Calcium dependency and free calcium concentrations during insulin secretion in a hamster beta cell line.

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Research Article

Using a glucose-responsive beta cell line, we tested the hypothesis that the free cytosolic Ca2+ concentration ([Ca2+]i) is the primary signal that couples a stimulus to insulin secretion, and examined the involvement of the extracellular Ca2+ pool in this process. Glucose or depolarization of the beta cell with 40 mM K+ stimulated a monophasic release of insulin directly proportional to the extracellular Ca2+ concentration. 40 mM K+ increased 45Ca2+ uptake and increased [Ca2+]i, which was measured with quin 2, 4.7-fold, from 56 +/- 3 to 238 +/- 17 nM. With high glucose, 45Ca2+ uptake did not increase, and [Ca2+]i was unchanged or fell slightly. There was a striking correlation between inhibitory effects of verapamil, the Ca2+ channel blocker, on insulin secretion and the rise in [Ca2+]i evoked by K+. Higher concentrations of verapamil were required to inhibit glucose- than K+-stimulated insulin secretion (dose giving half-maximal effect of 1.4 X 10(-4) M vs. 6.0 X 10(-7) M). Incubation in Ca2+-free, 1 mM EGTA buffer for 30 min lowered [Ca2+]i to 14 +/- 2 nM, and inhibited acute insulin release to both secretagogues. If high glucose was present in the Ca2+-free period, reintroduction of 2.5 mM Ca2+ in high glucose restored insulin secretion only to the basal rate. However, if low glucose was present during the Ca2+-free period, high glucose and 2.5 […]

Calcium Dependency and Free Calcium Concentrations during Insulin Secretion in a Hamster Beta Cell Line

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Abstract

Using a glucose-responsive beta cell line, we tested the hypothesis that the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is the primary signal that couples a stimulus to insulin secretion, and examined the involvement of the extracellular Ca^{2+} pool in this process. Glucose or depolarization of the beta cell with 40 mM K^+ stimulated a monophasic release of insulin directly proportional to the extracellular Ca^{2+} concentration. 40 mM K^+ increased $^{45}Ca^{2+}$ uptake and increased $[Ca^{2+}]_i$, which was measured with quin 2, 4.7-fold, from 56±3 to 238±17 nM. With high glucose, $^{45}Ca^{2+}$ uptake did not increase, and $[Ca^{2+}]_i$ was unchanged or fell slightly. There was a striking correlation between inhibitory effects of verapamil, the Ca^{2+} channel blocker, on insulin secretion and the rise in $[Ca^{2+}]_i$ evoked by K^+. Higher concentrations of verapamil were required to inhibit glucose- than K^+-stimulated insulin secretion (dose giving half-maximal effect of 1.4×10^{-4} M vs. 6.0×10^{-7} M). Incubation in Ca^{2+}-free, 1 mM EGTA buffer for 30 min lowered $[Ca^{2+}]_i$ to 14±2 nM, and inhibited acute insulin release to both secretagogues. If high glucose was present in the Ca^{2+}-free period, reintroduction of 2.5 mM Ca^{2+} in high glucose restored insulin secretion only to the basal rate. However, if low glucose was present during the Ca^{2+}-free period, high glucose and 2.5 mM Ca^{2+} triggered a full first-phase insulin response. These data suggest that high glucose generates a non-Ca^{2+} signal that turns over rapidly and provide direct evidence that K^+ triggers insulin release by drawing extracellular Ca^{2+} into the beta cell through verapamil-sensitive Ca^{2+} channels. However, an increase $[Ca^{2+}]_i$ is not the primary signal that evokes glucose-stimulated insulin release in this beta cell line.

Introduction

We have been using an SV40 transformed beta cell line (HIT cells) as a model system to further define the molecular mechanisms of insulin secretion (1, 2). In perifusions of HIT cells, high glucose or 40 mM K^+ evoke a monophasic, unsustained burst of hormone secretion. Extensive studies in islets and insulinomas suggest that the free cytosolic Ca^{2+} concentration, ($[Ca^{2+}]_i$),[1] is the major intracellular signal that triggers exocytosis. Until recently, the evidence to support this view was inferred from either $^{45}Ca^{2+}$ flux studies (3), or the extracellular Ca^{2+} requirement for insulin release (4, 5). One secretagogue where Ca^{2+} appears to be the signal for release is high K^+ concentrations that depolarize the plasma membrane opening voltage-dependent Ca^{2+} channels, allowing Ca^{2+} to enter the beta cell (4–8). How the extracellular Ca^{2+} pool is involved and the identity of the intracellular signal for glucose-stimulated insulin release are still unclear (9–13).

With the fluorescent Ca^{2+}-indicator, quin 2 (14), it is now possible to measure $[Ca^{2+}]_i$ in small cells. Quin 2 is highly charged and does not cross the plasma membrane; however, a nonpolar methyl-ester derivative, quin 2/AM, readily enters cells where it is hydrolyzed by esterases to quin 2. Upon binding to Ca^{2+}, the intensity of quin 2 fluorescence increases and fluorescence is monitored continuously in cell suspensions to measure $[Ca^{2+}]_i$. Recently Wollheim and Pozzan (7) and Rorsman et al. (15, 16) reported that K^+-stimulated insulin secretion in a rat insulinoma cell line (RINm5f) is accompanied by a rise in $[Ca^{2+}]_i$. In Wollheim's studies, the metabolizable secretagogues, alanine and glyceraldehyde, also raise $[Ca^{2+}]_i$. Although the initial studies of RINm5f cells showed glucose responsiveness (17), subsequent investigations from a number of laboratories (7, 18), including our own (19), have not found this cell line, when grown in vitro, to be acutely responsive to glucose. Furthermore, $[Ca^{2+}]_i$ falls in RIN cells exposed to glucose (15). Since other insulinomas also do not respond acutely to glucose, the HIT cells offer a unique opportunity to study the glucose signaling mechanisms.

To directly test the hypothesis that a rise in $[Ca^{2+}]_i$ is a key signal for insulin secretion, we have compared the involvement of the extracellular Ca^{2+} pool in glucose- and K^+-stimulated insulin secretion and the effect of both secretagogues on $[Ca^{2+}]_i$.

Methods

Reagents. We obtained bovine serum albumin (fraction V, RIA grade), Hepes, and EGTA from Sigma Chemical Co., St. Louis, MO; fetal bovine serum and RPMI-1640 medium from Gibco, Grand Island, NY; ^{125}I from Amersham Corp., Arlington Heights, IL; $^{45}CaCl_2$ from New England Nuclear, Boston, MA; and porcine insulin from Eli Lilly & Co., Indianapolis, IN. Quin 2 and the acetoxymethyl ester of quin 2 (quin 2/AM) were purchased from Calbiochem-Behring Corp., San Diego, CA. Verapamil was a gift from G. D. Searle and Co., Skokie, IL.

Insulin assay. Insulin was measured using a double-antibody radioimmunoassay modified from that of Hales and Randle (20) as previously described (21). The anti-insulin antibody was raised in guinea pigs against porcine insulin.

Cell culture and perifusion. The HIT cell subclone T15 was obtained from Dr. R. Santerre of Eli Lilly & Co. (22). The cells were grown at 37°C in 5% CO_2 and air, and maintained in RPMI-1640 medium with 10% fetal bovine serum as previously described (1, 2). The perifusion system has also been described in detail (1, 2).

Insulin secretion in static incubations. HIT cells were plated into 12-well Costar plates (3×10^5 cells/25-mm well) and grown for 3 d. On the day of the experiment, growth medium was removed and the cells were

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1. *Abbreviations used in this paper:* $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; DMSO, dimethylsulfoxide; ED_{50}, dose giving 50% maximal effect; F_{max}, maximal fluorescence; F_{min}, minimal fluorescence; KRB, Krebs-Ringer bicarbonate; quin 2/AM, the acetoxymethyl ester of quin 2; r^2, coefficient of determination.

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preincubated for 45 min in Krebs-Ringer bicarbonate (KRB) basal buffer. This was followed by a second incubation in basal buffer for 15 min, and a third incubation for 15 min in buffer containing the secretagogue. Test agents were present throughout the experiment. The buffer was saved and insulin content measured by radioimmunoassay. In a number of experiments, protein determinations were also performed. No differences were found when the data was expressed as insulin secretion per milligram of protein or per well.

$^{45}Ca^{2+}$ uptake. $^{45}Ca^{2+}$ uptake was measured by the method of Tan and Tashjian (23), decreasing the $CaCl_2$ content of the buffer to 1 mM to increase the $^{45}Ca^{2+}$ uptake. The cells were incubated with a buffer containing 1–2 μCi $^{45}Ca^{2+}$/ml and Ca^{2+} uptake determined at 2.5, 5, 15, 30, and 60 min. Stimulation was terminated by quickly washing the cells three times with ice-cold buffer and solubilizing the cells with 0.1 N NaOH. Aliquots were counted on a Packard Tri Carb Scintillation counter using ACS scintillation fluid from Amersham Corp.

Loading with quin 2. On day 3 or 4 after subculture, the cells were detached with trypsin (0.1%) in Puck's saline A, counted, centrifuged for 2 min at 300 *g*, and resuspended at a concentration of 20×10^6 cells/ml in modified KRB (KRB with 20 mM Hepes, 1.5 mM $CaCl_2$, 1.67 mM glucose, and 5 mg/ml bovine serum albumin). The cells were then preincubated for 15 min at 37°C with gentle agitation. Quin 2/AM, stored desiccated at −20°C as a 20-mM stock in dimethylsulfoxide (DMSO), was added to a final concentration of either 50 or 100 μM. The final concentration of DMSO was 0.5%. The cell suspension was mixed, incubated at 37°C with gentle agitation for 30 min, then diluted with modified KRB to $4–10 \times 10^6$ cells/ml. The incubation was continued for another 50 min. The cells were then centrifuged for 2 min at 300 *g* and resuspended in modified KRB to the same concentration of $4–10 \times 10^6$ cells/ml, and this washing procedure repeated once. The cell suspension was equilibrated to room temperature and the pH adjusted to 7.4. In each experiment, viability was determined by trypan blue exclusion and was always >90% for up to 3 h.

Fluorescence measurements. The cell suspension in 1.5 ml (usually 4×10^6 cells/ml) was placed in a square quartz cuvette (1×1 cm) and stirred continuously. In each experiment hydrolysis of the quin 2/AM was determined by monitoring the emission spectrum, which shifted to a peak at 492–495 nm in 75–80 min. The excitation wavelength was 340 nm and emission was measured at 492 nm. The band pass was 10 nm for both excitation and emission. A Perkin-Elmer LS-5 fluorescence spectrophotometer equipped with a 3600 LS data station and a model 660 printer were used. Test agents were added from concentrated solutions. The ionized free Ca^{2+} concentration in the buffer measured using a Ca^{2+} electrode was 1.2 mM.

Calibration of quin 2 fluorescence. Loading efficiency, defined as the percent of quin 2/AM molecules hydrolyzed and trapped as quin 2, was determined by measuring total fluorescence of cells in loading buffer with quin 2/AM and measuring fluorescence of cells alone after centrifugation (19,000 *g* for 60 s) and resuspension. The loading efficiency varied between 28 and 42% ($n = 4$). The cellular quin 2 concentration measured by loading 20×10^6 cells in 100 μM quin 2/AM, determining the cytocrit and calculating the intracellular concentration of quin 2, was 1.8±0.2 mM ($n = 6$).

Because of quin 2 leakage, which results from cell damage during centrifugation and resuspension (7), corrections for extracellular quin 2 concentrations were necessary. Leakage was greater at 37°C than at room temperature. We therefore measured $[Ca^{2+}]_i$ at room temperature. We corrected for leakage using the following procedure. EGTA in Tris buffer was added to the cell suspension while monitoring fluorescence. The rapid reduction in fluorescence represents extracellular quin 2. The cells were then lysed with 50 μM digitonin and fluorescence measured. This final measurement represents minimal fluorescence (F_{min}). Usually F_{min} was reached within 1.5 min after addition of digitonin. Maximum fluorescence (F_{max}) was determined by adding $CaCl_2$ to the cuvettes. The minimum amount of Ca^{2+} necessary to saturate quin 2 was determined for each experiment. This is necessary because it was found that addition of Ca^{2+} after quin 2 saturation was reached decreased fluorescence. Usually F_{max} was reached with a total $[Ca^{2+}]$ of 4.1 mM, which gives a calculated free $[Ca^{2+}]$ of 100 μM. The K_d (115 nM) for Ca^{2+} binding to quin 2 used in these experiments was the same as that determined by Tsien et al. (14).

Dependence of fluorescence on quin 2 concentration. To determine if the basal $[Ca^{2+}]_i$ was altered by the amount of quin 2 in the cells, measurements were made comparing fluorescence between cells loaded with 50 and 100 μM quin 2/AM. We found no significant differences between the basal $[Ca^{2+}]_i$ whether 50 or 100 μM quin 2/AM was loaded.

Insulin secretion in quin 2-loaded cells. Two groups of cell suspensions were prepared. Control cells treated with DMSO and experimental, quin 2-loaded cells were washed, resuspended in modified KRB buffer without glucose, incubated for 30 min at 37°C, centrifuged, and aliquots of 0.5×10^6 cells were placed into 12×75-mm test tubes. After a 15-min basal period in KRB containing 0.3 mg/ml glucose, the buffer was removed and the cells incubated for another 15 min in buffer containing the appropriate secretagogues. After centrifugation, the supernatant insulin concentration was measured. The cell pellet protein content was measured by the Lowry method (24).

Statistics. All values are expressed as the mean±SE. In perifusion, basal secretion is defined as the mean secretion rate in microunits per minute per plate during the final 5 min of the stabilization period; for static incubations, basal secretion is defined as the amount of insulin released during the final 15-min basal period. Statistical comparisons were made using the *t* test or one-way analysis of variance where appropriate (25). The coefficient of determination (r^2) was calculated for the simple linear regression.

Results

Glucose-stimulated insulin secretion: dependence on extracellular Ca^{2+}. As previously shown in perifusion (2), insulin secretion falls and reaches a stable rate (data not shown) during the 30-min stabilization period. Increasing the glucose concentration from 0 to 19.7 mM increased insulin secretion from 0.4±0.2 μU/min per plate to a peak of 8.9±0.8 μU/min per plate. Peak secretion in each chamber was reached 6–8 min after switching to high glucose. After a 30-min period in Ca^{2+}-free basal buffer (no added Ca^{2+} plus 1 mM EGTA), 19.7 mM glucose in Ca^{2+}-free KRB failed to stimulate insulin secretion (Fig. 1). Total insulin released in response to high glucose during the Ca^{2+}-free period (30–50 min) was 4.0±2.5 μU/plate compared with 94.4±7.8 μU/plate for the controls ($n = 4$, $P < 0.0001$). When a perifusion buffer containing 2.5 mM Ca^{2+} and high glucose replaced the Ca^{2+}-free buffer at 50 min, the insulin secretory

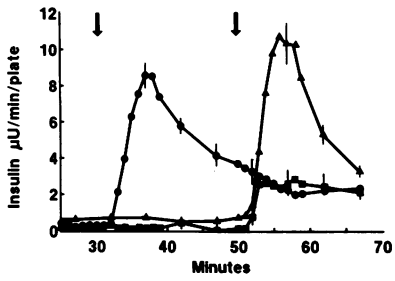

Figure 1. Calcium dependence of glucose-stimulated insulin secretion. Control HIT cells stimulated with 19.7 mM glucose (first arrow) in 2.5 mM Ca^{2+} buffer (●, $n = 4$) responded with a monophasic insulin response. Cells stimulated in Ca^{2+}-free buffer (no added Ca^{2+} plus 1 mM EGTA (■, $n = 4$) did not respond (30–50 min), while secretion returned only to the elevated basal levels of controls when 2.5 mM Ca^{2+} was returned (second arrow) (50–70 min). After 50 min in Ca^{2+}-free buffer in zero glucose, cells stimulated with glucose for the first time at 50 min with 2.5 mM Ca^{2+} demonstrated the full monophasic response of control (▲, $n = 8$).

rates returned only to control levels observed during the same period (50–70 min), 41.2±4.5 μU/plate vs. 46.1±4.7 μU/plate, but did not demonstrate the full early phase seen for control cells from 30 to 50 min (94.4±7.8 μU/plate, $n = 4$, $P < 0.005$). However, when HIT cells were perifused with zero glucose in the Ca^{2+}-free buffer during the 30-min basal period and the initial 20-min stimulation period, and then stimulated with 19.7 mM glucose in Ca^{2+}-containing buffer, a full secretory response occurred (Fig. 1). The total insulin released (106.8±11.0 μU/plate, $n = 8$, vs. 94.4±7.8 μU/plate for controls) and the peak secretory rate (11.1±1.1 μU/min per plate, $n = 8$, vs. 8.7±0.8 μU/plate per min for controls) were similar to the control rate during the initial stimulation with glucose (30–50 min). HIT cells perifused during the basal period in Ca^{2+}-containing buffer and then stimulated with 19.7 mM glucose in Ca^{2+}-free buffer (30–70 min) failed to respond to the glucose stimulation (data not shown).

In companion static incubations, glucose-stimulated insulin secretion increased as Ca^{2+} in the buffer increased from 0.5 to 2.5 mM (Fig. 2). However, insulin secretion was not stimulated above basal rates (equivalent Ca^{2+} concentrations and no glucose) when glucose was added in a buffer containing 0 or 0.1 mM added Ca^{2+}.

K^+-stimulated insulin secretion: dependence on extracellular Ca^{2+}. Increasing the K^+ concentration from 7 to 40 mM at 30 min produced a rapid, monophasic increase in insulin secretion (Fig. 3, controls). This spike of insulin release was completely eliminated when the cells were perifused in Ca^{2+}-free buffer during the basal and initial 15-min stimulation period (Fig. 3). When 2.5 mM Ca^{2+} in the presence of 40 mM K^+ was returned from 45 to 60 min, the secretory response was reduced to 60% of the control value for the initial 15 min stimulation (93.0±11.2 μU/plate vs. 150.7±10.0 μU/plate, $n = 6$, $P < 0.005$). If Ca^{2+}-free buffer was present during both the 30-min basal and initial 15-min stimulation period (0–45 min) in 7 mM K^+, and the cells were then stimulated for the first time with 40 mM K^+ at 45 min, a brisk spikelike secretory response was observed (Fig. 3). This response was, however, reduced to 80% of the control value (119.7±9.2 μU/plate vs. 150.7±10.0 μU/plate, $n = 6$, $P < 0.05$). If the basal buffer from 0 to 30 min contained 2.5 mM Ca^{2+}, but the cells were stimulated with 40 mM K^+ in Ca^{2+}-free buffer, no insulin secretion was elicited (data not shown).

In static incubations, 0.1 mM Ca^{2+} sustained a small secretory response to 40 mM K^+, but no stimulated insulin secretion was detected with no added Ca^{2+} (Fig. 4). The secretory response was directly proportional to the level of added Ca^{2+} from 0.1 to 2.5 mM.

$^{45}Ca^{2+}$ uptake. 40 mM K^+ stimulated a rapid, significant increase of $^{45}Ca^{2+}$ uptake from the external medium. By 2.5 min there was an increase in Ca^{2+} uptake (Fig. 5, $P < 0.001$, $n = 8$–12 per time point), which was fourfold higher than that seen at 2.5 min in basal media ($P < 0.001$). The $^{45}Ca^{2+}$ uptake rose rapidly in a linear fashion to 5 min, then decreased and was linear up to 60 min. In contrast, the $^{45}Ca^{2+}$ uptake in high glucose alone did not increase over the uptake seen in unstimulated cells. The K^+-stimulated $^{45}Ca^{2+}$ uptake was inhibited by verapamil (Fig. 5, *inset*). HIT cells preincubated for 30 min with 30 μM verapamil and then stimulated with 40 mM K^+ in the continued presence of verapamil had $^{45}Ca^{2+}$ uptake values of 8% of K^+-stimulation in the absence of verapamil at 1 min, 14% at 5 min, 28% at 10 min, and 17% at 15 min, (each point, $n = 4$, $P < 0.001$).

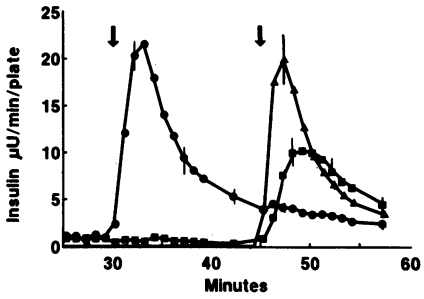

Figure 3. Calcium dependence of K^+-stimulated insulin secretion. K^+ (40 mM) stimulated a first-phase secretory response (●, $n = 6$) in 2.5 mM Ca^{2+} (first *arrow*) and failed to stimulate in Ca^{2+}-free buffer (30–45 min (■, $n = 6$). Returning the Ca^{2+} (second *arrow*) gave a response 60% of the initial response with Ca^{2+} (■, 45–60 min). First exposure to K^+ with 2.5 mM Ca^{2+} at 45 min after a Ca^{2+}-free period in low K^+ produced a monophasic response slightly less than the control response (▲, $n = 8$).

Insulin secretion from quin 2-loaded cells. The ability of suspensions of quin 2-loaded cells to release insulin in response to 40 mM K^+ and 19.6 mM glucose was tested (Fig. 6). The addition of quin 2 causes a drop in the basal secretory rates of the glucose-stimulated cells ($P < 0.005$, $n = 4$). In quin 2-loaded cells, an increase in the K^+ concentration of the buffer from 7 to 40 mM increased insulin release by 167% ($n = 4$, $P < 0.002$), whereas changing from 0 to 19.7 mM glucose increased insulin release by 97% ($n = 4$, $P < 0.005$). In control cells treated with DMSO alone, 40 mM K^+ caused a 224% increase ($n = 4$, $P < 0.002$) and 19.7 mM glucose a 91% increase ($n = 4$, $P < 0.01$). Quin 2 loading caused a significant reduction in the amount of insulin released in response to K^+-stimulation ($n = 4$, $P < 0.005$), but not glucose. The control and stimulated release in cells in suspension is much less than that of cells grown in monolayer.

Fluorescence measurements with K^+ stimulation. Fig. 7 shows a representative experiment illustrating the changes in $[Ca^{2+}]_i$ in cells treated with 40 mM K^+. Fig. 7 *A* shows the control experiment in which the resting $[Ca^{2+}]_i$ was 42 nM. The addition

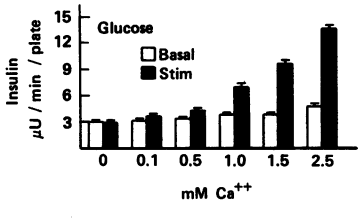

Figure 2. Glucose-stimulated insulin secretion in varying Ca^{2+} concentrations. HIT cells incubated in varying Ca^{2+} concentrations during a 60-min basal period (basal secretion assayed from the last 15 min, open bar, $n = 8$) followed by a 15-min stimulation period in high glucose, 19.7 mM (solid bar, $n = 8$), demonstrated a correlation between external Ca^{2+} concentration and insulin secretion.

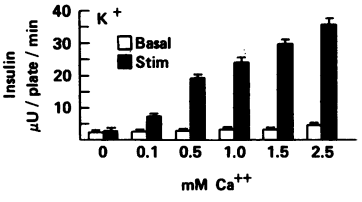

Figure 4. K^+-stimulated secretion with different calcium concentrations. Low concentrations of Ca^{2+} (0.1 and 0.5 mM) sustained a large portion of the insulin secretory response to K^+ (solid bar, $n = 8$) relative to rates in basal buffer with corresponding Ca^{2+} concentration (open bars, $n = 8$).

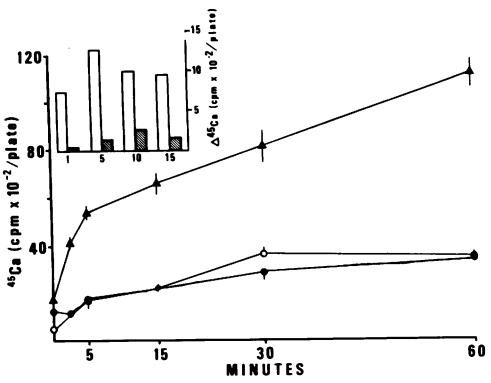

Figure 5. $^{45}Ca^{2+}$ uptake by HIT cells. HIT cells stimulated with 40 mM K^+ (▲, n = 9 per time point) demonstrated a rapid increase in $^{45}Ca^{2+}$ from 0 to 5 min and a slower linear increase from 5 to 60 min. High glucose (○, n = 4–6 per time point) failed to increase $^{45}Ca^{2+}$ uptake above basal values (●, n = 18 per time point). As shown in the *inset*, the change in $^{45}Ca^{2+}$ uptake above basal values ($\Delta^{45}Ca$) stimulated by K^+ (open bars) was significantly inhibited after a 30 min preexposure to 30 μM verapamil followed by stimulation with K^+ in the continued presence of verapamil (hatched bars) at each time point tested (n = 4).

of 40 mM K^+ resulted in a rapid rise in $[Ca^{2+}]_i$, which peaked at 40 s and remained above the basal $[Ca^{2+}]_i$ for up to 4.5 min. 40 mM K^+ stimulated an average 4.7-fold rise in $[Ca^{2+}]_i$ from 56±3 to 238±7 nM (n = 31, $P < 0.001$, Table I).

If verapamil, a blocker of voltage-sensitive, Ca^{2+} channels was added at concentrations of 30 μM or higher before the addition of 40 mM K^+, no rise in the $[Ca^{2+}]_i$ was seen (Fig. 7 *B*). Furthermore, the addition of 4 mM EGTA before addition of K^+ also completely blocked the increase in $[Ca^{2+}]_i$ (Fig. 7 *C*). The rapid fall in the basal $[Ca^{2+}]_i$ after addition of EGTA results from the quenching of extracellular quin 2 fluorescence by the binding of Ca^{2+} to EGTA.

Fluorescence measurements with glucose stimulation. Increasing glucose from 0 to 19.7 mM caused a slight, but significant fall in $[Ca^{2+}]_i$ (Fig. 8 *A*). In 11 experiments, $[Ca^{2+}]_i$ fell from 37±4 to 31±4 nM Ca^{2+} ($P < 0.001$, Table I). If cells were loaded in the presence of nonstimulatory concentrations of glucose (1.67 mM), there was no change in the basal $[Ca^{2+}]_i$, but

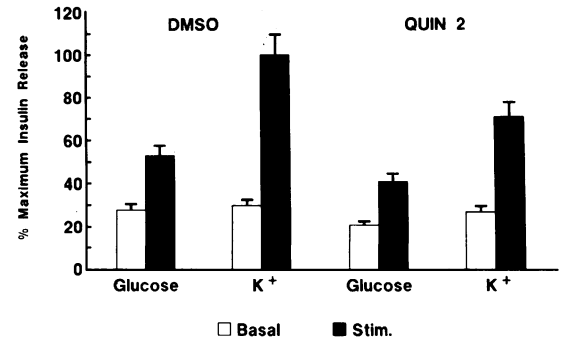

Figure 6. Insulin secretion from quin 2 loaded and control (sham loaded) HIT cells. Quin 2-loaded cells were treated as described in Methods. Control cells were not exposed to quin 2. After a 45-min basal preincubation period, insulin release was measured for a 15-min basal period in the presence of 0 or 1.67 mM glucose, and for 15 min after addition of 19.7 mM glucose or 40 mM K^+.

the slight drop in $[Ca^{2+}]_i$ was not observed upon addition of a stimulatory dose of glucose (Fig. 8 *B*). As shown in Figs. 1–4, 40 mM K^+ is a more potent stimulus of insulin secretion than 19.7 mM glucose. It is possible that with the smaller secretory signal a small increase of $[Ca^{2+}]_i$ might be lost in the rapid redistribution of Ca^{2+} in glucose-stimulated cells. Thus, we determined a concentration of K^+ that stimulated approximately the same threefold increase in insulin release as that of glucose. 15 mM K^+ increased insulin secretion 2.9-fold from 2.6±0.1 to 7.6±0.4 μU/min per plate ($P < 0.0005$, n = 8). With 15 mM K^+, $[Ca^{2+}]_i$ was still increased 1.5-fold from 43±2 to 66±5 nM ($P < 0.01$, n = 4).

Fluorescence measurements with 1 mM EGTA or verapamil alone. Since it was shown that perifusion of HIT cells for a 30-min period in the absence of extracellular Ca^{2+} plus 1 mM EGTA inhibited the acute release of insulin to both secretagogues, we tested the effect of this treatment on HIT cell Ca^{2+} homeostasis. During the 30-min period basal, $[Ca^{2+}]_i$ fell significantly from 42±5 nM (n = 5) to 14±2 nM (n = 6, $P < 0.001$, Table I). When 40 mM K^+ was added in the presence of Ca^{2+}, the increase in $[Ca^{2+}]_i$ to 380±51 nM was not significantly different from that found in control cells treated with 40 mM K^+ alone.

Since verapamil itself may have an effect on basal $[Ca^{2+}]_i$, 30 μM verapamil was incubated for 15 min with quin 2-loaded cells in the presence of low glucose and lowered the basal $[Ca^{2+}]_i$ slightly. At 15 min the $[Ca^{2+}]_i$ in control cells was 48±2 nM vs. 39±4 nM (n = 3, $P < 0.02$).

Actions of verapamil on high K^+- and glucose-stimulated insulin release and $[Ca^{2+}]_i$. Glucose- or K^+-stimulated insulin release was inhibited by verapamil in a dose-dependent manner (Fig. 9). Complete inhibition of glucose-stimulated insulin secretion was achieved at 100 μM verapamil. The dose giving the half-maximal effect (ED_{50}) for glucose-stimulated insulin secretion was 1.4×10^{-5} M. Verapamil inhibited 40 mM K^+-stimulated insulin release at much lower concentrations than those required to attenuate glucose-stimulated insulin release. At 1 μM verapamil, 40 mM K^+-stimulated insulin release was inhibited to 15% of control, whereas glucose-stimulated insulin release was still 88% of control. Over a narrow range of verapamil concentrations, both glucose- and K^+-stimulated insulin secretion are inhibited in a linear manner. The ranges of verapamil concentrations that produce these effects vary between the two secretagogues. From 100 nM to 7.5 μM verapamil, K^+-stimulated insulin secretion was inhibited and gave a coefficient of determination (r^2) of 0.98, whereas verapamil inhibition of glucose-stimulated insulin secretion in the range from 2.5 to 100 μM verapamil produced an r^2 of 0.97. At every concentration of verapamil tested, there was a remarkable agreement between the rise in $[Ca^{2+}]_i$ and insulin secretion stimulated by 40 mM K^+. At concentrations of 10 nM or less there was no effect of verapamil on insulin secretion or the rise in $[Ca^{2+}]_i$ triggered by 40 mM K^+. The inhibitory effect of the drug was completely reversible. In cells treated with 100 μM of verapamil and then returned to growth media overnight and restimulated, insulin secretion was similar to cells handled in the same manner, but not exposed to verapamil (data not shown).

Discussion

The functional integrity of quin 2-loaded HIT cells was established by cell viability and secretory studies. During a 15-min

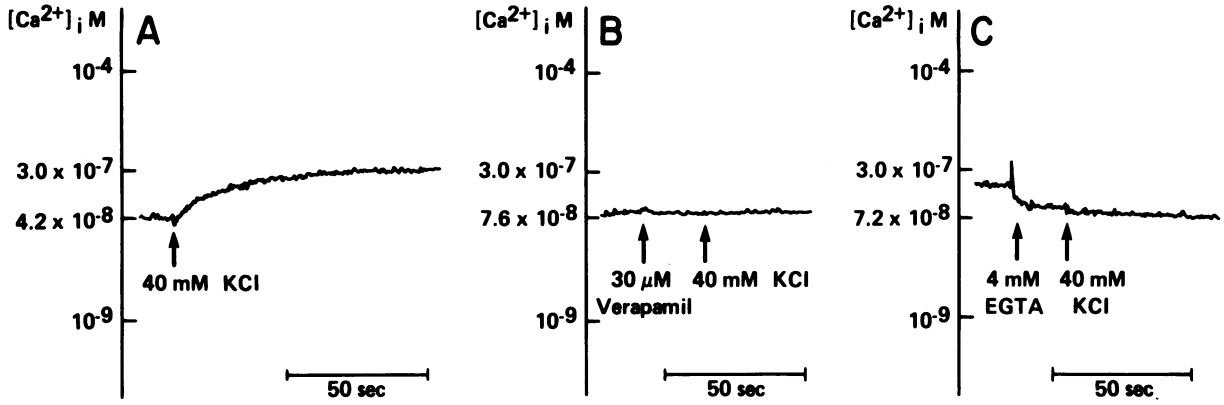

Figure 7. $[Ca^{2+}]_i$ in HIT cells, and response to K^+. *A* shows that K^+ increased the $[Ca^{2+}]_i$ from 42 to 243 nM. The effect of K^+ was completely blocked by prior addition of 30 μM verapamil (*B*), or 4 mM EGTA (*C*).

period in no glucose, the basal insulin secretory rate of quin 2-loaded cells was less than that of control cells. It is not surprising that the addition of quin 2, a Ca^{2+} chelator, into the cytoplasm of a beta cell has some effect on cell function. Similar findings have been reported in the RINm5f insulinoma cells where both basal and stimulated insulin release are decreased by quin 2 loading (7). However, in both cell lines, insulin secretion in quin 2-loaded cells is still maintained, making it possible to study the role of $[Ca^{2+}]_i$ in stimulus-secretion coupling.

These experiments in HIT cells corroborate many earlier reports in islets, demonstrating an absolute requirement for extracellular Ca^{2+} for K^+- or glucose-stimulated insulin release. In these studies the magnitude of the secretory responses to each stimuli was dependent on the extracellular Ca^{2+} concentration. Although both secretagogues demonstrated a dependence on extracellular Ca^{2+}, several differences were also apparent. Only K^+ increased $^{45}Ca^{2+}$ uptake and $[Ca^{2+}]_i$. With high glucose the $^{45}Ca^{2+}$ uptake was similar to the control rate in unstimulated cells, and $[Ca^{2+}]_i$ did not increase. The 40 mM K^+ studies establish that, as in pancreatic islets (4), a rise in $[Ca^{2+}]_i$ in the HIT cell can trigger insulin release without the presence of glucose. High concentrations of K^+ are a standard means of depolarizing the plasma membrane and opening voltage-dependent, Ca^{2+} channels (23). In RINm5f cells, high K^+ simultaneously alters the membrane potential (7) and increases $[Ca^{2+}]_i$. In either cell line, blocking the Ca^{2+} channel with verapamil, or chelating extracellular Ca^{2+} with EGTA, abolishes the effects of K^+ on $[Ca^{2+}]_i$ and insulin secretion. K^+ did not stimulate insulin release in the absence of extracellular Ca^{2+} and the insulin secretory response was linear from 0.1 to 2.5 mM Ca^{2+}. The minimum concentration of extracellular Ca^{2+} required for glucose-stimulated insulin secretion from HIT cells was similar to that seen in vitro in islets and was above 0.1 mM (26). In perifusion no insulin secretion was detected in Ca^{2+}-free buffer containing 1 mM EGTA. As shown with quin 2 measurements, exposure to a Ca^{2+}-free, EGTA buffer disrupts cellular Ca^{2+} homeostasis, lowers the basal $[Ca^{2+}]_i$, and may deplete Ca^{2+} stores. However, the lack of stimulated insulin release to both secretagogues by the Ca^{2+}-free, EGTA buffer was not due to irreversible damage to the cells, since replacing the Ca^{2+} elicited a recovery of secretion after the Ca^{2+}-free period.

The insulin secretory recoveries observed when Ca^{2+} was returned after exposing the HIT cells to glucose or K^+ in Ca^{2+}-free medium also differed. Reintroduction of 40 mM K^+ in 2.5 mM Ca^{2+} restored insulin secretion to 80% of the control period. If high glucose was present during the Ca^{2+}-free period, reintroduction of Ca^{2+} with high glucose restored insulin secretion only to the basal rate. However, if no glucose was present during the Ca^{2+}-free period, a full first phase insulin response was triggered by reintroduction of high glucose and 2.5 mM Ca^{2+}.

These observations are somewhat similar to data reported by Frankel et al. (27), who compared glucose-stimulated insulin release with the membrane potentials of single beta cells at vary-

Table I. Basal and Stimulated $[Ca^{2+}]_i$ in HIT Cell Line

Glucose	$[Ca^{2+}]_i$ Basal	40 mM K^+	19.7 mM glucose	Fold stimulation
0	37±4 (11)		31±4 (11)*	
1.67 mM	37±5 (9)		37±5 (09)	
1.67 mM	56±3 (31)	238±17 (31)*		4.7
1.67 mM + 1 mM EGTA	14±2 (6)‡	380±51 (6)*		27.1
1.67 mM	42±5 (5)	507±103 (5)*		12.1

Cells were incubated in basal buffer for 20 s, and the basal $[Ca^{2+}]_i$ given is the average $[Ca^{2+}]_i$ over the first 10 s. The stimulatory substance was then added as a concentrated bolus, and cell fluorescence monitored for 2 min. The $[Ca^{2+}]_i$ listed is the average $[Ca^{2+}]_i$ for the last 10 s of each stimulatory period. Data are given as means±SE (*n*).
* $P < 0.001$ from basal.
‡ $P < 0.001$ from basal in presence of Ca^{2+}.

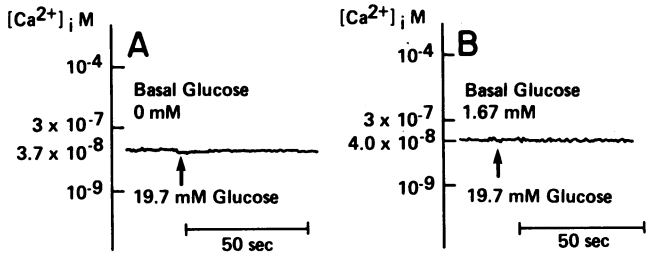

Figure 8. $[Ca^{2+}]_i$ in HIT cells, and response to glucose. HIT cells incubated in basal medium without glucose responded to the addition of 19.7 mM glucose with a decrease in $[Ca^{2+}]_i$ from 37 to 31 nM (*A*). When the basal incubation is carried out in buffer containing 1.67 mM glucose, no change in $[Ca^{2+}]_i$ is seen upon addition of 19.7 mM glucose (*B*).

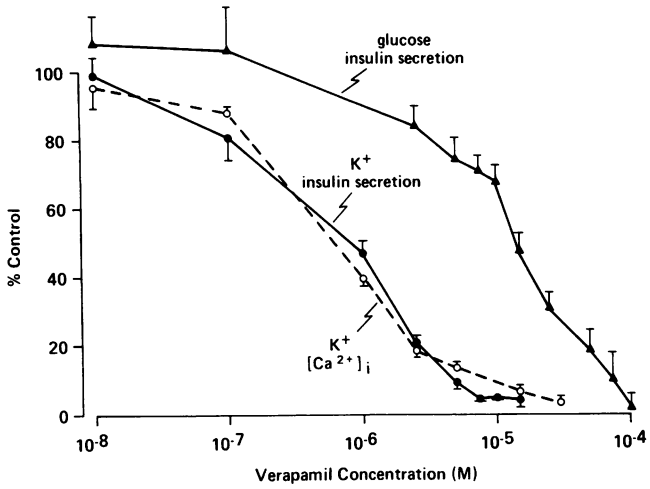

Figure 9. Effect of verapamil on $[Ca^{2+}]_i$ and insulin secretion. Cells were exposed to verapamil at the indicated concentrations. Results are plotted as percentage of control, where control is the response in the absence of inhibitor. Glucose-stimulated insulin secretion was inhibited by verapamil (▲, $n = 8$) at higher concentrations than those needed to inhibit K^+-stimulated insulin secretion (●, $n = 8$). Inhibition of the rise in $[Ca^{2+}]_i$ stimulated by verapamil (○, $n = 5$–6) paralleled the effect of verapamil on K^+-stimulated secretion.

ing Ca^{2+} and Mg^{2+} concentrations. Depolarization of the cell was associated with insulin release, and hyperpolarization with its suppression, irrespective of the Ca^{2+} concentration. Readdition of Ca^{2+} after a Ca^{2+}-Mg–free period caused hyperpolarization of the cell membrane and suppression of glucose-stimulated insulin release. They postulated that the suppression of glucose-stimulated insulin release by readdition of extracellular Ca^{2+} occurs because the rise in $[Ca^{2+}]_i$ increases intracellular K^+ permeability, which would hyperpolarize the cell membrane. Frankel's study (27) also shows that the relative concentrations of extracellular Ca^{2+} and Mg^{2+} are more important in glucose-stimulated secretion than the absolute level of either.

One could also speculate that another explanation for these inhibitory effects is that at stimulatory concentrations, glucose could generate a transitory intracellular signal or "second" messenger. The quin 2 data indicates this signal is not a rise in $[Ca^{2+}]_i$. When Ca^{2+} alone was reintroduced in the presence of high glucose, the glucose signal appears to have already been inactivated or metabolized in the 20 min Ca^{2+}-free period, since insulin secretion was not stimulated and only returned to the basal state. In cells not exposed to high glucose during the Ca^{2+}-free perifusion, the return of Ca^{2+} plus high glucose triggered a full secretory response. However, the lack of insulin secretion in Ca^{2+}-free media cannot be used as evidence that Ca^{2+} is the intracellular signal that triggers insulin release. In the absence of extracellular Ca^{2+}, exocytosis to all HIT cell secretagogues tested is blocked, including those which elevate intracellular cAMP levels that also trigger the acute release of insulin without increasing $[Ca^{2+}]_i$ (Hill, R. S., J. M. Oberwetter, and A. E. Boyd III, submitted for publication). The glucose-induced signal could result from the metabolism of glucose itself or be a glycolytic intermediate, or enzyme (28), or involve the hydrolysis of phosphoinositides with the generation of diacylglycerol and inositol trisphosphate (29).

A number of previous studies using isolated islets suggest that glucose activates insulin secretion by increasing $[Ca^{2+}]_i$ via an effect on voltage-dependent Ca^{2+} channels. Glucose-stimulated insulin secretion is associated with an increase in the frequency of action potentials (30). High concentrations of D600, an analogue of verapamil, inhibit this electrical spike activity (31), and the Ca^{2+} channel blocker also causes a dose-dependent inhibition of first-phase insulin secretion (32). The dependency of glucose-stimulated insulin secretion on extracellular Ca^{2+} (4, 5) and the glucose-mediated increase in $^{45}Ca^{2+}$ uptake into islets (reviewed in reference 4) also has been used as evidence that glucose may cause $[Ca^{2+}]_i$ to rise by triggering an influx of Ca^{2+} through voltage-dependent Ca^{2+} channels.

The present experiments and Wollheim's work (12) now clearly establish that Ca^{2+} uptake through voltage-dependent Ca^{2+} channels does not activate the immediate release of insulin stimulated by glucose. Although, in isolated islets, glucose increases $^{45}Ca^{2+}$ uptake, 5 μM verapamil completely abolishes this rapid uptake, but does not significantly decrease the first phase of insulin secretion (12). Thus, it is possible to dissociate the effect of glucose on Ca^{2+} uptake from first-phase insulin secretion. In the HIT cells we saw no increase in $^{45}Ca^{2+}$ uptake during glucose-stimulated insulin release. In isolated islets, verapamil inhibited the second phase of insulin secretion by 55%, suggesting that verapamil-sensitive Ca^{2+} channels are important in regulating the sustained release of insulin (12). We did not address that point in the HIT cells, since glucose alone does not stimulate a biphasic secretory pattern in this model system.

There is remarkable agreement between the inhibitory effects of verapamil on K^+-stimulated insulin secretion observed by Wollheim (12) in islets and our data in HIT cells. 5 μM verapamil decreases insulin secretion by 60% in islets (12), and 2.5 μM verapamil caused a similar degree of inhibition of secretion from HIT cells. Wollheim (12) explains some of the discrepancies in the literature on the inhibitory effects of verapamil or its analogues on the first phase of glucose-stimulated insulin release, by pointing out that in previous studies the islets were exposed to the drugs for lengthy periods before addition of the glucose stimulus. This could allow the drug time to gain access to intracellular sites or result in Ca^{2+} depletion before the glucose stimulus. In cell lines, the inhibitory effect of verapamil on the $[Ca^{2+}]_i$ rise and insulin secretion triggered by K^+ occurs almost immediately, and can be seen if both the secretagogue and inhibitor are added simultaneously.

There is a striking correlation between the inhibitory effects of verapamil on insulin release and the rise in $[Ca^{2+}]_i$ stimulated by K^+. The ED_{50} was 6.0×10^{-7} M for both processes. The coordinate inhibition by verapamil of Ca^{2+} uptake into heart muscle and pig coronary strip contractility shows a similar relationship and has a similar ED_{50} of 1.5×10^{-7} M (33, 34). In contrast, the inhibitory effect of verapamil on glucose-stimulated insulin secretion required much higher concentrations, with an ED_{50} of 1.4×10^{-5} M. This ED_{50} compares with that observed when verapamil is used to inhibit thyrotropin-releasing hormone (TRH)-stimulated prolactin release of 3×10^{-5} M (23). Since the immediate release of prolactin by TRH is thought to be triggered when Ca^{2+} is released from a "superficial," internal pool of Ca^{2+} (35), taken together these data suggest that at high concentrations verapamil may have intracellular inhibitory effects on secretion that are not related to an action of the drug on the voltage-dependent channel in the plasma membrane.

In these studies, a change in the total $[Ca^{2+}]_i$ was not linked to glucose-stimulated insulin release. Recently, studies using the patch-clamp method to study periodic electrical activity of pan-

creatic islets cell membranes suggest an electrical explanation for the differences in the signaling mechanisms between glucose and 40 mM K^+. Two laboratories have independently identified a new, K^+-selective channel that is insensitive to varying concentrations of intracellular-free Ca^{2+} and pH (36), but is inhibited by ATP or the metabolism of glucose (37). These K^+-selective channels may link the metabolism of glucose and the signaling of insulin secretion by mechanisms independent of the voltage-dependent Ca^{2+} channel. It is also clear from the patch clamp studies that there is a second distinct class of Ca^{2+} channels in the islet that are activated by membrane depolarization by K^+ as well as by cytoplasmic Ca^{2+} (38). These are the verapamil-sensitive, Ca^{2+} channels that allow Ca^{2+} influx into the HIT cell that activates insulin release by 40 mM K^+.

Our experiments could miss a small, but physiologically significant, localized increase in $[Ca^{2+}]_i$ in HIT cells exposed to glucose. The quin 2 technique measures the average $[Ca^{2+}]_i$ in the entire population of cells. Based on marked differences in $[Ca^{2+}]_i$ measurements in platelets loaded with either quin 2 or the Ca^{2+}-sensitive photoprotein, aequorin, Johnson et al. (39) have suggested that quin 2 may not detect a rise in $[Ca^{2+}]_i$ in a localized area of the cytoplasm where only a small fraction of the total intracellular quin 2 would be available for binding to Ca^{2+}.

Our laboratory (19), as well as others (40, 41), have shown that calmodulin appears to be involved in glucose-stimulated insulin release. The K_d for calmodulin is 2.5 μM (42), and quin 2 is insensitive to changes in Ca^{2+} between 1 and 10 μM Ca^{2+} (14). Using a morphologic technique that quantitates Ca^{2+} by precipitation of cations with pyroantimonate, Lenzen et al. (43) have examined localized changes in the distribution of Ca^{2+} in beta cells of perifused pancreases of mice. At the internal surface of the beta cell membrane they find a pool of Ca^{2+} that rapidly shifts into the cytoplasm in association with the release of insulin triggered by glucose. Two other observations suggest a need for caution in considering a Ca^{2+}-independent signal for glucose-stimulated insulin release. First, in a preliminary report, Prentki et al. (8) showed that glucose caused a rise in $[Ca^{2+}]_i$ in suspensions of normal beta cells. Second, the triose glyceraldehyde, which enters glycolysis at a step later than glucose, alters the handling of Ca^{2+} by the RINm5f cells and increases $[Ca^{2+}]_i$ (7). Using EGTA and verapamil, Wollheim concluded, like we did in these studies, that the extracellular Ca^{2+} pool played only a minor part in the increase in $[Ca^{2+}]_i$ with glyceraldehyde and presumably glucose (7). However, they suggested that an intracellular shift of Ca^{2+} is the signal for glucose-stimulated insulin release. Thus, further studies are necessary to determine if the signal that triggers glucose-stimulated insulin secretion in the HIT cell line is a localized increase in Ca^{2+}, and if it is similar to the physiologic signaling process in the normal islet beta cell.

In conclusion, the experiments presented here show that while HIT cell insulin secretion to high K^+ and glucose is dependent on extracellular Ca^{2+}, it is only for K^+-stimulated insulin release that Ca^{2+} has an intracellular role as a trigger for exocytosis. The amount of insulin secreted in response to stimulation with 40 mM K^+ is directly proportional to the amount of Ca^{2+} taken into the HIT cell through voltage-dependent Ca^{2+} channels. Glucose stimulation, on the other hand, does not trigger an uptake of extracellular Ca^{2+} or an increase of $[Ca^{2+}]_i$. While we cannot rule out the possibility that a glucose-stimulated change in $[Ca^{2+}]_i$ is either compartmentalized, too small, or too transient to be detected by the quin 2 technique, this does not seem likely. Furthermore, while K^+-stimulation produces a long-lasting intracellular secretory signal, the glucose signal is transitory.

Acknowledgments

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