneous $[Ca^{2+}]_i$ measurements.

EXPERIMENTAL PROCEDURES

Cellular FLIPglu Expression

MIN6 and COS cells were grown in DMEM supplemented with 25 mM glucose, 10% (vol/vol) FCS, penicillin, and streptomycin in a humidified atmosphere at 37°C and 5% CO_2. The pcDNA 3.1 vector was used for expression of FLII^{12}Pglu-700µ-δ6 (FLIPglu) (Addgene plasmid 17866) (Takanaga *et al.* 2008a). COS cells were transiently transfected using jetPEI (Qbiogene, Montreal, Canada). Stable MIN6 clones (MIN6-FLIPglu) were selected through resistance against G418 (250 µg/ml). Pancreatic islets were isolated from 6 to 10-week-old female NMRI mice by collagenase digestion in bicarbonate-buffered Krebs-Ringer solution. Beta cells were obtained using calcium-free Krebs-Ringer solution and kept in RPMI-1640 medium supplemented with 5 mM glucose. Cells were transduced at a multiplicity of infection of 10 for 2 h with adenoviral FLIPglu stock solution. Recombinant FLIPglu adenovirus was generated using the Ad-Easy viral vector system provided by B. Vogelstein (Baltimore, MD) (He *et al.* 1998). The FLIPglu coding cDNA was subcloned as a SalI-NotI fragment into the pShuttle-CMV vector, and the recombinant adenoviral plasmid was generated by homologous recombination with the pAdEasy-1 plasmid in *E. coli* BJ5183 cells. Adenoviruses were produced in 293 cells and purified by CsCl gradient centrifugation.

Recombinant FLIPglu Generation

The full coding sequence of FLIPglu (Takanaga *et al.* 2008a) was subcloned in-frame into the BamHI and NotI sites of the pGEX-6P-1

expression vector and expressed in the dark in *E. coli* BL21 using the glutathione S-transferase (GST) Gene Fusion System (Amersham Pharmacia Biotech, Freiburg, Germany). The GST-tag was cleaved by final incubation with PreScission protease. A total of 5µg protein was incubated in the dark for 5 min with 0, 0.1, 0.2, 0.5, 1, 2, 4, 5, 6, 8, 10, 25, 50, or 100 mM glucose in 100 µl Krebs-Ringer solution. Finally, ECFP and citrine fluorescence emission at 460 nm and 544 nm, respectively, were recorded in a microplate in a Labsystems Fluoroskan Ascent Counter (Thermo Fisher Scientific, Waltham, MA), and ECFP was excited at 430 nm.

Western Blot

MIN6-FLIPglu and COS cells were homogenized in PBS (pH 7.4), and insoluble material was pelleted by centrifugation. Protein concentration was quantified by Bradford protein assay. A total of 40 µg protein was fractionated by reducing 10% SDS-PAGE and electroblotted onto polyvinylidine difluoride membranes. Non-specific binding sites were blocked with Odyssey Blocking Buffer (Licor Biosciences, Lincoln, NE, USA) for 1 h at room temperature. Blots were incubated with GLUT1 (sc-7903, diluted 1:500, Santa Cruz Biotechnology, Santa Cruz, CA), GLUT2 (ab-54460, diluted 1:500, Abcam, Cambridge, U.K.), GK (sc-7908, diluted 1:200, Santa Cruz Biotechnology), or HK (sc-28885, diluted 1:200, Santa Cruz Biotechnology) antibody overnight at 4°C and then with the appropriate IRDye 800CW (Licor Biosciences) secondary antibody for 30 min at room temperature. Immunoreactivity was visualized using the Licor Infrared Imaging System (Licor Biosciences).

Fluorescence Microscopy

A cellR/Olympus IX81 (Olympus, Hamburg, Germany) inverted microscope system equipped with a Cellcubator (Olympus) to maintain 60% humidity, 37°C, and 5% CO_2 was used. ECFP was excited with a D436/10 filter (AHF Analysentechnik, Tübingen, Germany). ECFP and citrine emission were detected simultaneously using a DV-CC Dual View System (Optical Insights LLC, Tucson, AZ) equipped with a 505 dcxr beam splitter and D465/30 and HQ535/30 emission filters or with 455DCLP-D480/40 and 530DCLP-D560/40 filter sets (AHF Analysentechnik). Glass coverslips were mounted in a chamber. Perifusion was performed with Krebs-Ringer solution at a flow rate of 1 ml/min using a peristaltic pump (Ismatec, Zürich, Switzerland). Images were taken every 2 seconds with an UPLSAPO 60 x 1.35 numerical aperture oil-immersion objective (Olympus). For combined analysis of Ca^{2+} and FLIPglu, MIN6-FLIPglu cells were loaded with fura-2 acetoxymethyl ester by incubation in Krebs-Ringer solution containing 25 mM HEPES for 30 min at 37°C. HC340/26-HC387/11-BS409-HC510/84 filter sets were used for fura-2 acetoxymethyl ester, and images were obtained simultaneously every 5 seconds. Automated solution switching by the perifusion system entailed a lag time of 70 sec until the compound concentration was reached.

Recordings shown in the figures are corrected for this delay. For multiple image analysis of MIN6-FLIPglu cells on 6-well plates, an UPLSAPO 20 x 0.75 numerical aperture objective and scanR software (Olympus) were used. The image focus was automatically determined from transmitted light images using a gradient method. After background correction, individual cells were automatically detected using an edge detection method and tracked over time.

Measurement of Glucose and Pyruvate

Batches of 2 x 10^6 cells were incubated for 1, 2, 3, 5, 7, 9, or 10 min at 37°C and 300 rpm in 1 ml Krebs-Ringer solution in the presence of 10 mM glucose. Batches incubated for 10 min were centrifuged, washed, and incubated for 1, 2, 3, 5, 7, or 10 min in Krebs-Ringer solution without glucose. At the end point, all samples were centrifuged and the supernatant removed. After addition of 1 ml Krebs-Ringer solution, the samples were heated for 5 min to 95°C and finally centrifuged. Glucose was determined in the supernatant using the glucose-oxidase/peroxidase/o-dianisidin method and pyruvate by measuring the oxidation of reduced nicotinamide adenine dinucleotide in the presence of lactate dehydrogenase.

Insulin Secretion

For static incubation, MIN6-FLIPglu cells were seeded in 6-well microplates at a density of 3 x 10^5 cells and grown for 3 days. Thereafter, cells were incubated for 1 h in bicarbonate-buffered Krebs-Ringer solution without glucose supplemented with 0.1% albumin and subsequently incubated for 1 h without glucose, with 10 mM glucose or 40 mM KCl. Thereafter, 1 ml of the incubation buffer from each well was collected and gently centrifuged to remove detached cells. The secreted insulin was measured in the final supernatants radioimmunologically.

Statistical Analysis

Statistical analyses were performed using the Prism analysis program (Graphpad, San Diego, CA, USA).

RESULTS

Measurement of Intracellular Glucose using FLIPglu

Glucose concentration was evaluated by two-channel fluorescence analysis of the FLII^{12}Pglu-700µ-δ6 (FLIPglu) nanosensor. Fluorescence of citrine emission upon ECFP excitation and ECFP emission upon ECFP excitation was determined and the $citrine_{em}/ECFP_{em}$ upon ECFP excitation ratio calculated. For direct comparison of individual experiments, the ratio calculated at 0 mM glucose was set to 1 and values were presented as normalized ratios.

The *in vitro* titration of recombinant purified FLIPglu exhibited a linear range for the change in $citrine_{em}/ECFP_{em}$ ratio between 1 and 10 mM glucose (Fig. 1A). The binding constant for glucose was determined to be 0.572 ± 0.099 mM with a maximal normalized $citrine_{em}/ECFP_{em}$

ratio change of 0.42. MIN6 cells stably expressing FLIPglu were analyzed using a medium throughput microscopy approach with automated cell detection and calculation of the $citrine_{em}/ECFP_{em}$ ratio. MIN6-FLIPglu cells showed a significantly higher $citrine_{em}/ECFP_{em}$ ratio when incubated at 10 mM (1.402 ± 0.035) than at 1 mM (1.084 ± 0.014) glucose (Fig. 1B-G). The ratio change was stable over time (Fig. 1B-D) and independent from the individual cell morphology (Fig. 1E-F).

Perifusion experiments revealed, in greater detail, the correlation between the change in $citrine_{em}/ECFP_{em}$ ratio and the external glucose concentration in MIN6-FLIPglu cells (Fig. 2A). Glucose increased uniformly in the cytoplasm of MIN6 cells (Fig. 2B). Glucose influx was evaluated by quantifying the first 30 sec of the increase in the $citrine_{em}/ECFP_{em}$ ratio when external glucose was increased. Finally, the rate of cytosolic glucose accumulation was compared to that of extracellular glucose (Fig. 2C). The K_m for glucose was determined to be 9.6 mM in MIN6-FLIPglu cells.

Effects of 3-OMG and MH on the Intracellular Glucose Concentration

MIN6-FLIPglu cells were perifused with or without 10 mM glucose and the intracellular glucose concentration measured every 2 seconds (Fig. 3A). The addition of glucose to the medium evoked a rapid initial increase of glucose in MIN6 cells. Thereafter, the increase in intracellular glucose decelerated and stabilized. Removal of glucose from the medium resulted in an immediate intracellular decrease in MIN6 cells (Fig. 3A). The glucose and pyruvate content in MIN6 cells was calculated by end-point measurements (Fig. 3B). Glucose increased over 3 minutes directly after external supply, and pyruvate followed with a delay of 2 minutes. After removal from the medium, glucose decreased rapidly within one minute, whereas pyruvate declined slowly over 3 minutes in MIN6 cells (Fig. 3B).

Primary mouse beta cells exhibited an intracellular glucose concentration trace after glucose supply and removal comparable to that of MIN6 cells (Fig. 3C). Addition of 3-OMG (10 mM) or MH (10 mM) in the absence of glucose did not change the $citrine_{em}/ECFP_{em}$ ratio, confirming that the compounds did not interact with the $FLII^{12}Pglu\text{-}700\mu\text{-}\delta6$ nanosensor. Addition of 3-OMG (10 mM) in the presence of glucose (10 mM) resulted in a deceleration of the initial increase in the intracellular glucose concentration, whereas the steady state glucose concentration remained unchanged. In contrast, MH (10 mM) reduced the steady state glucose concentration. About that a slower decrease in the intracellular glucose concentration was detected after the removal of glucose from the medium (Fig. 3C)

Beta cells were compared to COS cells with respect to glucose uptake and metabolism. Glucose influx, efflux, and metabolism, as well as the intracellular glucose concentration, were calculated from the $citrine_{em}/ECFP_{em}$ ratio traces

(Fig. 3A and C) as follows. Glucose influx was evaluated by quantifying the first 30 sec of the increase in $citrine_{em}/ECFP_{em}$ ratio when external glucose was increased from 0 to 10 mM (Fig. 4A). Mouse beta cells exhibited a 60% faster glucose influx, whereas COS cells exhibited a 78% slower glucose influx compared to MIN6 cells. Glucose influx was diminished in mouse beta cells, MIN6 cells, and COS cells by 40%, 89%, and 85%, respectively, in response to 3-OMG (10 mM) (Fig. 4A). Supplementation with MH (10 mM) did not affect glucose influx. Glucose efflux and metabolism were evaluated by quantifying the first 60 sec of the decrease in the $citrine_{em}/ECFP_{em}$ ratio when external glucose was reduced from 10 to 0 mM (Fig. 4B). Glucose efflux and metabolism were comparable in mouse beta cells and MIN6 cells. COS cells exhibited an 82% slower glucose efflux and metabolism compared to insulin-secreting cells. The glucose efflux/metabolism rate was significantly reduced in mouse beta cells and MIN6 cells in the presence of MH (10 mM), by 49% and 45%, respectively. This reduction was not observed in COS cells. Addition of 3-OMG (10 mM) evoked a decrease (45%) in the glucose efflux rate in COS cells. This decrease was not observed in insulin-secreting cells. The intracellular glucose concentration at 10 mM glucose was evaluated by calculating the difference in the $citrine_{em}/ECFP_{em}$ ratio before and after equilibration with 10 mM glucose (Δ $citrine_{em}/ECFP_{em}$) (Fig. 4C). Overall, mouse beta cells and MIN6 cells had significantly higher intracellular glucose concentrations than COS cells (Fig. 4C). Supplementation with MH (10 mM) resulted in a significant decrease in the intracellular glucose concentration in mouse beta cells and MIN6 cells by 35% and 36%, respectively, but not in COS cells. In contrast, 3-OMG reduced the intracellular glucose concentration in COS cells by 55%, but only by 7% and 14% in mouse beta cells and MIN6 cells, respectively.

Glucokinase, HK1, GLUT1, and GLUT2 are key regulators of glucose uptake and metabolism. In MIN6 cells, both GLUT1 and GLUT2 were expressed at the protein level, whereas only GLUT1 was produced in COS cells (Fig. 3). Glucokinase was detected only in MIN6 cells (Fig. 3). In contrast, HK1 was produced in both cell types, but at a much higher level in COS cells (Fig. 3).

To evaluate the observed differences in intracellular glucose concentration, the effect of 3-OMG (10 mM) and MH (10 mM) on insulin secretion was determined in MIN6-FLIPglu cells. Glucose-induced insulin secretion was significantly lowered by MH (10 mM), but not by 3-OMG (Fig. 6). KCl-induced insulin secretion in the presence of 3-OMG or MH was comparable to that of control (Fig. 6).

Concomitant Analysis of Glucose Flux and Calcium Dynamics

The excitation and emission spectrum of the Ca^{2+} sensor fura-2 acetoxymethyl ester did not interfere with measurement of the

$citrine_{em}/ECFP_{em}$ ratio of the FLIPglu glucose nanosensor. Thus, loading of MIN6-FLIPglu cells with the fura-2 acetoxymethyl ester allowed simultaneous analysis of intracellular glucose and $[Ca^{2+}]_i$ in real-time.

In a cluster of 10 MIN6-FLIPglu cells, intracellular glucose simultaneously increased after glucose supply (Fig. 7A, upper panel). An increase in $[Ca^{2+}]_i$ occurred within 1 min (Fig. 7A, I-III), when glucose reached a threshold of ~4 mM (Fig. 7B). However, synchronized high amplitude oscillations were not observed until the intracellular glucose concentration reached ~7 mM (Fig. 7B). After the removal of glucose from the medium, the intracellular glucose concentration decreased simultaneously in the cell cluster (Fig. 7A, upper panel). At glucose concentrations below ~7 mM (Fig. 7B), $[Ca^{2+}]_i$ declined. Subsequent perifusion with 40 mM KCl did not provoke changes in the $citrine_{em}/ECFP_{em}$ ratio of FLIPglu. $[Ca^{2+}]_i$ increased instantly when KCl was added to the medium (Fig. 7A). However, the amplitude of the oscillations was less than that of the glucose stimulus.

Addition of the K_{ATP} channel closing agent tolbutamide (500 µM) alone did not provoke noticeable changes in the intracellular glucose concentration (Fig. 8, upper panel). The mean increase in $[Ca^{2+}]_i$ in the presence of tolbutamide was as high as with 10 mM glucose (Fig. 7 and 8). However, tolbutamide induced only small amplitude $[Ca^{2+}]_i$ oscillations (Fig. 8, lower panel). Addition of glucose (10 mM) to the medium resulted in an efficient increase in intracellular glucose and about that in $[Ca^{2+}]_i$ oscillations with high amplitude (Fig. 8).

DISCUSSION

The ability of beta cells to translate changes in blood glucose concentration to insulin secretion is crucial for glucose homeostasis (Lenzen & Panten 1988b; Lenzen 1992; Thorens 1992; Matschinsky 1996; Schuit 1997; Rorsman & Renstrom 2003; Baltrusch & Tiedge 2006b; Henquin 2009; Thorens & Mueckler 2010). To elucidate defects in glucose uptake, metabolism, and signal recognition under diabetic conditions, real-time glucose measurements in beta cells are of crucial importance. Measuring the extracellular glucose concentration is easy, whereas determining the intracellular glucose concentration requires sophisticated techniques (Yamada *et al.* 2000; Fehr *et al.* 2005a; Lalonde *et al.* 2005; Yamada *et al.* 2007). A new fluorescence-based method to determine the intracellular glucose concentration became available through the development of FRET-based glucose sensor molecules, so-called glucose nanosensors (Fehr *et al.* 2005a; Lalonde *et al.* 2005). The initially engineered glucose nanosensors detected the sugar in only a narrow concentration range and, therefore, were not suited for beta-cell-specific approaches (Fehr *et al.* 2003b; Fehr *et al.* 2004c). In contrast, $FLII^{12}Pglu\text{-}700\mu\text{-}\delta6$ is a glucose nanosensor with a broad detection range, to millimolar glucose concentrations (Takanaga *et al.* 2008a; Hou *et al.* 2009; Takanaga & Frommer 2010). In the

present study, we applied this glucose nanosensor for the first time in primary mouse beta cells by means of adenoviral transduction.

To avoid experimental variability in the $citrine_{em}/ECFP_{em}$ ratio due to transfection efficiency, we generated a stable $FLII^{12}Pglu$-700µ-δ6-producing MIN6 cell clone. Because insulin secretion of MIN6-$FLII^{12}Pglu$-700µ-δ6 cells and MIN6 control cells upon glucose stimulation was comparable, interference of glucose metabolism by the nanosensor can be excluded. The $citrine_{em}/ECFP_{em}$ ratio observed by incubation without glucose was set to 1 in each experiment, allowing comparability between experimental groups. A similar $FLII^{12}Pglu$-700µ-δ6 $citrine_{em}/ECFP_{em}$ ratio change in response to 10 mM glucose was determined in MIN6 cells by two different approaches: static incubation in medium throughput experiments and single cell perifusion experiments. Consistent with findings in HepG2 cells by Takanaga and co-workers (Takanaga *et al.* 2008a), we observed a good correlation between the in vitro glucose-binding affinity of $FLII^{12}Pglu$-700µ-δ6 and the in vivo normalized ratio change in MIN6 cells. Thus, estimation of the intracellular glucose concentration from the quantified $citrine_{em}/ECFP_{em}$ ratio (Fehr *et al.* 2003b; Fehr *et al.* 2004c; Takanaga *et al.* 2008a; Hou *et al.* 2009; Takanaga & Frommer 2010) seems to be justified in MIN6 cells.

Recent studies have suggested that a reliable estimation of the intracellular glucose accumulation rate can be achieved by calculating from the initial increase in the time-dependent ratio plots (Yamada *et al.* 2000; Takanaga *et al.* 2008a). In our approach, the rate of glucose accumulation in the cytosol of MIN6 cells had a K_m of ~10 mM. This observation can best be explained by the mixed expression of GLUT2 and GLUT1 in MIN6 cells (Thorens 1992; Schuit 1997; Thorens & Mueckler 2010). The K_m for glucose of GLUT2 has been reported to be ~17 mM, whereas GLUT1 has a very high affinity for glucose (Thorens & Mueckler 2010). In the HepG2 hepatoma cell line, a K_m of ~1.5 for glucose was determined using the $FLII^{12}Pglu$-700µ-δ6 nanosensor. In agreement GLUT1 has been shown to be highly expressed in HepG2 cells, whereas GLUT2, which is mainly present in primary hepatocytes, was down-regulated (Takanaga *et al.* 2008a). However, the intracellular glucose concentration is not simply determined by glucose uptake via facilitative transport, but reflects at least the sum of influx, efflux, synthesis, and metabolism.

Our results suggest that the observed plateau of the $citrine_{em}/ECFP_{em}$ ratio after glucose supply displays the steady state between glucose influx and metabolism in MIN6 cells. Under these conditions, glucose efflux and synthesis can be neglected in beta cells. After the initial increase in response to the glucose supply, intracellular glucose values determined by conventional end-point measurements showed high variability, whereas the high time resolution of the $FLII^{12}Pglu$-700µ-δ6 nanosensor kinetics

revealed a further increase in intracellular glucose with decreasing slope, which finally reached a stable plateau. During the slow increase in glucose concentration, the pyruvate concentration increased, indicating amplified glucose metabolism. After glucose removal, the intracellular concentration rapidly declined. This process is mediated by glucose phosphorylation and subsequent metabolism, as indicated by pyruvate production. Because GLUT proteins catalyze the facilitative transfer in a bidirectional manner (Thorens & Mueckler 2010), glucose efflux likewise contributes to the decrease in the intracellular glucose concentration.

At low concentrations, the non-metabolizable glucose analogue 3-OMG is thought to delay the equilibration of glucose across the plasma membrane in beta cells without significantly affecting glucose-induced insulin secretion (Sener *et al.* 1999b). In agreement, we observed a retardation of glucose influx during perifusion of MIN6 cells with 10 mM glucose in the presence of 10 mM 3-OMG, and only a minor reduction in the intracellular glucose concentration and glucose-induced insulin secretion. Additional support for the explanation that the first initial increase in the $citrine_{em}/ECFP_{em}$ ratio reflects glucose influx was obtained by analyzing primary mouse beta cells. After glucose supply, this fast initial increase in the $citrine_{em}/ECFP_{em}$ ratio was detectable, but absent in the presence of 3-OMG. The slow glucose influx rate and higher responsiveness of MIN6 cells to 3-OMG compared to primary mouse beta cells can be explained by differences in the density of GLUT1 and GLUT2 in the plasma membrane (Heimberg *et al.* 1995; Schuit 1997; Thorens & Mueckler 2010).

In non-insulin-producing COS cells, the level of glucose influx, efflux, and metabolism was significantly lower compared to primary mouse beta cells and MIN6 cells. This finding is consistent with the low level of GLUT1 expression and absence of GLUT2. Because 3-OMG strongly reduced intracellular glucose in COS cells, our results emphasize the rate-limiting character of glucose uptake via GLUT1 (Thorens & Mueckler 2010). In contrast, the rate of glucose metabolism in GLUT2-expressing cells is controlled at the glucose phosphorylation step (Thorens & Mueckler 2010). Glucokinase, the glucose-phosphorylating enzyme in beta cells, acts as a glucose sensor (Lenzen & Panten 1988b; Lenzen 1992; Matschinsky 1996; Baltrusch & Tiedge 2006b). Accordingly, in our studies in primary mouse beta cells and MIN6 cells, the competitive glucokinase inhibitor MH (Tiedge *et al.* 1997; Rasschaert *et al.* 2001) significantly diminished the intracellular glucose concentration and glucose-induced insulin secretion.

Oscillations in glucose levels have been previously reported using a glucose enzyme electrode implanted into single isolated primary islets (Jung *et al.* 2000b; Kennedy *et al.* 2002). Therefore, glucose oscillations were proposed to be an early intrinsic trigger for the oscillatory

nature of the stimulus-secretion pathway in beta cells (Jung *et al.* 2000b; Kennedy *et al.* 2002). Oscillations in the intracellular glucose concentration, however, have not been determined in mouse beta cells using FLII^{12}Pglu-700µ-δ6 nanosensor-based analyses. Because random fluctuations within a narrow range were observed rather than synchronized glucose oscillations, we propose downstream metabolites, such as ATP, as pacemakers of oscillations. Recent studies support a direct interplay between ATP and $[Ca^{2+}]_i$ beyond the K_{ATP} channel pathway (Detimary *et al.* 1998; Kennedy *et al.* 1999a; Heart *et al.* 2006a).

Concomitant real-time analyses of intracellular glucose and $[Ca^{2+}]_i$ have not been explored in beta cells previously. In agreement with studies in primary beta cells (Lenzen & Peckmann 2001a; Dyachok *et al.* 2006; Baltrusch & Lenzen 2007b; Dyachok *et al.* 2008b; Henquin 2009), $[Ca^{2+}]_i$ showed an initial increase with subsequent pronounced oscillations in response to glucose in MIN6 cells. We observed a threshold of ~4 mM glucose for the $[Ca^{2+}]_i$ increase. This glucose concentration is close to the inflection point of the sigmoidal activity curve of glucokinase, facilitating a fast switch to a higher rate of glucose metabolism. Interestingly, the calculated threshold for the $[Ca^{2+}]_i$ decrease after glucose removal was ~7 mM glucose. Therefore, a higher intracellular glucose concentration than in the initial phase can be hypothesized to be necessary for retaining $[Ca^{2+}]_i$ oscillations and maintaining the second phase of insulin secretion. Taking into account that glucose, but not tolbutamide, is able to mediate large amplitude $[Ca^{2+}]_i$ oscillations, our study provides further evidence of glucose-induced amplifying signals for $[Ca^{2+}]_i$ that bypass K_{ATP} channels (Henquin 2000; Lenzen & Peckmann 2001a; Henquin 2009).

In conclusion, we have demonstrated that FLII^{12}Pglu-700µ-δ6 nanosensor-based intracellular glucose analysis is well suited for beta-cell-specific approaches. Concomitant real-time analysis with $[Ca^{2+}]_i$, and in future studies other key regulators, such as ATP, will provide further insight into the regulation of glucose-induced insulin secretion in pancreatic beta cells and may help elucidate defects in diabetic conditions.

REFERENCES

1. Schuit, F. C. (1997) *Diabetologia* **40**, 104-111
2. Thorens, B. (1992) *Int Rev Cytol* **137**, 209-238
3. Thorens, B., and Mueckler, M. (2010) *Am J Physiol Endocrinol Metab* **298**, E141-145
4. Baltrusch, S., and Tiedge, M. (2006) *Diabetes* **55 Suppl 2**, S55-64
5. Lenzen, S. (1992) Glucokinase: signal recognition enzyme for glucose-induced insulin secretion. in *Nutrient regulation of insulin secretion* (Flatt, P. R. e. ed.), Portland Press,London and Chapel Hill. pp 101-125
6. Lenzen, S., and Panten, U. (1988) *Biochem Pharmacol* **37**, 371-378
7. Matschinsky, F. M. (1996) *Diabetes* **45**, 223-241
8. Henquin, J. C. (2009) *Diabetologia* **52**, 739-751
9. Baltrusch, S., and Lenzen, S. (2007) *Biochem Biophys Res Commun* **363**, 1038-1043
10. Lenzen, S., Lerch, M., Peckmann, T., and Tiedge, M. (2000) *Biochim Biophys Acta* **1523**, 65-72
11. Rorsman, P., and Renstrom, E. (2003) *Diabetologia* **46**, 1029-1045
12. Henquin, J. C. (2000) *Diabetes* **49**, 1751-1760
13. Miyawaki, A., Llopis, J., Heim, R., McCaffery, J. M., Adams, J. A., Ikura, M., and Tsien, R. Y. (1997) *Nature* **388**, 882-887
14. Adams, S. R., Harootunian, A. T., Buechler, Y. J., Taylor, S. S., and Tsien, R. Y. (1991) *Nature* **349**, 694-697
15. Dyachok, O., Isakov, Y., Sagetorp, J., and Tengholm, A. (2006) *Nature* **439**, 349-352
16. Berg, J., Hung, Y. P., and Yellen, G. (2009) *Nat Methods* **6**, 161-166
17. Heart, E., Corkey, R. F., Wikstrom, J. D., Shirihai, O. S., and Corkey, B. E. (2006) *Am J Physiol Endocrinol Metab* **290**, E143-E148
18. Kennedy, H. J., Pouli, A. E., Ainscow, E. K., Jouaville, L. S., Rizzuto, R., and Rutter, G. A. (1999) *J Biol Chem* **274**, 13281-13291
19. Fehr, M., Okumoto, S., Deuschle, K., Lager, I., Looger, L. L., Persson, J., Kozhukh, L., Lalonde, S., and Frommer, W. B. (2005) *Biochem Soc Trans* **33**, 287-290
20. Lalonde, S., Ehrhardt, D. W., and Frommer, W. B. (2005) *Curr Opin Plant Biol* **8**, 574-581
21. Tiedge, M., and Baltrusch, S. (2011) *Dtsch Med Wochenschr* **136**, 1130-1134
22. Baltrusch, S., Langer, S., Massa, L., Tiedge, M., and Lenzen, S. (2006) *Endocrinology* **147**, 5768-5776
23. Heimberg, H., De Vos, A., Pipeleers, D., Thorens, B., and Schuit, F. (1995) *J Biol Chem* **270**, 8971-8975
24. Rasschaert, J., Kadiata, M. M., and Malaisse, W. J. (2001) *Mol Cell Biochem* **226**, 77-81

25. Sener, A., Malaisse-Lagae, F., and Malaisse, W. J. (1993) *Biochimica et biophysica acta* **1177**, 54-60
26. Sener, A., Scruel, O., Louchami, K., Jijakli, H., and Malaisse, W. J. (1999) *Mol Cell Biochem* **194**, 133-145
27. Yamada, K., Nakata, M., Horimoto, N., Saito, M., Matsuoka, H., and Inagaki, N. (2000) *J Biol Chem* **275**, 22278-22283
28. Yamada, K., Saito, M., Matsuoka, H., and Inagaki, N. (2007) *Nat Protoc* **2**, 753-762
29. Fehr, M., Lalonde, S., Ehrhardt, D. W., and Frommer, W. B. (2004) *J Fluoresc* **14**, 603-609
30. Fehr, M., Lalonde, S., Lager, I., Wolff, M. W., and Frommer, W. B. (2003) *J Biol Chem* **278**, 19127-19133
31. Hou, B. H., Takanaga, H., Griesbeck, O., and Frommer, W. B. (2009) *Cell Calcium* **46**, 130-135
32. Takanaga, H., Chaudhuri, B., and Frommer, W. B. (2008) *Biochimica et biophysica acta* **1778**, 1091-1099
33. Takanaga, H., and Frommer, W. B. (2010) *FASEB J* **24**, 2849-2858
34. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998) *Proc Natl Acad Sci U S A* **95**, 2509-2514
35. Tiedge, M., Krug, U., and Lenzen, S. (1997) *Biochimica et biophysica acta* **1337**, 175-190
36. Jung, S. K., Kauri, L. M., Qian, W. J., and Kennedy, R. T. (2000) *J Biol Chem* **275**, 6642-6650
37. Kennedy, R. T., Kauri, L. M., Dahlgren, G. M., and Jung, S. K. (2002) *Diabetes* **51 Suppl 1**, S152-161
38. Detimary, P., Gilon, P., and Henquin, J. C. (1998) *Biochem J* **333**, 269-274
39. Dyachok, O., Idevall-Hagren, O., Sagetorp, J., Tian, G., Wuttke, A., Arrieumerlou, C., Akusjarvi, G., Gylfe, E., and Tengholm, A. (2008) *Cell Metab* **8**, 26-37
40. Lenzen, S., and Peckmann, T. (2001) *Biochem Pharmacol* **62**, 923-928

FOOTNOTES

We thank Dr. W. Frommer and Dr. H. Takanaga (Stanford, CA, USA) for providing the FLII^{12}Pglu-700µ-δ6 construct (Addgene plasmid 17866), and Dr. B. Vogelstein (Baltimore, MD) for providing the vectors of the adenoviral expression system. We thank M. Funk, J. Kresse, B. Leß, and R. Waterstradt for skillful technical assistance. This work has been supported by the European Union (Integrated Project EuroDia LSHM-CT-2006-518153 in the Framework Programme 6 [FP6] of the European Community).

FIGURE LEGENDS

Figure 1. Glucose analysis using the FLIPglu nanosensor. (A) Recombinant FLIPglu was analyzed using a microplate fluorimeter in the presence of different glucose concentrations. Mean normalized $citrine_{em}/ECFP_{em}$ ratios ± SEM of three individual experiments are shown. (B - G) MIN6-FLIPglu cells were analyzed by medium throughput microscopy. Fluorescence images were taken automatically either hourly over 4 h (B-D) or after 1 h (E-G). Images show the $citrine_{em}/ECFP_{em}$ ratio as a green/red merged image in a representative cell over time (C, D) or cell cluster (F, G) incubated in 1 mM (B, black circles; C, E, striped bar; F) or 10 mM (B, black squares; D, E, black bar; G) glucose. Shown are mean values ± SEM from two individual experiments with a total of 201 – 235 traced cells (B) or 3753 - 5534 cells (E) analyzed; *** $p < 0.001$ (Student's t test).

Figure 2. Intracellular glucose concentration in MIN6 cells. MIN6-FLIPglu cells were perifused in turn without or with increasing glucose concentrations as indicated by the bars. Fluorescence images were acquired every 2 seconds. (A) Normalized mean ratio traces of two individual experiments analyzing a total of 8 cells are shown. (B) Pseudocolored ratio images illustrate the ratio change at the three indicated time points (I-III) in two representative cells. (C) Fitted Michaelis-Menten kinetics of glucose influx as measured from the initial $citrine_{em}/ECFP_{em}$ ratio change when glucose was added in the indicated concentration.

Figure 3. Real-time intracellular glucose traces in mouse beta cells and MIN6 cells. (A) MIN6-FLIPglu cells were perifused with or without glucose (10 mM) as indicated. Fluorescence images were acquired every 2 seconds. A mean normalized $citrine_{em}/ECFP_{em}$ ratio trace ± SEM of 10 individual cells is shown. (B) Glucose (black circles) and pyruvate (grey square) content was determined in MIN6 cells by end-point measurements after incubation in Krebs-Ringer solution for 1, 2, 3, 5, 7, 9, or 10 min with 10 mM glucose or after 10 min with 10 mM glucose plus 1, 2, 3, 5, 7, or 10 min without glucose. Shown are mean values ± SEM for four individual experiments. (C) FLIPglu-expressing mouse beta cells were perifused with or without glucose (10 mM) (grey line) as indicated. In addition, glucose perifusion was performed in the presence of MH (10 mM), (red line) or 3-OMG (10 mM), (black line) Fluorescence images were acquired every 2 seconds. A mean normalized $citrine_{em}/ECFP_{em}$ ratio trace for 8 individual cells is shown. For clarity, SEM is only shown for control conditions.

Figure 4. Effects of 3-OMG and MH on changes in the intracellular glucose concentration in mouse beta cells, MIN6 cells, and COS cells. Real-time intracellular glucose traces as shown in Fig. 3 were used to calculate glucose influx, efflux, and metabolism in mouse beta cells, MIN6 cells, and COS cells. (A) The glucose influx rate was calculated from the initial change in $citrine_{em}/ECFP_{em}$ ratio when the glucose concentration in the perifusion medium was changed from 0 to 10 mM glucose. (B) Glucose efflux/ metabolism rate was calculated from the change in the $citrine_{em}/ECFP_{em}$ ratio when the

glucose concentration in the perfusion medium was changed from 10 to 0 mM glucose. (C) The intracellular glucose concentration was determined as Δ $citrine_{em}/ECFP_{em}$ ratio before and after equilibration with 10 mM glucose. Cells were analyzed with glucose alone (10 mM) (black bars) or with 3-OMG (10 mM) (striped bars) or MH (10 mM) (open bars). Mean values ± SEM of 10 - 26 individual cell traces are shown. *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to controls; $^{\#}$ $p < 0.05$; $^{\#\#\#}$ $p < 0.001$ compared to MIN6 cells (ANOVA/Bonferroni's multiple comparison test).

Figure 5. Expression of glucose transporters and glucose phosphorylating enzymes in MIN6 and COS cells. Protein was isolated from MIN6-FLIPglu cells and COS cells, and 40 µg was loaded per lane. Glucokinase, HK1, GLUT1, and GLUT2 expression were analyzed by immunoblotting. Representative blots from three independent experiments are shown.

Figure 6. Effects of MH and 3-OMG on insulin secretion in MIN6 cells. MIN6-FLIPglu cells were incubated after starvation without glucose (open bars), with glucose (10 mM) (black bars), or with KCl (40 mM) (striped bars) and MH (10 mM) or 3-OMG (10 mM). Insulin secretion is expressed per DNA content. Data are means ± SEM of four independent experiments. ** $p < 0.01$; *** $p < 0.001$ compared to control cells; ### $p < 0.001$ compared to cells without compound (ANOVA/Bonferroni's multiple comparison test).

Figure 7. Concomitant real-time analysis of the intracellular glucose concentration and calcium dynamics in MIN6 cells. MIN6-FLIPglu cells loaded with fura-2 acetoxymethyl ester were perifused with or without glucose (10 mM) or KCl (40 mM) as indicated by the bars. Fluorescence images were acquired simultaneously every 5 seconds for glucose dynamics and $[Ca^{2+}]_i$. (A) An intracellular glucose trace ± SEM averaged from 10 clustered cells attaching each other (upper panel) and corresponding $[Ca^{2+}]_i$ oscillations from three representative cells (I-III) within this cluster are shown. (B) Depicted is an overlay of the intracellular glucose (black) and $[Ca^{2+}]_i$ (red) trace ± SEM from clustered cells. Typical recordings from five independent experiments are shown.

Figure 8. Effects of tolbutamide on the intracellular glucose concentration and calcium dynamics in MIN6 cells. MIN6-FLIPglu cells loaded with fura-2 acetoxymethyl ester were perifused with tolbutamide (500 µM) in the presence or absence of glucose (10 mM) as indicated by the bars. Fluorescence images were acquired simultaneously every 5 seconds for glucose dynamics and $[Ca^{2+}]_i$. An intracellular glucose trace ± SEM averaged from 10 clustered cells attaching each other (upper panel) and corresponding $[Ca^{2+}]_i$ oscillations from a representative cell within this cluster (lower panel) is shown. Typical recordings from four independent experiments are shown.

Fig. 1

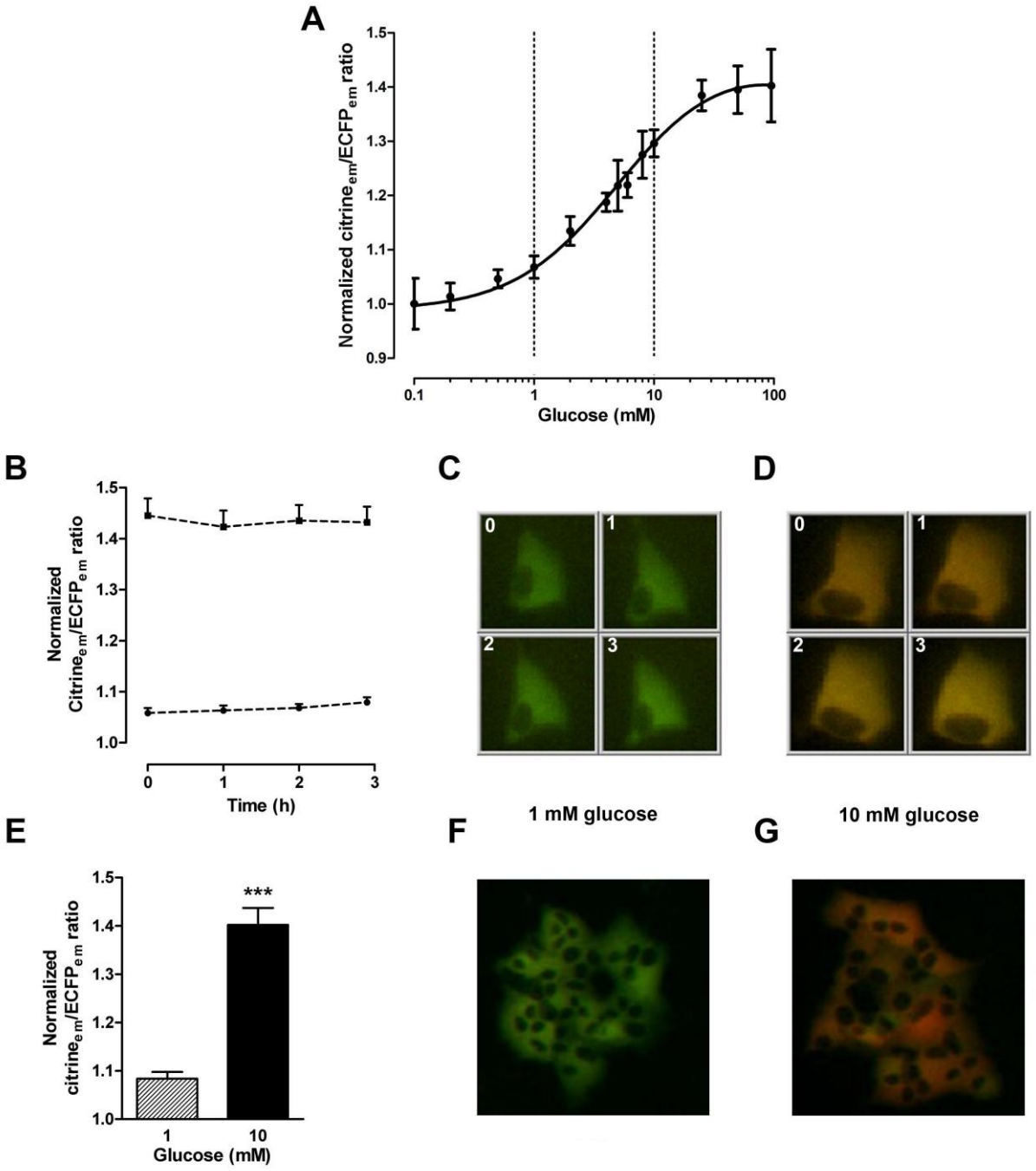

A
Normalized citrine$_{em}$/ECFP$_{em}$ ratio
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.1
1
10
100
Glucose (mM)
B
Normalized Citrine$_{em}$/ECFP$_{em}$ ratio
1.5
1.4
1.3
1.2
1.1
1.0
0
1
2
3
Time (h)
C
D
0
1
2
3
1 mM glucose
10 mM glucose
E
Normalized citrine$_{em}$/ECFP$_{em}$ ratio
1.5
1.4
1.3
1.2
1.1
1.0
***
1
10
Glucose (mM)
F
G

Fig. 2

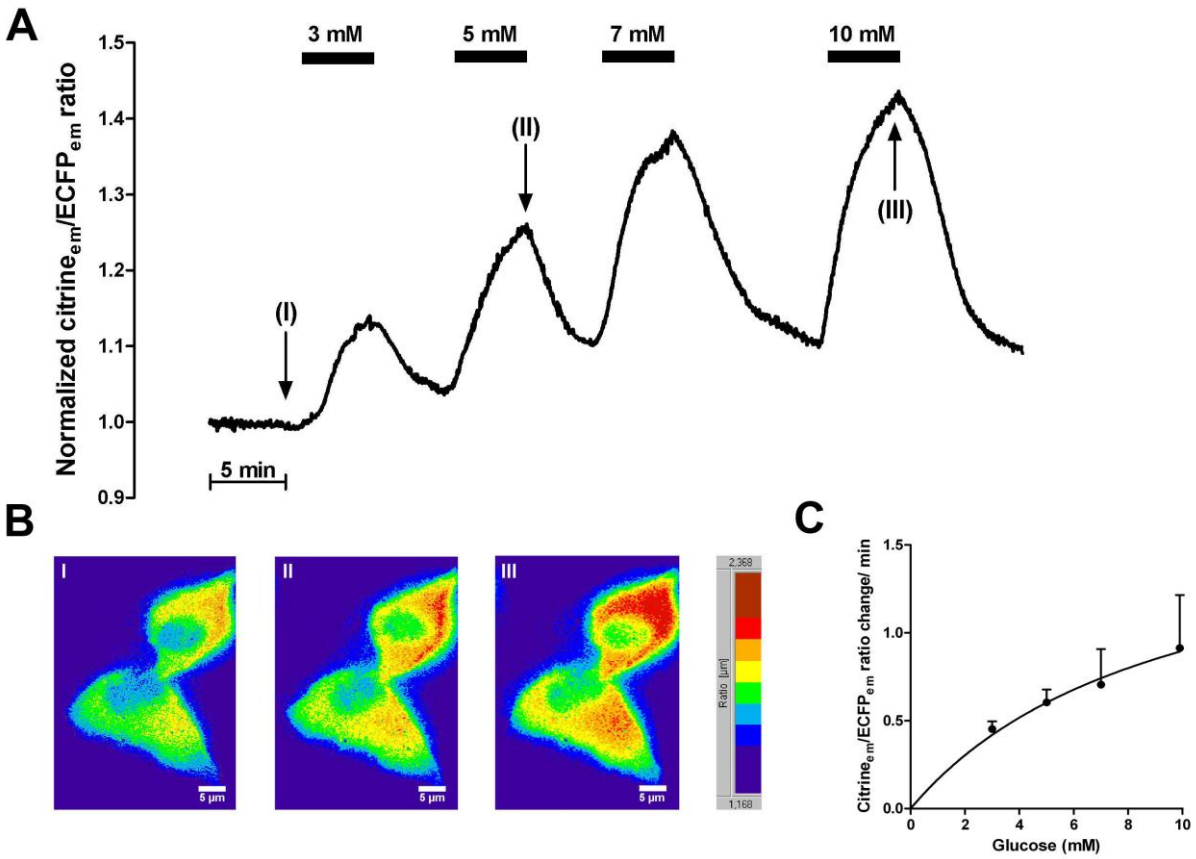

A
3 mM
5 mM
7 mM
10 mM
(I)
(II)
(III)
5 min
Normalized citrine$_{em}$/ECFP$_{em}$ ratio
1.5
1.4
1.3
1.2
1.1
1.0
0.9
B
I
II
III
5 µm
2.368
Ratio [µm]
1.168
C
Citrine$_{em}$/ECFP$_{em}$ ratio change/ min
1.5
1.0
0.5
0.0
0
2
4
6
8
10
Glucose (mM)

Fig. 3

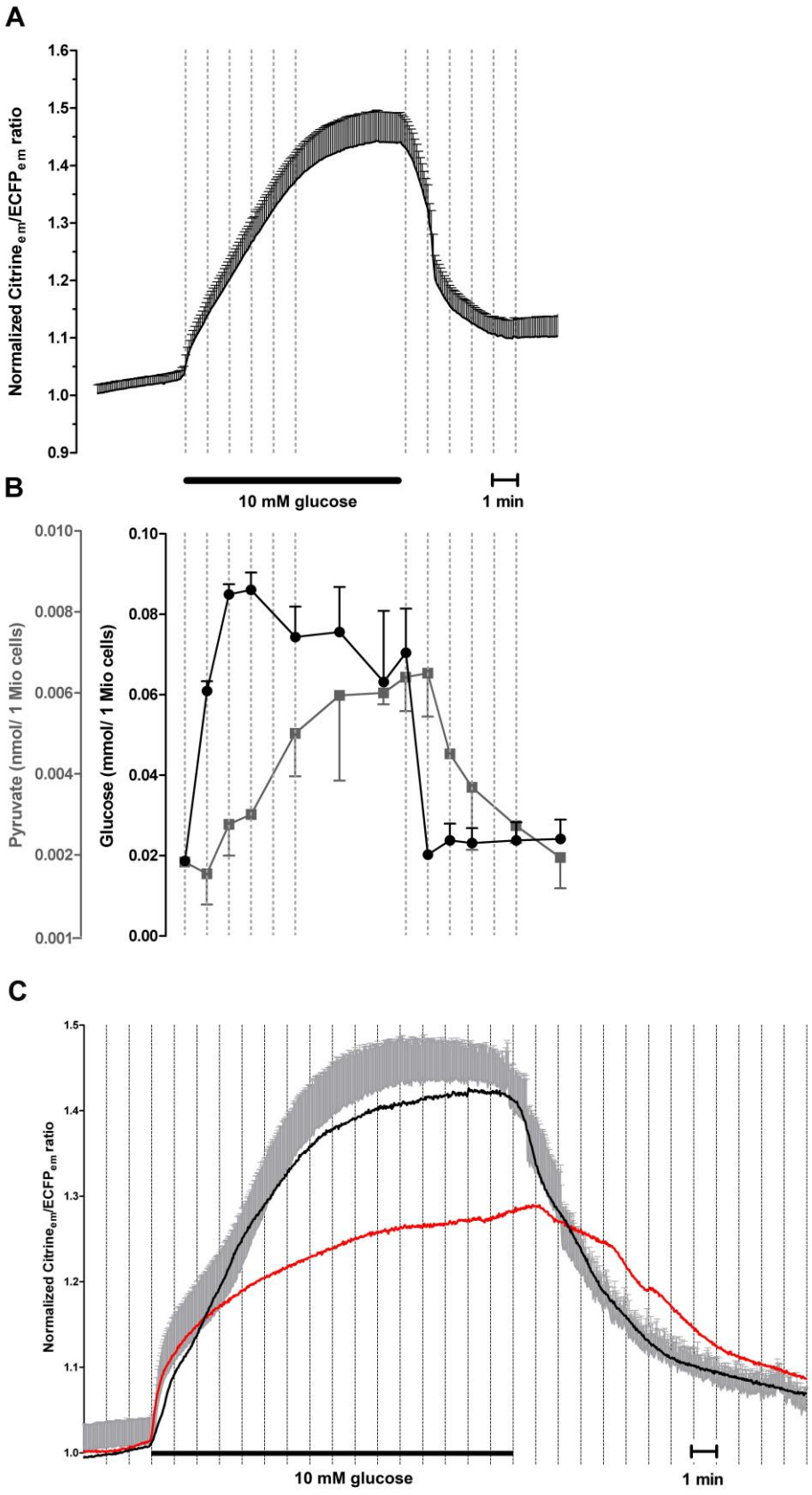

A
Normalized Citrine$_{em}$/ECFP$_{em}$ ratio
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
10 mM glucose
1 min
B
Pyruvate (nmol/ 1 Mio cells)
Glucose (mmol/ 1 Mio cells)
0.010
0.008
0.006
0.004
0.002
0.001
0.10
0.08
0.06
0.04
0.02
0.00
C
Normalized Citrine$_{em}$/ECFP$_{em}$ ratio
1.5
1.4
1.3
1.2
1.1
1.0
10 mM glucose
1 min

Fig. 4

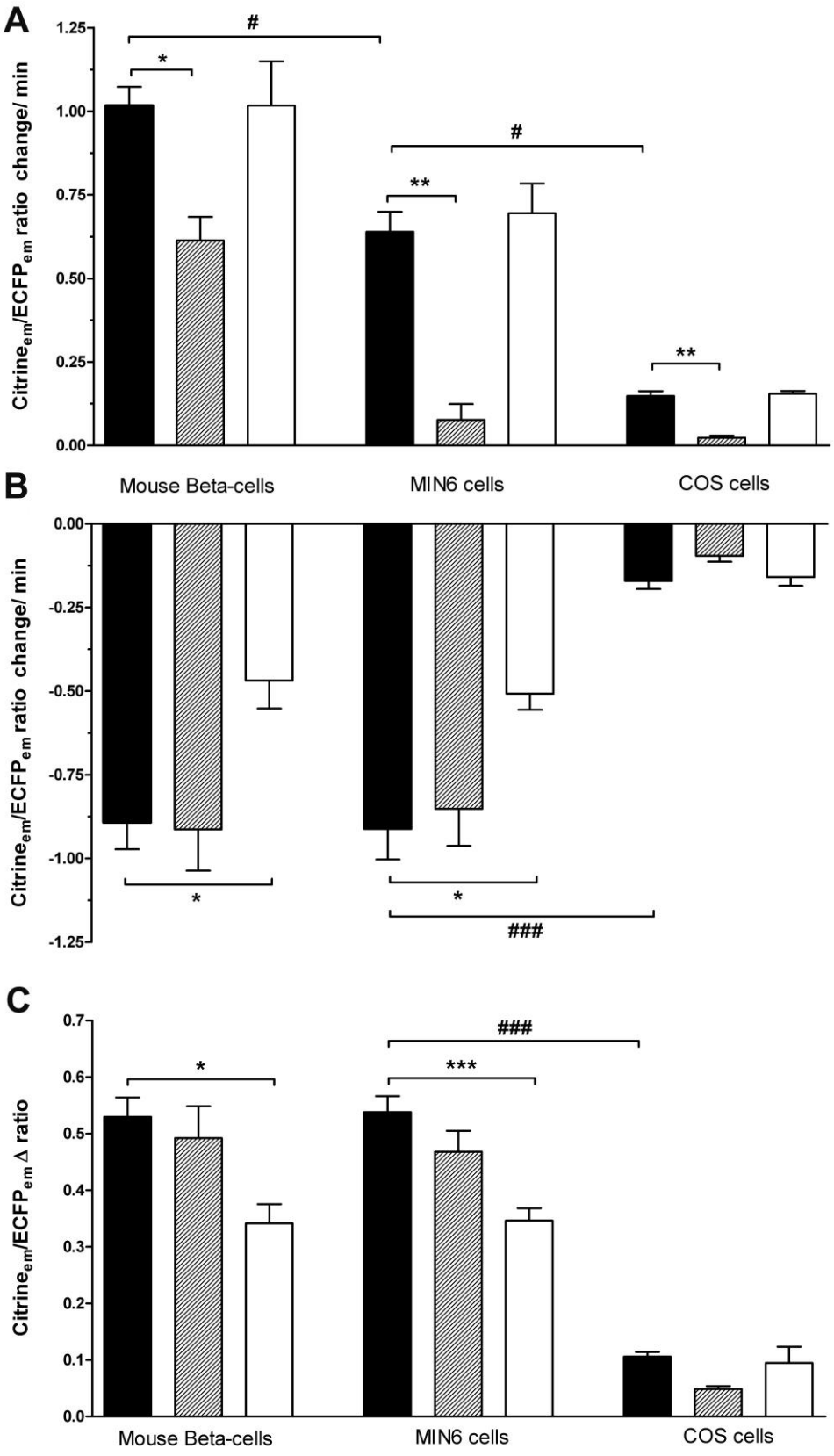

A
1.25
1.00
0.75
0.50
0.25
0.00
Citrine$_{em}$/ECFP$_{em}$ ratio change/ min
#
*
#
**
**
Mouse Beta-cells
MIN6 cells
COS cells
B
0.00
-0.25
-0.50
-0.75
-1.00
-1.25
Citrine$_{em}$/ECFP$_{em}$ ratio change/ min
*
*
###
C
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Citrine$_{em}$/ECFP$_{em}$ Δ ratio
*
###
***
Mouse Beta-cells
MIN6 cells
COS cells

Fig. 5

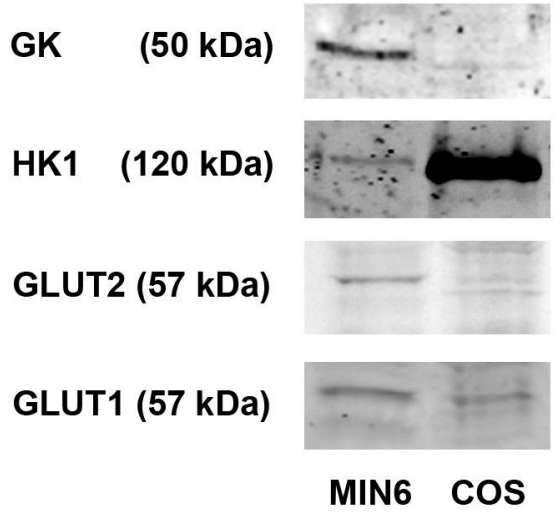


Fig. 6

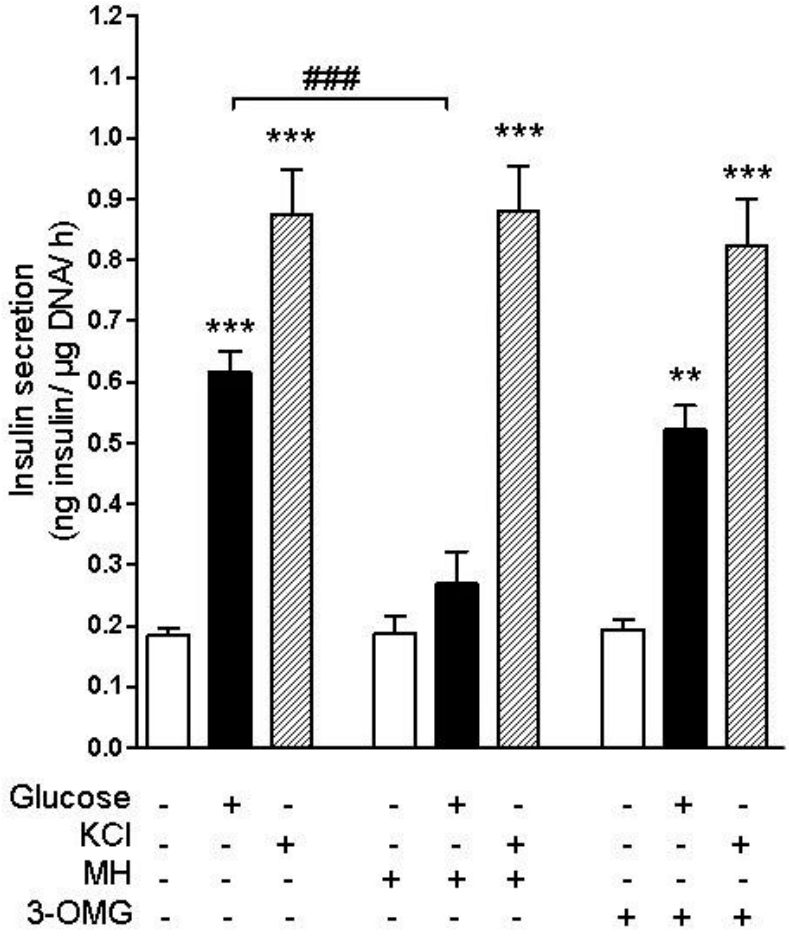
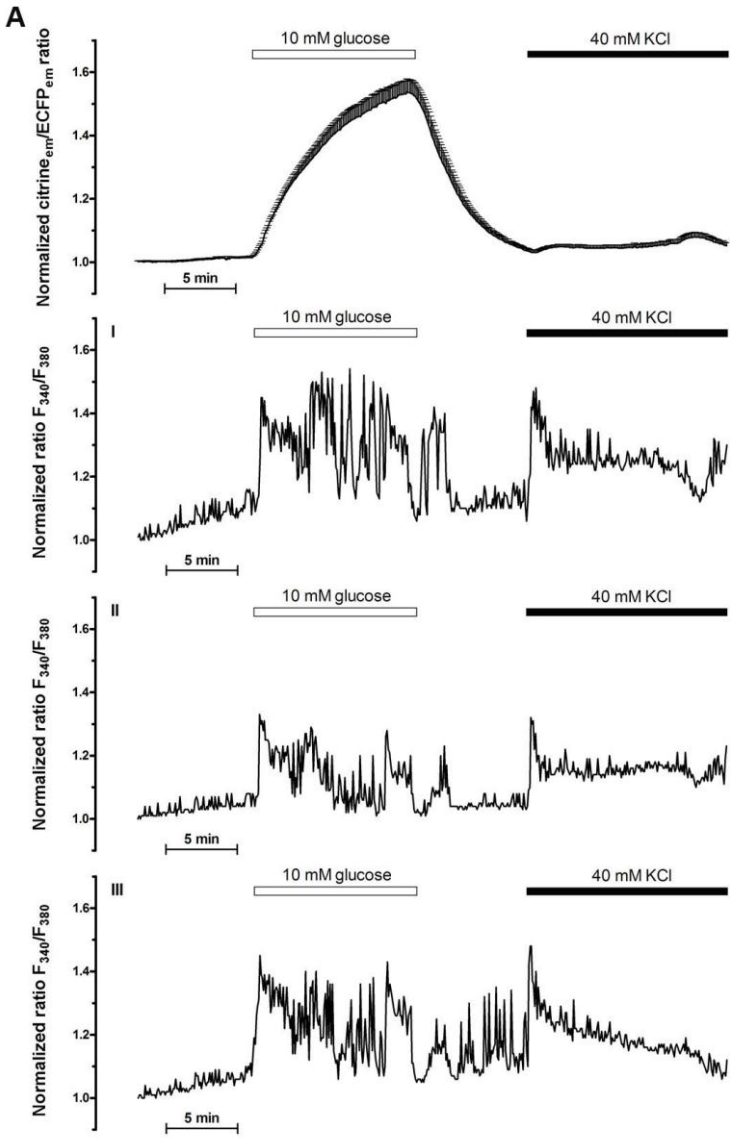

Insulin secretion
(ng insulin/ µg DNA/ h)
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
###
***
***
***
***
**
Glucose - + - - + - - + -
KCl - - + - - + - - +
MH - - - + + + - - -
3-OMG - - - - - - + + +

Fig. 7


A
10 mM glucose
40 mM KCl
Normalized citrine$_{em}$/ECFP$_{em}$ ratio
1.6
1.4
1.2
1.0
5 min
I
II
III
Normalized ratio F_{340}/F_{380}

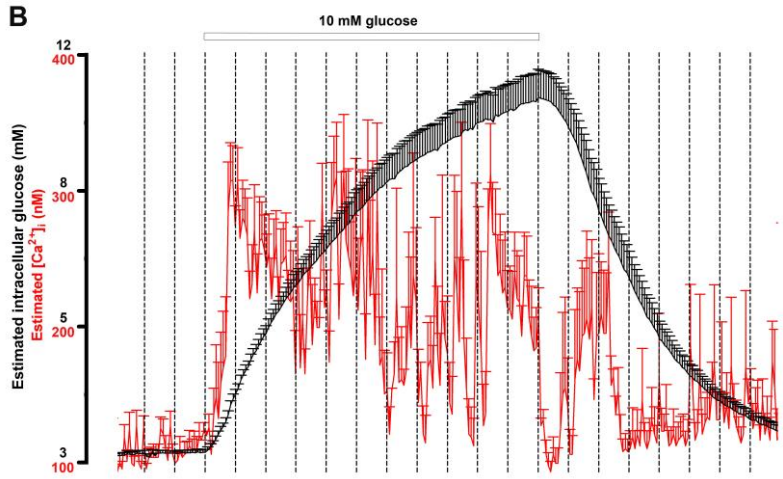
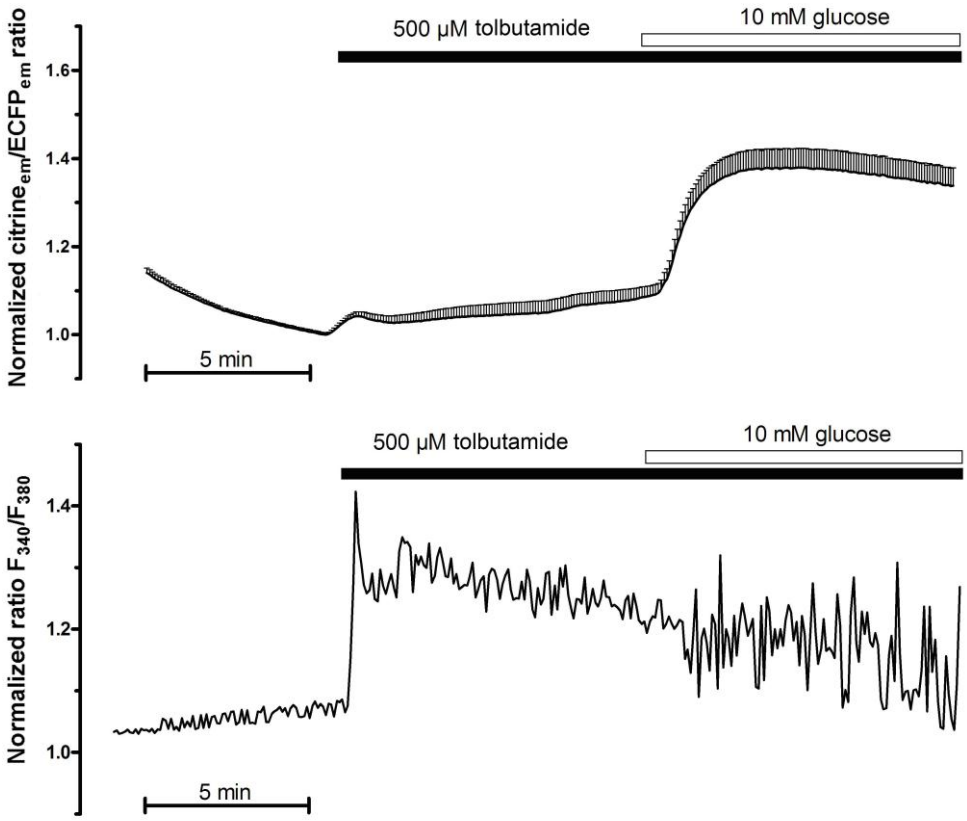

B
10 mM glucose
Estimated intracellular glucose (mM)
Estimated $[Ca^{2+}]_i$ (nM)
12
400
8
300
5
200
3
100

Fig. 8


500 µM tolbutamide
10 mM glucose
Normalized citrine$_{em}$/ECFP$_{em}$ ratio
1.6
1.4
1.2
1.0
5 min
500 µM tolbutamide
10 mM glucose
Normalized ratio F$_{340}$/F$_{380}$
1.4
1.2
1.0
5 min

Molecular Endocrinology

Glucose causes dissociation of the glucokinase-glucokinase regulatory protein complex already in the nucleus of hepatocytes

Abbreviated title: Glucokinase-GRP interaction

Authors: Martin Tobias Kaminski[1], Julia Schultz[2], Rica Waterstradt[2], Markus Tiedge[2], Sigurd Lenzen[1] and Simone Baltrusch[2]

Affiliations: [1]Institute of Clinical Biochemistry, Hannover Medical School, 30623 Hannover, Germany
[2]Institute of Medical Biochemistry and Molecular Biology, University of Rostock, 18057 Rostock, Germany

Address all correspondence and requests for reprints to:

Prof. Simone Baltrusch
Institute of Medical Biochemistry and Molecular Biology
University of Rostock
18057 Rostock
Germany
Tel. 0049-381-494-5760
Fax 0049-381-494-5752
E-mail: simone.baltrusch@med.uni-rostock.de

Grant Support: This work was supported by the European Union (Integrated Project EuroDia LSHM-CT-2006-518153 in the Framework Programme 6 [FP6] of the European-Community) and a grant from the German Diabetes Association (S.B.).

Disclosure summary: The authors have nothing to disclose.

Key words: glucokinase; glucokinase regulatory protein; hepatocytes; pancreatic beta cell; fluorescence microscopy

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EYFP, enhanced yellow fluorescent protein; FRET, fluorescence resonance energy transfer; FRAP, Fluorescence recovery after photobleaching; GK, glucokinase; GRP, glucokinase regulatory protein

Abstract

The glucose phosphorylating enzyme glucokinase regulates glucose metabolism in the liver. Glucokinase enzyme activity is modulated by a liver specific competitive inhibitor, the glucokinase regulatory protein (GRP). This protein mediates nuclear import of glucokinase at low glucose concentrations in hepatocytes. It remains unknown, however, whether the GRP is also involved in the nuclear export of glucokinase. In the nucleus of primary hepatocytes the interaction between glucokinase and the GRP, measured by acceptor photobleaching fluorescence resonance energy transfer, was significantly lower at 20 than at 5 mmol/l glucose. Interestingly, the nuclear to cytoplasmic ratio of glucokinase was significantly reduced at 20 mmol/l compared to 5 mmol/l glucose, whereas that of the GRP was only marginally lower. Using selective photoconversion and fluorescence recovery after photobleaching of glucokinase and GRP fluorescence fusion proteins, we directly show the translocation process of the proteins in primary hepatocytes. The mobility of the GRP was independent from the glucose concentration. In contrast, the nuclear export rate of glucokinase was significantly higher at 20 than at 5 mmol/l glucose. Using a nuclear targeted version of the FLII^{12}Pglu-700µ-δ6 glucose nanosensor, we investigate for the first time the nuclear glucose flux in primary hepatocytes. Nuclear glucose influx and efflux was significantly higher in hepatocytes compared to beta cells, another glucokinase expressing cell type. Thus, high glucose in the nucleus releases active glucokinase from the complex with the GRP. Our findings suggest that the glucokinase nuclear export in hepatocytes is depending on glucose, but not the GRP.

Introduction

Glucokinase regulates glycolytic flux at physiological millimolar glucose concentration in the liver and pancreatic beta cells (Lenzen & Panten 1988b; Iynedjian 1993; Matschinsky 1996; Baltrusch & Tiedge 2006c). Transcriptional mechanisms and on the posttranslational level interaction with regulatory proteins modulate glucokinase enzyme activity (Lenzen & Panten 1988b; Iynedjian 1993; Matschinsky 1996; Baltrusch & Tiedge 2006c). The glucokinase regulatory protein (GRP) is liver specific and plays in heaptocytes the most important role in glucokinase regulation (Van Schaftingen 1989b; Vandercammen & Van Schaftingen 1991; van Schaftingen *et al.* 1992; van Schaftingen *et al.* 1997; de la Iglesia *et al.* 2000).

The GRP binds glucokinase in the absence of glucose, when the enzyme resides in the super-opened conformation (van Schaftingen *et al.* 1997; de la Iglesia *et al.* 2000; Baltrusch *et al.* 2005; Baltrusch & Tiedge 2006c; Futamura *et al.* 2006; Heredia *et al.* 2006b; Zhang *et al.* 2006; Antoine *et al.* 2009). Glucose causes transition of glucokinase to a closed conformation, in which the enzyme is active and not accessible for interaction with the GRP (Kamata *et al.* 2004; Zhang *et al.* 2006; Antoine *et al.* 2009). We observed evidence, that the L58/N204 motif in the hinge region of glucokinase confers binding to GRP, whereas the L355/N350 motif modulates the binding affinity for GRP in the super-opened conformation (Baltrusch *et al.* 2005). However, further regions might be involved in binding, as demonstrated by naturally occurring activating and inactivating glucokinase mutations with reduced susceptibility to competitive inhibition by the GRP (Veiga-da-Cunha *et al.* 1996a; Veiga-da-Cunha *et al.* 1996b; Heredia *et al.* 2006b). Glucokinase:GRP complex formation is stimulated by fructose-6-phosphate and suppressed by fructose-1-phosphate (Van Schaftingen 1989b; Niculescu *et al.* 1997; van Schaftingen *et al.* 1997; Mukhtar *et al.* 1999; Veiga-da-Cunha & Van Schaftingen 2002; Baltrusch & Tiedge 2006c). However, species differences exist in these regulatory properties of the GRP (Brocklehurst *et al.* 2004a).

The binding to the GRP mediates translocation of glucokinase to the nucleus in hepatocytes. In the absence of the GRP, in pancreatic beta cells, glucokinase is exclusively localized in the cytoplasm (Vandercammen & Van Schaftingen 1993; Baltrusch & Tiedge 2006c). Thus, in liver, but not in pancreatic beta cells, nuclear to cytoplasmic compartmentation regulates glucokinase enzyme activity (Vandercammen & Van Schaftingen 1993; Baltrusch & Tiedge 2006c). In response to an increase in the glucose concentration, glucokinase rapidly translocates from the nucleus to the cytoplasm (Toyoda *et al.* 1995b; Fernandez-Novell *et al.* 1999; de la Iglesia *et al.* 2000; Jetton *et al.* 2001; Baltrusch *et al.* 2005; Wolff *et al.* 2008). Likewise small molecule glucokinase activators evoked an increase in cytoplasmic glucokinase in hepatocytes (Brocklehurst *et al.* 2004b; Futamura *et al.* 2006; Wolff *et al.* 2008). The mechanism of this glucokinase nuclear export, however, is less understood (Vandercammen & Van Schaftingen 1993; Agius *et al.* 1995; Toyoda *et al.* 1995b; Brown *et al.* 1997; van Schaftingen *et al.* 1997; de la Iglesia *et al.* 1999; Mukhtar *et al.* 1999; Shiota *et al.* 1999a; Bosco *et al.* 2000b; Jetton *et al.* 2001; Baltrusch & Tiedge 2006c).

Glucokinase and the GRP have a size, which is clearly above the limit of the nuclear pore complexes. Thus, both proteins require RanGTP dependent nuclear transport receptors (Güttler & Gorlich 2011). It was proposed, that the glucokinase nuclear export is mediated by a leucine rich nuclear export signal in the protein (Shiota *et al.* 1999a). However, leptomycin B, an inhibitor of this exportin 1 system did not inhibit the glucokinase nuclear export (Mukhtar *et al.* 1999). Other nuclear transport receptors (Güttler & Gorlich 2011) or a GRP dependent nuclear export for glucokinase, therefore, have to taken also into account. Thus, the aim of the study was to investigate the nuclear export of glucokinase in hepatocytes in consideration of the GRP translocation and the nuclear glucose flux.

Results

Interaction and localisation of glucokinase and the GRP in primary rat hepatocytes

To investigate direct interaction between glucokinase and GRP using acceptor photobleaching FRET, the endogenous proteins were labelled with Cy3 and Cy5, respectively. By binding of the target proteins, FRET occurs between Cy3 and Cy5, and photobleaching of Cy5 fluorescence results in enhanced Cy3 fluorescence. Interaction between glucokinase and GRP was tenfold higher at 5 mmol/l glucose than at 20 mmol/l glucose (Fig. 1). Fructose-6-phosphate (F-6-P) evoked a significantly enhanced in interaction at 5 mmol/l glucose (2.03 ± 0.32 vs. 4.37 ± 0.86), whereas fructose-1-phosphate (F-1-P) lowered the binding at 20 mmol/l glucose (0.21 ± 0.40 vs. 0.12 ± 0.18) (Fig. 1).

To study the sub-cellular localization of endogenous glucokinase and GRP the nuclear to cytoplasmic ratio was determined in primary rat hepatocytes. At 5 mmol/l glucose both proteins showed a comparable nuclear to cytoplasmic ratio. At 20 mmol/l glucose, the nuclear to cytoplasmic ratio of glucokinase was significantly reduced (3.42 ± 0.19 vs. 2.00 ± 0.11) (Fig. 2). In contrast, only a minor reduction in the nuclear to cytoplasmic ratio of the GRP was observed (3.44 ± 0.17 vs. 3.05 ± 0.13) (Fig.2).

Translocation of glucokinase and the GRP in primary rat hepatocytes

Selective photoconversion of Dendra2 in the nucleus of rat hepatocytes was used to detect the nuclear export of glucokinase and GRP (Fig. 3A). The nuclear export rate of the photoconverted red Dendra2 alone was 20-fold higher than those determined for Dendra2-GK and Dendra2-GRP fusion proteins (Fig. 3A). With a size of 28 kDa, Dendra2 can translocate through the nuclear pore complexes. In contrast, translocation of Dendra2-GK and Dendra2-GRP requires active transport by the exportin system. While the nuclear export rate of the

GRP was comparable at 20 and 5 mmol/l glucose, glucokinase showed a significantly lower nuclear export rate at 5 mmol/l glucose (-0.0056 ± 0.0061 vs. -0.0509 ± 0.0081) (Fig. 3A).

To confirm these results fluorescence recovery after photobleaching of EYFP-GK and EYFP-GRP fusion proteins was measured in the nucleus (Fig. 3B) and cytoplasm (Fig. 3C). Consistently, glucokinase, but not the GRP showed an alteration in the recovery rate as a function of the glucose concentration. At 20 mmol/l glucose with 65 ± 2 % a significantly lower percentage of EYFP-GK was measured in the nucleus compared to 5 mmol/l glucose (73 ± 2 %) (Fig. 3B). The adverse effect was observed in the cytoplasm. At 20 mmol/l glucose with 91 ± 1 % a significantly higher percentage of EYFP-GK was measured compared to 5 mmol/l glucose (84 ± 1 %) (Fig. 3C).

Glucokinase enzyme activity and immunoreactivity in the cytoplasm and nucleus of primary rat hepatocytes and MIN6 beta cells

Glucokinase immunoreactivity and enzyme activity was detectable in the nucleus of rat hepatocytes (Fig. 4A and B). MIN6 beta cells not endogenously expressing GRP were transiently transfected with EYFP-GRP (Fig. 4D) or as a control with the fluorescence protein EYFP alone (Fig. 4C). Like endogenous GRP in hepatocytes (Fig. 2), EYFP-GRP was predominantly detected inside the nucleus with a smaller fraction still present in the cytosol. Glucokinase immunoreactivity was measurable inside the nucleus of EYFP-GRP expressing cells and glucokinase enzyme activity was significantly decreased in cytosolic fractions, whereas being markedly increased in nuclear fractions compared to control cells (Fig. 4A and B).

Real-time analysis of the glucose concentration in the nucleus and cytoplasm of primary rat hepatocytes and MIN6 beta cells

Real-time glucose measurements were performed using FLII^{12}Pglu-700µ-δ6 (FLIPglu) (Kaminski *et al.* 2011). Generation of a nuclear targeted version of FLII^{12}Pglu-700µ-δ6 (FLIPglu-NUC) has enabled nucleus specific measurements. Finally, the glucose influx rate (Fig. 5A) and the glucose efflux/ metabolism rate (Fig. 5B) were quantified from the time-dependent plots (Kaminski *et al.* 2011). In the cytoplasm a comparable glucose influx was measured in MIN6 beta cells and hepatocytes. In contrast, in the nucleus a significantly higher glucose influx was determined in hepatocytes compared to MIN6 beta cells (0.565 ± 0.093 vs. 0.230 ± 0.036) (Fig. 5A). Expression of the GRP did not change the nuclear glucose influx in MIN6 beta cells. In hepatocytes a slightly lower cytoplasmic glucose efflux and metabolism were detectable compared to MIN6 beta cells. The nuclear glucose efflux/ metabolism rate of MIN6 beta cells was the same as determined in non-glucokinase expressing COS cells, whereas hepatocytes showed a significantly higher rate (0.239 ± 0.024 vs. 0.101 ± 0.023). Importantly, expression of the GRP significantly increased the nuclear efflux/ metabolism rate in MIN6 beta cells.

Discussion

Glucokinase is a key regulator of glucose metabolism in the liver (Baltrusch & Tiedge 2006c). Glucose induces a closed conformation of glucokinase, in which the enzyme is active. In the absence of glucose, glucokinase relaxes to a super-opened conformation (Kamata *et al.* 2004; Zhang *et al.* 2006; Antoine *et al.* 2009). This glucokinase conformation allows interaction with the liver specific GRP (van Schaftingen *et al.* 1997; Kamata *et al.* 2004; Baltrusch & Tiedge 2006c; Zhang *et al.* 2006; Antoine *et al.* 2009). In addition to the competitive inhibition (Vandercammen & Van Schaftingen 1991), the GRP sequesters glucokinase to the nucleus (Vandercammen & Van Schaftingen 1993; Agius *et al.* 1995; Toyoda *et al.* 1995b; Brown *et al.* 1997; van Schaftingen *et al.* 1997; de la Iglesia *et al.* 1999; Mukhtar *et al.* 1999; Shiota *et al.* 1999a; Bosco *et al.* 2000b; Jetton *et al.* 2001; Baltrusch & Tiedge 2006c). The presence of glucokinase in the nucleus is dependent upon the GRP, because in beta cells, where the GRP is not expressed, glucokinase is exclusively localized in the cytoplasm (Baltrusch & Tiedge 2006c). In agreement with previous experiments (de la Iglesia *et al.* 1999; Shiota *et al.* 1999a; Bosco *et al.* 2000b; Baltrusch *et al.* 2005; Langer *et al.* 2010), we have shown that overexpression of the GRP in MIN6 beta cells evokes translocation of glucokinase to the nucleus. Thus, glucokinase nuclear import is obviously mediated by the GRP.

However, the mechanism of the glucokinase nuclear export and the involvement of the GRP in this process are controversially discussed (Vandercammen & Van Schaftingen 1993; Agius *et al.* 1995; Toyoda *et al.* 1995b; Brown *et al.* 1997; van Schaftingen *et al.* 1997; de la Iglesia *et al.* 1999; Mukhtar *et al.* 1999; Shiota *et al.* 1999a; Bosco *et al.* 2000b; Jetton *et al.* 2001; Baltrusch & Tiedge 2006c). We have investigated for the first time direct interaction of endogenous proteins by acceptor photobleaching FRET in primary rat hepatocytes. In the nucleus, interaction between glucokinase and GRP was significant at 5 mmol/l glucose, but

nearly lost at 20 mmol/l glucose. In line with other studies, the nuclear to cytoplasmic ratio of glucokinase was significantly reduced at 20 mmol/l compared to 5 mmol/l glucose. The nuclear to cytoplasmic ratio of the GRP was at 20 mmol/l glucose slightly lower than at 5 mmol/l glucose (Rees *et al.* 2011), however, in comparison with glucokinase not significantly changed as a function of the glucose concentration.

Our results suggest that nuclear export of glucokinase is glucose dependent, whereas the GRP shuttles continuously between the nucleus and cytoplasm in hepatocytes. Kinetic analyses of nuclear export of glucokinase and GRP by selective photoconversion of Dendra2 fusion proteins (Gurskaya *et al.* 2006; Baltrusch & Lenzen 2007a; Baltrusch & Lenzen 2008) have not been explored previously in primary hepatocytes. Nuclear export of Dendra2-glucokinase and Dendra2-GRP was slow compared to the fluorescence protein Dendra2 alone. This observation can best be explained by the size of the fusion proteins, which is above the exclusion size for crossing the nuclear pore complexes (Güttler & Gorlich 2011). The role of the putative leucine rich nuclear export signal in the glucokinase (Shiota *et al.* 1999a) could not be investigated by this approach, because mutations of these leucine residues showed a loss of interaction with the GRP, thus of nuclear import (Baltrusch *et al.* 2005) (and S.B. unpublished observation). Therefore, masking of the glucokinase nuclear export signal in the complex with the GRP can be hypothesized (Shiota *et al.* 1999a).

We demonstrated for the first time nuclear export of glucokinase and GRP in living primary rat hepatocytes. Interestingly, nuclear export of Dendra2-GRP was comparable at 5 and 20 mmol/l glucose, whereas nuclear export of glucokinase was only detectable at 20 mmol/l glucose. Further support for the difference in nuclear export of glucokinase and the GRP was obtained from our fluorescence recovery after photobleaching experiments using EYFP-glucokinase and EYFP-GRP. Glucokinase, but not the GRP showed changes in the fluorescence recovery rate as a function of the glucose concentration.

Therefore, a glucokinase release from the GRP in the nucleus of hepatocytes can be assumed, if glucose rapidly increased in the nucleus after an external supply. Using the recently established FLII^{12}Pglu-700µ-δ6 based real-time method (Fehr *et al.* 2004c; Takanaga *et al.* 2008a; Kaminski *et al.* 2011) we in fact observed a comparable increase of the glucose concentration in the nucleus and in the cytoplasm of hepatocytes. The fast nuclear glucose influx was specific for hepatocytes. MIN6 beta cells showed a significantly lower nuclear glucose influx, whereas the cytoplasmic glucose influx was comparable to hepatocytes. Interestingly, overexpression of the GRP did not increase the nuclear glucose influx in MIN6 beta cells. Thus, our study provides first evidence that hepatocytes have a unique nuclear glucose influx capacity.

Glucose metabolism in the nucleus of hepatocytes, however, is dependent on the GRP expression (Niculescu *et al.* 1997). The nuclear glucose metabolism rate of MIN6 beta cells was comparable to that of COS cells, but significantly lower than in hepatocytes. Overexpression of the GRP in MIN6 cells resulted in a significant elevation of the nuclear glucose metabolism. This observation can best be explained by sequestration of glucokinase into the nucleus by the GRP and subsequent release at increasing nuclear glucose concentration. Accordingly, glucokinase enzyme activity could be measured in the nucleus of hepatocytes and GRP overexpressing MIN6 beta cells, but not in control MIN6 beta cells.

In conclusion, we have demonstrated that the increase in the nuclear glucose concentration releases glucokinase from the complex with the GRP and mediates a GRP independent glucokinase export. This observation provides new knowledge for the development of liver specific glucokinase activators as therapeutics for the treatment of type 2 diabetes mellitus and to the understanding of the consequence of genetic variants of the GRP on diabetes risk (Rees *et al.* 2011).

Materials and Methods

Cell culture

MIN6 and COS cells were grown in DMEM supplemented with 25 mmol/l glucose, 10% (vol/vol) FCS, penicillin, and streptomycin in a humidified atmosphere at 37°C and 5% CO_2. Cells were transfected with the vector DNA by the use of jetPEI (Qbiogene, Montreal, Canada) as described in the manufacturer's manual. Isolation and culture of primary rat hepatocytes was performed as described (Baltrusch *et al.* 2005). Hepatocytes were either transfected with jetPEI-Hepatocyte (Qbiogene) as described (Baltrusch *et al.* 2005) or transduced at a multiplicity of infection of 10 for 2 h with adenoviral FLIPglu or FLIPglu-NUC stock solution, respectively.

Generation of plasmids and adenoviruses

The pcDNA 3.1 vector was used for expression of FLII^{12}Pglu-700µ-δ6 (FLIPglu) (Addgene plasmid 17866) (Takanaga *et al.* 2008a). Recombinant FLIPglu adenoviruses were generated previously (Kaminski *et al.* 2011). A nuclear targeted version of FLIPglu was made by subcloning the coding cDNA as a SalI-NotI fragment into the pCMV/myc/NUC vector (Invitrogen, Carlsbad, CA). Recombinant nuclear targeted FLIPglu adenoviruses were generated by subcloning the coding cDNA from the pCMV/myc/NUC as a SalI-XbaI fragment into the pShuttle-CMV vector. Generation of EYFP-GK and EYFP-GRP has been described previously (Baltrusch *et al.* 2005). The EYFP in both was replaced by Dendra2 using AgeI and BsrGI restriction sites. pTagRFP-GRP was generated by subcloning the coding cDNA of GRP as a BglII-EcoRI fragment into the pTagRFP-N vector.

Assay of glucokinase enzyme activity and western blot analyses

Nuclear and cytoplasmic fractions of MIN6 cells and hepatocytes were isolated by a stepwise fractionation. Cells were incubated in 0.5 ml buffer containing 20 mmol/l HEPES (pH 7.9),

10 mmol/l KCL, 0.2 % NP40, 10 % Glycerol, 1 mmol/l EDTA, 1 mmol/l DTT, 1µg/ml pepstatin, 1 mmol/l PMSF, and proteases for 10 minutes on ice. The cytoplasmic fraction was collected as supernatant after centrifugation at 13,000 rpm for 30 sec. The pellet was re-suspended in 100µl buffer containing 20 mmol/l HEPES, 10 mmol/l KCL, 350 mmol/l NaCL, 20% Glycerol, 1 mmol/l EDTA, 1 mmol/l DTT, 1µg/ml pepstatin, 1mM PMSF, 1 mmol/l vanadate and proteases for 20minutes on ice. The nuclear fraction was collected as supernatant after centrifugation at 13,000 rpm for 30 sec. The protein concentration was quantified by Bradford protein assay. Glucokinase enzyme activity was measured by an enzyme-coupled photometric assay as described (Baltrusch *et al.* 2005). A total of 40 µg Protein was fractionated by reducing 10% SDS-PAGE and electroblotted to polyvinylidine difluoride membranes. Western blotting was performed as described (Kaminski *et al.* 2011)with GK antibody (sc-7908, diluted 1:500, or sc-1980, diluted 1:500 Santa Cruz Biotechnology, Santa Cruz, CA) for liver and beta cell GK, respectively.

Immunostaining

Primary rat hepatocytes were seeded on glass coverslips and incubated for 2 h as indicated. Thereafter, cells were fixed overnight with 4% paraformaldehyde in PBS at 4°C and immunostained as described (Baltrusch & Lenzen 2007a) with glucokinase antibody (sc-7908) diluted 1:100 in PBS and GRP antibody (sc-6340) diluted 1:100 in PBS (Santa Cruz Biotechnology, Santa Cruz, CA). Thereafter, cells were incubated with the appropriate secondary antibody, Cy3 or Cy5, all diluted 1:200 in PBS (Jackson Immuno Research, West Grove, PA). Finally, coverslips were washed three times with PBS and fixed onto slides.

Fluorescence microscopy

FRAP and fluorescence distribution after photoconversion measurements were taken with an Olympus Fluoview1000 confocal inverted microscope (Olympus, Hamburg, Germany) using the multiline argon laser (515 nm) to excite EYFP and the krypton-argon laser (561 nm) to

excite red Dendra2. Scanning was performed with 2 µs/pixel sampling speed and 128×128 image format for 30 sec. Photoactivation and photoconversion were done for one sampling interval during imaging with simultaneous scanner and a 405 nm laser diode (25 mW) using circular scanning (tornado mode). Intracellular glucose measurements using FLIPglu were carried out with a cellR/Olympus IX81 microscope system (Olympus, Hamburg, Germany) as described (Kaminski *et al.* 2011). Cells were maintained at 60 % humidity, 37°C and 5 % CO_2 with a Cellcubator (Olympus) during experiments. The procedure of acceptor photobleaching FRET experiments has been described previously (Baltrusch & Lenzen 2007a).

Statistical analyses

Statistical analyses were performed using the Prism analysis program (Graphpad, San Diego, CA, USA).

Acknowledgements

We thank Dr. W. Frommer and Dr. H. Takanaga (Stanford, CA, USA) for providing the FLII^{12}Pglu-700µ-δ6 construct (Addgene plasmid 17866) and Dr. B. Vogelstein (Baltimore, MD) for providing the vectors of the adenoviral expression system. We thank M. Funk, J. Kresse, and B. Leß for skilful technical assistance.

References

1. Baltrusch S, Tiedge M 2006 Glucokinase Regulatory Network in Pancreatic Beta-Cells and Liver. Diabetes 55 Suppl 2:S55-64

2. Iynedjian PB 1993 Mammalian glucokinase and its gene. The Biochemical journal 293:1-13

3. Lenzen S, Panten U 1988 Signal recognition by pancreatic Beta-cells. Biochemical pharmacology 37:371-378

4. Matschinsky FM 1996 Banting Lecture 1995. A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. Diabetes 45:223-241

5. de la Iglesia N, Mukhtar M, Seoane J, Guinovart JJ, Agius L 2000 The role of the regulatory protein of glucokinase in the glucose sensory mechanism of the hepatocyte. The Journal of biological chemistry 275:10597-10603

6. Van Schaftingen E 1989 A protein from rat liver confers to glucokinase the property of being antagonistically regulated by fructose 6-phosphate and fructose 1-phosphate. European journal of biochemistry /FEBS 179:179-184

7. van Schaftingen E, Vandercammen A, Detheux M, Davies DR 1992 The regulatory protein of liver glucokinase. Advances in enzyme regulation 32:133-148

8. van Schaftingen E, Veiga-da-Cunha M, Niculescu L 1997 The regulatory protein of glucokinase. Biochemical Society transactions 25:136-140

9. Vandercammen A, Van Schaftingen E 1991 Competitive inhibition of liver glucokinase by its regulatory protein. European journal of biochemistry /FEBS 200:545-551

10. Antoine M, Boutin JA, Ferry G 2009 Binding kinetics of glucose and allosteric activators to human glucokinase reveal multiple conformational states. Biochemistry 48:5466-5482

11. Baltrusch S, Francini F, Lenzen S, Tiedge M 2005 Interaction of glucokinase with the liver regulatory protein is conferred by leucine-asparagine motifs of the enzyme. Diabetes 54:2829-2837

12. Futamura M, Hosaka H, Kadotani A, Shimazaki H, Sasaki K, Ohyama S, Nishimura T, Eiki J, Nagata Y 2006 An allosteric activator of glucokinase impairs the interaction of glucokinase and glucokinase regulatory protein and regulates glucose metabolism. The Journal of biological chemistry 281:37668-37674

13. Heredia VV, Carlson TJ, Garcia E, Sun S 2006 Biochemical basis of glucokinase activation and the regulation by glucokinase regulatory protein in naturally occurring mutations. The Journal of biological chemistry 281:40201-40207

14. Zhang J, Li C, Chen K, Zhu W, Shen X, Jiang H 2006 Conformational transition pathway in the allosteric process of human glucokinase. Proceedings of the National Academy of Sciences of the United States of America 103:13368-13373

15. Kamata K, Mitsuya M, Nishimura T, Eiki J, Nagata Y 2004 Structural basis for allosteric regulation of the monomeric allosteric enzyme human glucokinase. Structure 12:429-438

16. Veiga-da-Cunha M, Courtois S, Michel A, Gosselain E, Van Schaftingen E 1996 Amino acid conservation in animal glucokinases. Identification of residues implicated in the interaction with the regulatory protein. The Journal of biological chemistry 271:6292-6297

17. Veiga-da-Cunha M, Xu LZ, Lee YH, Marotta D, Pilkis SJ, Van Schaftingen E 1996 Effect of mutations on the sensitivity of human beta-cell glucokinase to liver regulatory protein. Diabetologia 39:1173-1179

18. Mukhtar M, Stubbs M, Agius L 1999 Evidence for glucose and sorbitol-induced nuclear export of glucokinase regulatory protein in hepatocytes. FEBS letters 462:453-458

19. Niculescu L, Veiga-da-Cunha M, Van Schaftingen E 1997 Investigation on the mechanism by which fructose, hexitols and other compounds regulate the translocation of glucokinase in rat hepatocytes. The Biochemical journal 321:239-246

20. Veiga-da-Cunha M, Van Schaftingen E 2002 Identification of fructose 6-phosphate- and fructose 1-phosphate-binding residues in the regulatory protein of glucokinase. The Journal of biological chemistry 277:8466-8473

21. Brocklehurst KJ, Davies RA, Agius L 2004 Differences in regulatory properties between human and rat glucokinase regulatory protein. The Biochemical journal 378:693-697

22. Vandercammen A, Van Schaftingen E 1993 Species and tissue distribution of the regulatory protein of glucokinase. The Biochemical journal 294:551-556

23. Fernandez-Novell JM, Castel S, Bellido D, Ferrer JC, Vilaro S, Guinovart JJ 1999 Intracellular distribution of hepatic glucokinase and glucokinase regulatory protein during the fasted to refed transition in rats. FEBS letters 459:211-214

24. Jetton TL, Shiota M, Knobel SM, Piston DW, Cherrington AD, Magnuson MA 2001 Substrate-induced nuclear export and peripheral compartmentalization of hepatic

glucokinase correlates with glycogen deposition. International journal of experimental diabetes research 2:173-186

25. Toyoda Y, Miwa I, Satake S, Anai M, Oka Y 1995 Nuclear location of the regulatory protein of glucokinase in rat liver and translocation of the regulator to the cytoplasm in response to high glucose. Biochemical and biophysical research communications 215:467-473

26. Wolff M, Kauschke SG, Schmidt S, Heilker R 2008 Activation and translocation of glucokinase in rat primary hepatocytes monitored by high content image analysis. J Biomol Screen 13:837-846

27. Brocklehurst KJ, Payne VA, Davies RA, Carroll D, Vertigan HL, Wightman HJ, Aiston S, Waddell ID, Leighton B, Coghlan MP, Agius L 2004 Stimulation of hepatocyte glucose metabolism by novel small molecule glucokinase activators. Diabetes 53:535-541

28. Agius L, Peak M, Van Schaftingen E 1995 The regulatory protein of glucokinase binds to the hepatocyte matrix, but, unlike glucokinase, does not translocate during substrate stimulation. The Biochemical journal 309:711-713

29. Bosco D, Meda P, Iynedjian PB 2000 Glucokinase and glucokinase regulatory protein: mutual dependence for nuclear localization. The Biochemical journal 348:215-222

30. Brown KS, Kalinowski SS, Megill JR, Durham SK, Mookhtiar KA 1997 Glucokinase regulatory protein may interact with glucokinase in the hepatocyte nucleus. Diabetes 46:179-186

31. de la Iglesia N, Veiga-da-Cunha M, Van Schaftingen E, Guinovart JJ, Ferrer JC 1999 Glucokinase regulatory protein is essential for the proper subcellular localisation of liver glucokinase. FEBS letters 456:332-338

32. Shiota C, Coffey J, Grimsby J, Grippo JF, Magnuson MA 1999 Nuclear import of hepatic glucokinase depends upon glucokinase regulatory protein, whereas export is due to a nuclear export signal sequence in glucokinase. The Journal of biological chemistry 274:37125-37130

33. Güttler T, Gorlich D 2011 Ran-dependent nuclear export mediators: a structural perspective. EMBO J 30:3457-3474

34. Kaminski MT, Lenzen S, Baltrusch S 2011 Real-time analysis of intracellular glucose and calcium in pancreatic beta cells by fluorescence microscopy. The Journal of biological chemistry *submitted*

35. Langer S, Kaminski MT, Lenzen S, Baltrusch S 2010 Endogenous activation of glucokinase by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase is glucose dependent. Mol Endocrinol 24:1988-1997

36. Rees MG, Wincovitch S, Schultz J, Waterstradt R, Beer NL, Baltrusch S, Collins FS, Gloyn AL 2011 Cellular characterisation of the GCKR P446L variant associated with type 2 diabetes risk. Diabetologia *published online*

37. Baltrusch S, Lenzen S 2007 Novel insights into the regulation of the bound and diffusible glucokinase in MIN6 beta-cells. Diabetes 56:1305-1315

38. Baltrusch S, Lenzen S 2008 Monitoring of glucose-regulated single insulin secretory granule movement by selective photoactivation. Diabetologia 51:989-996

39. Gurskaya NG, Verkhusha VV, Shcheglov AS, Staroverov DB, Chepurnykh TV, Fradkov AF, Lukyanov S, Lukyanov KA 2006 Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light. Nat Biotechnol 24:461-465

40. Fehr M, Lalonde S, Ehrhardt DW, Frommer WB 2004 Live imaging of glucose homeostasis in nuclei of COS-7 cells. J Fluoresc 14:603-609

41. Takanaga H, Chaudhuri B, Frommer WB 2008 GLUT1 and GLUT9 as major contributors to glucose influx in HepG2 cells identified by a high sensitivity intramolecular FRET glucose sensor. Biochim Biophys Acta 1778:1091-1099

Figure Legends

Fig. 1. Direct interaction between glucokinase and the GRP in rat hepatocytes. Primary rat hepatocytes were seeded on glass coverslips and incubated for 2 h with 5 mmol/l (white bars) or 20 mmol/l (black bars) glucose in the absence or presence of 200 µmol/l fructose-6-phosphate (F-6-P) or fructose-1-phosphate (F-1-P), respectively. Finally, the cells were fixed and immunostained for glucokinase and glucokinase regulatory protein. The appropriate secondary antibody was labeled with Cy3 for glucokinase and Cy5 for glucokinase regulatory protein. FRET was measured as an increase in Cy3 fluorescence after Cy5 photobleaching. Data are expressed as means ± SEM from three individual experiments with a total of 17-19 hepatocytes analyzed. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to cells incubated with 5 mmol/l glucose in the presence of fructose-6-phosphate (ANOVA/Bonferroni's test).

Fig. 2. Localization of GRP and glucokinase in rat hepatocytes. Primary rat hepatocytes were seeded on glass coverslips and incubated for 2 h with 5 mmol/l (A, C (white bars)) or 20 mmol/l (B, C (black bars)) glucose. Finally, the cells were fixed and immunostained for glucokinase and GRP. (A, B) Representative images for the GRP are shown. Scale bar 2 µm (C). The nuclear/cytoplasmic ratio was calculated for glucokinase and GRP. Data are expressed as means ± SEM from three individual experiments with a total of 11 hepatocytes analyzed. ***, $p < 0.001$ (ANOVA/Bonferroni's test).

Fig. 3. Shuttling of glucokinase and GRP in rat hepatocytes. Primary hepatocytes were transfected with Dendra2, Dendra2-glucokinase or Dendra2-GRP (A), or EYFP, EYFP-glucokinase or EYFP-GRP (B, C) and incubated for 2 h with 5 mmol/l (white bars) or 20 mmol/l (black bars). (A) Green Dendra2 was exclusively photoconverted in the nucleus and the decrease of the red Dendra2 was determined over 15 sec. EYFP was bleached in a region in the nucleus (B) or the cytoplasm (C). Finally the recovery of EYFP 15 sec after photobleaching was determined in the same region. Data are expressed as means ± SEM from

three individual experiments with a total of 9 hepatocytes analyzed. *, $p < 0.05$ (ANOVA/Bonferroni's test).

Fig. 4. Glucokinase activity and immunoreactivity in rat hepatocytes and MIN6 beta cells expressing GRP. MIN6 cells were transiently transfected with EYFP-GRP or with the fluorescence protein EYFP alone as control. (A) Glucokinase activity was analyzed in cytosolic (white bars) and nuclear (black bars) fractions. Data are expressed as means ± SEM from three individual experiments. ***, $p < 0.001$ (ANOVA/Bonferroni's test) EYFP expressing MIN6 cells compared to EYFP-GRP expressing MIN6 cells. (B) Glucokinase immunoreativity was determined in cytosolic and nuclear fractions. A total of 40 µg cellular protein was loaded. Representative blots of three individual experiments are shown. Localization of EYFP (C) and EYFP-GRP (D) in MIN6 cells were visualized by fluorescence microscopy. Representative images of three individual experiments are shown. Scale bar 20 µm.

Fig. 5. Nuclear and cytoplasmic glucose influx and efflux in rat hepatocytes and MIN6 beta cells compared to COS cells. COS cells, MIN6 cells and primary rat hepatocytes were transfected with either cytosolically FLIPglu-Cyt (white bars) or the nuclear localized FLIPglu-Nuc (black bars). In addition, MIN6 cells were co-transfected with pTagRFP-GRP and FLIPglu-Cyt (white striped bars) or FLIPglu-Nuc (black striped bars). During real-time fluorescence microscopy cells were perifused without glucose (0-20 min), thereafter with 10 mmol/l glucose (20-40 min), and finally without glucose. The FRET ratio was calculated from $citrine_{em}$/ $ECFP_{em}$ at $ECFP_{ex}$. Glucose influx (A) was quantified by calculating the ratio increase upon glucose stimulation. Glucose metabolism and efflux (B) was quantified by calculating the subsequent ratio decrease after glucose deprivation. Data are expressed as means ± SEM from 7-20 individual experiments. *, $p < 0.05$ compared to MIN6 and COS 7

cells expressing FLIPglu-Nuc (ANOVA/Bonferroni's test). ###, $p < 0.001$ compared to MIN6 cells expressing FLIPglu-Nuc (student's t test).

Fig. 1

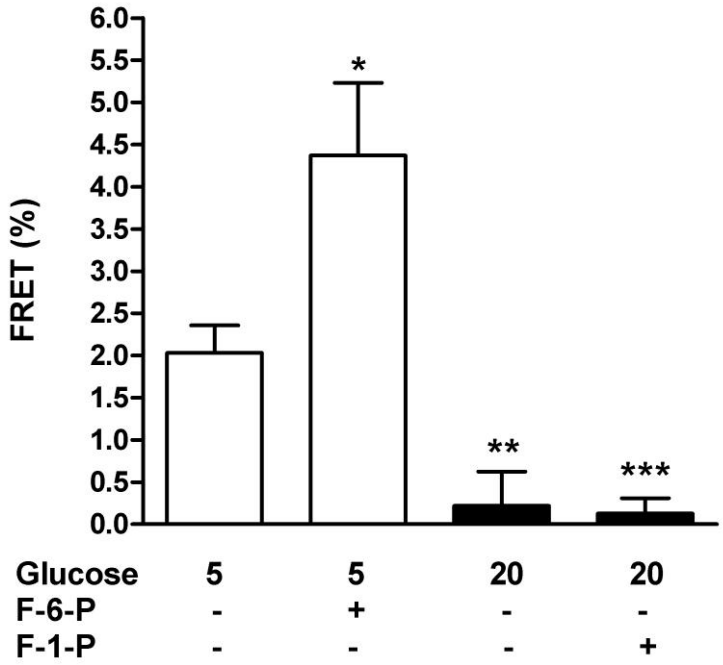


Fig. 2

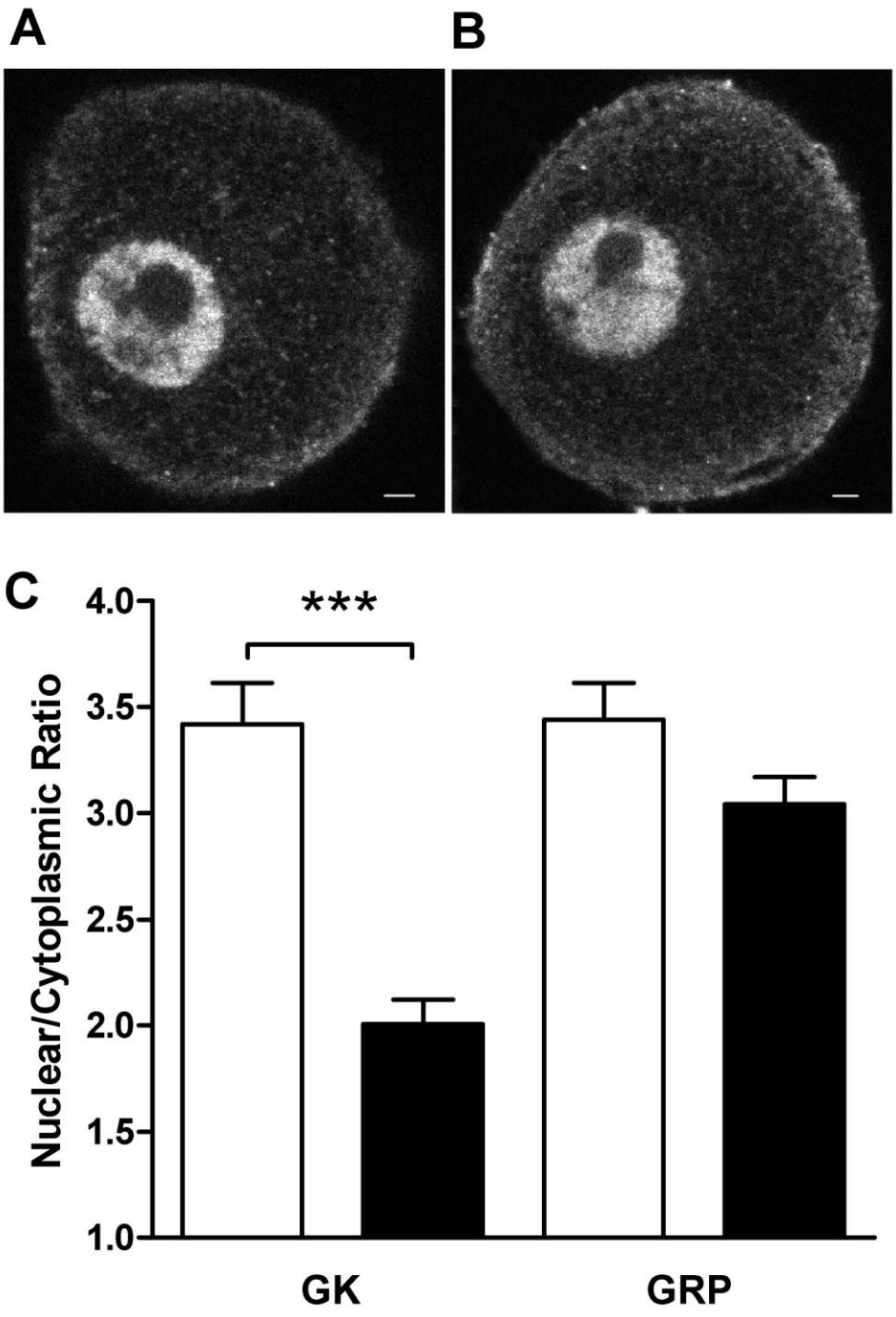


Fig. 3

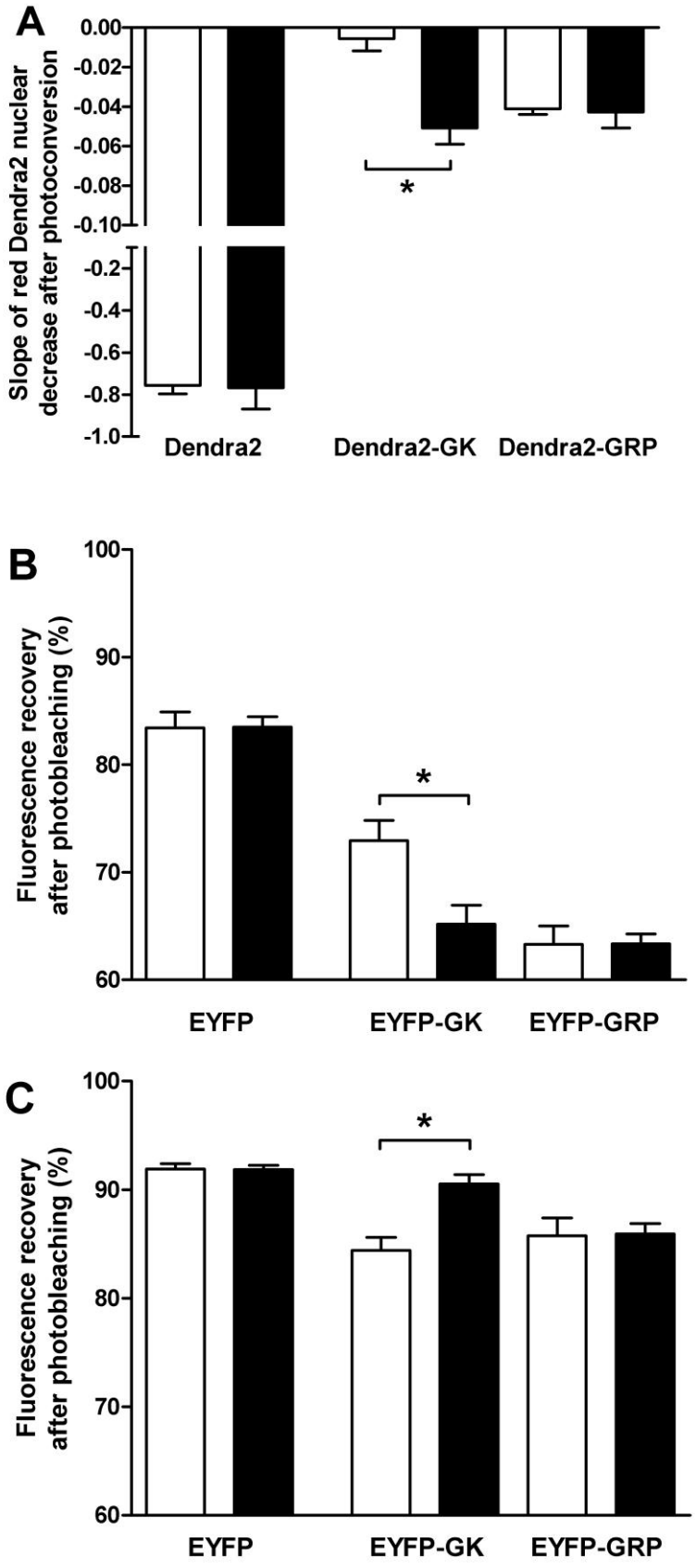

A
Slope of red Dendra2 nuclear decrease after photoconversion
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
-0.2
-0.4
-0.6
-0.8
-1.0
*
Dendra2
Dendra2-GK
Dendra2-GRP
B
Fluorescence recovery after photobleaching (%)
100
90
80
70
60
*
EYFP
EYFP-GK
EYFP-GRP
C
Fluorescence recovery after photobleaching (%)
100
90
80
70
60
*
EYFP
EYFP-GK
EYFP-GRP

Fig. 4

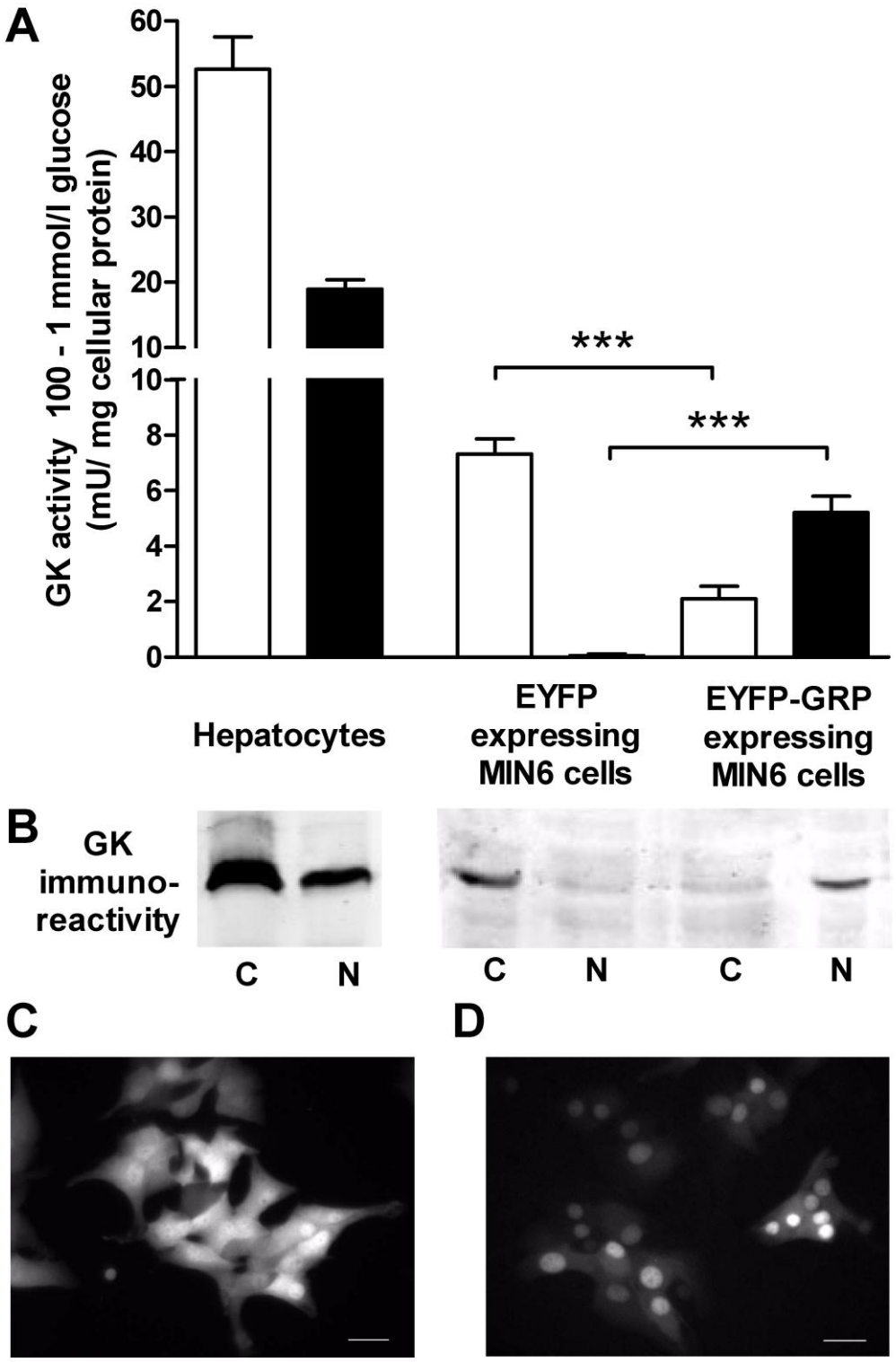
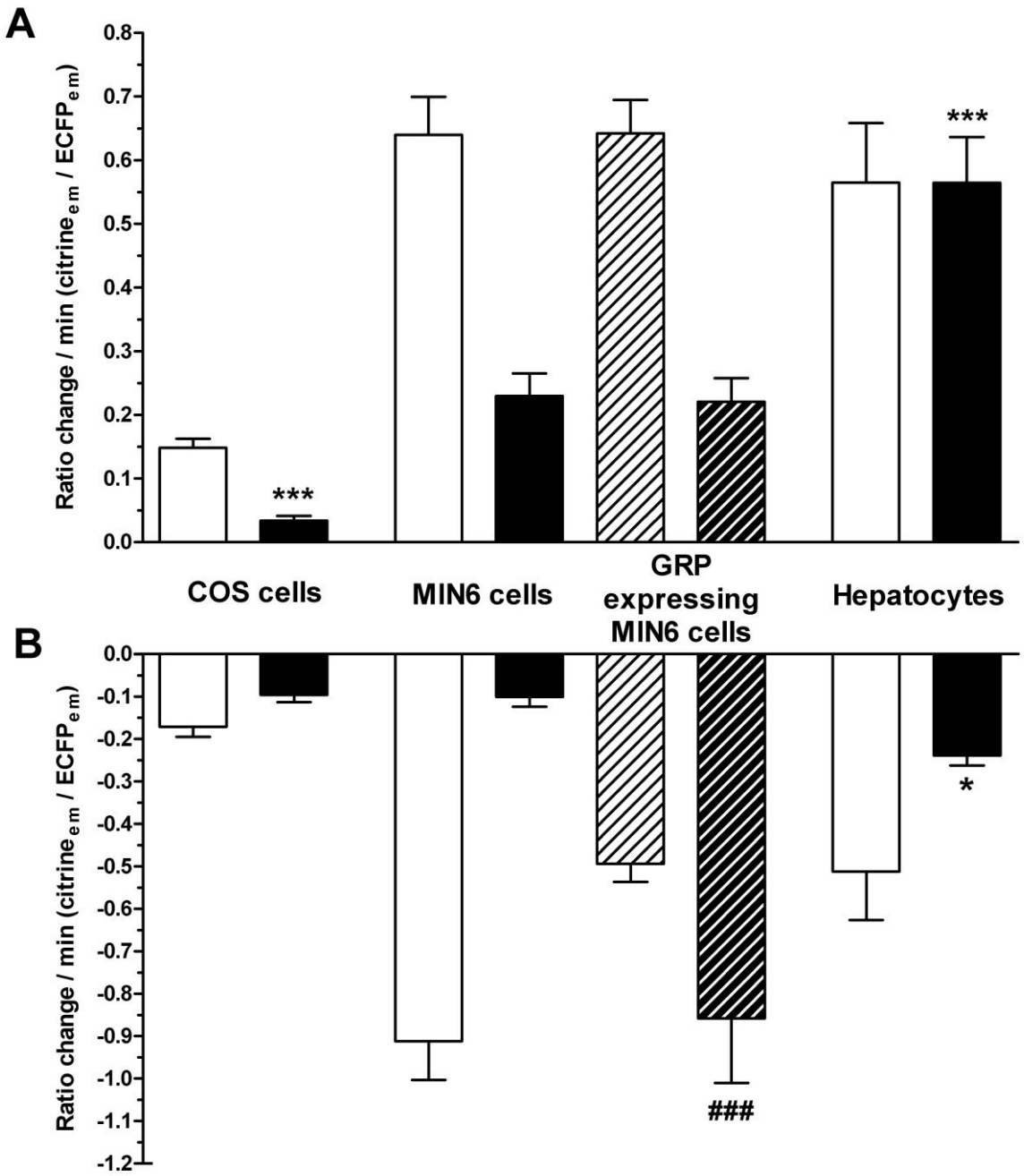

A
GK activity 100 - 1 mmol/l glucose
(mU/ mg cellular protein)
60
50
40
30
20
10
10
8
6
4
2
0
***
***
Hepatocytes
EYFP
expressing
MIN6 cells
EYFP-GRP
expressing
MIN6 cells
B
GK
immuno-
reactivity
C
N
C
N
C
N
C
D

Fig. 5


A
Ratio change / min (citrine em / ECFP em)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
***
COS cells
MIN6 cells
GRP expressing MIN6 cells
Hepatocytes
B
Ratio change / min (citrine em / ECFP em)
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
-0.9
-1.0
-1.1
-1.2
*
###

3 Diskussion

3.1 Etablierung fluoreszenzbasierter Glucosesensoren in der Aufklärung des intrazellulären Glucosefluxes in Beta-Zellen

In den Beta-Zellen des Pankreas ist die Glucoseaufnahme und -verstoffwechselung der bestimmende Faktor für die bedarfsgerechte Insulinsekretion und die Aufrechterhaltung der Blutglucosehomöostase. Eine direkte zeitaufgelöste Analyse des intrazellulären Glucosefluxes kann dabei wertvolle Erkenntnisse über die nachfolgenden Signalwege und insbesondere über das Zusammenspiel des *triggering pathway* und des *amplifying pathway* liefern (Panten 1989; Henquin 2000; Henquin 2009).

Die Entwicklung von fluoreszenzbasierten Metabolitsensoren innerhalb der letzten Jahre eröffnet neue Möglichkeiten zur Untersuchung von Stoffwechselwegen. So stehen Sensoren für Ca^{2+} (Miyawaki *et al.* 1997), cAMP (Adams *et al.* 1991; Dyachok *et al.* 2008a) und ATP (Heart *et al.* 2006b) zur Verfügung, die direkte ratiometrische Messungen mit Hilfe der Fluoreszenzmikroskopie ermöglichen. Neuere Studien beschäftigten sich mit der Generierung spezifischer FRET-basierter „Nanosensoren“ für die Detektion intrazellulärer Glucosekonzentrationen (Fehr *et al.* 2003a; Fehr *et al.* 2004a; Fehr *et al.* 2004b; Deuschle *et al.* 2005a; Chaudhuri *et al.* 2007; Frommer *et al.* 2009; Bittner *et al.* 2010; Chaudhuri *et al.* 2011). Diese Sensoren wurden bereits erfolgreich für unterschiedliche Fragestellungen zum Einsatz gebracht. Dabei wurden diese Konstrukte sowohl in Säugerzellen als auch in Pflanzen und Hefen exprimiert (Fehr *et al.* 2003a; Fehr *et al.* 2004a; Fehr *et al.* 2004b; Deuschle *et al.* 2005b; Fehr *et al.* 2005b; Bermejo *et al.* 2010; Prebil *et al.* 2011). Für weiterführende Studien konnten zudem die Eigenschaften dieser Sensoren in Bezug auf die Detektionsbreite und die Affinität zum Substrat durch gezielte Mutationen verbessert werden (Deuschle *et al.* 2005b; Fehr *et al.* 2005b).

Da die Funktion der Beta-Zellen wesentlich an die Anwesenheit und Verstoffwechselung der Glucose gebunden ist, war es das Ziel der vorliegenden Arbeit, den intrazellulären Glucoseflux weiterführend zu untersuchen. Zu diesem Zweck wurde die Expression eines Glucosesensors in verschiedenen klonalen und primären Beta-Zellen etabliert. Von besonderem Interesse sind des Weiteren die Hepatozyten, die ebenfalls entscheidend zur Glucosehomöostase beitragen.

Die Durchführung fluoreszenzmikroskopischer Echtzeitanalysen der intrazellulären Glucosekonzentration erfolgte in der vorliegenden Arbeit unter Anwendung des FRET-basierten Glucosesensors FLII^{12}Pglu-700µ-δ6 (Takanaga *et al.* 2008b). Der Anstieg der Ratio der Citrin/ECFP Emission nach Anregung des ECFP war bei diesem Glucosesensor in einem Bereich zwischen 1 mmol/l und 10 mmol/l Glucose linear. Somit konnten Veränderungen der Glucose in einem für Beta-Zellen physiologisch relevanten Bereich aufgelöst werden. Die Dissoziationskonstante des Glucosesensors liegt bei 660 ± 160 µM (Takanaga *et al.* 2008b). In den eigenen Messungen konnte ein vergleichbarer Wert von 572 ± 99 µM ermittelt werden. Der Sensor wurde daher nachfolgend erstmals in MIN6-Zellen sowohl transient als auch stabil überexprimiert. Es konnte in den durchgeführten Versuchen kein Einfluss des Sensors auf den Glucosestoffwechsel festgestellt werden. Die stabil transfizierten MIN6-Zellen zeigten im Vergleich zu untransfizierten Zellen keine Veränderungen in der glucosestimulierten Insulinsekretion. Um weiterführend kompartimentspezifische Untersuchungen zum Glucoseflux durchführen zu können, wurde die cDNA des FLIPglu Glucosesensors mit einer kernspezifischen *targeting*-Sequenz versehen, die eine gezielte Expression des Sensors im Nukleus ermöglichte.

Die Expression dieser Sensoren in isolierten primären Zellen wurde mit Hilfe einer zu diesem Zwecke generierten adenoviralen Expressionskassette erreicht. Neben dem zytoplasmatisch exprimierten FLIPglu Glucosesensor wurde auch der nukleär exprimierte FLIPglu Glucosesensor exprimiert. Im Gegensatz zur liposomalen Transfektion erlauben Adenoviren eine effiziente Einbringung des Sensors in isolierten primären Gewebezellen. Mit Hilfe dieses rekombinanten Transduktionssystems war es möglich, die Glucosesensoren auch in isolierten Beta-Zellen der Maus und Hepatozyten der Ratte erfolgreich zur Expression zu bringen und den Glucoseflux zu analysieren. Die in dieser Arbeit generierten Transfektionsvektoren ermöglichen zukünftig Studien in nahezu allen primären Zellen.

Die Quantifizierung der Glucoseaufnahme in primären Beta-Zellen der Maus, MIN6 Zellen und COS-Zellen erbrachte signifikante Unterschiede in der Aufnahmekapazität dieser Zellen. Verantwortlich hierfür ist das jeweilige Expressionsmuster der Glucosetransporter. Der in den COS-Zellen und allen Geweben ubiquitär exprimierte Glucosetransporter GLUT1 vermittelt eine kontinuierliche, aber von der Kapazität niedrige Glucoseaufnahme (Fehr *et al.* 2003a; Fehr *et al.* 2004b). Die Expression des Glucosetransporters GLUT2 in Beta-Zellen und MIN6 Zellen führt hingegen zu einer wesentlich höheren Glucoseaufnahmekapazität. Bei einer Glucosekonzentration von 10 mmol/l erreicht dieser niedrigaffine Glucosetransporter eine maximale Transportkapazität. Die Aufnahmekapazität

war in den isolierten primären Maus-Beta-Zellen im Vergleich zu den MIN6 Zellen weiter gesteigert. Die klonale Beta-Zelllinie MIN6 besitzt zwar wesentliche Eigenschaften von primären Beta-Zellen im Hinblick auf die glucosestimulierte Insulinsekretion (Ishihara *et al.* 1993), stellt jedoch als Tumorzelllinie eine Mischform verschiedener endokriner Zellen des Pankreas dar (Nakashima *et al.* 2009) und exprimiert den GLUT2 auf einem niedrigen Niveau (Schuit 1997; Uldry & Thorens 2004). Der Vergleich der insulinsezernierenden Zellen untereinander zeigte allerdings, dass die Glucosephosphorylierung, bzw. der nachfolgende Metabolismus vergleichbar waren. Im Gegensatz zu den COS-Zellen, die ausschließlich die Hexokinase exprimieren, war der Glucosemetabolismus in den Glucokinase exprimierenden Zellen signifikant erhöht. Dies unterstreicht die Bedeutung der Glucokinase als Glucosesensor in den insulinproduzierenden Zellen (Meglasson & Matschinsky 1984; Lenzen & Panten 1988b; Matschinsky 1990; Thorens 1992; Baltrusch & Tiedge 2006a). Durch den Einsatz des kompetitiven Glucokinaseinhibitors Mannoheptulose konnte dies weiterführend bestätigt werden. Auf COS-Zellen hatte Mannoheptulose keine Wirkung. Mannoheptulose verringerte die Eliminierungsrate der Glucose nur in den Glucokinase-exprimierenden Zellen durch Reduktion der Glucokinaseenzymaktivität (Tiedge *et al.* 1997; Lenzen & Peckmann 2001b). Final führte dies zu einer Verringerung der glucosestimulierten Insulinsekretion. Die Glucoseaufnahme hingegen wurde in allen untersuchten Zellen durch Zugabe äquimolarer Konzentrationen von 3-O-Methylglucose (3-OMG) signifikant verringert. 3-OMG als Kompetitor der Glucoseaufnahme (Tiedge *et al.* 1993) reduzierte zusätzlich in den COS-Zellen die Eliminierungsrate der Glucose. Im Gegensatz zu den insulinproduzierenden Zellen ist daher die Aufnahme der Glucose über den GLUT1 Transporter geschwindigkeitsbestimmend für die intrazelluläre Glucosehomöostase.

3.2 Metabolische Oszillationen und ihre Bedeutung für die Insulinsekretion

In der glucoseinduzierten Insulinsekretion spielen metabolische Oszillationen eine wichtige Rolle (Kennedy *et al.* 2002). Charakteristisch sind hierbei besonders Oszillationen des Calciums nach Schließen der ATP abhängigen K_{ATP} Kanäle und nachgeschaltetem Öffnen der spannungsabhängigen Ca^{2+}-Kanäle. Überdies konnten bereits Oszillationen des zytosolischen cAMPs nachgewiesen werden, die in Phase mit denen des Calciums auftreten

(Dyachok *et al.* 2008a). Oszillationen der Glucose wurden als möglicher erster Schritt dieser Signalkaskade postuliert (Jung *et al.* 2000a; Kennedy *et al.* 2002). Die Messungen, die zu dieser Aussage führten, wurden unter Verwendung von Enzymelektroden durchgeführt, die in den Extrazellularraum einzelner isolierter Inseln eingeführt wurden. Es konnte daher keine Aussage über den intrazellulären Glucoseflux erfolgen (Jung *et al.* 2000a; Kennedy *et al.* 2002). In der vorliegenden Arbeit konnten jedoch, im Gegensatz zu den synchronisierten Oszillationen des Calciums, keine solchen Oszillationen der intrazellulären Glucose unter Anwendung des FLIPglu Glucosesensors nachgewiesen werden. Dies deutet darauf hin, dass die Generierung des oszillierenden Signals dem Glucoseflux nachgeschaltet erfolgt, nicht aber durch das Zusammenspiel von Glucosetransport und -phosphorylierung beeinflusst wird.

Die Anwendung FRET basierter intrazellulärer Nanosensoren ermöglicht die direkte Analyse des Glucoseflux in zeitlichem und räumlichem Zusammenhang. Das hierbei verwendete FRET-Paar ECFP/Citrin ermöglicht überdies eine simultane Analyse mit anderen fluoreszenzbasierten Indikatoren für weitere zelluläre Metabolite. In der vorliegenden Arbeit wurde der Glucoseflux simultan mit dem Calciumflux gemessen. Es wurde erstmals eine simultane Analyse mit Hilfe des FLIPglu Glucosesensors und des Calciumindikators FURA durchgeführt. Damit konnte ein primärer und distaler Schritt der glucosestimulierten Insulinsekretion in derselben Zelle mit hoher zeitlicher Auflösung dargestellt werden. Die Stimulation von MIN6 Zellen mit Glucose führte zu einem nachfolgenden Einstrom von Calcium in das Zytoplasma (Lenzen & Peckmann 2001b; Baltrusch & Lenzen 2007b). Nach dem initialen Anstieg des Calciums kam es zu synchronisierten Calciumoszillationen mit hoher Amplitude. Es konnte ein Schwellenwert von etwa 4 mmol/l Glucose bestimmt werden, der den initialen Einstrom des Calciums bewirkt. Dieser Wert liegt in der Nähe des Wendepunkts der sigmoidalen Reaktionskinetik der Glucokinase und kann daher auch als Schwellenwert für das Auslösen der Insulinsekretion betrachtet werden (Lenzen & Panten 1988b; Thorens 1992; Baltrusch & Tiedge 2006a). Oszillationen des Calciums konnten nur bei einer intrazellulären Glucosekonzentration von ca. 6-7 mmol/l beobachtet werden. Dies ist ein Indiz dafür, dass eine gleichbleibend hohe intrazelluläre Glucosekonzentration die Voraussetzung für die zweite Phase der Insulinsekretion ist, während der Anstieg der Glucose über den beschriebenen Schwellenwert die Insulinsekretion der ersten Phase auslöst. Der Anstieg der intrazellulären Glucose konnte durch Zugabe von 3-OMG (Sener *et al.* 1999a) signifikant verringert werden und bewirkte einen verspäteten Calciumeinstrom. Die Insulinsekretion,

gemessen nach zweistündiger statischer Inkubation, blieb jedoch nahezu unbeeinflusst nach Erreichen des Schwellenwertes. Der Einsatz des kompetitiven Glucokinaseinhibitors Mannoheptulose hingegen führte einerseits zu einem verspäteten Einsetzen des Calciumeinstroms, andererseits aber auch zu einer Verringerung der Calciumkonzentration nach Stimulation und einer verminderten Insulinsekretion in der statischen Messung (Johnson *et al.* 2007).

3.3 Auslösen der Insulinsekretion (*triggering*) und verstärkende Signale (*amplification*) der Insulinsekretion

Der Einsatz von Mannoheptulose verringerte ebenfalls die Amplitude der Calciumoszillationen im Vergleich zu der Kontrollsituation (Lenzen & Peckmann 2001b). Die Calciumoszillationen wurden weiterhin durch den Einsatz von Tolbutamid charakterisiert. Die Inkubation mit dem K_{ATP}-Kanal schließenden Wirkstoff führte ohne Anstieg der intrazellulären Glucosekonzentration zu einem Anstieg der zytoplasmatischen Calciumkonzentration. Jedoch blieben die durch den Glucosestimulus induzierten Oszillationen aus (Grapengiesser *et al.* 1990; Lenzen & Peckmann 2001b). Diese konnten erst nach Anstieg der intrazellulären Glucosekonzentration durch Zugabe von Glucose in das Umströmungsmedium beobachtet werden. Der Einsatz von Diazoxid hingegen führte zur Öffnung des K_{ATP}-Kanals und damit zu einer dem Tolbutamid entgegengesetzten Wirkung (Trube *et al.* 1986). Diazoxid bewirkt nicht nur ein Ausbleiben des Calciumeinstroms, sondern auch Veränderungen des mitochondrialen Stoffwechsels (Grimmsmann & Rustenbeck 1998). Die Inkubation mit Diazoxid führte zur einer signifikanten Verringerung sowohl des Calciumeinstroms als auch der Insulinsekretion in Gegenwart stimulatorischer Glucosekonzentrationen (Johnson *et al.* 2007).

Es kann daher postuliert werden, dass die Glucosekonzentration in den Beta-Zellen einen Schwellenwert erreichen muss, der dann den Calciumeinstrom über den K_{ATP}-Kanal bewirkt. Die typischen Oszillationen des Calciums werden nach den Ergebnissen dieser Arbeit nicht durch die Glucosephosphorylierung, sondern erst durch nachfolgend generierte Schlüsselmoleküle ausgelöst und verstärkt (Henquin 1988; Panten *et al.* 1988; Gembal *et al.* 1992; Henquin 2000; Henquin 2009). Eine Reihe solcher Moleküle werden als potentielle Faktoren für die Amplifizierung der Insulinsekretion diskutiert, darunter das NADPH

(Ivarsson *et al.* 2005; Panten & Rustenbeck 2008) und das cAMP (Dyachok *et al.* 2006; Dyachok *et al.* 2008a). Oszillationen des cAMP werden ebenfalls durch die Anwesenheit der Glucose hervorgerufen und durch Ca^{2+} verstärkt. Die Oszillationen des cAMP korrelieren zudem mit Schwankungen der Insulinsekretion. Auch die Translokation von Insulingranula (Rorsman & Renstrom 2003; Baltrusch & Lenzen 2008) kann zu Oszillationen beitragen, die im Verlauf der glucosestimulierten Insulinsekretion generiert werden.

Der mitochondriale Metabolismus wird als wichtiger Amplifikator der Insulinsekretion erachtet (Kennedy *et al.* 1999b; Jung *et al.* 2000a; Kennedy *et al.* 2002; Heart *et al.* 2006b; Henquin 2009). Dieser Verstärkungsmechanismus steht in direktem Zusammenhang mit der Verstoffwechselung der Glucose, kann aber auch durch externe Stimuli die Insulinsekretion beeinflussen. Insbesondere Intermediate des Citratzyklus wie das Citrat wurden als Schlüsselfaktoren beschrieben (Urban & Panten 2005; Panten & Rustenbeck 2008).

Die Ergebnisse dieser Studien zeigen, dass der Glucoseflux durch das Schrittmacherenzym Glucokinase im Zusammenspiel mit dem Glucosetransporter GLUT2 kontrolliert wird. Allerdings kann die Glucokinase nicht als Schrittmacher für die Entstehung metabolischer Oszillationen angesehen werden. Die durchgeführte kombinierte Analyse von Glucose und dem intrazellulären Ca^{2+} deutet auf generierende und amplifizierende Signale im nachfolgenden mitochondrialen Stoffwechsel hin.

3.4 Einfluss der Glucosekonzentration auf die Bindung des endogenen Aktivators PFK2 an die Glucokinase.

Die glucosestimulierte Insulinsekretion in den Beta-Zellen des Pankreas sowie die Speicherung bzw. Bereitstellung der Glucose in der Leber werden beide entscheidend durch das Zusammenspiel des Glucosesensors Glucokinase und den Glucosetransporter GLUT2 vermittelt. Während der GLUT2 es den Zellen ermöglicht, ein schnelles Equilibrium zwischen extra- und intrazellulärer Glucose herzustellen, übernimmt das Sensorenzym Glucokinase die geschwindigkeitsbestimmende initiale Phosphorylierung des Zuckers und damit dessen Einschleusung in den Metabolismus. Während das Leberisoenzym der Glucokinase durch Insulin auf transkriptionellem Wege reguliert wird, ist die Expression der Glucokinase in den pankreatischen Beta-Zellen von der Nahrungsverfügbarkeit weitgehend unbeeinflusst (Iynedjian *et al.* 1989; Tiedge *et al.* 1999).

Im Wesentlichen erfolgt jedoch die Regulation der Glucokinaseaktivität sowohl in den Beta-Zellen des Pankreas als auch in der Leber auf posttranslationaler Ebene. Neben der Glucose selbst als primärem Regulator der Glucokinaseaktivität wird die Enzymaktivität dabei durch Kompartimentierung und Protein-Protein-Interaktionen beeinflusst (Van Schaftingen 1989a; de la Iglesia *et al.* 1999; Tiedge *et al.* 1999; Bosco *et al.* 2000a; Rizzo *et al.* 2002; Baltrusch & Lenzen 2007a). In beiden Geweben tritt besonders das bifunktionelle Enzym 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase (PFK-2/FBPase-2) als endogener Aktivator der Glucokinase auf (Baltrusch *et al.* 2001). Die Erhöhung der intrinsischen Glucokinaseaktivität erfolgt dabei im Gegensatz zu pharmakologischen Aktivatoren ohne eine Beeinflussung der Affinität und der Kooperativität zum Substrat Glucose (Massa *et al.* 2004). Nur in der Leber erfolgt die posttranslationale Regulation der Glucokinase zusätzlich durch Bindung des leberspezifischen GRP und eine Translokation der GK in den Nukleus bei niedrigen Glucosekonzentrationen.

Die PFK-2/FBPase-2 aktiviert die Glucokinase über die FBPase-2 - Untereinheit (Baltrusch *et al.* 2001; Massa *et al.* 2004; Baltrusch *et al.* 2006; Smith *et al.* 2007). Sie verfügt hierbei in beiden Isoformen über eine spezifische Aminosäuresequenz (LKVWT), die die Bindung an die GK ermöglicht (Baltrusch *et al.* 2001). Eine Überexpression der PFK-2/FBPase-2 führte in insulinproduzierenden Zellen nur bei stimulatorischen Glucosekonzentrationen zu einer signifikanten Steigerung der Enzymaktivität der Glucokinase und final zu einer erhöhten Insulinsekretion. In der vorliegenden Arbeit galt daher der Frage besondere Bedeutung, inwieweit die Glucose selbst die Interaktion zwischen der Glucokinase und den endogenen Aktivatoren beeinflusst. Der Einsatz der FRET-Technik mit fluoreszierenden Fusionskonstrukten der GK, des GRP und der PFK-2/FBPase-2 ermöglichte die intrazelluläre Analyse der Bindung. Dadurch konnte gezeigt werden, dass die PFK-2/FBPase-2 nur unter hohen Glucosekonzentrationen an die GK bindet. Im Gegensatz dazu verstärkte sich die Interaktion zwischen GRP und Glucokinase bei niedrigen Glucosekonzentrationen.

3.5 Glucokinaseregulation in der Leber - Die Rolle der GRP vermittelten Kerntranslokation

Die Interaktion der Glucokinase mit dem GRP stellt den wichtigsten Regulationsmechanismus der Enzymaktivität in der Leber dar. Es vermittelt die

glucoseabhängige Translokation der Glucokinase in den Nukleus bei gleichzeitiger Inaktivierung des Enzyms (Shiota *et al.* 1999b). Die Abwesenheit eines Kernimportsignals für die GK bedingt die notwendige Bindung an das GRP für den Kernimport. Ohne das Vorhandensein des GRP zeigte die Glucokinase eine zytoplasmatische Verteilung. Die Lokalisierung der beiden Bindungspartner spielt dabei in Abhängigkeit von der aktuellen intrazellulären Glucosekonzentration eine wesentliche Rolle. Das GRP ist unabhängig von der Glucosekonzentration zum größeren Anteil im Kern lokalisiert und nur zu einem geringen Anteil im Zytoplasma. Hingegen ist die Veränderung der Kern/Zytoplasma Verteilung für die Glucokinase allgemein akzeptiert. Das Enzym ist nur bei niedriger Glucosekonzentration vermehrt im Kern nachweisbar, während unter 25 mM Glucose eine deutliche zytoplasmatische Lokalisation zu beobachten war (Fernandez-Novell *et al.* 1999). Die Ergebnisse dieser Studie deuten darauf hin, dass das GRP in diesem Mechanismus eine Transporterfunktion übernimmt, während es selbst einem kontinuierlichen Austausch zwischen nukleärer und zytoplasmatischer Fraktion unterliegt. Die Bindung der Glucokinase an das GRP scheint damit im Wesentlichen durch die Glucosekonzentration determiniert zu werden (Mukhtar *et al.* 1999). Im Metabolismus generierte Fructose-Phosphate nehmen überdies eine modulatorische Funktion ein. Während Fructose-1-Phosphat die Bindung verstärkt, wird diese durch Fructose-6-Phosphat geschwächt (Niculescu *et al.* 1997). Nicht endogene kompetitive Inhibitoren der Glucokinase, wie die Mannoheptulose, die wie die Glucose eine Konformationsänderung des Enzyms bewirken, können dagegen die Dissoziation der Glucokinase erleichtern (Niculescu *et al.* 1997). Die konnte auch eindrucksvoll durch den Einsatz der synthetischen Glucokinaseaktivatoren gezeigt werden. Diese stabilisieren die geschlossene Konformation der Glucokinase, die keine Bindung an das GRP zulässt (Futamura *et al.* 2006; Heredia *et al.* 2006a).

Eine erhöhte Motilität der Glucokinase und die damit verbundene Translokation aus dem Zellkern ins Zytoplasma sind als Antwort auf eine erhöhte Blutglucosekonzentration von besonderem Interesse. Die Mechanismen, welche zur Dissoziation der Glucokinase und zum Export aus dem Nukleus heraus führen, stehen daher im Fokus der vorliegenden Arbeit. Bislang ist es unklar, ob der Kernexport der Glucokinase unabhängig von der GRP Bindung verläuft, oder ob diese Bindung auch für den Export zwingend erforderlich ist (Mukhtar *et al.* 1999). Die Glucokinase könnte auf Grund ihrer Größe den Zellkern unabhängig von spezifischen Transportmechanismen verlassen. Vorangehende Studien postulierten allerdings ein Kernexportmotif in der Glucokinase als Ursache für eine GRP-unabhängige Translokation aus dem Zellkern (Shiota *et al.* 1999b). Da der Kernexport der GK jedoch

nicht durch den Einsatz von Leptomycin B verhindert werden konnte, stellte sich die Frage, inwieweit ein Nachweis für die Dissoziation der Glucokinase vom GRP innerhalb des Zellkerns tatsächlich erbracht werden konnte.

Um diese Fragestellung weiter untersuchen zu können, wurden Fusionskonstrukte des GRP in insulinproduzierenden MIN6 Zellen zur Expression gebracht. Diese Zellen exprimieren, wie primäre Beta-Zellen, nur die Glucokinase, jedoch nicht das GRP. Somit konnte unter Kontrollbedingungen keine Enzymaktivität der GK in nukleären Fraktionen nachgewiesen werden. Dagegen war die GK in den EYFP-GRP transfizierten Zellen im Zellkern nachweisbar und zudem GK Enzymaktivität messbar. Dies bestätigt die Rolle des GRP für den nukleären Import der Glucokinase in einem artifiziellen System (Bosco *et al.* 2000a; Baltrusch *et al.* 2005). Überdies zeigen diese Versuche, dass der Import der GK in den Nukleus nicht von weiteren leberspezifischen Faktoren abhängig ist.

In primären Hepatozyten konnte mittels Photoaktivierung und der FRAP-Technik (*Fluorescence Recovery After Photobleaching*) erstmalig gezeigt werden, dass die Motilität und der Kernexport der Glucokinase, nicht aber des GRP, von der Glucosekonzentration abhängig ist. Während bei niedriger Glucosekonzentration nur eine sehr geringe Abnahme der nukleären GK-Fluoreszenz zu beobachten war, war dieser bei hohen Glucosekonzentrationen gesteigert. Interessanterweise konnte im Zellkern von Hepatozyten mit Hilfe des FLIPglu Sensors im Vergleich zu den MIN6 Zellen sowohl eine gesteigerte Aufnahmekapazität für die Glucose als auch ein erhöhter Glucosemetabolismus beobachtet werden.

Während ein gesteigerter Glucosemetabolismus durch Überexpression des GRP auch in MIN6 Zellen erreicht werden konnte, blieb die Glucoseaufnahme in den Zellkern unverändert. Diese Ergebnisse liefern den direkten Hinweis auf einen vom GRP unabhängigen nukleären Export der Glucokinase. Das Enzym ist nachweislich bereits im Zellkern aktiv und kann daher nicht mehr an das GRP gebunden vorliegen. Es lässt sich spekulieren, dass durch die besonders effiziente Glucoseaufnahme in den Zellkern von Hepatozyten die Dissoziation des GK-GRP-Komplexes beschleunigt wird. Es gilt jedoch zu klären, ob die aktive Glucokinase weitere physiologische Funktionen im Zellkern von Hepatozyten erfüllt.

4 Zusammenfassung

Die Aufrechterhaltung der Blutglucosehomöostase erfordert das konzertierte Zusammenspiel mehrerer Organe, denen die Expression von glucosesensorischen Elementen gemein ist. Insbesondere sind dies die Beta-Zellen des Pankreas und die Leber. In beiden Geweben wird zum einen das Enzym Glucokinase exprimiert, welches als Glucosesensor eine Schlüsselfunktion in der Erhaltung der Blutglucosehomöostase einnimmt. Zum anderen exprimieren beide Gewebe den niedrigaffinen Glucosetransporter GLUT2, welcher einen schnellen Ausgleich zwischen extra- und intrazellulärer Glucose bei hohen Glucosekonzentrationen gewährleistet. Das Zusammenspiel dieser beiden Faktoren bestimmt die Kopplung extrazellulärer Glucosekonzentrationen an intrazelluläre metabolische Signalkaskaden.

In den Beta-Zellen des Pankreas wird das Peptidhormon Insulin gebildet und durch eine Stimulus-Sekretions-Kopplung nach Anstieg der Blutglucose bedarfsgerecht sezerniert. Diese Signalkaskade kann im Wesentlichen in zwei Vorgänge geteilt werde, den sogenannten *triggering pathway*, welcher primär die glucoseinduzierte Insulinsekretion auslöst, und den *amplifying pathway*, der sekundär durch Stoffwechselintermediate die Insulinsekretion verstärkt. Für beide Wege ist jedoch die Glucose und ihre Verstoffwechselung notwendig. Eine zeitaufgelöste Analyse der intrazellulären Glucose kann daher wertvolle Informationen über den Zusammenhang von Glucoseflux und Insulinsekretion liefern. In der hier vorliegenden Studie konnten erstmals sogenannte FRET-basierte Nanosensoren für Glucose in insulinproduzierenden klonalen und primären Beta-Zellen zur Expression gebracht werden. Hierfür wurden zum einen eine MIN6 Zelllinie stabil transfiziert und zum anderen ein adenovirales Expressionssystem für die Transduktion primärer Zellen generiert. Die Glucosesensoren basieren auf einer für die Glucose spezifischen Bindungsdomäne, an die ein FRET-Paar aus ECFP und Citrin fusioniert wurde. Mittels Fluoreszenzmikroskopie kann so die intrazelluläre Glucosekonzentration in Echtzeit dargestellt werden.

Die Bedeutung des GLUT2 Glucosetransporters und der Glucokinase für die Beta-Zellen konnte durch die Echtzeitanalyse der intrazellulären Glucosekonzentration eindrucksvoll bestätigt werden. So zeigte sich bei Beta-Zellen im Vergleich zu nicht-insulinproduzierenden COS-Zellen, die den GLUT1 Glucosetransporter und die Hexokinase exprimieren, eine deutlich erhöhte Glucoseaufnahmerate und -verstoffwechselung. Der Einsatz von Mannoheptulose als kompetitiver Inhibitor der Glucokinase verlangsamte die

Eliminierungsrate der Glucose in den Beta-Zellen und verminderte die Insulinsekretion. Auf COS-Zellen hatte Mannoheptulose jedoch keinen Einfluss. Durch 3-O-Methylglucose konnte in den Beta-Zellen nur die Glucoseaufnahme verringert werden. In COS-Zellen war dagegen auch der Glucosemetabolismus herabgesetzt. Während in COS-Zellen die Glucoseaufnahme die Rate des Glucosemetabolismus bestimmt, übernimmt die Glucokinase diese zentrale Rolle in Beta-Zellen. Die Phosphorylierung der Glucose ist somit geschwindigkeitsbestimmend in den Beta-Zellen.

Metabolische Oszillationen spielen in der glucoseinduzierten Insulinsekretion eine wichtige Rolle. Eine wesentliche Frage ist hierbei, an welcher Stelle in der Signalkaskade diese Oszillationen generiert werden. Im Gegensatz zu vorangegangenen Publikationen, die Oszillationen der intrazellulären Glucosekonzentration postulierten, konnten in der hier vorliegenden Arbeit mittels direkter intrazellulärer Messung keine solchen Oszillationen nachgewiesen werden. Dahingegen sind Oszillationen des Calciums als unmittelbares Signal für die Exozytose von Insulingranula allgemein akzeptiert. Um Veränderungen in der intrazellulären Glucose- und Calciumkonzentration in der selben Zelle in Echtzeit beobachten zu können, wurden erstmals simultane fluoreszenzmikroskopische Analysen mit dem Glucosesensor FLIPglu und dem Calciumindikator FURA durchgeführt. Es zeigte sich hierbei, dass der initiale Calciumanstieg bei einer Glucosekonzentration von 4 mmol/l erreicht wurde. Oszillationen des intrazellulären Calciums mit großer Amplitude traten aber erst bei einer Glucosekonzentration von 6-7 mmol/l auf. Tolbutamid als K_{ATP} schließender Wirkstoff erzeugte ebenfalls einen Calciumeinstrom, jedoch keine Oszillationen. Diese Ergebnisse unterstützen daher die Hypothese, dass der initiale Calciumeinstrom über den *triggering pathway* ausgelöst wird, wohingegen die Oszillationen über den *amplifying pathway* vermittelt werden. Beide Wege sind dabei eindeutig von der intrazellulären Glucosekonzentration abhängig.

Weiterhin war es ein Ziel der vorliegenden Arbeit, den Einfluss der Glucose auf die Interaktion zwischen der Glucokinase und dem Glucokinase Regulatorprotein zu untersuchen. Das GRP übernimmt in der Leber die Funktion eines Inhibitors der Glucokinaseenzymaktivität, in dem es bei niedriger Glucose an die Glucokinase bindet und eine Translokation des Enzyms in den Zellkern vermittelt. Es konnte mit Hilfe von FRET-Analysen gezeigt werden, dass das GRP nur unter niedrigen Glucosekonzentrationen an die Glucokinase bindet, während der sowohl in Leber als auch in den Beta-Zellen exprimierte endogene Aktivator PFK-2/FBPase-2 nur bei hoher Glucosekonzentration eine verstärkte Bindung an die Glucokinase zeigte. Dies hebt die Bedeutung des GRP für die

Glucokinaseregulation ausschließlich in der Leber hervor. Die Kompartimentierung der Glucokinase zwischen Zellkern und Zytoplasma verändert sich in Abhängigkeit von der Glucosekonzentration. Das GRP ist hingegen unabhängig von der Glucosekonzentration zum größten Teil im Kern lokalisiert und nur zu einem geringen Teil im Zytoplasma. Die Ergebnisse dieser Studie zeigen für das GRP die Rolle eines Transporters auf, der einem kontinuierlichen Austausch zwischen Zellkern und Zytoplasma unterliegt und die Glucokinase nur unter niedrigen Glucosekonzentrationen bindet und translozierte. Die Dissoziation und Relokalisierung der GK ins Zytoplasma nach Wiederanstieg der Blutglucose wurde bislang kontrovers diskutiert. Mit Hilfe von GRP-Fusionskonstrukten konnte in dieser Arbeit zum einen nachgewiesen werden, dass die Motilität der Glucokinase, nicht jedoch des GRP entscheidend von der Glucosekonzentration abhängig ist. Des Weiteren wurde mit Hilfe des neu generierten FLIPglu-NUC Glucosesensors, welcher eine Zielsequenz für ein nukleäres *targeting* enthält, erstmals der nukleäre Glucosestoffwechsel analysiert. Damit konnte gezeigt werden, dass der Zellkern isolierter Hepatozyten eine höhere Glucoseaufnahmekapazität und eine höhere Glucosephosphorylierungskapazität aufweist als COS- und Beta-Zellen. Dies zeigt, dass die Glucokinase nach Anstieg der Glucosekonzentration im Zellkern enzymatisch aktiv ist und damit nicht mehr im Komplex mit dem GRP vorliegen kann. Der Kernexport der Glucokinase erfolgt also unabhängig vom GRP.

Die in dieser Arbeit durchgeführten Echtzeitanalysen der intrazellulären Glucosekonzentration liefern detaillierteres Wissen über die glucosestimulierte Insulinsekretion in den Beta-Zellen des Pankreas. Dadurch lassen sich Defekte in diesem Mechanismus zukünftig besser darstellen und ermöglichen ein vertieftes Verständnis der Pathogenese des Typ 2 Diabetes mellitus. Zum anderen konnte erstmals gezeigt werden, dass der nukleäre Export der Glucokinase von der Glucosekonzentration, nicht aber vom GRP abhängig ist.

5 Literaturverzeichnis

Adams, S. R., Harootunian, A. T., Buechler, Y. J., Taylor, S. S. & Tsien, R. Y. (1991). Fluorescence ratio imaging of cyclic AMP in single cells. *Nature* **349**: 694-697.

Agius, L., Peak, M. & Van Schaftingen, E. (1995). The regulatory protein of glucokinase binds to the hepatocyte matrix, but, unlike glucokinase, does not translocate during substrate stimulation. *Biochem J* **309 (Pt 3)**: 711-713.

Aguilar-Bryan, L., Nichols, C. G., Wechsler, S. W., Clement, J. P. t., Boyd, A. E., 3rd, Gonzalez, G., Herrera-Sosa, H., Nguy, K., Bryan, J. & Nelson, D. A. (1995). Cloning of the beta cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. *Science* **268**: 423-426.

Antoine, M., Boutin, J. A. & Ferry, G. (2009). Binding kinetics of glucose and allosteric activators to human glucokinase reveal multiple conformational states. *Biochemistry* **48**: 5466-5482.

Arden, C., Baltrusch, S. & Agius, L. (2006). Glucokinase regulatory protein is associated with mitochondria in hepatocytes. *FEBS Lett* **580**: 2065-2070.

Arden, C., Harbottle, A., Baltrusch, S., Tiedge, M. & Agius, L. (2004). Glucokinase is an integral component of the insulin granules in glucose-responsive insulin secretory cells and does not translocate during glucose stimulation. *Diabetes* **53**: 2346-2352.

Ashcroft, F. M., Harrison, D. E. & Ashcroft, S. J. (1984). Glucose induces closure of single potassium channels in isolated rat pancreatic beta-cells. *Nature* **312**: 446-448.

Ashcroft, S. J. H. (1981). Metabolic Controls of Insulin Secretion. In The Islets of Langerhans, Academic Press 117-149.

Baltrusch, S., Francini, F., Lenzen, S. & Tiedge, M. (2005). Interaction of glucokinase with the liver regulatory protein is conferred by leucine-asparagine motifs of the enzyme. *Diabetes* **54**: 2829-2837.

Baltrusch, S., Langer, S., Massa, L., Tiedge, M. & Lenzen, S. (2006). Improved metabolic stimulus for glucose-induced insulin secretion through GK and PFK-2/FBPase-2 coexpression in insulin-producing RINm5F cells. *Endocrinology* **147**: 5768-5776.

Baltrusch, S. & Lenzen, S. (2007a). Novel insights into the regulation of the bound and diffusible glucokinase in MIN6 beta-cells. *Diabetes* **56**: 1305-1315.

Baltrusch, S. & Lenzen, S. (2007b). Regulation of [Ca2+]i oscillations in mouse pancreatic islets by adrenergic agonists. *Biochem Biophys Res Commun* **363**: 1038-1043.

Baltrusch, S. & Lenzen, S. (2008). Monitoring of glucose-regulated single insulin secretory granule movement by selective photoactivation. *Diabetologia* **51**: 989-996.

Baltrusch, S., Lenzen, S., Okar, D. A., Lange, A. J. & Tiedge, M. (2001). Characterization of glucokinase-binding protein epitopes by a phage-displayed peptide library. Identification of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase as a novel interaction partner. *J Biol Chem* **276**: 43915-43923.

Baltrusch, S. & Tiedge, M. (2006). Glucokinase Regulatory Network in Pancreatic {beta}-Cells and Liver. *Diabetes* **55 Suppl 2**: S55-64.

Bell, G. I., Kayano, T., Buse, J. B., Burant, C. F., Takeda, J., Lin, D., Fukumoto, H. & Seino, S. (1990). Molecular biology of mammalian glucose transporters. *Diabetes Care* **13**: 198-208.

Berg, J., Hung, Y. P. & Yellen, G. (2009). A genetically encoded fluorescent reporter of ATP:ADP ratio. *Nat Methods* **6**: 161-166.

Bermejo, C., Haerizadeh, F., Takanaga, H., Chermak, D. & Frommer, W. B. (2010). Dynamic analysis of cytosolic glucose and ATP levels in yeast using optical sensors. *Biochem J* **432**: 399-406.

Bittner, C. X., Loaiza, A., Ruminot, I., Larenas, V., Sotelo-Hitschfeld, T., Gutierrez, R., Cordova, A., Valdebenito, R., Frommer, W. B. & Barros, L. F. (2010). High resolution measurement of the glycolytic rate. *Front Neuroenergetics* **2**.

Bjorkhaug, L., Molnes, J., Sovik, O., Njolstad, P. R. & Flatmark, T. (2007). Allosteric activation of human glucokinase by free polyubiquitin chains and its ubiquitin-dependent cotranslational proteasomal degradation. *J Biol Chem* **282**: 22757-22764.

Bosco, D., Meda, P. & Iynedjian, P. B. (2000). Glucokinase and glucokinase regulatory protein: mutual dependence for nuclear localization. *Biochem J* **348 Pt 1**: 215-222.

Brocklehurst, K. J., Davies, R. A. & Agius, L. (2004a). Differences in regulatory properties between human and rat glucokinase regulatory protein. *Biochem J* **378**: 693-697.

Brocklehurst, K. J., Payne, V. A., Davies, R. A., Carroll, D., Vertigan, H. L., Wightman, H. J., Aiston, S., Waddell, I. D., Leighton, B., Coghlan, M. P. & Agius, L. (2004b). Stimulation of hepatocyte glucose metabolism by novel small molecule glucokinase activators. *Diabetes* **53**: 535-541.

Brown, K. S., Kalinowski, S. S., Megill, J. R., Durham, S. K. & Mookhtiar, K. A. (1997). Glucokinase regulatory protein may interact with glucokinase in the hepatocyte nucleus. *Diabetes* **46**: 179-186.

Cardenas, M. L. (1997). Kinetic behaviour of vertebrate hexokinases with emphasis on hexokinase D (IV). *Biochem Soc Trans* **25**: 131-135.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. & Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263**: 802-805.

Chaudhuri, B., Hormann, F. & Frommer, W. B. (2011). Dynamic imaging of glucose flux impedance using FRET sensors in wild-type Arabidopsis plants. *J Exp Bot* **62**: 2411-2417.

Chaudhuri, B., Niittyla, T., Hormann, F. & Frommer, W. B. (2007). Fluxomics with ratiometric metabolite dyes. *Plant Signal Behav* **2**: 120-122.

Cook, D. L. & Hales, C. N. (1984). Intracellular ATP directly blocks K+ channels in pancreatic B-cells. *Nature* **311**: 271-273.

Cornish-Bowden, A. & Storer, A. C. (1986). Mechanistic origin of the sigmoidal rate behaviour of rat liver hexokinase D ('glucokinase'). *Biochem J* **240**: 293-296.

Danial, N. N., Gramm, C. F., Scorrano, L., Zhang, C. Y., Krauss, S., Ranger, A. M., Datta, S. R., Greenberg, M. E., Licklider, L. J., Lowell, B. B., Gygi, S. P. & Korsmeyer, S. J. (2003). BAD and glucokinase reside in a mitochondrial complex that integrates glycolysis and apoptosis. *Nature* **424**: 952-956.

de la Iglesia, N., Mukhtar, M., Seoane, J., Guinovart, J. J. & Agius, L. (2000). The role of the regulatory protein of glucokinase in the glucose sensory mechanism of the hepatocyte. *J Biol Chem* **275**: 10597-10603.

de la Iglesia, N., Veiga-da-Cunha, M., Van Schaftingen, E., Guinovart, J. J. & Ferrer, J. C. (1999). Glucokinase regulatory protein is essential for the proper subcellular localisation of liver glucokinase. *FEBS Lett* **456**: 332-338.

Detimary, P., Gilon, P. & Henquin, J. C. (1998). Interplay between cytoplasmic Ca2+ and the ATP/ADP ratio: a feedback control mechanism in mouse pancreatic islets. *Biochem J* **333 (Pt 2)**: 269-274.

Deuschle, K., Fehr, M., Hilpert, M., Lager, I., Lalonde, S., Looger, L. L., Okumoto, S., Persson, J., Schmidt, A. & Frommer, W. B. (2005a). Genetically encoded sensors for metabolites. *Cytometry A* **64**: 3-9.

Deuschle, K., Okumoto, S., Fehr, M., Looger, L. L., Kozhukh, L. & Frommer, W. B. (2005b). Construction and optimization of a family of genetically encoded metabolite sensors by semirational protein engineering. *Protein Sci* **14**: 2304-2314.

Dooley, C. T., Dore, T. M., Hanson, G. T., Jackson, W. C., Remington, S. J. & Tsien, R. Y. (2004). Imaging dynamic redox changes in mammalian cells with green fluorescent protein indicators. *J Biol Chem* **279**: 22284-22293.

Dyachok, O., Idevall-Hagren, O., Sagetorp, J., Tian, G., Wuttke, A., Arrieumerlou, C., Akusjarvi, G., Gylfe, E. & Tengholm, A. (2008). Glucose-induced cyclic AMP oscillations regulate pulsatile insulin secretion. *Cell Metab* **8**: 26-37.

Dyachok, O., Isakov, Y., Sagetorp, J. & Tengholm, A. (2006). Oscillations of cyclic AMP in hormone-stimulated insulin-secreting beta-cells. *Nature* **439**: 349-352.

Farrelly, D., Brown, K. S., Tieman, A., Ren, J., Lira, S. A., Hagan, D., Gregg, R., Mookhtiar, K. A. & Hariharan, N. (1999). Mice mutant for glucokinase regulatory protein exhibit decreased liver glucokinase: a sequestration mechanism in metabolic regulation. *Proc Natl Acad Sci U S A* **96**: 14511-14516.

Fehr, M., Ehrhardt, D. W., Lalonde, S. & Frommer, W. B. (2004a). Minimally invasive dynamic imaging of ions and metabolites in living cells. *Curr Opin Plant Biol* **7**: 345-351.

Fehr, M., Frommer, W. B. & Lalonde, S. (2002). Visualization of maltose uptake in living yeast cells by fluorescent nanosensors. *Proc Natl Acad Sci U S A* **99**: 9846-9851.

Fehr, M., Lalonde, S., Ehrhardt, D. W. & Frommer, W. B. (2004). Live imaging of glucose homeostasis in nuclei of COS-7 cells. *J Fluoresc* **14**: 603-609.

Fehr, M., Lalonde, S., Lager, I., Wolff, M. W. & Frommer, W. B. (2003). In vivo imaging of the dynamics of glucose uptake in the cytosol of COS-7 cells by fluorescent nanosensors. *J Biol Chem* **278**: 19127-19133.

Fehr, M., Okumoto, S., Deuschle, K., Lager, I., Looger, L. L., Persson, J., Kozhukh, L., Lalonde, S. & Frommer, W. B. (2005a). Development and use of fluorescent nanosensors for metabolite imaging in living cells. *Biochem Soc Trans* **33**: 287-290.

Fernandez-Novell, J. M., Castel, S., Bellido, D., Ferrer, J. C., Vilaro, S. & Guinovart, J. J. (1999). Intracellular distribution of hepatic glucokinase and glucokinase regulatory protein during the fasted to refed transition in rats. *FEBS Lett* **459**: 211-214.

Förster, V. T. (1948). Zwischenmolekulare Energiewanderung und Fluoreszenz. *Annalen der Physik*55-75.

Frommer, W. B., Davidson, M. W. & Campbell, R. E. (2009). Genetically encoded biosensors based on engineered fluorescent proteins. *Chem Soc Rev* **38**: 2833-2841.

Futamura, M., Hosaka, H., Kadotani, A., Shimazaki, H., Sasaki, K., Ohyama, S., Nishimura, T., Eiki, J. & Nagata, Y. (2006). An allosteric activator of glucokinase impairs the interaction of glucokinase and glucokinase regulatory protein and regulates glucose metabolism. *J Biol Chem* **281**: 37668-37674.

Gembal, M., Gilon, P. & Henquin, J. C. (1992). Evidence that glucose can control insulin release independently from its action on ATP-sensitive K+ channels in mouse B cells. *J Clin Invest* **89**: 1288-1295.

Grapengiesser, E., Gylfe, E. & Hellman, B. (1990). Sulfonylurea mimics the effect of glucose in inducing large amplitude oscillations of cytoplasmic Ca2+ in pancreatic beta-cells. *Mol Pharmacol* **37**: 461-467.

Grimmsmann, T. & Rustenbeck, I. (1998). Direct effects of diazoxide on mitochondria in pancreatic B-cells and on isolated liver mitochondria. *Br J Pharmacol* **123**: 781-788.

Grimsby, J., Coffey, J. W., Dvorozniak, M. T., Magram, J., Li, G., Matschinsky, F. M., Shiota, C., Kaur, S., Magnuson, M. A. & Grippo, J. F. (2000). Characterization of glucokinase regulatory protein-deficient mice. *J Biol Chem* **275**: 7826-7831.

Grimsby, J., Sarabu, R., Corbett, W. L., Haynes, N. E., Bizzarro, F. T., Coffey, J. W., Guertin, K. R., Hilliard, D. W., Kester, R. F., Mahaney, P. E., Marcus, L., Qi, L., Spence, C. L., Tengi, J., Magnuson, M. A., Chu, C. A., Dvorozniak, M. T., Matschinsky, F. M. & Grippo, J. F. (2003). Allosteric activators of glucokinase: potential role in diabetes therapy. *Science* **301**: 370-373.

Gurskaya, N. G., Verkhusha, V. V., Shcheglov, A. S., Staroverov, D. B., Chepurnykh, T. V., Fradkov, A. F., Lukyanov, S. & Lukyanov, K. A. (2006). Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light. *Nat Biotechnol* **24**: 461-465.

Güttler, T. & Gorlich, D. (2011). Ran-dependent nuclear export mediators: a structural perspective. *EMBO J* **30**: 3457-3474.

Gylfe, E. (1976). Comparison of the effects of leucines, non-metabolizable leucine analogues and other insulin secretagogues on the activity of glutamate dehydrogenase. *Acta Diabetol Lat* **13**: 20-24.

Gylfe, E., Ahmed, M., Bergsten, P., Dansk, H., Dyachok, O., Eberhardson, M., Grapengiesser, E., Hellman, B., Lin, J. M., Sundsten, T., Tengholm, A., Vieira, E. & Westerlund, J. (2000). Signaling underlying pulsatile insulin secretion. *Ups J Med Sci* **105**: 35-51.

He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W. & Vogelstein, B. (1998). A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A* **95**: 2509-2514.

Heart, E., Corkey, R. F., Wikstrom, J. D., Shirihai, O. S. & Corkey, B. E. (2006). Glucose-dependent increase in mitochondrial membrane potential, but not cytoplasmic calcium, correlates with insulin secretion in single islet cells. *Am J Physiol Endocrinol Metab* **290**: E143-E148.

Heimberg, H., De Vos, A., Pipeleers, D., Thorens, B. & Schuit, F. (1995). Differences in glucose transporter gene expression between rat pancreatic alpha- and beta-cells are correlated to differences in glucose transport but not in glucose utilization. *J Biol Chem* **270**: 8971-8975.

Henquin, J. C. (1988). ATP-sensitive K+ channels may control glucose-induced electrical activity in pancreatic B-cells. *Biochem Biophys Res Commun* **156**: 769-775.

Henquin, J. C. (2000). Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* **49**: 1751-1760.

Henquin, J. C. (2009). Regulation of insulin secretion: a matter of phase control and amplitude modulation. *Diabetologia* **52**: 739-751.

Heredia, V. V., Carlson, T. J., Garcia, E. & Sun, S. (2006). Biochemical basis of glucokinase activation and the regulation by glucokinase regulatory protein in naturally occurring mutations. *J Biol Chem* **281**: 40201-40207.

Honda, A., Adams, S. R., Sawyer, C. L., Lev-Ram, V., Tsien, R. Y. & Dostmann, W. R. (2001). Spatiotemporal dynamics of guanosine 3',5'-cyclic monophosphate revealed by a genetically encoded, fluorescent indicator. *Proc Natl Acad Sci U S A* **98**: 2437-2442.

Hou, B. H., Takanaga, H., Griesbeck, O. & Frommer, W. B. (2009). Osmotic induction of calcium accumulation in human embryonic kidney cells detected with a high sensitivity FRET calcium sensor. *Cell Calcium* **46**: 130-135.

Inagaki, N., Gonoi, T., Clement, J. P. t., Namba, N., Inazawa, J., Gonzalez, G., Aguilar-Bryan, L., Seino, S. & Bryan, J. (1995). Reconstitution of IKATP: an inward rectifier subunit plus the sulfonylurea receptor. *Science* **270**: 1166-1170.

Inouye, S. & Tsuji, F. I. (1994). Aequorea green fluorescent protein. Expression of the gene and fluorescence characteristics of the recombinant protein. *FEBS Lett* **341**: 277-280.

Ishihara, H., Asano, T., Tsukuda, K., Katagiri, H., Inukai, K., Anai, M., Kikuchi, M., Yazaki, Y., Miyazaki, J. I. & Oka, Y. (1993). Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. *Diabetologia* **36**: 1139-1145.

Ivarsson, R., Quintens, R., Dejonghe, S., Tsukamoto, K., in 't Veld, P., Renstrom, E. & Schuit, F. C. (2005). Redox control of exocytosis: regulatory role of NADPH, thioredoxin, and glutaredoxin. *Diabetes* **54**: 2132-2142.

Iynedjian, P. B. (1993). Mammalian glucokinase and its gene. *Biochem J* **293 (Pt 1)**: 1-13.

Iynedjian, P. B., Pilot, P. R., Nouspikel, T., Milburn, J. L., Quaade, C., Hughes, S., Ucla, C. & Newgard, C. B. (1989). Differential expression and regulation of the glucokinase gene in liver and islets of Langerhans. *Proc Natl Acad Sci U S A* **86**: 7838-7842.

Jetton, T. L., Liang, Y., Pettepher, C. C., Zimmerman, E. C., Cox, F. G., Horvath, K., Matschinsky, F. M. & Magnuson, M. A. (1994). Analysis of upstream glucokinase promoter activity in transgenic mice and identification of glucokinase in rare neuroendocrine cells in the brain and gut. *J Biol Chem* **269**: 3641-3654.

Jetton, T. L., Shiota, M., Knobel, S. M., Piston, D. W., Cherrington, A. D. & Magnuson, M. A. (2001). Substrate-induced nuclear export and peripheral compartmentalization of hepatic glucokinase correlates with glycogen deposition. *Int J Exp Diabetes Res* **2**: 173-186.

Johnson, D., Shepherd, R. M., Gill, D., Gorman, T., Smith, D. M. & Dunne, M. J. (2007). Glucose-dependent modulation of insulin secretion and intracellular calcium ions by GKA50, a glucokinase activator. *Diabetes* **56**: 1694-1702.

Jung, S. K., Kauri, L. M., Qian, W. J. & Kennedy, R. T. (2000). Correlated oscillations in glucose consumption, oxygen consumption, and intracellular free Ca(2+) in single islets of Langerhans. *J Biol Chem* **275**: 6642-6650.

Kamata, K., Mitsuya, M., Nishimura, T., Eiki, J. & Nagata, Y. (2004). Structural basis for allosteric regulation of the monomeric allosteric enzyme human glucokinase. *Structure* **12**: 429-438.

Kayano, T., Burant, C. F., Fukumoto, H., Gould, G. W., Fan, Y. S., Eddy, R. L., Byers, M. G., Shows, T. B., Seino, S. & Bell, G. I. (1990). Human facilitative glucose transporters. Isolation, functional characterization, and gene localization of cDNAs encoding an isoform (GLUT5) expressed in small intestine, kidney, muscle, and adipose tissue and an unusual glucose transporter pseudogene-like sequence (GLUT6). *J Biol Chem* **265**: 13276-13282.

Kennedy, H. J., Pouli, A. E., Ainscow, E. K., Jouaville, L. S., Rizzuto, R. & Rutter, G. A. (1999). Glucose generates sub-plasma membrane ATP microdomains in single islet beta-cells. Potential role for strategically located mitochondria. *J Biol Chem* **274**: 13281-13291.

Kennedy, R. T., Kauri, L. M., Dahlgren, G. M. & Jung, S. K. (2002). Metabolic oscillations in beta-cells. *Diabetes* **51 Suppl 1**: S152-161.

Kerner, W. B., J. (2009). Definition, Klassifikation und Diagnostik des Diabetes mellitus. *Diabetologie und Stoffwechsel Supplement 2 Praxis-Leitlinien der Deutschen Diabetes-Gesellschaft*115-117.

Kim, W. H., Lee, J. W., Suh, Y. H., Hong, S. H., Choi, J. S., Lim, J. H., Song, J. H., Gao, B. & Jung, M. H. (2005). Exposure to chronic high glucose induces beta-cell apoptosis through decreased interaction of glucokinase with mitochondria: downregulation of glucokinase in pancreatic beta-cells. *Diabetes* **54**: 2602-2611.

Lager, I., Fehr, M., Frommer, W. B. & Lalonde, S. (2003). Development of a fluorescent nanosensor for ribose. *FEBS Lett* **553**: 85-89.

Lalonde, S., Ehrhardt, D. W. & Frommer, W. B. (2005). Shining light on signaling and metabolic networks by genetically encoded biosensors. *Curr Opin Plant Biol* **8**: 574-581.

Langer, S., Kaminski, M. T., Lenzen, S. & Baltrusch, S. (2010). Endogenous activation of glucokinase by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase is glucose dependent. *Mol Endocrinol* **24**: 1988-1997.

Lenzen, S. (1990). Hexose recognition mechanisms in pancreatic B-cells. *Biochem Soc Trans* **18**: 105-107.

Lenzen, S. (1992). Glucokinase: signal recognition enzyme for glucose-induced insulin secretion. In Nutrient regulation of insulin secretion. Flatt, P. R. e., Portland Press,London and Chapel Hill101-125.

Lenzen, S., Lerch, M., Peckmann, T. & Tiedge, M. (2000). Differential regulation of [Ca2+]i oscillations in mouse pancreatic islets by glucose, alpha-ketoisocaproic acid, glyceraldehyde and glycolytic intermediates. *Biochim Biophys Acta* **1523**: 65-72.

Lenzen, S. & Mirzaie-Petri, M. (1992). Inhibition of aconitase by alloxan and the differential modes of protection of glucose, 3-O-methylglucose, and mannoheptulose. *Naunyn Schmiedebergs Arch Pharmacol* **346**: 532-536.

Lenzen, S. & Panten, U. (1988). Signal recognition by pancreatic B-cells. *Biochem Pharmacol* **37**: 371-378.

Lenzen, S. & Peckmann, T. (2001). Effects of tolbutamide and N-benzoyl-D-phenylalanine (NBDP) on the regulation of [Ca2+]i oscillations in mouse pancreatic islets. *Biochem Pharmacol* **62**: 923-928.

Lenzen, S., Schmidt, W., Rustenbeck, I. & Panten, U. (1986). 2-ketoglutarate generation in pancreatic B-cell mitochondria regulates insulin secretory action of amino acids and 2-keto acids. *Biosci Rep* **6**: 163-169.

Lenzen, S. & Tiedge, M. (1994). Regulation of pancreatic B-cell glucokinase and GLUT2 glucose transporter gene expression. *Biochem Soc Trans* **22**: 1-6.

MacDonald, P. E., Joseph, J. W. & Rorsman, P. (2005). Glucose-sensing mechanisms in pancreatic beta-cells. *Philos Trans R Soc Lond B Biol Sci* **360**: 2211-2225.

Magnuson, M. A., Andreone, T. L., Printz, R. L., Koch, S. & Granner, D. K. (1989). Rat glucokinase gene: structure and regulation by insulin. *Proc Natl Acad Sci U S A* **86**: 4838-4842.

Magnuson, M. A. & Shelton, K. D. (1989). An alternate promoter in the glucokinase gene is active in the pancreatic beta cell. *J Biol Chem* **264**: 15936-15942.

Massa, L., Baltrusch, S., Okar, D. A., Lange, A. J., Lenzen, S. & Tiedge, M. (2004). Interaction of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) with glucokinase activates glucose phosphorylation and glucose metabolism in insulin-producing cells. *Diabetes* **53**: 1020-1029.

Massa, M. L., Gagliardino, J. J. & Francini, F. (2011). Liver glucokinase: An overview on the regulatorymechanisms of its activity. *IUBMB Life*.

Matschinsky, F., Liang, Y., Kesavan, P., Wang, L., Froguel, P., Velho, G., Cohen, D., Permutt, M. A., Tanizawa, Y., Jetton, T. L. & et al. (1993). Glucokinase as pancreatic beta cell glucose sensor and diabetes gene. *J Clin Invest* **92**: 2092-2098.

Matschinsky, F. M. (1990). Glucokinase as glucose sensor and metabolic signal generator in pancreatic beta-cells and hepatocytes. *Diabetes* **39**: 647-652.

Matschinsky, F. M. (1996). Banting Lecture 1995. A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. *Diabetes* **45**: 223-241.

Matschinsky, F. M., Magnuson, M. A., Zelent, D., Jetton, T. L., Doliba, N., Han, Y., Taub, R. & Grimsby, J. (2006). The network of glucokinase-expressing cells in glucose homeostasis and the potential of glucokinase activators for diabetes therapy. *Diabetes* **55**: 1-12.

Matthaei, S. B., R. Fritsche, A. Gallwitz, B. Häring, H.-U. Joost, H.-G. Kellerer, M. & Kloos, C. K., T. Nauck, M. & Schernthaner, G. S., E. Thienel, F. (2009). Behandlung des Diabetes mellitus Typ2. *Diabetologie und Stoffwechsel Supplement 2 Praxis-Leitlinien der Deutschen Diabetes-Gesellschaft*138-143.

Meglasson, M. D. & Matschinsky, F. M. (1984). New perspectives on pancreatic islet glucokinase. *Am J Physiol* **246**: E1-13.

Meglasson, M. D. & Matschinsky, F. M. (1986). Pancreatic islet glucose metabolism and regulation of insulin secretion. *Diabetes Metab Rev* **2**: 163-214.

Middleton, R. J. (1990). Hexokinases and glucokinases. *Biochem Soc Trans* **18**: 180-183.

Miyawaki, A., Llopis, J., Heim, R., McCaffery, J. M., Adams, J. A., Ikura, M. & Tsien, R. Y. (1997). Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin. *Nature* **388**: 882-887.

Mochizuki, N., Yamashita, S., Kurokawa, K., Ohba, Y., Nagai, T., Miyawaki, A. & Matsuda, M. (2001). Spatio-temporal images of growth-factor-induced activation of Ras and Rap1. *Nature* **411**: 1065-1068.

Mukhtar, M., Stubbs, M. & Agius, L. (1999). Evidence for glucose and sorbitol-induced nuclear export of glucokinase regulatory protein in hepatocytes. *FEBS Lett* **462**: 453-458.

Nagai, T., Sawano, A., Park, E. S. & Miyawaki, A. (2001). Circularly permuted green fluorescent proteins engineered to sense Ca2+. *Proc Natl Acad Sci U S A* **98**: 3197-3202.

Nakashima, K., Kanda, Y., Hirokawa, Y., Kawasaki, F., Matsuki, M. & Kaku, K. (2009). MIN6 is not a pure beta cell line but a mixed cell line with other pancreatic endocrine hormones. *Endocr J* **56**: 45-53.

Niculescu, L., Veiga-da-Cunha, M. & Van Schaftingen, E. (1997). Investigation on the mechanism by which fructose, hexitols and other compounds regulate the translocation of glucokinase in rat hepatocytes. *Biochem J* **321 (Pt 1)**: 239-246.

Okar, D. A., Manzano, A., Navarro-Sabate, A., Riera, L., Bartrons, R. & Lange, A. J. (2001). PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2,6-bisphosphate. *Trends Biochem Sci* **26**: 30-35.

Panten, U. (1989). Mechanism of insulin secretion and its modulation by sulfonylureas. *Contrib Nephrol* **73**: 16-21; discussion 22-13.

Panten, U. & Rustenbeck, I. (2008). Fuel-induced amplification of insulin secretion in mouse pancreatic islets exposed to a high sulfonylurea concentration: role of the NADPH/NADP+ ratio. *Diabetologia* **51**: 101-109.

Panten, U., Schwanstecher, M., Wallasch, A. & Lenzen, S. (1988). Glucose both inhibits and stimulates insulin secretion from isolated pancreatic islets exposed to maximally effective concentrations of sulfonylureas. *Naunyn Schmiedebergs Arch Pharmacol* **338**: 459-462.

Parton, L. E., Ye, C. P., Coppari, R., Enriori, P. J., Choi, B., Zhang, C. Y., Xu, C., Vianna, C. R., Balthasar, N., Lee, C. E., Elmquist, J. K., Cowley, M. A. & Lowell, B. B. (2007). Glucose sensing by POMC neurons regulates glucose homeostasis and is impaired in obesity. *Nature* **449**: 228-232.

Perrin, F. (1932). Theorie quantique des transferts d'activation entre molécules de même espèce. Cas de solutionsfluorescentes. *Ann Chim Physsique*283-313.

Ponsioen, B., Zhao, J., Riedl, J., Zwartkruis, F., van der Krogt, G., Zaccolo, M., Moolenaar, W. H., Bos, J. L. & Jalink, K. (2004). Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator. *EMBO Rep* **5**: 1176-1180.

Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G. & Cormier, M. J. (1992). Primary structure of the Aequorea victoria green-fluorescent protein. *Gene* **111**: 229-233.

Prebil, M., Chowdhury, H. H., Zorec, R. & Kreft, M. (2011). Changes in cytosolic glucose level in ATP stimulated live astrocytes. *Biochem Biophys Res Commun* **405**: 308-313.

Printz, R. L., Magnuson, M. A. & Granner, D. K. (1993). Mammalian glucokinase. *Annu Rev Nutr* **13**: 463-496.

Rasschaert, J., Kadiata, M. M. & Malaisse, W. J. (2001). Effects of D-mannoheptulose upon D-glucose metabolism in tumoral pancreatic islet cells. *Mol Cell Biochem* **226**: 77-81.

Rees, M. G., Wincovitch, S., Schultz, J., Waterstradt, R., Beer, N. L., Baltrusch, S., Collins, F. S. & Gloyn, A. L. (2011). Cellular characterisation of the GCKR P446L variant associated with type 2 diabetes risk. *Diabetologia*.

Rizzo, M. A., Magnuson, M. A., Drain, P. F. & Piston, D. W. (2002). A functional link between glucokinase binding to insulin granules and conformational alterations in response to glucose and insulin. *J Biol Chem* **277**: 34168-34175.

Rorsman, P. & Renstrom, E. (2003). Insulin granule dynamics in pancreatic beta cells. *Diabetologia* **46**: 1029-1045.

Rorsman, P. & Trube, G. (1985). Glucose dependent K+-channels in pancreatic beta-cells are regulated by intracellular ATP. *Pflugers Arch* **405**: 305-309.

Rustenbeck, I., Baltrusch, S. & Tiedge, M. (2010). Do insulinotropic glucose-lowering drugs do more harm than good? The hypersecretion hypothesis revisited. *Diabetologia* **53**: 2105-2111.

Sato, M., Ozawa, T., Inukai, K., Asano, T. & Umezawa, Y. (2002). Fluorescent indicators for imaging protein phosphorylation in single living cells. *Nat Biotechnol* **20**: 287-294.

Schaufele, F., Demarco, I. & Day, R. N. (2005). FRET Imaging in the Wide-Field Microscope. In Molecular Imaging. Periasamy, A. & Day, R. N., American Physiological Society.

Schuit, F. C. (1997). Is GLUT2 required for glucose sensing? *Diabetologia* **40**: 104-111.

Sener, A., Malaisse-Lagae, F. & Malaisse, W. J. (1993). Hexose metabolism in pancreatic islets: time-course of the oxidative response to D-glucose. *Biochim Biophys Acta* **1177**: 54-60.

Sener, A., Scruel, O., Louchami, K., Jijakli, H. & Malaisse, W. J. (1999). Inhibition of glucose-induced insulin release by 3-O-methyl-D-glucose: enzymatic, metabolic and cationic determinants. *Mol Cell Biochem* **194**: 133-145.

Shimomura, O., Johnson, F. H. & Saiga, Y. (1962). Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. *J Cell Comp Physiol* **59**: 223-239.

Shiota, C., Coffey, J., Grimsby, J., Grippo, J. F. & Magnuson, M. A. (1999b). Nuclear import of hepatic glucokinase depends upon glucokinase regulatory protein, whereas export is due to a nuclear export signal sequence in glucokinase. *J Biol Chem* **274**: 37125-37130.

Smith, W. E., Langer, S., Wu, C., Baltrusch, S. & Okar, D. A. (2007). Molecular coordination of hepatic glucose metabolism by the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase:glucokinase complex. *Mol Endocrinol* **21**: 1478-1487.

St Charles, R., Harrison, R. W., Bell, G. I., Pilkis, S. J. & Weber, I. T. (1994). Molecular model of human beta-cell glucokinase built by analogy to the crystal structure of yeast hexokinase B. *Diabetes* **43**: 784-791.

Takanaga, H., Chaudhuri, B. & Frommer, W. B. (2008). GLUT1 and GLUT9 as major contributors to glucose influx in HepG2 cells identified by a high sensitivity intramolecular FRET glucose sensor. *Biochim Biophys Acta* **1778**: 1091-1099.

Takanaga, H. & Frommer, W. B. (2010). Facilitative plasma membrane transporters function during ER transit. *Faseb J* **24**: 2849-2858.

Thorens, B. (1992). Molecular and cellular physiology of GLUT-2, a high-Km facilitated diffusion glucose transporter. *Int Rev Cytol* **137**: 209-238.

Thorens, B. (2008). Glucose sensing and the pathogenesis of obesity and type 2 diabetes. *Int J Obes (Lond)* **32 Suppl 6**: S62-71.

Thorens, B. (2010). Central control of glucose homeostasis: the brain--endocrine pancreas axis. *Diabetes Metab* **36 Suppl 3**: S45-49.

Thorens, B. & Mueckler, M. (2010). Glucose transporters in the 21st Century. *Am J Physiol Endocrinol Metab* **298**: E141-145.

Tiedge, M. & Baltrusch, S. (2011). [New perspectives of imaging techniques in islet research]. *Dtsch Med Wochenschr* **136**: 1130-1134.

Tiedge, M., Hohne, M. & Lenzen, S. (1993). Insulin secretion, insulin content and glucose phosphorylation in RINm5F insulinoma cells after transfection with human GLUT2 glucose-transporter cDNA. *Biochem J* **296 (Pt 1)**: 113-118.

Tiedge, M., Krug, U. & Lenzen, S. (1997). Modulation of human glucokinase intrinsic activity by SH reagents mirrors post-translational regulation of enzyme activity. *Biochim Biophys Acta* **1337**: 175-190.

Tiedge, M. & Lenzen, S. (1991). Regulation of glucokinase and GLUT-2 glucose-transporter gene expression in pancreatic B-cells. *Biochem J* **279 (Pt 3)**: 899-901.

Tiedge, M. & Lenzen, S. (1995). Effects of glucose refeeding and glibenclamide treatment on glucokinase and GLUT2 gene expression in pancreatic B-cells and liver from rats. *Biochem J* **308 (Pt 1)**: 139-144.

Tiedge, M., Steffeck, H., Elsner, M. & Lenzen, S. (1999). Metabolic regulation, activity state, and intracellular binding of glucokinase in insulin-secreting cells. *Diabetes* **48**: 514-523.

Toyoda, Y., Miwa, I., Kamiya, M., Ogiso, S., Nonogaki, T., Aoki, S. & Okuda, J. (1995a). Tissue and subcellular distribution of glucokinase in rat liver and their changes during fasting-refeeding. *Histochem Cell Biol* **103**: 31-38.

Toyoda, Y., Miwa, I., Satake, S., Anai, M. & Oka, Y. (1995b). Nuclear location of the regulatory protein of glucokinase in rat liver and translocation of the regulator to the cytoplasm in response to high glucose. *Biochem Biophys Res Commun* **215**: 467-473.

Trube, G., Rorsman, P. & Ohno-Shosaku, T. (1986). Opposite effects of tolbutamide and diazoxide on the ATP-dependent K+ channel in mouse pancreatic beta-cells. *Pflugers Arch* **407**: 493-499.

Tsien, R. Y. (1998). The green fluorescent protein. *Annu Rev Biochem* **67**: 509-544.

Tsien, R. Y., Bacskai, B. J. & Adams, S. R. (1993). FRET for studying intracellular signalling. *Trends Cell Biol* **3**: 242-245.

Uldry, M. & Thorens, B. (2004). The SLC2 family of facilitated hexose and polyol transporters. *Pflugers Arch* **447**: 480-489.

Unger, R. H. (1991). Diabetic hyperglycemia: link to impaired glucose transport in pancreatic beta cells. *Science* **251**: 1200-1205.

Urban, K. A. & Panten, U. (2005). Selective loss of glucose-induced amplification of insulin secretion in mouse pancreatic islets pretreated with sulfonylurea in the absence of fuels. *Diabetologia* **48**: 2563-2566.

Van Schaftingen, E. (1989a). A protein from rat liver confers to glucokinase the property of being antagonistically regulated by fructose 6-phosphate and fructose 1-phosphate. *Eur J Biochem* **179**: 179-184.

Van Schaftingen, E. (1994). Short-term regulation of glucokinase. *Diabetologia* **37 Suppl 2**: S43-47.

Van Schaftingen, E., Detheux, M. & Veiga da Cunha, M. (1994). Short-term control of glucokinase activity: role of a regulatory protein. *Faseb J* **8**: 414-419.

van Schaftingen, E., Vandercammen, A., Detheux, M. & Davies, D. R. (1992). The regulatory protein of liver glucokinase. *Adv Enzyme Regul* **32**: 133-148.

van Schaftingen, E., Veiga-da-Cunha, M. & Niculescu, L. (1997). The regulatory protein of glucokinase. *Biochem Soc Trans* **25**: 136-140.

Van Schaftingen, E. & Veiga da Cunha, M. (2004). Discovery and Role of Glucokinase Regulatory Protein. In Glucokinase and Glycemic Disease: From Basics to Novel Therapeutics, Frontiers in Diabetes. Basel, Karger. **16:** 193-207.

Vandercammen, A. & Van Schaftingen, E. (1991). Competitive inhibition of liver glucokinase by its regulatory protein. *Eur J Biochem* **200**: 545-551.

Vandercammen, A. & Van Schaftingen, E. (1993). Species and tissue distribution of the regulatory protein of glucokinase. *Biochem J* **294 (Pt 2)**: 551-556.

Veiga-da-Cunha, M., Courtois, S., Michel, A., Gosselain, E. & Van Schaftingen, E. (1996a). Amino acid conservation in animal glucokinases. Identification of residues implicated in the interaction with the regulatory protein. *J Biol Chem* **271**: 6292-6297.

Veiga-da-Cunha, M. & Van Schaftingen, E. (2002). Identification of fructose 6-phosphate- and fructose 1-phosphate-binding residues in the regulatory protein of glucokinase. *J Biol Chem* **277**: 8466-8473.

Veiga-da-Cunha, M., Xu, L. Z., Lee, Y. H., Marotta, D., Pilkis, S. J. & Van Schaftingen, E. (1996). Effect of mutations on the sensitivity of human beta-cell glucokinase to liver regulatory protein. *Diabetologia* **39**: 1173-1179.

Waldhäusl, W. & Lenzen, S. (2007). Kohlenhydrat-Stoffwechsel. In Pathophysiologie: Molekulare, zelluläre, systemische Grundlagen von Krankheiten. Wien, Wilhelm Maudrich Verlag.

Wolff, M., Kauschke, S. G., Schmidt, S. & Heilker, R. (2008). Activation and translocation of glucokinase in rat primary hepatocytes monitored by high content image analysis. *J Biomol Screen* **13**: 837-846.

Yamada, K., Nakata, M., Horimoto, N., Saito, M., Matsuoka, H. & Inagaki, N. (2000). Measurement of glucose uptake and intracellular calcium concentration in single, living pancreatic beta-cells. *J Biol Chem* **275**: 22278-22283.

Yamada, K., Saito, M., Matsuoka, H. & Inagaki, N. (2007). A real-time method of imaging glucose uptake in single, living mammalian cells. *Nat Protoc* **2**: 753-762.

Zhang, J., Campbell, R. E., Ting, A. Y. & Tsien, R. Y. (2002). Creating new fluorescent probes for cell biology. *Nat Rev Mol Cell Biol* **3**: 906-918.

Zhang, J., Li, C., Chen, K., Zhu, W., Shen, X. & Jiang, H. (2006). Conformational transition pathway in the allosteric process of human glucokinase. *Proc Natl Acad Sci U S A* **103**: 13368-13373.

6 Publikationen

Teilergebnisse der vorliegenden Dissertation sind bereits veröffentlicht.

Kurzbeiträge:

Kaminski M.T., Lenzen S., Baltrusch S. (2007) Aufklärung der Glucokinaseregulation in der Leber durch sensitive fluoreszenzmikroskopische Methoden. *Diabetologie und Stoffwechsel* **16,** Suppl. 1, S117, P 341, 42. Jahrestagung der Deutschen Diabetes-Gesellschaft, Hamburg, 2007

Kaminski M.T., Lenzen S., Baltrusch S. (2007) Live imaging of glucose uptake and metabolism in insulin-producing MIN6 cells by a fluorescent sensor. *Diabetologia* **50,** Suppl. 1, S 227, 541, 43. EASD Annual Meeting, Amsterdam, 2007

Kaminski M.T., Lenzen S., Baltrusch S. (2008) Einsatz eines fluoreszenzbasierten Sensors zur Untersuchung der Glucosehomöostase in Insulin produzierenden MIN6 Zellen. *Diabetologie und Stoffwechsel* **17,** Suppl. 1, S 32, 86, 43. Jahrestagung der Deutschen Diabetes-Gesellschaft, München, 2008

Kaminski M.T., Lenzen S., Baltrusch S. (2008) Live imaging of glucose homeostasis in MIN6 cells and primary beta cells by fluorescence microscopy. *Diabetologia* **51,** Suppl. 1, S 78, 175, 44. EASD Annual Meeting, Rome, 2008

Kaminski M.T., Lenzen S., Baltrusch S. (2009) Fluoreszenzmikroskopische Analyse der Glucosehomöostase in primären pankreatischen Beta-Zellen. *Diabetologie und Stoffwechsel* **18,** Suppl. 1, S 20, FV 59, 44. Jahrestagung der Deutschen Diabetes-Gesellschaft, Leipzig, 2009

Kaminski M.T., Lenzen S., Baltrusch S. (2009) Real-Time Analyse der Glucosehomöostase in Beta-Zellen mittels Fluoreszenzmikroskopie. Präsentiert auf dem 9. Inselworkshop, Greifswald, 2009

Kaminski M.T., Lenzen S., Baltrusch S. (2009) Real-time analysis of glucose homeostasis in beta cells by fluorescence microscopy – The FLIPglu technology. Präsentiert bei der Summer School on Endocrinology, Bregenz, 2009

Kaminski M.T., Schmitt H., Lenzen S., Baltrusch S. (2009) Fluorescence based real-time analysis of stimulus-secretion coupling in mouse beta cells. *Diabetologia* **52**, Suppl. 1, S 53, 115, 45. EASD Annual Meeting, Vienna, 2009

Langer S., Kaminski M.T., Lenzen S., Baltrusch S. (2009) Regulation of hepatic glucose homeostasis by glucokinase localization. *Diabetologia* **52**, Suppl. 1, S 239, 602, 45. EASD Annual Meeting, Vienna, 2009

Kaminski M.T., Lenzen S., Baltrusch S. (2010) Fluoreszenzmikroskopische Echtzeitanalyse der glucoseinduzierten Insulinsekretion in Beta-Zellen der Maus. *Diabetologie und Stoffwechsel* **19**, Suppl. 1, S 91, P 273, 45. Jahrestagung der Deutschen Diabetes-Gesellschaft, Stuttgart, 2010

Kaminski M.T., Lenzen S., Baltrusch S. (2010) New aspects of hepatic glucose phosphorylation by intracellular glucokinase localization. *Diabetologia* **53**, Suppl. 1, S 253, 629, 46. EASD Annual Meeting, Stockholm, 2010

Kaminski M.T., Lenzen S., Baltrusch S. (2011) Die hepatische Glucokinasetranslokation und Glucosephosphorylierung ist direkt an die Blutglucosekonzentration gekoppelt. *Diabetologie und Stoffwechsel* **20**, Suppl. 1, S 15, FV 35, 46. Jahrestagung der Deutschen Diabetes-Gesellschaft, Leipzig, 2011

Veröffentlichungen:

Langer S., Kaminski M.T., Lenzen S., Baltrusch S. (2010) Endogenous activation of glucokinase by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase is glucose dependent *Molecular Endocrinology* **24**(10):1988-1997

Kaminski M.T., Lenzen S., Baltrusch S. Real-time analysis of intracellular glucose and calcium in pancreatic beta cells by fluorescence microscopy
Zur Publikation eingereicht.

Kaminski M.T., Schultz J., Waterstradt R., Tiedge M., Lenzen S., Baltrusch S. Glucose causes dissociation of the glucokinase-glucokinase regulatory protein complex already in the nucleus of hepatocytes
Zur Publikation vorbereitet

7 Curriculum Vitae

Persönliche Daten

Name:	Martin Tobias Kaminski
Geburtsdatum und -ort:	6. Mai 1980 in Hannover
Staatsangehörigkeit:	deutsch

Schulausbildung

1986 – 1990	Grundschule Kardinal-Bertram Schule Hannover
1990 – 1992	Orientierungsstufe Döhren, Hannover
1992 – 1999	Elsa Brändström Gymnasium, Hannover Abschluss: Allgemeine Hochschulreife

Zivildienst

08/1999 – 06/2000	Deutsches Rotes Kreuz, Kreisverband Hannover-Stadt e.V.

Studium

10/2000 – 05/2006	Studium der Biologe an der Universität Hannover Abschluss: Diplom-Biologe, Abschlussnote: sehr gut. Diplomarbeit mit dem Thema: „Untersuchungen zur Proteininteraktion des Galectin-3 mit Modellproteinen während des intrazellulären Transportes“ am Institut für Physiologische Chemie, Tierärztliche Hochschule Hannover.
09/2003 – 05/2004	Auslandsaufenthalt, Northeastern University, Boston, MA, USA, DAAD-Stipendiat.

Promotion

06/2006 – 02/2012	Promotion mit dem Thema: „Fluoreszenzmikroskopische Untersuchungen zur intrazellulären Glucosehomöostase in Beta-Zellen und in der Leber“ am Institut für Klinische Biochemie, Medizinische Hochschule Hannover.

Die vorliegende Dissertation wurde im Juni 2006 unter Anleitung von Herrn Professor Dr. S. Lenzen am Institut für Klinische Biochemie der Medizinischen Hochschule Hannover begonnen und im Dezember 2011 abgeschlossen.

8 Erklärung

Hierdurch erkläre ich, dass die Dissertation

Fluoreszenzmikroskopische Untersuchungen zur intrazellulären Glucosehomöostase in Beta-Zellen und in der Leber

selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, den 15.12.2011

Martin Tobias Kaminski

9 Danksagung

Herrn Prof. Dr. Sigurd Lenzen
danke ich für die Überlassung des interessanten Dissertationsthemas, für die Bereitstellung von Arbeitsplatz und –materialien, seine hilfreiche Unterstützung und sein großes Interesse am Fortgang der Arbeit. Insbesondere danke ich Herrn Prof. Lenzen für das mir entgegengebrachte Vertrauen und sein außerordentliches Engagement im Laufe der Dissertation.

Frau Prof. Dr. Simone Baltrusch
danke ich für die außerordentlich gute Betreuung während der Durchführung meiner Arbeit. Ihre Unterstützung bei der Konzeption und Durchführung der Arbeiten, sowie ihre stetige Diskussionsbereitschaft waren eine Bedingung für das Gelingen der Arbeit.

Allen Mitarbeitern des Instituts für Klinische Biochemie der Medizinischen Hochschule Hannover möchte ich für die ständige Hilfsbereitschaft und das nette Arbeitsklima danken. Mein ganz besonderer Dank gilt Dr. Wiebke Gehrmann, Dr. Ilir Mehmeti, Dr. Heike Schmitt, Dr. Sara Langer, Maren Böger und Anke Brix, sowie Jasmin Kresse, Britta Leß, Monika Funck, Martin Wirth, Dr. Matthias Elsner, Dr. Stephan Lortz und Dr. Ortwin Naujok. Auch Rica Waterstradt aus dem Institut für Institut für Medizinische Biochemie und Molekularbiologie der Universität Rostock möchte ich danken.