Triggering and Augmentation Mechanisms, Granule Pools, and Biphasic Insulin Secretion

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The insulin secretory response by pancreatic β-cells to an acute "square wave" stimulation by glucose is characterized by a first phase that occurs promptly after exposure to glucose, followed by a decrease to a nadir, and a prolonged second phase. The first phase of release is due to the ATP-sensitive K^+ (K_{ATP}) channel-dependent (triggering) pathway that increases $[Ca^{2+}]_i$ and has been thought to discharge the granules from a "readily releasable pool." It follows that the second phase entails the preparation of granules for release, perhaps including translocation and priming for fusion competency before exocytosis. The pathways responsible for the second phase include the K_{ATP} channel-dependent pathway because of the need for elevated $[Ca^{2+}]_i$ and additional signals from K_{ATP} channel-independent pathways. The mechanisms underlying these additional signals are unknown. Current hypotheses include increased cytosolic long-chain acyl-CoA, the pyruvate-malate shuttle, glutamate export from mitochondria, and an increased ATP/ADP ratio. In mouse islets, the β-cell contains some 13,000 granules, of which ~100 are in a "readily releasable" pool. Rates of granule release are slow, e.g., one every 3 s, even at the peak of the first phase of glucose-stimulated release. As both phases of glucose-stimulated insulin secretion can be enhanced by agents such as glucagon-like peptide 1, which increases cyclic AMP levels and protein kinase A activity, or acetylcholine, which increases diacylglycerol levels and protein kinase C activity, a single "readily releasable pool" hypothesis is an inadequate explanation for insulin secretion. Multiple pools available for rapid release or rapid conversion of granules to a rapidly releasable state are required. *Diabetes* 51 (Suppl. 1):S83–S90, 2002

Our knowledge of the mechanisms involved in stimulus-secretion coupling in the pancreatic β-cell has increased remarkably in the last 10–15 years. In 1984, the ATP-sensitive K^+ (K_{ATP}) channel was identified (1) and found to be responsible for glucose-induced depolarization of the β-cell (2). Closure of this channel increases Ca^{2+} entry and $[Ca^{2+}]_i$ and thereby stimulates insulin release (3,4). This pathway is usually described as the K_{ATP} channel-dependent pathway. In 1992, three papers reported the existence of a glucose signaling pathway that was "independent" of the K_{ATP} channel (5–7). This pathway does not increase $[Ca^{2+}]_i$ but strongly augments the secretory response when $[Ca^{2+}]_i$ is increased by other means. This pathway (and there may be more than one) is usually described as the "K_{ATP} channel-independent" pathway and works in synergy with the K_{ATP} channel-dependent pathway (5–8). This means, of course, that the pathway is not strictly independent of the K_{ATP} channel. The latter (the K_{ATP} channel-dependent pathway) provides the elevation of $[Ca^{2+}]_i$, the former (the K_{ATP} channel-independent pathway) augments the response to the increased $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ requirement for both pathways is in accord with the long-held conviction that an elevation of $[Ca^{2+}]_i$ is critical to the stimulation of insulin secretion (3). Subsequently, however, we found that glucose could augment insulin secretion in Ca^{2+}-depleted rat islets in the complete absence of extracellular Ca^{2+} and in the absence of any rise in $[Ca^{2+}]_i$ (9). This glucose augmentation effect operates best when β-cell protein kinase (PK) A and C are simultaneously maximally activated. Under these conditions, glucose augmentation can be demonstrated at very low $[Ca^{2+}]_i$ (10). A physiological role for this novel pathway is possible, though not yet proven, because the combination of pituitary adenylyl cyclase activating peptide (PACAP) and carbachol, which activate PKA and PKC, respectively, promotes glucose augmentation of insulin release in the absence of any rise in $[Ca^{2+}]_i$ in both HIT cells (11) and rat islets (10). The pancreatic islet in vivo is stimulated not by glucose alone or single agonists, but concurrently by multiple agonists, including amino acids, fatty acids, acetylcholine, PACAP, glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide 1 (GLP-1) (3,12–15), and several other agonists. Therefore, this pathway may have a physiological role in the control of insulin secretion. The pathway can be described as K_{ATP}

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Accepted for publication 18 June 2001.

CAPS, Ca^{2+}-dependent activator protein for secretion; DAG, diacylglycerol; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucogon-like peptide 1; K_{ATP}, ATP-sensitive K^+; NSF, *N*-ethylmaleimide-sensitive factor; PAC, pituitary adenylyl cyclase activating peptide; PK, protein kinase; SNAP-25, synaptosomal-associated protein 25; SNARE, NSF attachment protein receptor; t-SNARE, target-SNARE; VAMP-2, vesicle associated membrane protein 2; VIP, vasoactive intestinal peptide; v-SNARE, vesicle-SNARE.

The symposium and the publication of this article have been made possible by an unrestricted educational grant from Servier, Paris.

channel-independent, Ca^{2+}-independent in contrast to the K_{ATP} channel-independent, Ca^{2+}-dependent pathway discovered in 1992. It has also been shown that the K_{ATP} channel-independent pathway augments insulin secretion stimulated by mastoparan in both rat and human islets (16). It seems likely that in these diverse situations, the augmentation pathways have similar mechanisms.

THERE ARE FOUR WELL-ESTABLISHED PATHWAYS OF β-CELL STIMULUS-SECRETION COUPLING

1. Depolarization (triggering)

By the K_{ATP} channel-dependent pathway. Increased concentrations of glucose and other nutrients cause depolarization via closure of the K_{ATP} channel, increased Ca^{2+} entry via voltage-dependent Ca^{2+} channels, increased $[Ca^{2+}]_i$, and increased rates of exocytosis (1–4).

By increased concentrations of arginine. The depolarization in this case results from entry of the positively charged amino acid via CAT2A, a cationic amino acid transporter (17).

2. Augmentation

By the K_{ATP} channel-independent Ca^{2+}-dependent pathway of glucose action (5–7). This pathway acts at a site distal to the elevation of $[Ca^{2+}]_i$. The mechanisms of action have not been defined, and several candidate mechanisms exist. Among these are a glucose-induced increase in the concentration of malonyl CoA, inhibition of carnitine palmitoyl transferase I, decreased fatty acid oxidation, and an increase in cytosolic long-chain fatty acids (18,19). The latter have the potential to act directly (20) or indirectly as signal moieties, e.g., to activate PKC isoforms that can stimulate exocytosis or to act via palmitoylation or other acylation reactions. The malonyl CoA hypothesis is currently controversial (21,22), and other candidate mechanisms exist. These include the pyruvate-malate shuttle (23), glutamate (24), and the ATP/ADP ratio (25).

By the K_{ATP} channel-independent Ca^{2+}-independent pathway of glucose action (9,10). The question arises as to whether this pathway is the same as the K_{ATP} channel-independent (Ca^{2+}-dependent) pathway that was described earlier (5–8) or whether it is a novel and distinct pathway that only exerts its full effect in the presence of maximally activated PKC and PKA. If the former is the case, then there are at least two possible consequences: *1*) combined activation of PKA and PKC is mimicking the effect of elevated $[Ca^{2+}]_i$; or *2*) the augmentation pathways act on different targets, i.e., those involved in exocytosis that are not triggered by Ca^{2+}. The most obvious possibility for the latter would be GTP-dependent exocytosis (26–28). Ca^{2+}-dependent augmentation by glucose is resistant to a reduction in cellular GTP content by mycophenolic acid, whereas Ca^{2+}-independent augmentation is abolished by such treatment (29). This finding suggests two situations: *1*) the mechanisms of augmentation involve GTP-dependent steps; and *2*) there is only one glucose-augmentation pathway, but it acts on the two separate mechanisms by which Ca^{2+} and GTP can independently stimulate exocytosis in the β-cell (30–35). The concept of a G protein (Ge) controlling exocytosis, first postulated in 1986 (27), is well developed (28), despite the fact that Ge has yet to be identified. Both heterotrimeric and low molecular weight GTP-binding proteins are involved in control of translocation and exocytosis (28,36–41). In addition, mastoparan, a tetradecapeptide purified from wasp venom with the ability to activate G proteins, stimulates insulin release in a Ca^{2+}-independent manner (16,42) and this stimulation is also augmented by glucose (16).

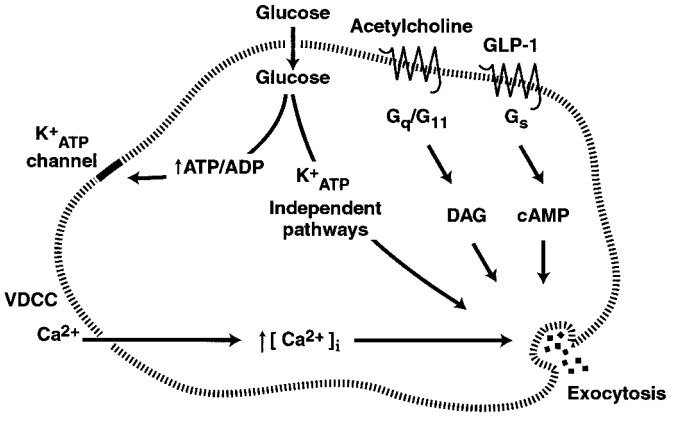

FIG. 1. Major signaling pathways for glucose and hormones in the pancreatic β-cell. The figure illustrates the effect of glucose to depolarize the β-cell via activation of the K_{ATP} channel-dependent pathway and to augment Ca^{2+}-stimulated insulin release via the K_{ATP} channel-independent pathways. Additionally, pathways that potentiate insulin secretion via increased levels of cyclic AMP and DAG are shown. VDCC, voltage-dependent calcium channel.

3. Activation of phospholipases and PKC.

These pathways are activated by hormones such as acetylcholine. Increased phosphoinositide turnover results in mobilization of stored calcium to increase $[Ca^{2+}]_i$ and increased production of diacylglycerol (DAG), which activates PKC isoforms. This pathway has important enhancing effects on stimulated release (12).

4. Stimulation of adenylyl cyclase activity and activation of PKA.

These pathways are activated by hormones such as vasoactive intestinal peptide (VIP), PACAP, GLP-1, and GIP. These hormones, acting via Gs, stimulate adenylyl cyclase and cause a rise in cyclic AMP and activation of PKA. The increased activity of PKA potentiates insulin secretion (13). It should be noted, however, that there might be additional signaling pathways for agonists that activate Gs, as has been shown for VIP, PACAP, and GIP (14,15) (A general scheme is shown in Fig. 1).

READILY RELEASABLE AND RESERVE GRANULE POOLS IN β-CELLS

Obviously, the stimulation of insulin release by even one secretagogue, such as glucose, is due to a strictly coordinated interplay of many factors with bearing on granule movements: docking at the plasma membrane, preparation for release (priming), and exocytosis. In the β-cell, the total number of insulin-containing granules is in large excess over the number required to control the glycemia of a single meal. Typically, only a small percentage of the granules, and therefore of the total insulin content of the β-cell, is secreted in response to a glucose stimulus. The complexity of the granule population in the β-cell is not yet understood but can be described in terms of at least three pools: a reserve pool, a morphologically docked pool (of granules that are in contact with the plasma membrane), and a readily releasable pool (36). In this model, the docked pool contains granules in different states of

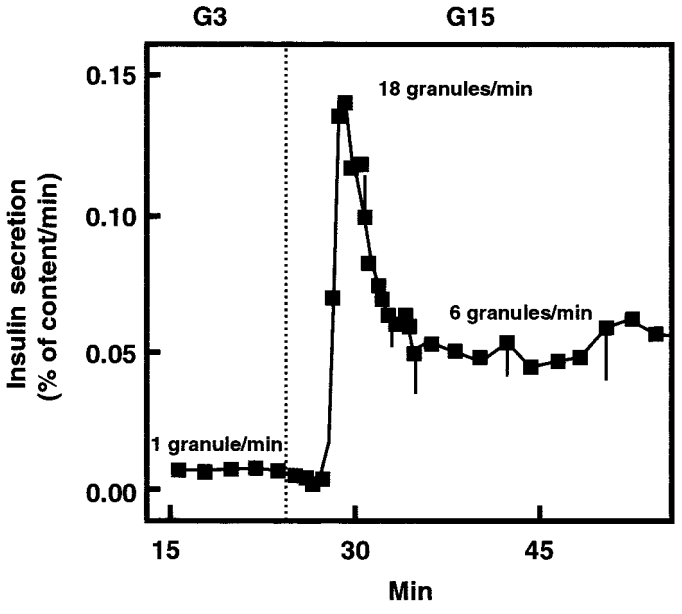

FIG. 2. The biphasic insulin secretory response to 16.7 mmol/l glucose by mouse islets. The results are expressed as percent of total insulin content released per minute. Also shown are the number of granules released per β-cell per minute under basal conditions, at the peak of the first phase, and during the second phase. The figure is adapted from the paper by Anello et al. (45).

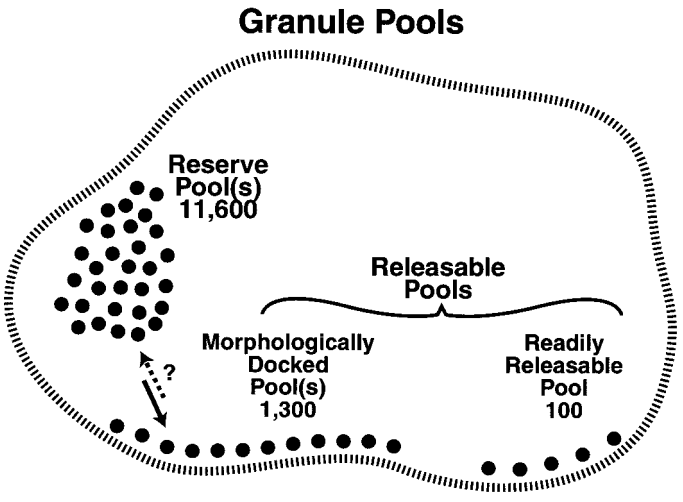

FIG. 3. Schematic representation of the reserve, morphologically docked, and readily releasable granule pools in the mouse pancreatic β-cell. The numbers of granules in the pools are derived from papers by Dean (44), Anello et al. (45), and Rorsman et al. (46).

readiness for secretion (primed or nonprimed) and includes the readily releasable pool. The reserve pool complexity can be deduced from the fact that granules containing newly synthesized insulin appear to be preferentially secreted relative to other granules (43). Nevertheless, given the state of our current knowledge, it is useful to describe the granules as being either in a readily releasable pool or in reserve granule pools. The reserve granule pools are large compared with the readily releasable pool. In the mouse β-cell, the total granule population has been estimated by quantitative morphometry as 13,000 (44). The number of rapidly releasable granules has been estimated by capacitance studies to range from 40 to 100 granules or only 0.3–0.7% of the total. As the readily releasable pool is associated with the first phase of glucose-stimulated insulin release, it is obvious that the sustained second phase of glucose-stimulated release must involve translocation of granules from reserve pools to the readily releasable pool or transformation of morphologically docked granules to release competency before exocytosis. While one can intuitively associate the first phase of release with the readily releasable pool, the idea is supported quantitatively from measurements of release rates.

Figure 2 shows the insulin secretory response to 16.7 mmol/l glucose by mouse islets (45). The secretion rates are expressed as the percentage of islet insulin content released per minute. As a result, it is also possible to calculate the rate of insulin secretion as the number of granules released by an "average" β-cell per minute. The latter method of expression is calculated from the total number of granules in the mouse β-cell (13,000), and the assumption that the cells contain the same number of granules and insulin content. Expressed in this manner, it can be seen that, at the peak of the first phase of insulin secretion, the mouse β-cell is releasing granules at a rate of approximately one every 3 s. During the second phase of sustained release, the β-cell is releasing granules at a rate of one every 10 s. Although the words fast and slow are subjective, there is little doubt that the rate of granule release by the β-cell is slow! In the sustained second phase of glucose-stimulated insulin secretion, only 6 of the 13,000 granules in the cell undergo exocytosis every minute. Knowledge of these rates is of importance to our understanding of the control of insulin secretion. Integrating the first phase of release in the data shown in Fig. 2 to determine the size of the readily releasable pool gives a value of ~100 granules, a value in close agreement with the value derived from capacitance studies.

In quantitative morphometric studies of electron micrographs of β-HC9 cells (a mouse-derived β-cell line), we found that >10% of the granules were located within one granule diameter of the plasma membrane (unpublished observations), i.e., the granules could be described as morphologically docked. It seems reasonable to assume that the small number of readily releasable granules are included in this pool and that the remaining morphologically docked granules are likely to proceed to the readily releasable state as required. It has been reported that a similar morphologically docked pool in the mouse β-cell also comprises 10% of the total number of granules in the cell (46). This percentage is used in Fig. 3 where the reserve, morphologically docked, and readily releasable pools are illustrated. If the 1,300 morphologically docked granules are the granules that provide the second phase of release following discharge of the readily releasable pool, then it can be calculated that they could sustain the second phase of release (at a rate of six granules per β-cell per minute) for over 3.5 h. It follows that to replace the secreted granules and to keep the morphologically docked pool filled, the translocation of only six granules per minute will suffice. These numbers make it clear that *1*) detecting the readily releasable pool by morphological criteria, in the absence of a specific marker, is near impossible; and *2*) that detecting the replacement granules (6 granules moving per minute out of a total of 11,000 in the reserve pool) is also near impossible.

EXOCYTOSIS IN THE PANCREATIC β-CELL

According to the soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) hypothesis (46–49), granule docking and exocytosis involves the formation of a "core complex" of syntaxin and synaptosomal-associated protein 25 (SNAP-25) from the plasma membrane, these being the target-SNAREs (t-SNAREs), and vesicle-associated membrane protein 2 (VAMP-2)/synaptobrevin-2 from the granule membrane, this being the vesicle-SNARE (v-SNARE). Additional processes include the mechanisms of priming or conversion to the readily releasable state. In cells with regulated secretion, exocytosis occurs upon cell activation. These processes have not been clearly defined and involve multiple interactions with accessory proteins, conformational changes of the complex, and Ca^{2+}- and ATP-dependent steps. Considerable uncertainty exists with respect to many of the interactions involved. This arises from the early state of our knowledge, the rapidity with which exocytosis of individual granules occurs after activation, the process of endocytosis that follows exocytosis and involves some of the same proteins, and most likely because of differences between secretory systems in the several cell types and species studied to date. Although there are common features in all of the secretory systems studied (including fast secretory systems such as that which exists at synapses and slow secretory systems such as the β-cell), the vastly different exocytotic rates alone imply that the control of exocytosis in various cell types must be different. Despite these differences, models and hypotheses can be developed and used as a basis for experimentation and to help us make progress. We have worked with a very simple model and hypothesis, i.e., that coimmunoprecipitation of VAMP-2 (a v-SNARE) by antisera against syntaxin (a t-SNARE) identifies the readily releasable pool of granules in the β-cell (50).

Exocytosis requires the interaction between the v-SNARE VAMP-2 and the t-SNAREs syntaxin and SNAP-25. These three proteins associate via coiled-coil interactions into an extremely stable complex before exocytosis (51). The individual roles of the multiple syntaxin isoforms in the β-cell are not known. Similarly, the role of SNAP-23, which can replace SNAP-25 but with lower efficiency, is not known (52). Proteins involved in the exo/endocytic cycle include the α- and β-SNAPs (53), NSF, and munc-18 among others. Ca^{2+} is thought to be required for several steps in docking, priming, and exocytosis, and candidate target proteins have been identified by their C2 Ca^{2+}-binding domains. Among these are synaptotagmin, rabphilin, munc-13, doc-2 (54), and the Ca^{2+}-dependent activator protein for secretion (CAPS) (55). Calmodulin and CaM kinase II, a multifunctional Ca^{2+}/calmodulin-dependent PK, have long been thought to be involved in Ca^{2+}-stimulated secretion, as reviewed by Easom (56). Although little is known about the precise roles these proteins play in exocytosis, there are intriguing hints as to their potential involvement. For example, Ca^{2+} binding to CAPS allows phospholipid binding and suggests a possible role in fusion (55); munc-13 binds Ca^{2+} and DAG and interacts with doc-2, suggesting a role in the potentiation of insulin release by activators of phospholipase C, such as acetylcholine (54). Calmodulin and CaM kinase II have multiple potential roles in the phosphorylation of key proteins (56). The importance of the potential Ca^{2+} sensor synaptotagmin has been demonstrated by deletion studies in mice and *Drosophila* where Ca^{2+}-mediated exocytosis is severely disrupted. In the rat pancreatic β-cell; syntaxin1A, 4, and 5; SNAP-25; VAMP-2; and munc-18 have been identified (57). In the β-TC6-f7 and HIT-T15 cell lines, and in the pancreatic islets, cellubrevin and VAMP-2 but not VAMP-1 have been identified, as have SNAP-25, syntaxin isoforms 1–4, synaptotagmin III, and munc-18 (58). Regazzi et al. (34) reported cellubrevin and VAMP-2 but not VAMP-1 in the β-cell. Other reports include synaptotagmin III (59,60), rabphilin and rab3A (61), and cellubrevin (62). Evidence is available that syntaxin 1 but not syntaxin 2 is involved in β-cell exocytosis (63–65). VAMP-2 and cellubrevin are required for Ca^{2+}-stimulated exocytosis but may not be involved in GTP-stimulated exocytosis (34). Also identified are NSF and α-SNAP (35), Noc2 (66), and cysteine string proteins (67,68). The fact that the isoforms of the interacting proteins are expressed differentially in various cell types emphasizes the complexity of the molecular interactions involved in exocytosis. Additionally, splice variants exist so that syntaxins 1–4 are further subdivided. SNAP-23, an isoform of SNAP-25, is not cleaved by botulinum neurotoxin E, as is SNAP-25, and can replace SNAP-25, though with less efficiency (52). All this variety makes it essential to take note of the specific isoforms and splice variants for each individual cell type under study. At least in part, this multiplicity of interacting proteins is one reason why the roles of the members of SNARE complexes have not been elucidated with any certainty in the β-cell. Obvious examples of this uncertainty include the exact role(s) of the isoforms of synaptotagmin, which is most certainly a Ca^{2+}-sensor, and munc-18, which is thought to prevent syntaxin from binding to SNAP-25 but may have more than one role in the control of exocytosis (69–71). Synaptotagmin isoforms III and VII are present in β-cells, and overexpression results in increased Ca^{2+}-sensitivity (72).

We have taken a combined physiological and biochemical approach to study the readily releasable granule pool and found that such a pool could be detected in cell lysates by immunoprecipitation of syntaxin and Western blotting for coimmunoprecipitated VAMP-2. When insulin is rapidly discharged by glucose during the 5–10 min that make up the first phase of release, the readily releasable pool, as judged by the Western blot for VAMP-2, is much reduced and sometimes undetectable when compared with nonstimulated cells. Subsequently, even in the continued presence of 16.7 mmol/l glucose, the readily releasable granule pool is gradually refilled over the second phase. These data suggest that the rate of insulin secretion during the second phase is not limited by the availability of granules that can be detected in this way, but by the rate at which the granules are prepared for release (primed). The readily releasable granule pool can also be discharged by other secretagogues that cause the rapid release of insulin, e.g., by a depolarizing concentration of KCl and by mastoparan, with similar reduction or loss of our ability to detect it. Discharge of the readily releasable pool by glucose and other stimulators with acute action is blocked by inhibitors of insulin release such as norepinephrine.

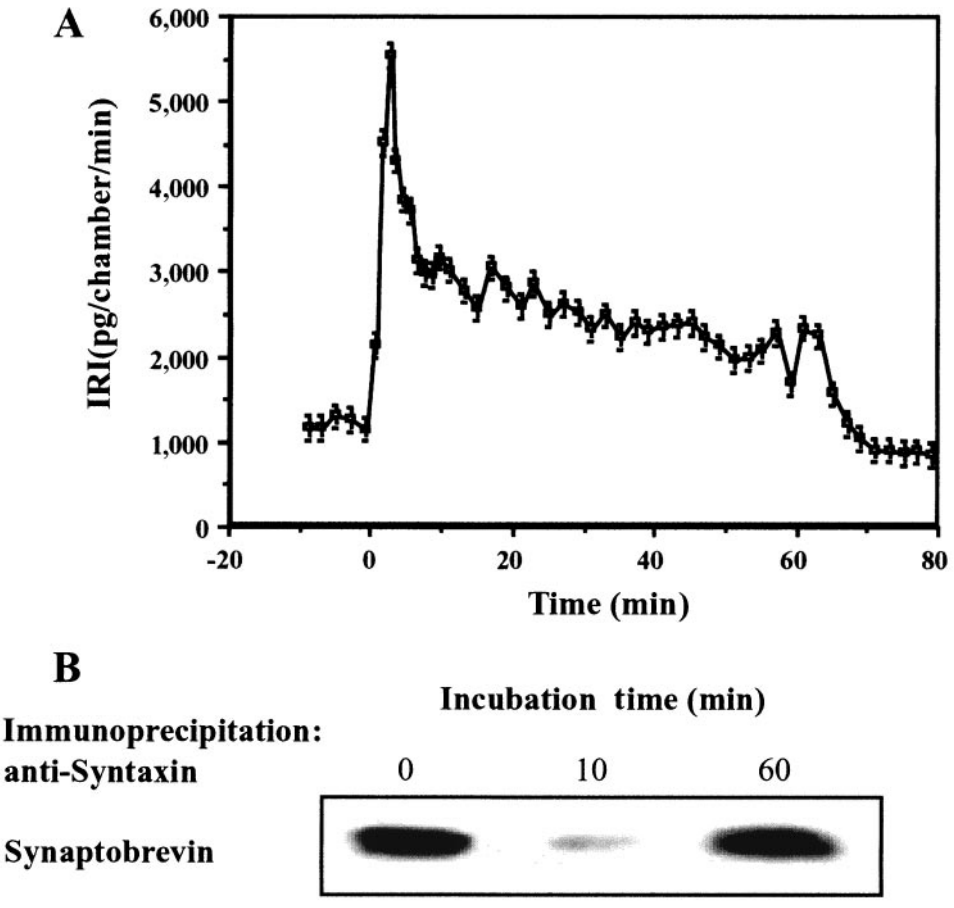

FIG. 4. Dynamic changes in insulin secretion and the readily releasable granule pool in response to stimulation by 30 mmol/l glucose. *A*: Insulin secretion from βHC9 cells was studied under perifusion conditions. Addition of 30 mmol/l glucose stimulated insulin secretion in a biphasic manner with a peak of first-phase release at 5 min and a subsequent plateau of second-phase release. Values are means ± SE of four experiments. IRI, immunoreactive insulin. *B*: In parallel experiments, βHC-9 cells were treated with 30 mmol/l glucose for 0, 10, and 60 min. Cell lysates were prepared and subjected to immunoprecipitation by an anti-syntaxin antibody bound to protein G agarose beads. After elution from the beads, the immunoprecipitated proteins were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were probed with the anti–VAMP-2 (vesicle-associated membrane protein 2) antibody. Representative blots are shown. Data shown are from reference 50.

However, it is clear from insulin release data under conditions different from those due to stimulation by glucose alone that the concept of a single readily releasable pool is inadequate. For example, exposure of islets to a stimulatory glucose concentration in the presence of GLP-1, PACAP, VIP, or GIP, or other activators of adenylyl cyclase, will result in a larger first phase of release than that due to glucose alone. Therefore, either there are multiple readily releasable granule pools or there are pools that can be converted to the readily releasable state (e.g., by cyclic AMP) with extreme rapidity. This will be referred to again in the next section, in the discussion of the readily releasable pool that is released by glucose stimulation.

TIME COURSE OF ACTION OF HIGH GLUCOSE CONCENTRATIONS ON INSULIN SECRETION AND ON THE READILY RELEASABLE POOL

In the experiments described here, βHC-9 cells were incubated in 0.1 mmol/l glucose and subsequently exposed to 30 mmol/l glucose for 60 min. The rate of insulin secretion was monitored at 1-min intervals. Cell lysates were prepared and immunoprecipitated by antibody against syntaxin at 0, 10, and 60 min, times which 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. 4. Also shown in Fig. 4 are the Western blots for VAMP-2 from the syntaxin immunoprecipitates at these time points. A readily releasable pool is detected 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 coimmunoprecipitation of VAMP-2 at 60 min, the granule pool has been refilled. However, the granule pool is no longer readily releasable, i.e., it is not released at the rate of the first phase. During the second phase of glucose-stimulated release, from 10–60 min, the rate of recruitment of granules from reserve pools to the pool that we detect by coimmunoprecipitation exceeds the rate of granule exocytosis and leads to the refilling of the granule pool. The refilled pool seen at 60 min, as detected by coimmunoprecipitation, is not immediately released by the glucose stimulus, which is still present and which has been shown to result in a continuously elevated intracellular Ca^{2+} concentration (73). It seems 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,

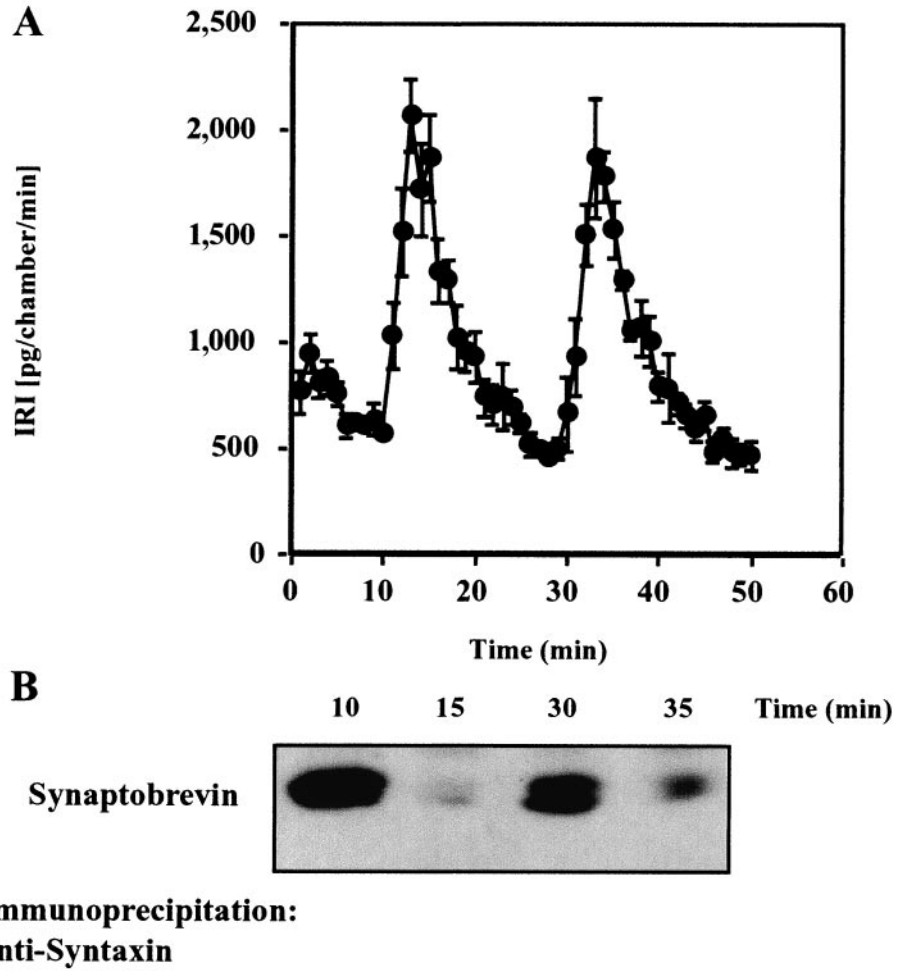

FIG. 5. Perifusion and immunoblot analyses of repeated glucose stimulation in βHC-9 cells. *A*: The results of perifusion studies on βHC-9 cells. During the incubation period, the cells were exposed twice to 30 mmol/l glucose, each time for a period of 5 min. The first incubation was followed by perifusion of the cells with Krebs Ringer bicarbonate buffer containing 0.1 mmol/l glucose for 15 min. IRI, immunoreactive insulin. Values are means ± SE of four experiments. *B*: βHC-9 cells were incubated in KRB solutions containing 0.1 mmol/l and 30 mmol/l glucose according to the protocol used for the perifusion experiments. Equal amounts of the cell lysates at the 10, 15, 30, and 35 min time points were immunoprecipitated with anti-syntaxin antibody conjugated to protein G agarose beads. After elution from the beads, the immunoprecipitated proteins were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were probed with the anti-VAMP-2 antibody. Representative blots are shown. Data shown are from reference 50.

despite the presence of a granule pool of apparently equal size to the readily releasable pool detected at 0 min, might be due to the time required to prime the granules for release after they have reached the pool. To test this possibility, βHC-9 cells were exposed to 30 mmol/l glucose for 5 min, to induce a first phase of release and discharge the readily releasable 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, respectively, and under stimulated conditions at 15 and 35 min, respectively. 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 the reduction of the insulin release rate to baseline levels. Restimulation by glucose at 30 min resulted in another "first phase" of secretion (Fig. 5). These data were correlated with the immunoprecipitation data (also shown in Fig. 5). A readily releasable granule pool was clearly detected under basal conditions at zero time, which was discharged by the 5-min stimulation with 30 mmol/l glucose, i.e., at the peak of the first phase of insulin secretion. After return of the cells to basal conditions (0.1 mmol/l glucose), the readily releasable pool was regenerated at 30 min and again discharged by the glucose stimulus. It seems most likely, therefore, that the first phase of insulin release is due to a pool of granules that is primed and "readily releasable." As this primed 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 (perhaps only transformed from the morphologically docked pool), and have to be primed before they can be released. Thus, the rate of second-phase insulin release is governed by the rate at which the granules can be primed. While these studies were carried out on a mouse β-cell line that mimics the mouse islet response, i.e., with a second-phase plateau beginning immediately after the nadir of the first phase, it is interesting to apply the same interpretation to the second-phase responses seen with rat islets or in humans, where the rate of insulin secretion increases from the nadir to a higher plateau. In these cases, the development

of this response would be due to a progressive increase in the rate at which the granules are primed after reaching what is a releasable, but not a "readily" releasable, pool.

As mentioned earlier, the concept of only one readily releasable pool of insulin-containing granules does not adequately explain the situation where the first phase of glucose-stimulated insulin secretion can be immediately potentiated by activators of adenylyl cyclase or PKC. It is still necessary to explain the immediately enhanced first-phase response under these circumstances. Possibilities include the following: *1*) multiple readily releasable granule pools that require either increased PKA or PKC activity combined with increased $[Ca^{2+}]_i$ (provided by the glucose stimulus) for rapid release; and *2*) the ability of PKA and PKC to convert specific granule pools (morphologically docked?) to the rapidly releasable state. This is a major issue for future research.

In summary, a readily releasable pool of insulin-containing granules is responsible for the first phase of glucose-stimulated insulin release. After discharge during the first phase, refilling occurs by translocation of granules from reserve pools at a rate that exceeds the rate of second-phase insulin release. Thus, the rate of release of insulin during the second phase is not determined by the rate of granule translocation. It is the rate of priming that is rate limiting.

Finally, it should be recognized that the mechanisms controlling the first phase of insulin secretion are no less complicated than those controlling the second phase because they are in fact the same. Although the readily releasable pool of insulin-containing granules is poised for release, the mechanisms involved in its preparation are the same as those that prepare granules for release during the second phase. Thus, granule translocation to the plasma membrane, morphological docking, preparation for release, priming, and exocytosis all follow a similar sequence of events. Most likely, it is only the rate constants that differ.

The messengers controlling the first phase of glucose-stimulated release are ATP and ADP, membrane potential, $[Ca^{2+}]_i$, and the Ca^{2+} sensors—whatever they may be. The messengers controlling the second phase of release are the same as for the first phase, ATP and ADP, membrane potential, $[Ca^{2+}]_i$, and the Ca^{2+} sensors. However, these are combined with additional glucose-induced signals that may include citrate and malonyl CoA, long-chain acyl-CoAs, diacyl glycerol, PKC isoforms, phospholipases, and phosphoinositides. The multiplicity of functions involved in the control of sustained second-phase secretion requires a multiplicity of coordinated signals.

ACKNOWLEDGMENTS

The work described here was supported by grants (to G.W.G.S.) from the National Institutes of Health (DK-42063, DK-54243, and DK-56737) and by Mentor-Based Postdoctoral Fellowships from the American Diabetes Association.

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