Metabolic amplification of insulin secretion by glucose is independent of β-cell microtubules

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Mourad NI, Nenquin M, Henquin JC. Metabolic amplification of insulin secretion by glucose is independent of β-cell microtubules. Am J Physiol Cell Physiol 300: C697–C706, 2011. First published December 22, 2010; doi:10.1152/ajpcell.00329.2010.—Glucose-induced insulin secretion (IS) by β-cells is controlled by two pathways. The triggering pathway involves ATP-sensitive potassium (KATP) channel-dependent depolarization, Ca2+ influx, and rise in the cytosolic Ca2+ concentration ([Ca2+]c), which triggers exocytosis of insulin granules. The metabolic amplifying pathway augments IS without further increasing [Ca2+]c. After exclusion of the contribution of actin microfilaments, we here tested whether amplification implicates microtubule-dependent granule mobilization. Mouse islets were treated with nocodazole or taxol, which completely depolymerized and polymerized tubulin. They were then perfused to measure [Ca2+]c and IS. Metabolic amplification was studied during imposed steady elevation of [Ca2+]c, by tolbutamide or KCl or by comparing [Ca2+]c and IS responses to glucose and tolbutamide. Nocodazole did not alter [Ca2+]c; or IS changes induced by the three secretagogues, whereas taxol caused a small inhibition of IS that is partly ascribed to a decrease in [Ca2+]c. When [Ca2+]c was elevated and controlled by KCl or tolbutamide, the amplifying action of glucose was unaffected by microtubule disruption or stabilization. Both phases of IS were larger in response to glucose than tolbutamide, although triggering [Ca2+]c was lower. This difference, due to amplification, persisted in nocodazole- or taxol-treated islets, even when IS was augmented fourfold by microfilament disruption with cytochalasin B or latrunculin B. In conclusion, metabolic amplification rapidly augments first and second phases of IS independently of insulin granule translocation along microtubules. We therefore extend our previous proposal that it does not implicate the cytoskeleton but corresponds to acceleration of the priming process conferring release competence to insulin granules.

biphasic insulin release; cytosolic calcium; exocytosis; insulin granules; pancreatic islets

Precise insulin secretion by the endocrine pancreas is essential for normal glucose homeostasis. At the β-cell level, glucose exerts its temporal control and amplitude regulation through a dual mechanism. Glucose metabolism turns on two distinct complementary sequences of events known as the triggering pathway and the metabolic amplifying pathway (reviewed in Ref. 22). The triggering pathway begins with the closure of ATP-sensitive potassium (KATP) channels, which permits depolarization of the plasma membrane, leading to Ca2+ influx via voltage-dependent calcium channels and resulting in an increase in the cytosolic free Ca2+ concentration ([Ca2+]c) that triggers exocytosis of insulin-containing granules (10, 13, 16, 22). The metabolic amplifying pathway does not directly implicate KATP channels (1, 21, 53) or any further rise in cytosolic global or subplasmalemmal [Ca2+] (21, 46) but augments the secretory response to the triggering Ca2+ signal by as yet unresolved mechanisms.

When the concentration of glucose is abruptly and steadily increased, in vitro or in vivo, insulin secretion displays a biphasic time course characterized by a prominent rapid first phase and a sustained second phase (reviewed in Refs. 18, 23, 41, 47, 61). Impairment of the first phase is one of the earliest signs of β-cell dysfunction in type 2 diabetic patients (5, 15). Understanding the cellular mechanisms of this peculiar kinetics has thus aroused much interest but remains a challenging problem for cell physiologists. The picture that emerges from numerous proposed models is that distribution of insulin granules in distinct pools and changes in the triggering Ca2+ signal are both involved (4, 13, 18, 23, 41, 47, 53, 60). The first phase is ascribed to Ca2+-induced exocytosis of a small pool of readily releasable insulin granules. The second phase is thought to require functional recruitment and physical mobilization of reserve granules to replenish the releasable pool near exocytotic sites. Several laboratories have suggested that metabolic amplification only contributes to second phase and corresponds to acceleration of the recruitment process through actions on the cytoskeleton of microfilaments and microtubules (4, 13, 47, 58, 60).

Pharmacological disruption of the web of actin microfilaments beneath the plasma membrane of β-cells facilitates insulin secretion (57), and remodeling of this web might play a role in the secretory response to glucose (60). However, we recently reported that metabolic amplification influences the first as well as the second phase independently of functional actin microfilaments (39).

The proposition that β-cell microtubules are active players in insulin secretion originates from experiments by Lacy et al. (33) and Malaisse et al. (8, 34, 51), who showed that drugs known to interfere with tubulin polymerization (vinblastine, vincristine, and colchicine) generally inhibited insulin secretion from pieces of rat pancreas, isolated islets, or the perfused pancreas. The changes in secretion were, however, variable, dependent on the duration of drug application and the type of stimulus (56), and sometimes small (38) or even absent (17). Nonetheless, the bulk of results supported models in which the second phase of glucose-induced insulin secretion is dependent on mobilization of secretory granules along microtubules (26, 35). This interpretation was supported by cinematographic evidence that vinblastine and colchicine inhibited glucose-induced granular movements in β-cells (30, 32, 50). More recently, conventional kinesin, which provides ATP-dependent motor activity to transport secretory vesicles along microtubules, has been identified in insulin-secreting cells lines and in islets (2, 37). It was then proposed that one consequence of the rise in β-cell [Ca2+]c, produced by glucose is a dephosphorylation of kinesin, with increase of its motor activity and...
acceleration of insulin granule movements along microtubules (9). This scenario was compatible with observations that inactivation of kinesin by antisense nucleotides or dominant-negative mutants inhibits insulin granule movement and insulin secretion (37, 58, 59). Glucose-induced granular movement along microtubules is thus a plausible mechanism of metabolic amplification.

The aim of the present study therefore was to investigate whether the metabolic amplifying pathway requires functional microtubules in β-cells. To this end, mouse islets were treated with nocodazole to depolymerize tubulin and disrupt microtubules or treated with taxol to polymerize tubulin and stabilize microtubules (28, 29). Insulin secretion and islet [Ca^{2+}], were measured in dynamic perfusion systems to distinguish between the two phases of secretion and ascertain that the observed changes in secretion were really due to the amplifying pathway and not to alterations of the triggering Ca^{2+} signal.

**MATERIALS AND METHODS**

The study was approved by, and the experiments were conducted in accordance with, the guidelines of the University of Louvain Animal Research Committee.

**Solutions and reagents.** The control medium was a bicarbonate-buffered solution containing 120 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 24 mM NaHCO₃, 10 mM glucose, and 1 mg/ml bovine serum albumin. The solution was gassed with 94% O₂-6% CO₂ to maintain a pH of 7.4. A similar solution was used as test medium in most experiments after adjustment of the glucose concentration and addition of the studied substances. When the concentration of KCl was increased to 30 mM, that of NaCl was decreased accordingly. Most reagents were from Sigma or Merck (Darmstadt, Germany). Diazoxide (a gift from Schering-Plough, Brussels) and tolbutamide were added from fresh stock solutions in 0.1 N NaOH. Nocodazole, taxol, cytochalasin B (Sigma), and latrunculin B (Calbiochem) added from a stock solution in water.

**Preparations.** Islets were aseptically isolated by collagenase digestion of the pancreas of female C57BL6 mice (8–10 mo) obtained from a local colony. After hand selection, the islets were cultured for about 20 h in RPMI 1640 medium (Invitrogen, Merelbeke, Belgium) kept at 37°C in a 95% air-5% CO₂ atmosphere. The culture medium contained 10 mM glucose, 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

**Measurements of insulin secretion.** Cultured islets were first preincubated for 90 min at 37°C in 2 ml control medium containing 10 mM glucose and supplemented or not with 10 μM nocodazole or 5 μM taxol. Batches of 20 preincubated islets were then perfused at 37°C (24) with test solutions, the composition of which was changed as indicated at the top of the figures. Effluent fractions were collected at 2-min intervals and saved for insulin assay, using rat insulin as a standard (24). At the end of the experiments, the islets were recovered, and their insulin content was determined after extraction in acid-ethanol (40). At the start of perfusions, the islet insulin content was (ng per islet) the following: controls, 102 ± 4.1; nocodazole, 98 ± 4.3; and taxol, 103 ± 5.7. Fractional insulin secretion rate was then calculated as the percentage of islet insulin content that was secreted per minute (40). The two phases of insulin secretion were computed over 7 and 20 or 30 min, respectively, as specified in the legend of the appropriate figures.

**Measurements of islet [Ca^{2+}].** Cultured islets were loaded with the Ca^{2+} indicator fura-PE3/AM (2 μM) for 2 h at 37°C, in 2 ml control medium containing 10 mM glucose, and supplemented or not with nocodazole, vinblastine, or taxol. After loading was completed, the islets were transferred into a chamber mounted on the stage of a microscope and maintained at 37°C. The fura-PE3 probe was excited at 340 and 380 nm, and emission was captured at 510 nm by a Quantum 512QC camera (Roper Scientific, Duluth, GA) and analyzed by the MetaFluor software (Universal Imaging, Downington, PA). Stimulus-induced [Ca^{2+}], changes were measured in individual islets and averaged for presentation as mean traces. Average [Ca^{2+}], during first and second phases were computed over the same periods as for insulin secretion.

**Measurements of islet polymerized and depolymerized tubulin.** The ratio of polymerized tubulin (microtubules) to unpolymerized (free) tubulin in control and treated islets was measured using a microtubules/tubulin assay kit (Cytoskeleton, Denver, CO), based on separation of the two fractions by ultracentrifugation and quantification by Western blot analysis. Batches of 130 islets were treated exactly as for the previous experiments and then processed as described by the manufacturer with one additional step. Proteins in the supernatant fraction were precipitated with the ProteoExtract kit (Calbiochem, San Diego, CA) because they were too diluted for loading on the gel. The immunoblots were visualized by the SuperSignal West Dura chemiluminescence system (Thermo, Rockford, IL), and the ratio of microtubules to tubulin in each sample was quantified using the Quantity One software (Bio-Rad Laboratories, Hercules, CA).

**Results**

**Effects of drug treatment on islet tubulin.** The role of β-cell microtubules in metabolic amplification of insulin secretion was studied by treating mouse islets with tubulin-polymerizing or -depolymerizing agents (28, 29). We first ascertained their effects on the polymerization state of islet tubulin. In control islets, 34% of tubulin was present in the form of microtubules (polymerized) and 66% in free (unpolymerized) form (Fig. 1). This proportion is similar to that previously found in rat islets (36, 44). After 90 min of preincubation with vinblastine or nocodazole (two microtubule disrupters), islet tubulin was almost completely depolymerized. Preincubation with taxol, a microtubule stabilizer, produced the opposite effect, causing virtually complete polymerization of islet tubulin (Fig. 1, left). These biochemical results in islets agree with the morphological disappearance and stabilization of microtubules in insulin-secreting HIT-cells treated with nocodazole and taxol, respectively (14). Figure 1 (right) also shows that latrunculin B and cytochalasin B, two drugs causing depolymerization of actin microfilaments (39), did not influence tubulin polymerization and did not interfere with the decrease and increase in tubulin polymerization produced by nocodazole and taxol, respectively.

**Inhibition of islet [Ca^{2+}] by vinblastine.** Islet treatment with 10 μM vinblastine inhibited glucose-induced insulin secretion by about 30%, as previously reported (33), but unexpectedly caused a similar decrease in [Ca^{2+}]. Vinblastine also inhibited KCl-induced [Ca^{2+}], rise by 25–30%. Because these changes in [Ca^{2+}], complicate the interpretation of secretion
changes, the drug was not further used, and microtubules were depolymerized by nocodazole (Fig. 1) that had little effect on [Ca\textsuperscript{2+}].

Impact of microtubule disruption and stabilization on metabolic amplification of insulin secretion during elevation of islet [Ca\textsuperscript{2+}], by KCl. Amplification of insulin secretion by glucose is classically studied under conditions where the triggering [Ca\textsuperscript{2+}] signal is stably elevated by a nonglucose stimulus and minimally affected by glucose itself (21). While K\textsubscript{ATP} channels were held open with diazoxide, islet cells were depolarized with KCl in the presence of low or high glucose (Fig. 2). The resulting increase in [Ca\textsuperscript{2+}], was similar in the presence of 1 and 15 mM glucose (Fig. 2B), but insulin secretion was larger in the presence of high glucose (Fig. 2A). The approximately twofold difference in secretion (Fig. 2F) for a similar [Ca\textsuperscript{2+}], (Fig. 2C) corresponds to the metabolic amplification.

Similar experiments were then performed after depolymerization of tubulin with 10 \mu M nocodazole that was present during the preincubation period and during [Ca\textsuperscript{2+}], and insulin measurements (Fig. 2D). Nocodazole slightly (15\%, P < 0.05) attenuated KCl-induced [Ca\textsuperscript{2+}], rise in 1 mM glucose but not in 15 mM glucose (Fig. 2C). When compared with controls, insulin secretion was not significantly affected, so that the amplifying action of high glucose (2.1-fold) was unaltered (Fig. 2, D and F).

We then tested the effects of 5 \mu M taxol, a microtubule-stabilizer (Fig. 2E). The time course of KCl-induced [Ca\textsuperscript{2+}], increase was unaffected (Fig. 2E, inset), but its magnitude was reduced by ~20\% in low and high glucose (Fig. 2C). This may contribute to the ~50\% inhibition of KCl-induced insulin secretion that taxol produced at both glucose concentrations (Fig. 2F). Importantly, the secretory response induced by a similar [Ca\textsuperscript{2+}], (Fig. 2C) was doubled by high glucose as in controls (Fig. 2F). Metabolic amplification was thus unaltered by microtubule disruption or stabilization under these conditions of depolarization with KCl.

Impact of microtubule disruption and stabilization on metabolic amplification of insulin secretion during elevation of islet [Ca\textsuperscript{2+}], by tolbutamide. The amplifying action of glucose can also be studied during forced closure of all K\textsubscript{ATP} channels with a high concentration of sulfonylurea (21). The experiments illustrated in Fig. 3 (A and B) start 10 min after addition of 500 \mu M tolbutamide to a medium containing 3 mM glucose. In control islets, [Ca\textsuperscript{2+}], was markedly elevated and insulin secretion was stimulated (Figure 3, compare with dotted lines labeled G3 and showing control data in the absence of tolbutamide). Subsequently raising the concentration of glucose to 15 mM caused a rapid but transient drop in [Ca\textsuperscript{2+}], which reflects Ca\textsuperscript{2+} uptake by the endoplasmic reticulum and transient \beta-cell membrane repolarization (11, 12). Concomitantly, with the reascension of [Ca\textsuperscript{2+}], to levels slightly higher than before glucose stimulation (48), insulin secretion increased markedly (Fig. 3B). The difference in [Ca\textsuperscript{2+}], between steady-state periods in tolbutamide + high glucose (10–30 min) and tolbutamide + low glucose (~20–0 min) was small, though significant (Fig. 3C), whereas the difference in secretory rate averaged 4.2-fold (Fig. 3D) and is thus largely attributable to amplification (48).

Islet treatment with nocodazole did not affect [Ca\textsuperscript{2+}], or insulin secretion in the presence of tolbutamide and 3 or 15 mM glucose (Fig. 3). The amplification of secretion (4.3-fold difference between responses in low and high glucose) was thus similar to that in control islets (Fig. 3D). Islet treatment with taxol slightly lowered [Ca\textsuperscript{2+}], (Fig. 3, A and C) and similarly inhibited (40\%) insulin secretion (Fig. 3, B and D) in the presence of tolbutamide and low or high glucose. The amplifying action of glucose on secretion therefore remained unaltered (4.2-fold) (Fig. 3D).

Impact of microtubule disruption and stabilization on metabolic amplification of insulin secretion studied without clamping of islet [Ca\textsuperscript{2+}],. The amplifying action of glucose was next studied by comparing the changes in [Ca\textsuperscript{2+}], and insulin secretion produced by separate stimulation of islets with either 15 mM glucose or 500 \mu M tolbutamide in 3 mM glucose (Fig. 4). In control islets, tolbutamide produced a rapid increase in [Ca\textsuperscript{2+}], characterized by an initial peak followed by a steady but slowly declining elevation (Fig. 4A, trace C) and triggered biphasic insulin secretion (Fig. 4D, filled circles). Glucose also produced a biphasic increase in [Ca\textsuperscript{2+}], that was slightly delayed when compared with the response to tolbutamide (Fig. 4B, trace C), because of a small initial
decrease due to uptake of Ca\(^{2+}\) in the endoplasmic reticulum (12, 19). In response to this rise in \([\text{Ca}^{2+}]_{c}\), biphasic insulin secretion occurred (Fig. 4E, filled circles), which was also delayed compared with the response to tolbutamide. Importantly, the effect of glucose on \([\text{Ca}^{2+}]_{c}\) was smaller (10%) than that on insulin (30%). However, despite this partial inhibition, the amplifying action of glucose on secretion was not altered (Fig. 4F). Altogether these results show that metabolic amplification does not depend on functional microtubules.

**Impact of concomitant perturbation of microtubule and microfilament functions on metabolic amplification of insulin secretion.** We recently reported that, while increasing insulin secretion, depolymerization of actin microfilaments did not impair metabolic amplification (39). We now investigated the impact of a concomitant inactivation of microtubules and microfilaments (Fig. 5). Islet microfilaments were disrupted by treatment with either cytochalasin B or latrunculin B (39). The lack of effect of these two drugs on tubulin polymerization has been described above (Fig. 1). We also ascertained that taxol and nocodazole had no effect on microfilaments. The proportion of depolymerized to polymerized actin (76%/24%) was produced by the two secretagogues. The effect on \([\text{Ca}^{2+}]_{c}\) was smaller (10%) than that on insulin (30%). However, despite this partial inhibition, the amplifying action of glucose on secretion was not altered (Fig. 4F). Altogether these results show that metabolic amplification does not depend on functional microtubules.

![Fig. 2. Effects of tubulin depolymerization and polymerization on glucose amplification of insulin secretion during clamping of the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{c}\)) in islets with KCl. Islets were first preincubated for 90 min with or without 10 \(\mu\)M Noco or 5 \(\mu\)M taxol. They were then used to measure insulin secretion (A, D, E, and F) or [Ca\(^{2+}\)]\(_{c}\) (B, C, and insets in D and E). Experiments were performed in the presence of 100 \(\mu\)M diazoxide (Dz) to prevent any effect of glucose on ATP-sensitive potassium (KATP) channels and either 1 or 15 mM glucose (G1 or G15). Islets were stimulated by a rise of the KCl concentration from 4.8 to 30 mM (K30) at 0 min. Noco and taxol were present throughout the experiments. A, B, D, and E (and insets): time course of insulin and [Ca\(^{2+}\)]\(_{c}\) changes induced by KCl. C and F: [Ca\(^{2+}\)]\(_{c}\) and insulin responses integrated over the 40 min of stimulation. *Significant difference (\(P < 0.05\) or less) with controls. Significant differences (\(P < 0.001\)) between responses in G1 and G15 are shown above pairs of columns, together with the fold-difference in insulin secretion (F). Values are means ± SE for 10 experiments of insulin secretion and 36–45 islets from 6 to 7 preparations for [Ca\(^{2+}\)]\(_{c}\).
control islets) was unaffected by taxol (78%/22%) or nocodazole (75%/25%) alone. Neither drug impaired the depolymerizing action of cytochalasin B (96%/4%) or latrunculin B (99%/1%).

In a first series, we tested the impact of a concomitant interference with microtubules and microfilaments. Islets were preincubated and then perfused with 10 μM cytochalasin B and 5 μM taxol, whereas controls were preincubated and perfused without drug or with only one of the two drugs (Fig. 5, A–D). When tested alone, cytochalasin B increased tolbutamide- and glucose-induced insulin secretion approximately fourfold (Fig. 5, A–C), despite a slight inhibition of $[Ca^{2+}]_i$ in the presence of glucose (Fig. 5D). When compared with untreated controls, the effect of cytochalasin B on insulin secretion induced by both secretagogues was slightly attenuated by taxol (increase by 3-fold only) (Fig. 5, A and B), probably because of an inhibition of the $[Ca^{2+}]_i$ rise (Fig. 5D). However, when the combined effects of taxol and cytochalasin B were compared with the inhibited response of islets treated with taxol alone, the difference was fourfold again.

In a second series, we interfered with microtubules before depolymerizing microfilaments. Islets were preincubated without drug or with 10 μM nocodazole to disrupt microtubules and then perfused with nocodazole, 1 μM latrunculin B, or both (Fig. 5, E–H). In islets preincubated without drug, latrunculin B alone increased tolbutamide- and glucose-induced insulin secretion approximately fourfold (Fig. 5, E–G) without affecting $[Ca^{2+}]_i$. (Fig. 5H). Similar increases in insulin secretion by latrunculin B were observed in islets pretreated with nocodazole. When islets were preincubated with taxol alone and then perfused with taxol and cytochalasin B, results were similar to those shown in Fig. 5, A–C, when both drugs were present during the preincubation period (data not shown). We can thus conclude that microtubules are unnecessary to sustain the high rates of insulin secretion occurring when the brake exerted by actin microfilaments is lifted.

As to the specific question addressed in the study, these results clearly establish that amplification of insulin secretion, estimated by the ratio of the responses to glucose versus tolbutamide, is neither augmented nor impaired by concomitant inactivation of microfilaments and microtubules in β-cells (Fig. 5, C and G). The same conclusion can also be reached from experiments similar to those shown in Fig. 3. Raising the concentration of glucose from 3 to 15 mM in the presence of tolbutamide augmented insulin secretion more than fourfold in islets treated with cytochalasin B + taxol or with latrunculin B + nocodazole (not illustrated).

DISCUSSION

Our results show that β-cell microtubules play little role in short-term control of insulin secretion in mouse islets and, more specifically, are not implicated in metabolic amplification. They also provide further support to our...
recent conclusions that amplification is a late event in stimulus-secretion coupling and influences both phases of secretion (39).

Experimental tools. Vinca alkaloids, such as vincristine and vinblastine, have previously been used to disrupt microtubules in β-cells and generally found to inhibit insulin secretion (8, 33, 34, 38). Except for the report that vincristine reduced the synchrony of $[\text{Ca}^{2+}]_c$ changes in clusters of MIN6 cells stimulated by tolbutamide or glucose (52), possible effects of this and related drugs on $[\text{Ca}^{2+}]_c$ have not been looked for. We found that vinblastine depolymerized tubulin and inhibited insulin secretion in mouse islets as expected, but also that it inhibited glucose- and KCl-induced $[\text{Ca}^{2+}]_c$ increases. We therefore elected to use another established tubulin-depolymerizing agent (28) nocodazole, which also completely disrupted islet microtubules but had virtually no effect on $[\text{Ca}^{2+}]_c$. Unlike vinblastine, nocodazole did not inhibit insulin secretion induced by three secretagogues, which suggests that previously observed inhibitions were at least partly secondary to changes in $[\text{Ca}^{2+}]_c$. We acknowledge, however, that nocodazole was reported to inhibit glucose-induced insulin secretion in rat islets (26) and HIT cells (14).

Although taxol is the most widely used agent to stabilize microtubules (29), its effects on β-cell function have only rarely been studied. Addition of taxol simultaneously with high glucose did not affect insulin secretion in HIT cells (14), but pretreatment with the drug inhibited glucose-induced insulin secretion in rat islets (26). Here, we found that pretreatment with taxol completely stabilized microtubules in mouse islets, inhibited insulin secretion induced by KCl, tolbutamide, or glucose, but also attenuated the rise in $[\text{Ca}^{2+}]_c$ produced by the three secretagogues. Microtubule-independent effects of taxol on $[\text{Ca}^{2+}]_c$ release from the endoplasmic reticulum have been described in neural cells and cardiomyocytes (3, 62), but we are not aware of effects on voltage-dependent calcium channels. Whatever the underlying mechanism, the small decrease of $[\text{Ca}^{2+}]_c$ pro-

Fig. 4. Effects of tubulin depolymerization and polymerization on glucose- and Tolb-induced insulin secretion. Islets were first preincubated for 90 min with or without 10 μM Noco or 5 μM taxol. They were then used to measure $[\text{Ca}^{2+}]_c$ (A–C) or insulin secretion (D–F). Noco or taxol was present throughout the indicated experiments. A and D: islets were stimulated with 500 μM Tolb in the presence of G3. B and E: islets were stimulated by an increase in the glucose concentration from 1 to 15 mM. A, B, D, and E: time course of $[\text{Ca}^{2+}]_c$ and insulin changes. C and F: $[\text{Ca}^{2+}]_c$ and insulin responses integrated over 7 min for first phase (2–9 min for Tolb stimulation and 3–10 min for glucose stimulation) and 20 min for second phase (10–30 min for both). *Significant difference ($P < 0.01$) with controls. Significant differences ($P < 0.01$) between responses to Tolb in G3 (open bars) and to G15 (filled bars) are shown above pairs of columns, together with the fold-difference in insulin secretion (C and F). Values are means ± SE for 8–11 experiments of insulin secretion and 33–45 islets from 5 to 7 preparations for $[\text{Ca}^{2+}]_c$. 

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duced by taxol probably contributed to the partial inhibition of secretion.

Microtubules and metabolic amplification. The major aim of the present study was to test the possibility that metabolic amplification of insulin secretion involves the microtubular system of \( H_9252 \)-cells. Our results refute the tested hypothesis. Amplification, defined as a larger secretion rate in face of a similar or lower islet \([\text{Ca}^{2+}]_c\), was not impaired by disruption or stabilization of microtubules. Importantly, neither the magnitude nor the rapidity of onset of amplification was altered by interference with microtubule function. Even when absolute levels of secretion were slightly reduced by taxol (an effect that we partly ascribe to inhibition of \([\text{Ca}^{2+}]_c\)), the relative change caused by amplification was unaffected. Metabolic amplification has long been regarded as a process that specifically contributes to sustain second phase of insulin secretion (4, 47, 53, 59), but our recent data have shown that it is a rapid phenomenon that expresses itself within just a few minutes of exposure to high glucose (22, 24, 39). This can be appreciated under control conditions (Fig. 4) where first-phase insulin secretion is larger in response to glucose than to tolbutamide in face of a lower triggering \([\text{Ca}^{2+}]_c\) signal. This can also be seen when the concentration of glucose is increased in the presence of a saturating concentration of tolbutamide (Fig. 3). Preservation of both rapidity and magnitude of amplification after islet treatment with nocodazole and taxol implies that the process neither involves an acute action of glucose on microtubules nor is conditioned by a microtubule-mediated particular organization of pools of insulin granules.

Metabolic amplification, microtubules, and pools of insulin granules. The ~10,000 granules present in rodent \( H_9252 \)-cells (7, 43, 55) are functionally and/or geographically distributed into pools: a small pool of 50–100 readily releasable granules, a pool of ~700 granules docked (tethered) to the plasma membrane, a pool of ~1,500 almost docked granules (near but not attached to the plasma membrane), and a large reserve pool of

![Fig. 5. Effects of combined inactivation of microtubules and microfilaments on glucose- and Tolb-induced insulin secretion.](http://ajpcell.physiology.org/)

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Models implicating microtubules in metabolic amplification view the amplification process as an accelerated refilling of the pool of readily releasable granules through mobilization of insulin granules along microtubules (13, 58). Early cinematographic studies of primary β-cells in monolayer cultures showed that glucose increased saltatory movements of insulin granules and that these movements were inhibited by vinblastine, vincristine, or colchicine (30, 32, 50). More recent studies imaged cell lines in which insulin granules were tagged with a targeted fluorescent protein (20, 27, 59). This improved technique more clearly distinguished between slow random movements of the majority of granules and fast directed movements of a small population of granules. Stimulation of Ca$^{2+}$ influx by either glucose or KCl selectively augmented directed granular movements, an effect that was suppressed by disruption of microtubules with nocodazole (20) or by inhibition of the microtubule-associated kinesin (58, 59). These observations prompted the proposal that these fast, microtubule-dependent movements serve to replenish the readily releasable pool of granules (20) and that their acceleration by glucose corresponds to the amplification process (58).

From our measurements of insulin secretion rates and insulin content of the islets, we can calculate, on the basis of 10,000 granules per cell, the number of granules released by each β-cell (39). We estimate that, over 30 min of stimulation, each β-cell released about 50 granules in response to tolbutamide in low glucose and released about 115 granules in response to high glucose. These numbers are similar to those of granules in the readily releasable pool but much smaller than those in the pools (be they docked or almost docked) that serve to refill the releasable pool. After disruption of the web of actin microfilaments with either cytochalasin B or latrunculin B, the numbers of released granules markedly increased to about 185 (tolbutamide) and 420 (high glucose). Importantly, these three- to fourfold increases also occurred in the absence of functional microtubules. Altogether, our results obtained in whole islets indicate that intact microtubules are not required for acute refilling of the releasable pool and for short-term control of insulin secretion. Interestingly, estimations of exocytosis by capacitance measurements in mouse β-cells treated with colcemid (45) or INS1 cells treated with vindesin (27) also suggested that microtubules are not necessary for replenishment of the readily releasable pool of insulin granules after its emptying by trains of depolarizations. Moreover, we can exclude that the microtubule-dependent acceleration of fast granular movements by glucose (20, 58) underlies metabolic amplification for a simple reason. Unlike insulin secretion (Fig. 2) (21), this acceleration of granular movements was not augmented further by high glucose in the presence of KCl (20). Kinesin-driven translocation of insulin granules along microtubules (9, 37, 58) probably contributes to maintenance of long-term β-cell secretory function but is unlikely to play an active role in acute regulation of insulin secretion, except perhaps in pathological degranulated β-cells or in inherently poorly granulated cell lines. Other functions of kinesin-1 are also possible. Thus targeted inactivation of kinesin-1 in β-cells led to inhibition of both phases of glucose-induced insulin secretion in vivo, independently of any alteration of the subcellular localization of insulin granules (6).

In conclusion, in sharp contrast with the increase in insulin secretion observed after disruption or stabilization of actin microfilaments (39, 57, 60), no acute secretory changes follow alterations of microtubule function in primary mouse β-cells. Together, our previous (39) and the present study show that metabolic amplification does not involve recruitment of reserve granules by the cytoskeleton. In agreement with a previously proposed model (54), we reinforce our recent conclusion that metabolic amplification corresponds to acceleration of the priming process conferring release competence to insulin granules (39). This should now help identifying the molecular mechanisms and cellular effectors of a quantitatively important (22) regulatory step of stimulus-secretion coupling in β-cells.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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