Rapid Publication

Identification of the Docked Granule Pool Responsible for the First Phase of Glucose-Stimulated Insulin Secretion

Samira Daniel, Mitsuhiko Noda, Susanne G. Straub, and Geoffrey W.G. Sharp

The mechanisms underlying the first phase of glucose-stimulated insulin release, the deterioration of which marks the early stages of both type 1 and type 2 diabetes, are essentially unknown. Among many hypotheses, one holds that the first phase is due to a readily releasable pool of insulin-containing granules. We used current knowledge of the mechanisms of exocytosis and the proteins involved in docking granules at the plasma membrane to test this hypothesis. A docked pool of readily releasable granules was identified by immunoprecipitation of the plasma membrane protein syntaxin with a specific antibody and by co-immunoprecipitation of soluble *N*-ethylmaleimide-sensitive factor attachment protein-25 (SNAP-25) and the granule proteins synaptobrevin and synaptotagmin. The four SNARE proteins co-immunoprecipitated each other, thus identifying the core complex associated with docked granules. Using co-immunoprecipitation as a marker for docked granules, we found that the docked pool was rapidly discharged during the first phase of glucose-stimulated insulin release and refilled during the second phase. Other secretagogues also released the pool, whereas the physiological inhibitor norepinephrine blocked its release. Further studies on the nature of this pool of granules should shed light on the causes of its deterioration in the early stages of diabetes and the reasons for deficient insulin release. *Diabetes* 48:XXX–XXX, 1999

From the Department of Molecular Medicine, College of Veterinary Medicine, Cornell University, Ithaca, New York; M.N.'s current address is the Department of Metabolic Diseases, Faculty of Medicine, University of Tokyo, Japan.

Address correspondence and reprint requests to Department of Molecular Medicine, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853-6401.

Received for publication 23 February 1999 and accepted in revised form 11 June 1999. Posted on the World Wide Web at www.diabetes.org/diabetes on <<date>>.

KRBB, Krebs-Ringer bicarbonate buffer; NSF, *N*-ethylmaleimide-sensitive factor; PVDF, polyvinylidene fluoride; SNAP, soluble *N*-ethylmaleimide-sensitive factor attachment protein; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; TBST, Tris-buffered saline with Tween; TKM, Tris-KCl-$MgCl_2$; t-SNARE, plasma membrane target protein; v-SNARE, vesicle membrane protein.

Several pathways are involved in stimulus-secretion coupling for glucose-stimulated insulin release. The convergence of these signaling pathways on the translocation of granules, granule docking, and exocytosis results in a complex but characteristic biphasic pattern of insulin release (1–3). Much effort has been expended and many theories have been developed in trying to understand the mechanisms of biphasicity, in part because of the relationship between the onset of diabetes and the loss of the first phase of glucose-stimulated release (4,5). Translocation and exocytosis are controlled by proteins that have been highly conserved from yeast to mammals (6,7), and detailed knowledge has been gained from studies on neurotransmitter release (8,9). The basic exocytotic machinery consists of soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein (SNAP) receptors (SNAREs) that associate to form a complex on which the essential components of the docking and fusion apparatus assemble (10). The vesicle membrane proteins (v-SNAREs) synaptobrevin and synaptotagmin combine with the plasma membrane target proteins (t-SNAREs) syntaxin and SNAP-25 to constitute the SNARE complex on which the SNAPs and NSF form a multimeric complex essential for granule docking and exocytosis (6–10). Although the details and timing of these events in the β-cell are unknown, it is clear that the core complex of SNARE proteins is associated with docked granules. We have taken advantage of this core complex to identify docked granules in the β-cell and subsequently tested their ready releasability, physiological relevance, and relationship with the two phases of insulin release. Studies were carried out on the βHC-9 cell line, which is an excellent model of the mouse pancreatic β-cell from which it is derived and which responds to the physiological range of glucose concentrations with biphasic insulin release (11).

RESEARCH DESIGN AND METHODS

Materials. Monoclonal antibodies to syntaxin (10H5) and synaptotagmin (1D12) were kindly provided by Dr. Masami Takahashi. Human monoclonal antibody to syntaxin (SP6) was purchased from Upstate Biotechnology (Lake Placid, NY) and the antibody to SNAP-25 from Transduction Laboratories (Lexington, KY). Dr. Reinhard Jahn kindly gave us the monoclonal antibody to synaptobrevin 2 (C1 69.1). Mouse immunoglobulin G (IgG) and the enhanced chemiluminescence reagents were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). The protein G agarose beads (from GpC streptococcus), the prestained SDS-PAGE standards, and mastoparan were purchased from Sigma (St. Louis, MO). Norepinephrine was obtained from Fluka (Milwaukee, WI).

Cell culture. βHC-9 cells were cultured in Dulbecco's modified Eagle's medium containing 25 mmol/l glucose, 1 mmol/l pyruvate, 15% horse serum, 2.5% fetal bovine serum, 100 µg/ml streptomycin, and 100 units/ml penicillin at 37°C in a 95% air and 5% CO_2 atmosphere. The cells were cultured in 75 cm^2 flasks for the cell lysate preparations, in wells of 16 mm diameter for the insulin secretion incubations and on glass coverslips for the perifusion incubations.

SDS-PAGE and immunoblot analysis. Samples were resuspended in Laemmli sample buffer (65 mmol/l Tris, 3% SDS, 10% glycerol, 0.025 mg/ml bromophenol blue and 5% 2-mercaptoethanol) and boiled for 5 min. The samples were separated by SDS-PAGE and then transferred electrophoretically to a polyvinylidene fluoride (PVDF) membrane. The transfer solution contained 25 mmol/l Tris, 192 mmol/l glycine, and 20% v/v methanol, pH 8.3. The membrane was then blocked in Tris-buffered saline with Tween (TBST) containing 13.24 mmol/l Tris-Cl, 1.94 mmol/l Tris-Base, 32.72 mmol/l NaCl, 1.79 mmol/l EDTA, 0.05% Tween 20, 1% bovine serum albumin, and 5% nonfat dried milk for at least 1 h at 4°C. Primary antibody incubations were carried out for 1 h at 4°C. After washing with TBST buffer, at least three times for 5 min each, the membrane was incubated with horseradish peroxidase-conjugated donkey antibodies to mouse IgG diluted 1:5,000 for 1 h at 4°C. Washing of the membrane was carried out as before and the immune complexes were visualized by enhanced chemiluminescence (ECL kit).

Fractionation of βHC-9 cells and immunoprecipitation. Membrane and cytosol protein fractions were prepared as follows. βHC-9 cells were washed twice with phosphate-buffered saline, scraped off the flasks and the samples collected in 1.5 ml Eppendorf tubes. After centrifugation at 4°C at 15,000 rpm, the pellets were stored at –80°C for at least 30 min and quickly thawed at room temperature. The samples were then suspended in Tris-KCl-$MgCl_2$ (TKM) buffer (25 mmol/l Tris HCl, pH 7.4, 25 mmol/l KCl, 5 mmol/l $MgCl_2$, 0.25 mol/l sucrose, and the protease inhibitors aprotinin, leupeptin, and phenylmethylsulfonyl fluoride) and centrifuged for 5 min at 1,000 rpm. The supernatant was centrifuged at 15,000 rpm for 1 h. The resulting supernatant contained the cytosolic fraction and was collected while the remaining pellet was solubilized on ice in TKM buffer containing 1% NP40. After centrifugation at 4°C for 15 min at 15,000 rpm, the supernatant was collected as the solubilized crude membrane fraction.

Immunoprecipitation reactions were carried out by first incubating the antibody with protein G agarose beads for a couple of hours at 4°C with shaking, followed by three washes with a lysis buffer [50–137 mmol/l NaCl, 15.7 mmol/l $NaH_2PO_4(H_2O)$, 1.47 mmol/l KH_2PO_4, 2.68 mmol/l KCl, 1% NP40, 1 mmol/l dithiothreitol (DTT), 15 mmol/l $MgCl_2$, protease inhibitors, pH 7.4]. The lysate samples were incubated with the antibody bound protein G agarose beads for 2 h at 4°C with shaking. After several washes with the lysis buffer, the beads were resuspended in Laemmli sample buffer.

Treatment of βHC-9 cells. Flasks (75 cm^2) of βHC-9 cells were washed twice in Krebs-Ringer bicarbonate buffer (KRBB) composed of 129 mmol/l NaCl, 5 mmol/l $NaHCO_3$, 4.8 mmol/l KCl, 1.2 mmol/l KH_2PO_2, 2.0 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, 0.2% bovine serum albumin, and 10 mmol/l 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (pH 7.4) containing 0.1 mmol/l glucose. Preincubation was carried out at 37°C in KRBB for 30 min. After aspirating out the medium, 10 ml of fresh KRBB containing 30 mmol/l glucose, 35 mmol/l KCl, or 15 µmol/l mastoparan was used for the incubation conditions, with KRBB alone serving as the control. During inhibitor treatment, norepinephrine was added along with glucose, KCl, and mastoparan during the incubation period. The incubations were carried out for 10 min for glucose and mastoparan and for 2 min for KCl. The reactions were stopped by removing the medium and washing the cells twice with cold phosphate-buffered saline. After completely aspirating out any leftover solution, the flasks were incubated in 700 µl of lysis buffer and rocked at 4°C for 30 min. The sample was collected in 1.5 ml Eppendorf tubes and centrifuged at 15,000 rpm for 5 min. The resulting supernatant was collected as cell lysate.

Insulin secretion under static incubation conditions. Wells of 16 mm in diameter of βHC-9 cells grown to the concentration of 0.4–1.6 × 10^6 were washed and then preincubated for 30 min at 37°C in 1.5 ml KRBB. After the medium was removed, 1 ml of fresh KRBB containing test substances was given to the cells for 60 min at 37°C or 30 min in the case of mastoparan. At the end of the incubations, the medium was collected from each of the samples and centrifuged briefly to sediment any detached cells. The supernatants were stored at –20°C until radioimmunoassay. After any leftover buffer was removed from the wells by aspiration, 1 ml of a solution containing 77% ethanol and 1% HCl was added to each well to extract the insulin and determine the insulin content of the cells. The plates were kept overnight at –20°C until assay. The data are expressed as means ± SE. Statistical significance was evaluated by one-way analysis of variance by Bonferroni's method.

Insulin secretion under perifusion conditions. The perifusion system is as described previously (12) with some modifications. In brief, βHC-9 cells were grown on glass coverslips, which were then placed in each of the four 0.7-ml perifusion chambers with the cells facing the inside of the chamber. The cells were perifused with KRBB and 0.1 mmol/l glucose at 37°C for 30- or 60-min equilabration periods prior to the test periods with 30 mmol/l glucose.The specific protocols are described in the text and figure legends. Samples were collected at 1- and 2-min intervals for the measurement of insulin secretion. At the end of the experiment, the coverslips were suspended in 1 ml of solution of 77% ethanol and 1% HCl to extract the insulin and kept at –20°C until assay to measure the residual insulin content.

RESULTS AND DISCUSSION

Identification of the core complex of docked granules. The presence of SNARE proteins in the βHC-9 cells was demonstrated by Western blotting. Four components of the core complex—synaptotagmin, syntaxin, SNAP-25 (data not shown), and synaptobrevin—were detected in a membrane fraction from the cells at molecular weights of 65, 35, and 18 kDa, respectively (Fig. 1*A*). These proteins could not be detected in cytosol fractions. The four antibodies used in the Western blots were then used to immunoprecipitate core complex proteins. All four antibodies immunoprecipitated the appropriate protein and co-immunoprecipitated the three

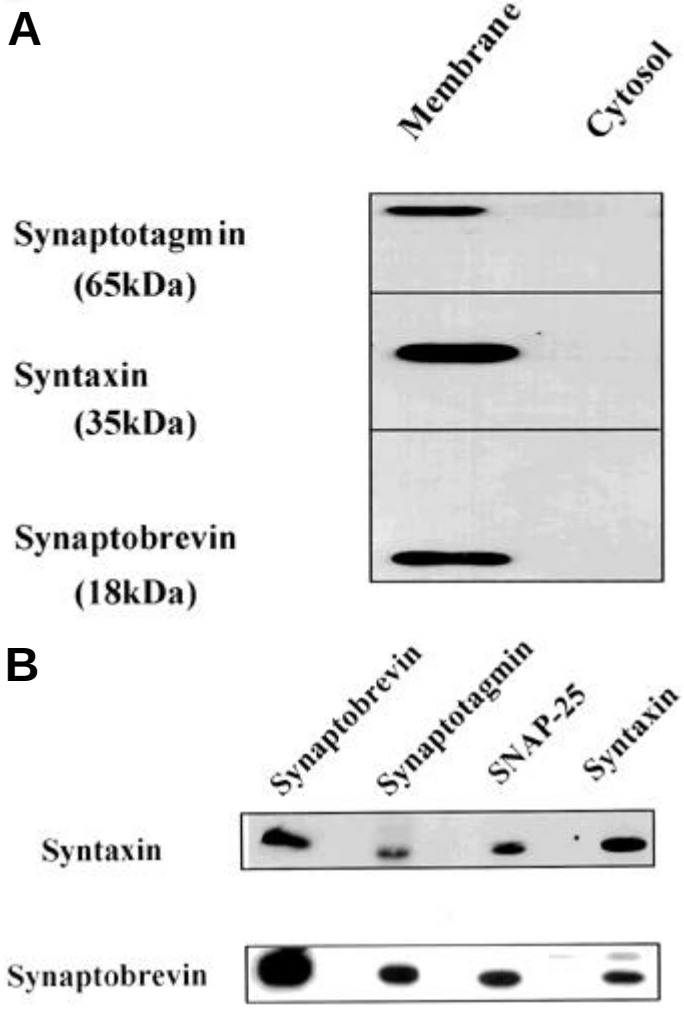

FIG. 1. Localization of SNARE proteins and co-immunoprecipitation of the core complex. *A*: Membrane and cytosol protein fractions were separated by a 15% SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies against synaptotagmin, syntaxin, and synaptobrevin. *B*: Cell lysates from βHC-9 cells prepared in TKM buffer were immunoprecipitated with antibodies against synaptobrevin, synaptotagmin, SNAP-25, and syntaxin. The immunoprecipitated proteins were separated by 15% SDS-PAGE. After transfer of the proteins to a PVDF membrane, the membrane was cut based on molecular weight and immunoblotted with the anti-syntaxin (*upper panel*) or anti-synaptobrevin antibodies (*lower panel*).

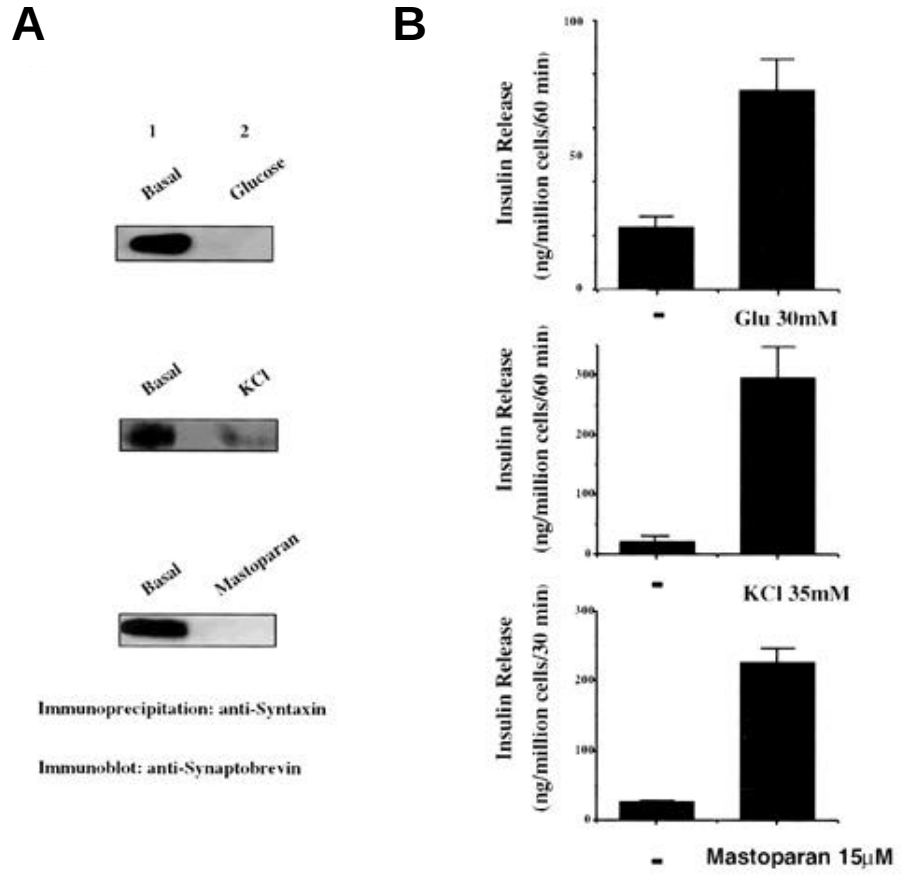

FIG. 2. Effects of secretagogues on insulin secretion and the core complex in βHC9 cells. *A*: βHC-9 cells grown in 75 cm^2 flasks were preincubated at 37°C in KRBB for 30 min. During the incubation period, KRBB containing 0.1 mmol/l glucose served as the control, and 30 mmol/l glucose, 35 mmol/l KCl, or 15 μmol/l mastoparan was used to stimulate insulin secretion. The incubations were carried out for 10 min for glucose and mastoparan and for 2 min for KCl. Samples were immunoprecipitated with protein G agarose-conjugated anti-syntaxin antibody and separated by 15% SDS-PAGE. After transfer of the proteins to PVDF membranes, the membrane was incubated with the anti-synaptobrevin antibody. *Lane 1* denotes the control or basal conditions; *lane 2* denotes the secretagogue-treated conditions. *B*: βHC-9 cells grown in wells of 16 mm in diameter were treated with KRBB containing the stimulators of insulin release, 30 mmol/l glucose and 35 mmol/l KCl for 60 min and 15 μmol/l mastoparan for 30 min, with KRBB alone serving as the control. The graphs in *B* denote the insulin release measured from the above treated cells. Values are mean ± SE for each condition. The number of wells (*n*) used for each condition was four. Statistical significance is *P* < 0.01 vs. basal for glucose, KCl, and mastoparan.

other proteins. Data showing the co-immunoprecipitation of syntaxin and synaptobrevin are depicted in Fig. 1*B*.

Effect of stimulators of insulin secretion—glucose, potassium chloride, and mastoparan—on the core complex. Because the co-immunoprecipitation of v-SNARE proteins by antibodies against t-SNARE proteins (and vice-versa) identifies the core complex of docked granules, and thereby the docked granules themselves, we used the interaction between the v-SNARE synaptobrevin and the t-SNARE syntaxin to characterize the pool of docked granules. We tested lysates prepared from βHC-9 cells that had been treated with stimulators and inhibitors of insulin secretion. Thus, the cells were exposed to high glucose concentrations (20–30 mmol/l), to a depolarizing concentration of KCl (35 mmol/l), and to the wasp venom peptide mastoparan (15 μmol/l). These were chosen because of the variety of mechanisms by which they stimulate insulin release (Fig. 2*B*), with glucose as the archetypical physiological stimulator, KCl to depolarize the cell and provide a "pure" Ca^{2+} stimulus, and mastoparan as the putative Ge-protein stimulator (13,14). The results shown in Fig. 2*A* are from cells exposed to glucose and mastoparan for 10 min (though we found subsequently that 5 min of exposure to glucose is sufficient to discharge the docked pool [Fig. 6]) and to KCl for only 2 min. The reason for the shorter period for KCl is that the secretory response to KCl is both faster and larger than the response to glucose. The antibody against syntaxin was used to co-immunoprecipitate synaptobrevin. Immuno-blots revealed that under basal nonstimulatory conditions, synaptobrevin co-precipitated with syntaxin, indicating the presence of docked granules. Exposure of the βHC-9 cells to the stimulators of insulin release resulted, in all three

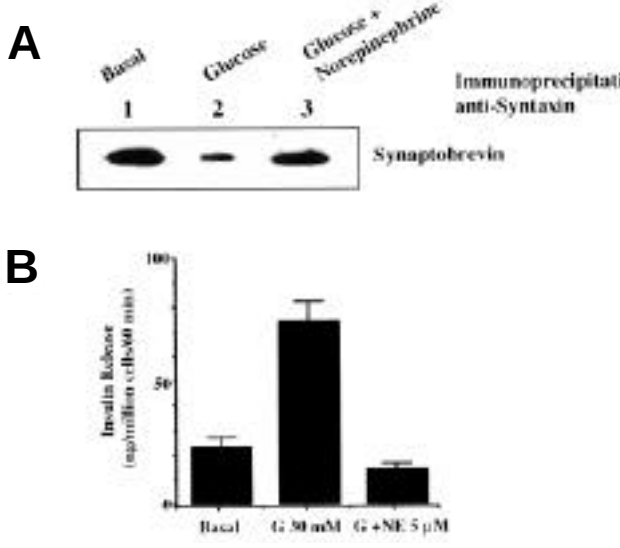

FIG. 3. Effect of the α2-adrenergic physiological inhibitor norepinephrine on glucose-stimulated insulin release. βHC-9 cells were stimulated with 30 mmol/l glucose with and without 10 μmol/l norepinephrine. Cells incubated in KRBB alone served as the control or basal conditions. The incubation and immunoblotting reactions shown in *A* were carried out as described in METHODS. *Lane 1* represents βHC-9 cells treated with only KRBB containing 0.1 mmol/l glucose; *lane 2* represents 30 mmol/l glucose-stimulated βHC-9 cells; and *lane 3* represents βHC-9 cells exposed to 30 mmol/l glucose in the presence of 10 μmol/l norepinephrine. *B* represents the insulin release measurements from βHC-9 cells that were treated as described for *lanes 1, 2,* and *3* in *A*. The insulin assay was performed as described in METHODS. Values are means ± SE of four experiments. The stimulation of release by 30 mmol/l glucose and inhibition by 10 μmol/l norepinephrine were both statistically significant (*P* < 0.01). The norepinephrine-treated cells had a release rate that was not significantly different from basal.

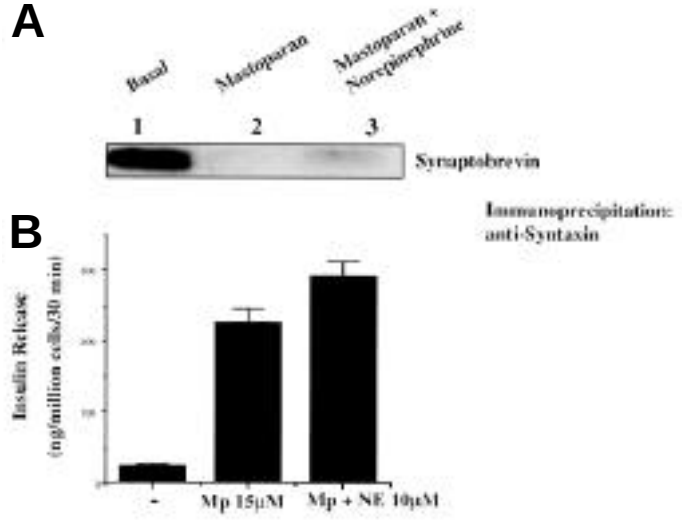

FIG. 4. Lack of effect of the inhibitor norepinephrine on mastoparan-stimulated insulin release. βHC-9 cells were stimulated with 15 μmol/l mastoparan with and without 10 μmol/l norepinephrine. Cells incubated in KRBB alone served as the control or basal conditions. As shown in *A*, the samples were subject to immunoprecipitation by the antibody against syntaxin, followed by SDS-PAGE and immunoblotting with the anti-synaptobrevin antibody. *Lane 1* represents βHC-9 cells treated with only KRBB containing 0.1 mmol/l glucose; *lane 2* represents 15 μmol/l mastoparan-stimulated βHC-9 cells; and *lane 3* represents the cells exposed to 15 μmol/l mastoparan and 10 μmol/l norepinephrine. *B* represents the insulin release measurements from βHC-9 cells that were treated as described for *lanes 1*, *2*, and *3* in *A*. The insulin assay was performed as described in METHODS. Values are means ± SE of four experiments. Stimulation of release in the presence of mastoparan was statistically significant compared with basal release in the absence and presence of norepinephrine ($P < 0.01$). There was no significant effect of norepinephrine.

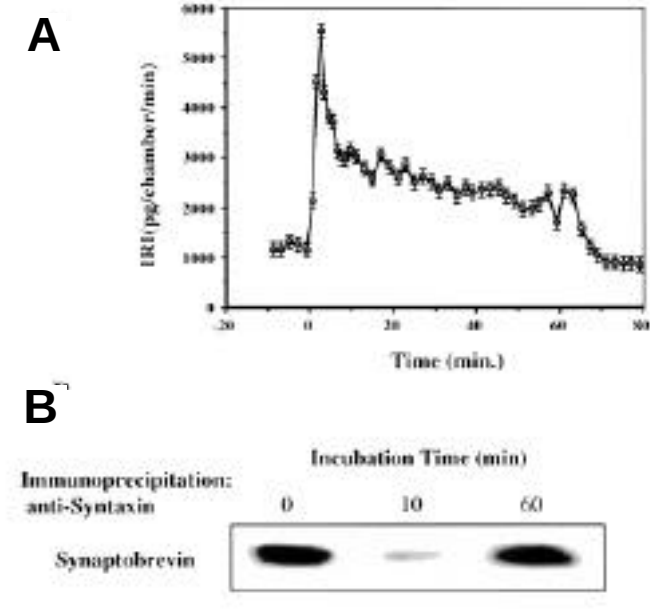

FIG. 5. Dynamic changes in insulin secretion and the core complex. *A*: Perifusion studies as described in METHODS were carried out with the addition of 30 mmol/l glucose during the incubation period. Values are means ± SE of four determinations. IRI, immunoreactive insulin. *B*: βHC-9 cells were treated with 30 mmol/l glucose for 0 min, 10 min, and 60 min. Cell lysates were prepared as described in METHODS, followed by immunoprecipitation of the samples by an anti-syntaxin antibody bound to protein G agarose beads. After SDS-PAGE and transfer of the proteins onto a PVDF membrane, the blots were probed with the anti-synaptobrevin antibody.

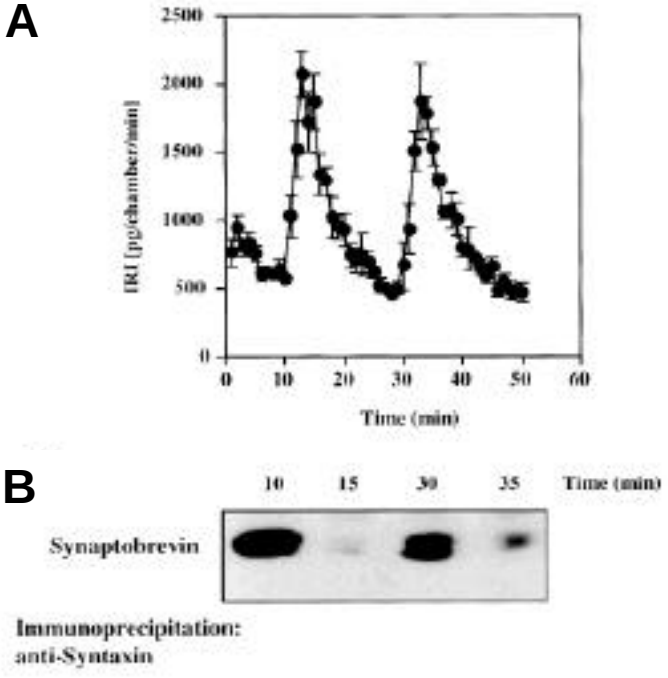

FIG. 6. Perifusion and immunoblot analyses of repeated glucose stimulation in βHC-9 cells. *A:* Perifusion studies on βHC-9 cells were carried out as described in METHODS. During the incubation period, two 30 mmol/l glucose treatments were given, each for a period of 5 min. The two 5-min stimulatory periods were separated by a 15-min perifusion in the presence of 0.1 mmol/l glucose. IRI, immunoreactive insulin. Values are means ± SE of four experiments. *B*: βHC-9 cells were incubated in KRBB containing 0.1 mmol/l or 30 mmol/l glucose according to the protocol described in METHODS. Equal amounts of the cell lysates at the 10-min, 15-min, 30-min, and 35-min time points were immunoprecipitated with the anti-syntaxin antibody conjugated to protein G agarose beads and separated by SDS-PAGE. The blots were then probed with the anti-synaptobrevin antibody.

cases, in the discharge of the core complex—as judged by failure of the antibody against syntaxin to immunoprecipitate synaptobrevin from the cell lysates (Fig. 2*A*). These results imply that the granules that are already docked are being stimulated by glucose, KCl, and mastoparan to fuse with the plasma membrane and release insulin.

Effect of a physiological inhibitor of insulin secretion, norepinephrine, on the core complex. To confirm that co-immunoprecipitation of the core complex proteins identifies the docked granules, further studies were performed by exposing the cells to the stimulators of insulin secretion, glucose or mastoparan, in the absence and presence of norepinephrine. Norepinephrine completely blocks insulin release in response to a high glucose concentration (Fig. 3*B*) but has no effect on mastoparan-stimulated release (Fig. 4*B*). As can be seen in Fig. 3*A*, as norepinephrine blocks glucose-stimulated insulin release, so norepinephrine blocks the effect of glucose to discharge the docked granule pool. Mastoparan, however, discharged the docked granule pool in both the absence and presence of norepinephrine, in accord with its ability to stimulate insulin secretion regardless of the presence of norepinephrine (Fig. 4*A*). Thus, the co-immunoprecipitation of synaptobrevin with syntaxin identifies a physiologically releasable pool of docked granules.

Two granule pools are responsible for the biphasic release of insulin. We then studied the time course of

action of high glucose concentrations on insulin secretion and on the docked pool. βHC-9 cells perifused in 0.1 mmol/l glucose were subsequently exposed to 30 mmol/l glucose for 60 min. The rate of insulin secretion was monitored at 1- or 2-min intervals. Cell lysates were prepared and immunoprecipitated by antibody against syntaxin at zero time, 10 min, and 60 min, times that correspond to basal conditions, the end of the first phase of insulin secretion, and late in the second phase of secretion. The pattern of the response, with a first phase of secretion that peaks at 5 min and is complete at 10 min and a prolonged plateau of elevated release, is seen clearly in Fig. 5*A*. Also shown in Fig. 5*B* is the Western blot for synaptobrevin from the syntaxin immunoprecipitate at these time points. The core complex is present at zero time (basal conditions) but has been largely discharged by the end of the first phase at 10 min. Subsequently, as judged by the presence of the core complex at 60 min, the docked pool of granules has been refilled. One conclusion that can be drawn from these studies is that the docked pool of granules, detected by co-immunoprecipitation of syntaxin and synaptobrevin, is the pool of granules that is responsible for the first phase of glucose-stimulated insulin secretion. A further conclusion is that during the second phase of glucose-stimulated release, the rate of recruitment of granules from the reserve pool to the docked pool exceeds the rate of granule exocytosis. This imbalance in rates leads to the refilling of the docked granule pool.

The question arises as to why the refilled pool, seen at 60 min, is not readily released by the glucose stimulus, which is still present and which has been shown to result in a continuously elevated intracellular Ca^{2+} concentration (11). We surmised that the inability of the glucose stimulus to raise the rate of secretion during the second phase to a level equal to that of the first phase, despite the presence of a docked pool of apparently equal size, might be due to the time required to prepare (prime) the granules for release after they have docked. In other words, the rate of second-phase insulin release is governed by the rate at which the docked granules can be primed. To test this possibility, the βHC-9 cells were exposed to 30 mmol/l glucose for 5 min to induce a first phase of release and discharge the docked pool; returned to 0.1 mmol/l glucose for the next 15 min (to take off the stimulus); and then restimulated with 30 mmol/l glucose for a further 5 min. Cell lysates were prepared from cells under basal conditions at 10 and 30 min and under stimulated conditions at 15 and 35 min. Exposure to 30 mmol/l glucose at 10 min resulted, as expected, in a first phase of insulin secretion. Removal of the glucose stimulus at 15 min resulted in a rapid reduction of the insulin release rate to baseline levels. Restimulation by glucose at 30 min resulted in another "first phase" of secretion (see Fig. 6*A*). These data were correlated with the immunoprecipitation data (shown in Fig. 6*B*). A pool of docked granules was clearly detected under basal conditions at zero time. The pool was discharged in 5 min at the peak of the first phase of insulin secretion. After return of the cells to basal conditions (0.1 mmol/l glucose), the docked pool was regenerated at 30 min and again discharged by the glucose stimulus. It seems probable, therefore, that the first phase of insulin release is due to a pool of granules that is primed and ready for release. As this primed, docked pool is rapidly discharged during the first phase, the second phase of release must be due to granules that are translocated from a reserve pool to the plasma membrane, and have to be primed before release. The rate at which the granules are primed after docking appears to be rate limiting for insulin exocytosis during the second phase.

In summary, we have identified the docked pool of granules that is responsible for the first phase of glucose-stimulated insulin release. After discharge during the first phase, the pool is refilled by translocation of granules from a reserve pool at a rate that exceeds the second-phase release rate. Thus, the rate of release of insulin during the second phase is not determined by the rate of translocation. Rather, it is the rate of priming after docking that is rate limiting. Interestingly, these results confirm a two-compartment model for the β-cell that was proposed 30 years ago by Grodsky et al. (15) and proposed again more recently on the basis of capacitance measurements (16).

ACKNOWLEDGMENTS

The work described here was supported by grants from the National Institutes of Health (RO1-DK-42063 and RO1-DK-54243) and by a Mentor-Based Postdoctoral Fellowship from the American Diabetes Association. The authors are grateful to Dr. Masami Takahashi for his generous gift of antibodies against syntaxin and synaptotagmin, to Dr. Reinhard Jahn for the antibody against synaptobrevin, and to Dr. Douglas Hanahan for βHC-9 cells. We are also grateful to Dr. Troitza Bratanova-Tochkova for maintaining βHC-9 cells.

REFERENCES

1. Newgard CB, McGarry JD: Metabolic coupling factors in pancreatic β-cell signal transduction. *Ann Rev Biochem* 64:689–719, 1995
2. Zawalich WS, Kelley GG: The pathogenesis of NIDDM: the role of the pancreatic beta cell. *Diabetologia* 38:986–991, 1995
3. Ashcroft FM, Proks P, Smith PA, Ammala C, Bokvist K, Rorsman P: Stimulus-secretion coupling in pancreatic beta cells. *J Cell Biochem* 55:54–65, 1994
4. Cerasi E, Luft R: The plasma insulin response to glucose infusion in healthy subjects and in diabetes mellitus. *Acta Endocrinol* 55:278–304, 1967
5. Cerasi E: Potentiation of insulin release by glucose in man. *Acta Endocrinol* 79:511–534, 1975
6. Finger FP, Novick P: Spatial regulation of exocytosis: lessons from yeast. *J Cell Biol* 142:609–612, 1998
7. Ferro-Novick S, Jahn R: Vesicle fusion from yeast to man. *Nature* 370: 191–193, 1994
8. Parsons TD, Coorssen JR, Horstmann H, Lee AK, Tse FW, Almers W: The last seconds in the life of a secretory vesicle. *Cold Spring Harb Symp Quant Biol* 60:389–396, 1995
9. Sudhof TC: The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature* 375:645–653, 1995
10. Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempts P, Rothman JE: SNAP receptors implicated in vesicle targeting and fusion. *Nature* 362:318–324, 1993
11. Noda M, Komatsu M, Sharp GWG: The βHC-9 pancreatic beta-cell line preserves the characteristics of progenitor mouse islets. *Diabetes* 45:1766–1773, 1996
12. Kikuchi M, Rabinovitch A, Blackard WG, Renold AE: Perifusion of pancreas fragments: a system for the study of dynamic aspects of insulin secretion. *Diabetes* 23:550–559, 1974
13. Gomperts BD: Ge: a GTP-binding protein mediating exocytosis. *Ann Rev Physiol* 52:591–606, 1990
14. Komatsu M, McDermott AM, Gillison SL, Sharp GWG: Mastoparan stimulates exocytosis at a Ca^{2+}-independent late site in stimulus-secretion coupling: studies with the RINm5F β-cell line. *J Biol Chem* 268:23297–23306, 1993
15. Grodsky GM, Curry D, Landahl H, Bennett L: Further studies on the dynamic aspects of insulin release *in vitro* with evidence for a two-compartmental storage system. *Acta Diabetol* 6:554–578, 1969
16. Proks P, Eliasson L, Ammala C, Rorsman P, Ashcroft FM: Ca^{2+}- and GTP-dependent exocytosis in mouse pancreatic β-cells involves both common and distinct steps. *J Physiol* 496:255–264, 1996