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Modes of exocytosis and electrogenesis underlying canine biphasic insulin secretion

Stanley Misler[1], Kevin D. Gillis[2]

[1]Departments of Medicine and Cell Biology/Physiology, Washington University School of Medicine, St. Louis MO 63110, [2]Dalton Cardiovascular Research Center and Department of Biological Engineering, University of Missouri-Columbia, Columbia MO 65211

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1. ABSTRACT

Biphasic insulin secretion in response to glucose consists of a transient first phase followed by a progressive second phase. It is a well described feature of whole perfused pancreases as well as isolated pancreatic islets of Langerhans. Applying to single cell assays of exocytosis (capacitance monitoring and amperometry) to single canine β-cells we have examined the time courses of granule exocytosis in response to voltage-clamp depolarizations that mimic two modes of glucose-induced electrical activity, and then compared these to biphasic insulin secretion. Action potentials evoked in short trains at frequencies similar those recorded during first phase insulin secretion trigger phasic exocytosis from a small pool of insulin granules that are likely docked near voltage-activated Ca^{2+} channels. In contrast, prolonged voltage-clamp pulses mimicking plateau depolarizations occur during second phase insulin secretion and trigger tonic or continuous exocytosis. Comparing the latter results with ones obtained using photorelease of caged Ca^{2+} in other insulin-secreting cells, we suggest that tonic exocytosis likely results from granule release from a highly Ca^{2+}-sensitive pool of insulin granules, likely located further from Ca^{2+} channels. Both phasic and tonic modes of exocytosis are enhanced by glucose, via its metabolism. Hence, in canine β-cells we propose that two distinct modes of exocytosis, tuned to two types of electrical activity, may underlay biphasic insulin secretion.

2. INTRODUCTION

β-cells of the pancreatic islets of Langerhans are endocrine secretory cells that release insulin in a complex, Ca^{2+} entry-dependent manner in response to glucose. Transport of glucose and other fuel metabolites into the cell, followed by aerobic metabolism of these fuels, drives the conversion of ADP to ATP, which closes ATP sensitive K^+, or K^+(ATP), channels. Against a background of open non-selective cation channels, closure of K^+(ATP) channels depolarizes the β-cell, opens voltage-dependent Ca^{2+} channels and triggers localized Ca^{2+} entry and Ca^{2+} -dependent exocytosis of insulin granules. As in the case of other excitable endocrine cells, the clustering of Ca^{2+} entry and Ca^{2+}- dependent K^+ channels can give rise to repeated brief bursts of electrical and exocytotic activity (e.g., 1). However, insulin secretion in β-cells also has complex kinetics occurring over time scales of several to many minutes. During a sustained rise in plasma glucose concentration occurring over 20-30 minutes, pancreatic islets of Langerhans often display a biphasic insulin secretion consisting of an initial, several minutes-long spike, or first phase, insulin secretion (FPIS). This is followed by a slowly developing but highly persistent dome, or second phase, insulin secretion (SPIS) (2-3). FPIS likely saturates insulin receptors on hepatocytes thereby (i) blocking release of stored or newly formed glucose and (ii) stimulating importation of glucose newly absorbed from the intestinal tract. SPIS provides the

sustained circulating concentrations of insulin needed to stimulate glucose uptake by resting myocytes and peripheral adipocytes. In obese individuals, defects in biphasic secretion, coupled with target cell insulin insensitivity, contribute to the over-stimulation of the β-cell with eventual loss of secretory capacity (4).

Based largely on work with rodent islets, biphasic insulin secretion has long been attributed to the existence of multiple pools of large, ~300 nm diameter, dense core insulin granules in β-cells (e.g., 5). In the simplest formulation of the granule pool hypothesis, one pool of granules is docked to the plasma membrane and released first in response to stimulation, whereas a second reserve pool of granules in the cytoplasm is recruited to the plasma membrane for release with prolonged stimulation (6). Ongoing work, however, suggests that granules may exist in a wide range of functional states that differ in their relationship to the plasma membrane and to Ca^{2+} channels, and vary in fusion competence and Ca^{2+} sensitivity (7-18). A current challenge is to map these functional states defined from powerful reductionist approaches to physiologically relevant phases of insulin release elicited by glucose in the native islets.

The present consensus is that an immediately releasable pool (IRP) of vesicles, likely docked nearby high voltage activated Ca^{2+} channels, is responsible for FPIS (5, 7-8). Release from this pool is triggered by an initial barrage of glucose-evoked action potentials (APs) that produce a local rise in cytosolic $[Ca^{2+}]$ to tens of μM, adequate for rapid release utilizing a mechanism with a low affinity for $[Ca^{2+}]$. However, the mechanism of release responsible for SPIS, remains the subject of intense inquiry.

(i) Does the transition from FPIS to SPIS involve physical translocation of "newcomer" granules from a cytoplasmic reserve pool to a readily releasable pool docked to the plasma membrane, as first suggested in 1968 (9)? Phrased in terms of modern cell biology, does the granule pool underlying SPIS consist of granules whose contents are undergoing progressive processing and aggregation while in transit from the Golgi to the plasma membrane? Do members of this granule pool finally attach to the plasma membrane via the formation of protein-protein and protein-lipid complexes involving SNARE proteins and other proteins such as synaptotagmins (e.g., 19), as proposed for granules from other endocrine cells (20, 21)? The approach, docking and fusion of fluorescently labeled granules can now be monitored using total internal reflectance fluorescence microscopy which allows selective imaging of the plasma membrane and submembrane cytoplasm (13, 22-25).

(ii) Do the granule pools underlying FPIS and SPIS, whether already docked, or arriving at the membrane as "newcomers", represent granules in fundamentally different states, in that their fusion is triggered by different Ca^{2+} levels and/or requires additional stimulants? Proposed candidates for these additional stimulants include slowly developing glucose-derived signal(s) such as the generation cAMP; long chain acyl CoA molelcules; or nitric oxide, all operating independently of closure of K^+(ATP) channels. Alternate, possibilities include enhancement of the pyruvate–malate shuttle and/or actin remodeling to facilitate handoff of newly synthesized granules to docking sites (10, 11, 14). Yet another candidate is Ca^{2+} entry through an alternate high voltage activated Ca^{2+} channel (Cav2.3 or R-type) (12). Lastly, it has also been suggested that granules underlying SPIS may have distinct SNARE protein binding affinities (13) or a higher Ca^{2+} sensitivity than those underlying FPIS, hence responding to a distinct spatio-temporal profile of cytosolic $[Ca^{2+}]$ (15-18).

One possibility that has not received much attention is that underlying the two phases of insulin secretion may be two phases of electrical activity. An examination of our data from canine islets prompted us to consider this possibility and its relationship to two pools of granules with differing Ca^{2+} sensitivities (26). Single canine β-cells display bursts of APs during the interval of glucose stimulated FPIS. On transition to SPIS, β-cells display plateau depolarizations to between -30 and -20 mV with few superposed APs (compare Figure 1A and B). To understand how these distinct patterns of electrical activity relate to insulin granule exocytosis from single cells, we measured depolarization-evoked increases in plasma membrane capacitance (ΔC_m) that are proportional to increases in plasma membrane surface induced by granule membrane insertion (27,28). In some experiments we measured simultaneously quantal release events (QREs), where released serotonin, preloaded into insulin granules, is oxidized on the surface of a carbon fiber electrode positioned at the cell surface to produce spikes of amperometric current (29-30). We propose that single APs, or those clustered in brief trains, provoke individual short pulses of Ca^{2+} entry. These raise restricted domains of submembrane cytosolic $[Ca^{2+}]$ as high as 10s of μM and trigger **phasic exocytosis** (steps of ΔC_m in synchrony with single QREs) from a small, easily exhaustible pool of insulin granules, likely an IRP docked near voltage activated Ca^{2+} channels. In contrast, we propose that plateau depolarizations resembling those seen during SPIS, provoke slower and lower-amplitude rises in $[Ca^{2+}]_i$. These smaller more global increases in cytosolic calcium trigger slower-to-start **tonic exocytosis** consisting of steady creeps in C_m and bursts of QREs, which outlast the actual depolarization, and likely occur from a highly Ca^{2+}-sensitive pool (HCSP) of granules located further from Ca^{2+} channels. The size of the HCSP is enhanced by glucose, as well as by activation of protein kinases A and C (16, 17).

It is worth noting that the joint contribution of both **phasic and tonic exocytosis** to quantal release during sustained activity is a well appreciated phenomenon in excitable cells. At the neuromuscular junction, increased asynchronous quantal release, recorded as miniature endplate potential activity in muscle, follows a train of endplate potentials synchronized with motor nerve action potentials (31, 32). Also, both phasic and tonic modes of exocytosis, measured as increases in C_m have previously been reported from peptidergic neurophypohyseal terminals and adrenal chromaffin cells (33-35). Hence, by analogy

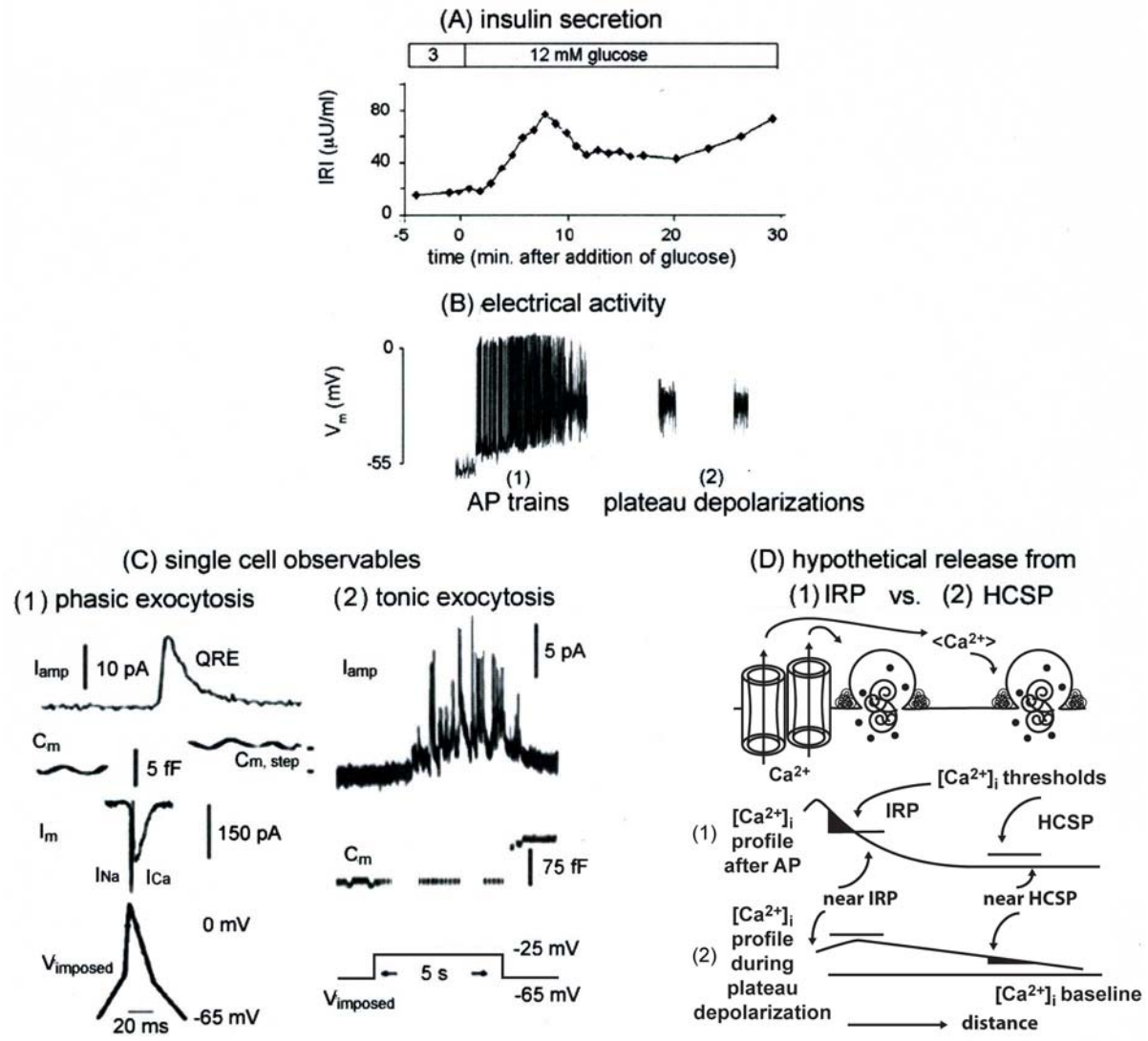

Figure 1. A model of biphasic insulin secretion based on dual modes of exocytosis tuned to dual modes of electrogenesis. (A) The time courses of biphasic insulin secretion measured by radioimmunoassay is compared with (B) perforated-patch current-clamp recording of early spiking (APs) (pattern 1) giving way to plateau depolarization (pattern 2) upon the transition from the FPIS to the SPIS. (C) Regarding single cell observable correlates of secretion, phasic exocytosis of likely one or two granules measured using capacitance and amperometry occurs immediately after a single action potential seen in a train (pattern 1) is mimicked by voltage clamp. In comparison, tonic exocytosis of multiple granules evoked by plateau depolarization (see pattern 2) begins ~ one second after onset of depolarization and continues for up to several seconds after the end of the voltage-clamp depolarization (note small stepwise tail of increases in C_m and QRE after repolarization). (D) Cartoon illustrating relative locations and Ca^{2+} thresholds for hypothetical release from the immediately releasable pool, IRP, versus the highly calcium sensitive pool, HCSP. The granules of IRP, proposed to be activated by action potentials, requires higher intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) to drive release (upper threshold), which is only found at sites near Ca^{2+} channels, where high local $[Ca^{2+}]_i$ occurs very quickly after the start of depolarization but also rapidly declines after the depolarization ends. In contrast, granules of HCSP granules, proposed to be activated by a plateau depolarization, should be released by lower $[Ca^{2+}]_i$ values (lower threshold) but may be located further from Ca^{2+} channels after Ca^{2+} entry. Therefore exocytosis should occur with a delay after depolarization begins, but $[Ca^{2+}]_i$ is sufficient to drive exocytosis well after the depolarization ends. The contents of the granule is denoted by molecules of various sizes to indicate large molecules such as insulin and C-peptide and small molecules such as serotonin loaded from the bath. Data traces adapted with permission from figures in refs 18 and 26.

we are proposing that two distinct components of exocytosis, tuned to two components of electrical activity contribute significantly to biphasic insulin secretion in dog, with the added aspect that each component is amplified by the time-dependent effects of glucose metabolism on granule refilling. In addition, we suggest that a similar

scheme may underlie at least a component of SPIS in human, pig, and rat β-cells, which have recently been shown to have plateau depolarizations and prolonged Ca^{2+} elevations (1, 18, 36).

3. OVERVIEW OF TWO DISTINCT MODES OF EXOCYTOSIS IN CANINE β-CELLS

3.1. FPIS, action potentials and phasic exocytosis vs. SPIS, plateau depolarizations and tonic exocytosis.

Here we present data suggesting that action potentials evoke phasic exocytosis and FPIS while plateau depolarizations evoke tonic exocytosis and SPIS (26).

Figure 1A shows that under conditions of near physiological ambient temperature and bath added forskolin, an activator of adenylyl cyclase, canine islets, dispersed largely to single cells, display biphasic glucose-induced insulin release. FPIS begins at 2-4 min after raising glucose from 3 to 12 mM, peaks at 7 -10 min, and then the declines to a plateau. SPIS emerges from the plateau by 20 min. Fig 1B shows under identical bath conditions biphasic electrical activity has a similar time course as biphasic insulin secretion. By 1-2 min after a similar rise in glucose the β-cell depolarizes from its resting potential of nearly -60 mV to trigger isolated APs that subsequently increase in frequency; by 5-7 min after elevating the glucose concentration a bursting mode peaking at ~5 APs/burst and ~4 bursts/min is evident. Thereafter the action potentials, which initially overshoot 0 mV, become smaller and aborted to give way to a plateau depolarization at -30 to -20 mV. This plateau depolarization is temporally correlated with the transition from FPIS to SPIS and continues for minutes into the SPIS.

Secretion and electrical activity are also pharmacologically related. The Na^+ channel blocker tetrodotoxin, which reduces AP frequency as well as amplitude, suppresses FPIS leaving a slow creep to SPIS; the Ca^{2+} channel agonist BAY K8644, which increases plateau depolarization by 5-7 mV, increases SPIS by 3-4 fold (26).

Fig 1C shows that the two patterns of electrical activity clearly evoke two components of exocytosis from voltage-clamped canine β-cells. In some cells, voltage clamp depolarizations that simulate individual or short bursts of APs often evoke a fast-on and fast-off **phasic** component of exocytosis, consisting of single QREs and small (3-5 fF) steps of membrane capacitance (left panel). The defining characteristic of this phasic exocytosis is that it is well-synchronized with the stimulus, terminating within ~50 ms of the AP stimulus. Based on these observations we propose that moderate-frequency AP activity, leading to a phasic component of exocytosis, underlies FPIS.

In contrast, in cells lacking phasic release, it is clear that cell depolarization to -25 mV evokes a **tonic** component of exocytosis, consisting of barrages of QREs contributing to a large increase in C_m seen at the end of the depolarization (Figure 1C, right panel). Here, however, exocytosis begins up to hundreds of ms after the start of a depolarization, and continues for up to several seconds after its conclusion. In cells where global cytosolic $[Ca^{2+}]$ was measured with a Ca^{2+} sensing dye, the onset of exocytosis correlated with persistent increases in global cytosolic Ca^{2+} to >500 nM. We propose that **tonic exocytosis** induced by plateau depolarizations underlies SPIS. Many cells demonstrate a combination of both phasic and tonic exocytosis.

Both phasic and tonic components of exocytosis elicited by voltage-clamp depolarization are enhanced 2-3 fold at a concentration of ambient glucose that provokes insulin secretion. This should help maintain or enhance exocytosis even in the face of slowly declining AP and plateau depolarization amplitudes.

3.2. Phasic secretion is proposed to be from an IRP whereas tonic secretion is proposed to be from a HCSP

Comparing these results with the finding of distinct pools of granules identified in response to flash photolysis of caged Ca^{2+} compounds added to the cytoplasm of native or clonal β-cells (16, 17), we suggest that it is likely that the granule pools underlying the phasic versus tonic components of exocytosis differ in their Ca^{2+} sensitivities for fusion, and their localization with respect to plasma membrane Ca^{2+} channels (see Figure 1D).

We propose that phasic exocytosis evoked by single or short trains of single APs occurs from an IRP closely co-localized with high-voltage-activated Ca^{2+} channels. This co-localization allows IRP granules to experience micro-domains of cytosolic $[Ca^{2+}]$ in the tens of μM during or immediately after APs; these micro-domains are sufficient to trigger exocytosis by a low-affinity Ca^{2+} sensor. In contrast, we propose that tonic exocytosis evoked by prolonged plateau depolarizations results from HCSP granules that are poorly co-localized with Ca^{2+} channels. During plateau depolarizations Ca^{2+} influx is smaller but more prolonged than in the case of APs, therefore HCSP granules experience much smaller (low μM) but prolonged rises in cytosolic $[Ca^{2+}]$. Nevertheless, these modest elevations in cytosolic $[Ca^{2+}]$ are sufficient to trigger release because HCSP granules display higher Ca^{2+} sensitivity than those of the IRP.

Two caveats merit mention here. First, the size of the HCSP may be artificially elevated under our experimental conditions because the HCSP is enhanced by protein kinase A as well as by increasing ambient [glucose] (17), in that our experiments were performed with continuous application of forskolin and often elevated bath [glucose]. Second, the HCSP may also make a small contribution to FPIS because trains of action potentials could result in the diffusive spread of the Ca^{2+} signal to granules of the HCSP (e.g. 15).

Whether both granule pools are docked to the plasma membrane and ready for release in resting cells is unclear. In voltage-clamp experiments at least 100 ms of continuous depolarization to voltages positive to -30 mV is needed to activate tonic release (26). Potentially, this is

enough time for granules to translocate to the plasma membrane or to undergo other maturation steps in preparation for release. Moreover, in canine β-cells a "priming" depolarization to -35 mV, which allows Ca^{2+} entry but is not sufficient to promote release, shortens the latency to release in response to subsequent depolarization to membrane voltages positive to -30 mV. Thus modest elevations of intracellular [Ca^{2+}] may mobilize granules into a release-ready state. This is consistent with the observation from caged Ca^{2+} experiments that the HCSP requires priming with moderate elevations in intracellular Ca^{2+} in the range of 300-400 nM to be prominent upon photo-elevation of intracellular Ca^{2+} into the μM range (17). In addition, both modes of exocytosis appear to rapidly "run down" during trains of depolarizations suggesting that recruitment of granules is necessary to replenish and maintain both pools of granules during and after intense secretion. In preliminary experiments it appears that both phasic and tonic exocytosis recover from depletion over ~ 2 min, apparently along exponential time courses that are accelerated by elevated glucose.

While we have not applied flash photolysis of caged Ca^{2+} to define the size of the IRP and HCSP in canine β-cells, using an indirect method of paired pulse stimulation (37) we tentatively estimate the maximum size of the IRP from the depression of the C_m steps seen in response to the first two 100 or 200 ms depolarizations applied at 1 Hz as 148 fF. This corresponds to ~60 -80 insulin granules of 2-2.5 fF each, and is comparable to the size of the IRP in mouse β-cells (17). Estimation of the size of the HCSP from depolarization experiments is more difficult because pool refilling presumably occurs during the depolarizing train. We note that the total C_m creep recorded during a short train was more than double the IRP.

In comparing multicellular insulin secretion by radioimmunoassay with single-cell electrophysiological assays of exocytosis it is critical to note that the electrophysiological assays only require fusion pore formation and/or the diffusion of a highly mobile small molecule into the extracellular space. Release of the compact, crystalline insulin core may not always occur with every membrane fusion event. In addition, even under conditions where both small and large molecules are successfully released, the kinetics of release of insulin might be significantly slower and its appearance in the bath delayed by up to several seconds. In principle, insulin sensing by amperometry would be an ideal technique for bridging the electrophysiological and immunological assays (38); however in practice, this technique is limited by measurement instability over the many minutes needed to measure biphasic insulin secretion. At present the most promising real-time approach to detecting native granule content release uses total internal reflection fluorescence microscopy to directly visualize granule approach to the plasma membrane and then discharge of its contents, (e.g. GFP-tagged insulin and/or C-peptide fragment or Zn^{2+}) (23-25), whose release is monitored with an extracellular fluorescent chelator. Assays of granule core release, though not critical to our qualitative arguments, might be important for any future quantitative reconciliation of results from the various approaches (38a).

4. SUMMARY AND PERSPECTIVE

We propose that canine β-cells may provide a relatively simple model for biphasic insulin secretion. Based on comparison of the time courses of biphasic insulin secretion measured by radioimmunoassay with electrical activity and exocytosis we have proposed a simple model of dual mode of electrical activity plus a dual mode of exocytosis to account for glucose-induced insulin release. Action potentials evoked in short trains at frequencies similar to those recorded during first phase insulin secretion trigger phasic exocytosis from a small pool of insulin granules that are likely docked near voltage activated Ca^{2+} channels. In contrast, prolonged voltage-clamp depolarizations designed to mimic plateau depolarization occurring during second phase insulin secretion, trigger tonic exocytosis from a highly Ca^{2+}-sensitive pool of insulin granules that are likely located further from Ca^{2+} channels. The model incorporates both the well-studied relationship of AP activity to exocytosis from the IRP, likely prominent in FPIS of many species, and a novel relationship of plateau depolarization to exocytosis from an HCSP. The scaling of these features by glucose-induced and time-dependent changes in the sizes of IRP and HCSP may counteract subtle time-dependent changes in distinct patterns of electrical activity, such as decreases in AP frequency or sags in plateau depolarization amplitude, and hence maintain and even enhance the distinct secretory phases. This new model is complementary to others of biphasic secretion. While de-emphasizing the potential involvement of distinct Ca^{2+} channels in the two phases of secretion (12), it emphasizes the importance of docking of different pools of insulin granules, here proposed to be of distinctly different Ca^{2+} sensitivity and located at varying distance from Ca^{2+} entry channels. In modified forms this model may also apply to humans (1) and other species, for example, pig (18) and rat (36) where intermittent periods of plateau depolarizations occur with prolonged glucose stimulation.

Comparison of the results from canine β-cells with the much more extensive sets of data from mouse β-cells suggests that the results are complementary. First the data from canine β-cells are consistent with glucose metabolism contributing to SPIS, though the metabolic intermediates or target exocytotic molecules are as yet unknown. Second, as the tonic exocytosis is readily exhaustible with seconds of intermittent depolarization, resupply of fusion-competent granules, either through physical translocation or biochemical maturation, is clearly important to sustain this mode of secretion. Third, though we have not had the opportunity to extensively "phenotype" the HVA Ca^{2+} channels in the dog β-cells, preliminary observations suggest that both L- type and non-L-type channels contribute to SPIS. In particular, high doses of nifedipine fail to completely block the whole cell HVA Ca^{2+} current, whereas the L-type channel opener BAYK 8644 dramatically increases SPIS. Finally, though mouse β-cells display bursting pacemaker activity (36)

rather than plateau depolarizations, long bursts at glucose concentrations greater than 10 mM might deplete phasic exocytosis leaving tonic exocytosis evoked by cumulative elevations of global intracellular [Ca^{2+}] that result from the prolonged burst.

This model leaves several key questions unanswered:

1. Where are HCSP granules located? Whereas it is clear that IRP granules are located close to Ca^{2+} channels, it is not yet clear if HCSP granules are preferentially targeted to sites with a paucity of Ca^{2+} channels or just simply randomly distributed on the cell surface.

2. What are the molecular determinants that distinguish the Ca^{2+} sensitivity of HCSP versus IRP granules? For example, do HCSP granules utilize different synaptotagmin isoforms as Ca^{2+}sensors (39, 40, 40a)?

3. Do HCSP and IRP granules have different molecular apparatus to target granules to specific docking sites? For example, are there differences in SNARE adaptor proteins such as MUNCs (41, 42) or different interactions with cytoskeletal elements (43)?

Do granules in the HCSP differ from the IRP in the mode of fusion pore opening and closure and thus differ in the time course and/or quantity of insulin that is released during an exocytotic event (44-46)?

In order to address these questions and test the basic model it will be important to develop and employ molecular tools to selectively manipulate FPIS versus SPIS and/or the IRP versus HCSP granule population. Selective three- to four-fold reduction of SPIS has been engineered by specific "knock-down" of one of two proteins. These are (i) **MUNC 18c**, a SNARE adaptor protein which interacts with syntaxin 4 in the docking of granules to the plasma membrane (42, 43) and (ii) **cdc42,** a Rho GTPase, whose glucose-dependent cycling appears critical for the remodeling of filamentous actin and the hand-off of granules to the syntaxin 4/MUNC18c complexes (47). The cdc42 knock-down is especially interesting because it suggests that molecules other than traditional motors, such as myosin Va (48, 49) may modulate granule supply and pool refilling.

In contrast, preferential enhancement of the HCSP compared to the IRP has been observed following expression of SNAP-25 mutations (e.g., S187E) that mimic phosphorylation of the protein by protein kinase C in INS-1 cells (50). Use of these mutations to study glucose-induced insulin secretion may help strengthen the link between the HCSP and SPIS.

Finally, there has also been expanding interest in (i) the molecular determinants controlling fusion pore dynamics, and (ii) the roles of pore dynamics in mediating differential release of peptides versus small molecular weight cargoes (e.g., catecholamines) in large, dense core endocrine granules. In the case of the insulin granules, the ultimate size of the fusion pore, as well as the crystalline state of the granule, may determine the relative release of monomeric vs. polymeric insulin, and perhaps the relative release of insulin for endocrine function vs. the release of ATP or GABA for paracrine function (25, 45). Hence, does the relative proportion of insulin to non-insulin secreting exocytotic events differ for phasic/IRP vs. tonic /HCSP pools and does knock-down of MUNC18c or cdc42, or expression of SNAP-25 S187E, change fusion pore properties?

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Send correspondence to: Stanley Misler, Departments of Medicine and Cell Biology/Physiology, Box 8126, Washington University School of Medicine, St. Louis MO 63110, Tel: 314-454-7719, Fax :314-454-5126, E-mail: smisler@dom.wustl.edu

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