Antidiabetic sulfonylurea stimulates insulin secretion independently of plasma membrane $K_{\text{ATP}}$ channels

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Geng X, Li L, Bottino R, Balamurugan AN, Bertera S, Densmore E, Su A, Chang Y, Trucco M, Drain P. Antidiabetic sulfonylurea stimulates insulin secretion independently of plasma membrane $K_{\text{ATP}}$ channels. Am J Physiol Endocrinol Metab 293: E293–E301, 2007. First published April 3, 2007; doi:10.1152/ajpendo.00016.2007.—Understanding mechanisms by which glibenclamide stimulates insulin release is important, particularly given recent promising treatment by glibenclamide of permanent neonatal diabetic subjects. Antidiabetic sulfonylureas are thought to stimulate insulin secretion solely by inhibiting their high-affinity ATP-sensitive potassium ($K_{\text{ATP}}$) channel receptors at the plasma membrane of $\beta$-cells. This normally occurs during glucose stimulation, where ATP inhibition of plasmalemmal $K_{\text{ATP}}$ channels leads to voltage activation of L-type calcium channels for rapidly switching on and off calcium influx, governing the duration of insulin secretion. However, growing evidence indicates that sulfonylureas, including glibenclamide, have additional $K_{\text{ATP}}$ channel receptors within $\beta$-cells at insulin granules. We tested nonpermeabilized $\beta$-cells in mouse islets for glibenclamide-stimulated insulin secretion mediated by granule-localized $K_{\text{ATP}}$ channels by using conditions that bypass glibenclamide action on plasmalemmal $K_{\text{ATP}}$ channels. High-potassium stimulation evoked a sustained rise in $\beta$-cell calcium level but a transient rise in insulin secretion. With continued high-potassium depolarization, addition of glibenclamide dramatically enhanced insulin secretion without affecting calcium. These findings support the hypothesis that glibenclamide, or an increased ATP/ADP ratio, stimulates insulin secretion in part by binding to granule-localized $K_{\text{ATP}}$ channels that functionally contribute to sustained second-phase insulin secretion.

Generally, $K_{\text{ATP}}$ channels couple glucose metabolism and membrane electrical signaling (1, 2, 10, 16, 27). $K_{\text{ATP}}$ channels are ideal receptors for ligands signaling changes in glucose metabolism, because they are designed as sensors of adenine nucleotide levels. ATP binding to the $K_{\text{Ca}}$ subunit of the $K_{\text{ATP}}$ channel inhibits the potassium efflux that otherwise maintains the electrically negative resting state of the cell. ADP binding to the sulfonylurea receptor 1 (SUR1) subunit of the $K_{\text{ATP}}$ channel can antagonize the inhibition gating and restore the resting state. Thus the inhibition gating by ATP and its antagonism by ADP allow glucose metabolism to tightly regulate the $\beta$-cell plasmalemmal potential, allowing L-type calcium channels and calcium influx to be rapidly switched on or off. This calcium-controlled signaling pathway initiating GSIS is the $\beta$-cell’s initial but transient response to curb rises in blood glucose levels.

A prolonged duration of high blood glucose levels, however, requires sustained insulin secretion. Experimentally, this sustained regulatory phase is observed as second-phase insulin release (36). Relatively little is known about the mechanisms coupling rates of high glucose metabolism to the second phase, even though the second phase is disrupted in most forms of diabetes. What is known is that calcium plays different roles in second-phase than it does in first-phase insulin secretion (36, 19, 40). In response to glucose stimulation, first-phase release is initiated by a rise in calcium level but is terminated by rapid depletion of a limited supply of calcium-releasable secretory granules at the plasmalemma. Importantly, the calcium level remains elevated, and after a characteristic delay, a second signal from high glucose metabolism stimulates the resupply of the calcium-releasable secretory granule pool at the plasmalemma. As for the first phase, ATP and ADP are candidate signals coupling high glucose metabolism and second-phase secretion (11, 14, 20, 38), but their receptor proteins in the $\beta$-cell are unknown.

Plasmalemmal $K_{\text{ATP}}$ channels are unlikely candidate receptors by which glucose stimulates second phase. If another signaling pathway is used to elevate intracellular calcium level, for example, raising extracellular KCl from 4.8 to 30 mM, then second-phase insulin secretion becomes plasmalemmal $K_{\text{ATP}}$ channel independent (19, 20, 40). Under these conditions, high glucose stimulates further increases in insulin release without further increasing intracellular calcium level (36). These stud-

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ies raise the question of whether additional \( \text{K}_{\text{ATP}} \) channels other than plasmalemmal \( \text{K}_{\text{ATP}} \) channels couple glucose metabolism to the resupply of calcium-releasable secretory granules for sustained second-phase release.

Glibenclamide also appears to have binding sites within the \( \beta \)-cell, in addition to its plasmalemmal \( \text{K}_{\text{ATP}} \) channel sites. Glibenclamide is distinguished from other antidiabetic sulfonylureas, not only by its superior secretagogue potency but also by its exceptional ability to be internalized within \( \beta \)-cells (23, 25, 26). Furthermore, glibenclamide has been shown to localize to high-affinity sites of the insulin secretory granule membrane (7, 34), which recently have been identified as \( \text{K}_{\text{ATP}} \) channel subunits (21, 47, 50). Of functional relevance, SUR1 knockout mice, which have no \( \text{K}_{\text{ATP}} \) channels, exhibit chronically elevated \( \beta \)-cell calcium level yet no detectable second-phase insulin release by high glucose, unless cholinergic modulatory pathways are stimulated (15, 44). These findings predict a second \( \text{K}_{\text{ATP}} \) channel-dependent pathway, beyond calcium influx and at insulin secretory granules, that resupplies the calcium-releasable granule pool. Otherwise, the high-glucose stimulation, in the permissive high-\( \beta \)-cell calcium, would stimulate insulin release.

In this study, we determined whether glibenclamide, which mimics ATP inhibition of \( \text{K}_{\text{ATP}} \) channels, has an effect on insulin secretion independent of plasmalemmal \( \text{K}_{\text{ATP}} \) channels. To bypass the signaling pathway involving the plasmalemmal \( \text{K}_{\text{ATP}} \) channel, we applied high-potassium depolarization of the \( \beta \)-cell plasmalemma, which activates the L-type channels, and monitored \( \beta \)-cell calcium level using either rhod-2 (12) or GCamp2 fluorescent indicators (46). Insulin release was assayed by both dynamic perfusion and live-cell imaging of fluorescently labeled insulin. The results show that in the presence of high-potassium depolarization, glibenclamide stimulates a sustained insulin secretion without further increasing calcium levels.

**MATERIALS AND METHODS**

**Islet preparation and culture.** Murine islets were isolated from male BALB/c mice (20–25 g; Taconic, Germantown, NY). Islets were isolated by intraductal collagenase injection, as previously described (6), and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 7.5 mM glucose, 100 mM streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine (Life Technologies, Grand Island, NY) in a humidified 5% CO\(_2\) incubator at 37°C.

**Islet dynamic perfusion and ELISA assay.** Groups of 75–100 isolated hand-picked and size-matched islets (diameter 100–125 \( \mu \)m) were used for each perfusion experiment, as previously reported (6). The islets were washed in Krebs-Ringer bicarbonate buffer (KRBB), pH 7.35, containing 20 mM HEPES, 0.1% bovine serum albumin (BSA), 7.5 mM glucose, and 4.8 mM KCl, except where indicated otherwise. Low glucose was set at 7.5 mM because it gave more sustained stimulated insulin release rates with glibenclamide stimulation compared with 5.6 mM glucose. After a 45-min equilibration with KRBB containing 7.5 mM glucose, elution fractions were collected for basal secretion. KRBB buffer containing 7.5 mM glucose and 30 mM KCl was then used to perfuse the islets, during which time elution fractions were collected for high potassium-stimulated first-phase secretion. Finally, islets were perfused with the following: KRBB with 7.5 mM glucose and 30 mM KCl (mock addition negative control); KRBB with 20 mM glucose, KRBB with 7.5 mM glucose, 30 mM KCl, and 4 \( \mu \)M glibenclamide (glibenclamide experiment); or 20 mM glucose (high-glucose positive control), during which time elution fractions were collected and stimulated and control second-phase secretion determined. High extracellular potassium first-phase secretory responses were comparable with and without diazoxide (\( n = 9 \); unpublished results), consistent with the highly effective depolarization of the \( \beta \)-cell plasmalemma by high potassium. Since diazoxide is known to have deleterious alterations in mitochondrial respiration (24, 37, 41) quite apart from the \( \text{K}_{\text{ATP}} \) channel, diazoxide was omitted. Insulin concentration of the elution samples was measured using ELISA (ALPCO, Windham, NH) with rat insulin as standard. At the end of the experiment, insulin was extracted from the islets and quantified to determine the insulin content remaining in the islets as well as the insulin in the secretory fractions. The average maximal insulin secretory rate was 125 pg/min-islet\(^{-1}\) or 0.25%\( \text{min}^{-1}\) of total islet insulin. The insulin secretory rates are given as fractions of the maximal rate observed in each experiment, to emphasize comparison of the time courses across experiments.

**Effective glibenclamide dose.** BSA was used at 0.1%, where it blocks nonspecific binding sites for insulin, BSA, however, also is well known to bind glibenclamide (25, 26). Therefore, for perfusion, we titrated glibenclamide to the minimum final total concentration in the superfusate (4 \( \mu \)M) that rapidly achieved secretory response rate amplitudes that were comparable to those achieved by 20 mM glucose. The actual glibenclamide within the \( \beta \)-cells in these transient experiments is therefore far less than 4 \( \mu \)M, due to binding to the BSA present, slow partitioning from peripheral to interior \( \beta \)-cells of the islet, and slow partitioning into the \( \beta \)-cell cytosol. For the confocal experiments, we obviated these problems by not using BSA and studying cells on the islet surface. Under these conditions, 400 nM glibenclamide maximally stimulated release, which better estimates the effective glibenclamide dose.

**No calcium control perfusions.** Extracellular calcium was removed by adding no calcium and buffering residual calcium with 1 mM EGTA in the KRBB, used during preincubation periods of 45 min and continued during the application of glibenclamide or high-potassium stimulus in the indicated experiments. Free extracellular calcium level was restored by the addition of 3.5 mM CaCl\(_2\).

**Confocal monitored release of Ins-C-GFP-labeled insulin secretory granules.** Islets were infected with Ins-C-GFP, a live-cell fluorescent reporter of insulin granules and, within 2 days, assayed in KRBB at 37°C as described previously (48). Solution changes were performed by superfusion using the BioLogic RSC-160 sewer pipe solution changer, pressurized by its associated BioLogic MS-4 four-syringe module (Molecular Kinetics, Pullman, WA). Glucose concentration was stepped from 7.5 to 20 mM, or glibenclamide was stepped from 0 to 400 nM in KRBB at 37°C with glucose maintained at 7.5 mM. Confocal microscopy was performed using an Olympus Fluoview 300 confocal laser scanning head with an Olympus IX70 inverted microscope (Melville, NY) as described previously (21, 48). Excitation of green fluorescent protein (GFP) was done using the 488-nm argon laser line at ~2% maximum power. Emission was detected using a 510PQ long-pass and BA530RIF short-pass filter. All images were obtained by using a Plan Apo \( \times \) 60 oil, NA 1.4, objective lens. Images were recorded from the bottom plasma membrane of a \( \beta \)-cell in an intact islet. Cytoplasmic regions were monitored using MetaMorph v6.1 analysis software from Universal Imaging (Downingtown, PA). Time course decay analyses were performed using IGOR Pro v5.05A (WaveMetrics, Lake Oswego, OR).

**Calcium level monitoring by rhod-2 and by GCamp2.** Membrane-permeant rhod-2 AM (2.5 mM; Ref. 12) was superfused onto islets at 37°C typically for 5 min or less, while Ins-C-GFP was initially imaged to identify peripherally located \( \beta \)-cells by their green fluorescent insulin granules. The \( \beta \)-cells were then monitored with the 543-nm excitation laser at <5% power until sufficient basal intracellular red rhod-2 fluorescence was clearly detectable, and then all extracellular rhod-2 AM was washed out by superfusion and the experiment initiated. In a minority of \( \beta \)-cells, significant nonuniform mitochondrial staining was detected, and these cells were not monitored. Three
rhod-2, 0.90 saturation would occur at values. With one-to-one binding stoichiometry of calcium and generally transits to within twofold on either side of these nM; Ref. 25) and that stimulated This is consistent with the fluorescence increased not only in response to the step from 4.8 to 30 mM. In each of the experiments, rhod-2 stepping extracellular potassium from 4.8 to 30 mM and then showing that the rhod-2 does not saturate in response to Figure 2 shows the results of eight control experiments level.

Statistical tests and data display. Pairwise statistical comparison of the perfusion time courses were performed using the parametric unpaired t-test and the nonparametric Kolmogorov-Smirnov test (9) with highly similar significance results in each experiment reported. For simplicity, only the results of the more popular t-test are shown. Mean (±SE) and box-plot time courses were constructed using IGOR Pro (v5.05A) and displayed using Adobe Illustrator (v11.0.0; Adobe Systems, San Jose, CA).

RESULTS

Glibenclamide in 7.5 mM glucose stimulates insulin release following maintained KCl stimulation. Figure 1 shows the results of experiments where live intact mouse islets were perfused in 7.5 mM glucose. The islets responded to a high-potassium stimulus with a transient first-phase insulin release, which then decayed toward basal levels. As the first secretory response subsided, the mouse islets were stimulated with either 4 μM glibenclamide or mock (no addition) control. The islets responded to 4 μM glibenclamide with a second-phase insulin release (n = 6 islet preparations) but not to the mock addition control (n = 6). The peak amplitudes of the first phase of the secretory response to the high-potassium depolarization and insulin islet content were within 30% of one another in all 12 perfusions. The rapid transient first-phase insulin secretory response to the high-potassium stimulus likely results from a rise in β-cell calcium level.

High-potassium depolarization but not subsequent glibenclamide stimulates a rapid and sustained rise in β-cell calcium level. Figure 2 shows the results of eight control experiments showing that the rhod-2 does not saturate in response to stepping extracellular potassium from 4.8 to 30 mM and then from 30 to 100 mM. In each of the experiments, rhod-2 fluorescence increased not only in response to the step from 4.8 to 30 mM but again in response to the step from 30 to 100 mM. This is consistent with the Kd for calcium of rhod-2 (500–700 nM; Ref. 25) and that stimulated β-cell intracellular calcium generally transits to within twofold on either side of these values. With one-to-one binding stoichiometry of calcium and rhod-2, 0.90 saturation would occur at ~5 μM, an order of magnitude above the peak β-cell calcium level.

In nine experiments, we then monitored β-cell calcium responses of rhod-2 to the same high-potassium first stimulus, followed by the second glibenclamide stimulus used in the insulin secretory assays. β-Cell calcium level rapidly rose immediately after the high-potassium stimulus but was not further changed by the subsequent glibenclamide stimulus. The results indicate that the initial high-potassium and subsequent glibenclamide stimulation are distinguished not only by transient vs. sustained insulin secretion but also by rapidly rising vs. unchanging β-cell calcium levels.

To corroborate that the high potassium was maintaining a constant β-cell calcium level, we independently monitored β-cell calcium responses of the fluorescent protein GCamP2 (46) to the same high-potassium and glibenclamide stimulus protocol. GCamP2 is a genetically encoded calcium sensor directed exclusively to the β-cell cytosol. In the five experiments performed, the β-cell calcium level sharply rose in
response to the first high-potassium depolarization. In the presence of sustained high potassium, subsequent glibenclamide stimulation did not alter the elevated β-cell calcium level. Thus, by two distinct measures under the same high-potassium conditions, glibenclamide stimulated insulin secretion without further raising β-cell calcium levels clamped by 30 mM potassium depolarization.

Glibenclamide-stimulated insulin release in 7.5 mM glucose mimics 20 mM glucose-stimulated insulin release following maintained high-K⁺ stimulation. Figure 3 shows results with 20 mM glucose used as second stimulus instead of glibenclamide. The high-potassium depolarization stimulated a transient first phase of insulin release, as before, and the second 20-mM glucose stimulus evoked a second-phase insulin secretory response (n = 4 islet preparations) that was similar in time course and amplitude to that obtained with glibenclamide.

The results so far suggest a model in which the glibenclamide stimulus and increased ATP from the high-glucose stimulus might each be acting through the same mechanism, binding to their granule KATP channel receptors. This interpretation is consistent with the observation that each stimulus applied alone results in secretory rates of similar amplitude and time course. However, an alternative model is that each stimulus acts through nonoverlapping mechanisms that coincidentally give rise to the similar secretory responses observed. These two models can be distinguished by experiments identical to those previously performed except with simultaneous application of glibenclamide and high glucose. As controls, experiments were performed in parallel in which each stimulus was applied alone. If the same mechanism is involved, the secretory response rates should be nonadditive. If separate mechanisms are brought into play, then the rates with both stimuli applied should be up to twice the rates with either stimulus applied alone. Figure 4 shows that the insulin secretory rates in response to simultaneous application of the glibenclamide and 20 mM glucose are nonadditive. In all pairwise comparisons (glibenclamide and glucose vs. glibenclamide, glibenclamide and glucose vs. glucose, and glibenclamide vs. glucose), there was no significant difference in insulin secretory response (P > 0.1; n = 3).

Glibenclamide-stimulated insulin release following sustained KCl stimulation by confocal monitoring of Ins-C-GFP expressed in mouse islets. The perfusion experiments provide an excellent population sample of the average behavior of β-cells but fail to provide single-cell information. From the perfusion assays alone, we cannot know whether β-cell re-
sponses to the glibenclamide following high-potassium stimulation occur heterogeneously or homogeneously within a single islet. For example, most β-cells might be refractory to the second glibenclamide stimulus, whereas a few β-cells might suddenly and completely degranulate. Alternatively, most β-cells might be incrementally responding to the glibenclamide stimulation, wherein each β-cell releases a minority fraction of its insulin secretory granules. In eight experiments, we therefore studied individual β-cell responses to glibenclamide after high-potassium depolarization by using the live-cell fluorescent reporter of insulin granules (Ins-C-GFP) and confocal microscopy (21, 48).

β-Cells expressing Ins-C-GFP were superfused with low-potassium control (4.8 mM), high potassium (30 mM), or high potassium (30 mM) and glibenclamide (400 nM). First, in control experiments, we maintained the low-potassium KRBB secretion buffer throughout a series of experiments, taking 50 images (every 20 s for 1,000 s) from the 0.5-μm optical section (n = 5). Figure 5 shows that over the entire time course of the low-potassium experiments, the fluorescence decay in single β-cells was ~0.05 that of the initial value. This is a measure of secretagogue-independent fluorescence decay (bleaching), which occurs initially with this fluorophore (43) and is minimal because of the high signal-to-noise property of the Ins-C-GFP reporter and consequent low excitation intensity used. Second, we switched from low potassium after five images, to high potassium for the remaining 45 images (n = 5). In this series of experiments, the fluorescence decayed more rapidly upon the switch to high potassium than in the controls and then paralleled the control fluorescence. This is a measure of first-phase release response to the potassium depolarization and elevation of β-cell calcium. Third, we switched from low potassium after five images, to high potassium after an additional five images, and then to high potassium plus 400 nm glibenclamide for the remaining 40 images (n = 8). In this series of experiments, the fluorescence decayed more rapidly than the control, first in response to the switch to high potassium and then in response to the switch to glibenclamide. The time constants show that the first- and second-response components are kinetically distinct and mimic the faster first and slower second phases in response to high glucose. Overall, the results indicate that β-cells rather uniformly responded to the high-potassium and glibenclamide secretagogues, as they do to high-glucose stimulation, with a gradual time-dependent release of a fraction of their insulin secretory content.

We next asked to what extent does the time course of decay in single β-cell fluorescence reflect insulin secretion. The time course of fluorescence decay in the stimulated β-cell should most closely relate to cumulative insulin release in the extra-

**Fig. 3.** Glibenclamide stimulation of insulin secretion mimics that of high glucose following high-K⁺ depolarization. A: time course of fractional peak insulin secretory rate of mouse islets in KRBB in 7.5 mM glucose with the stimuli indicated. First stimulus was 30 mM K⁺ depolarization to all perifusions beginning at 5 min and continued through the remainder of the perifusions. Second stimulus was 4 μM glibenclamide (solid line; n = 6; from Fig. 1) or 20 mM glucose positive control (dashed line; n = 4) and continued through the remainder of the perifusions along with the 30 mM potassium depolarization. B: relative effect of glibenclamide, compared with the 20 mM glucose positive control (R_{Glib}/R_{Gluc}). As in Fig. 1, baseline secretory rate was measured for each perifusion from 0 to 5 min. The secretory rate in response to increasing KCl from 4.8 to 30 mM KCl was then measured for each perifusion from 6 to 12 min. The 30 mM KCl was continued in each perifusion throughout the remainder of the perifusions. The secretory rate in response to a second 4 μM glibenclamide stimulus or a second 20 mM glucose stimulus was then measured from 13 to 35 min, as indicated. ELISA was used to determine the insulin content in the fractions of the collected secretion medium, and results are the fraction of the maximal secretory rate obtained within each perifusion, for easy comparison of time courses. Statistical comparison of the glibenclamide vs. 20 mM glucose positive control perifusions showed no significant difference (P > 0.1) during any of the basal or stimulus periods.

**Fig. 4.** Simultaneous stimulation of insulin secretion by glibenclamide and glucose is similar to that by either stimulus alone. Time course of fractional peak insulin secretory rate of mouse islets in KRBB in 7.5 mM glucose was determined in response to the stimuli indicated. As before, the first stimulus was 30 mM K⁺ depolarization at 5 min and continued throughout the experiments. The second stimulus was simultaneous application of 4 μM glibenclamide and 20 mM glucose (dashed-solid line; n = 3), 4 μM glibenclamide alone as control (solid line; n = 3), or 20 mM glucose alone as control (dashed line; n = 3) and continued through the remainder of the perifusions. All 3 experimental stimulus conditions were performed in parallel on each of 3 independent sets of islet preparations. ELISA was used as before to determine the insulin content in the perfusion fractions, and the results are the fraction of the maximal secretory rate obtained within each perifusion. Error bar cap widths increase from glibenclamide or glucose alone to glibenclamide and glucose combined stimulus conditions for clarity. Statistical comparisons showed no significant difference (P > 0.1) between any pairwise comparisons.
granules. For the glibenclamide response, Fig. 6 shows representative experiments on imaging the islet β-cell response to high-potassium depolarization and to the subsequent glibenclamide stimulation. For the response to the high-potassium depolarization, Fig. 6A presents images from one of the eight experiments showing the minor decays in cellular fluorescence and release of one to a few fluorescent granules. For the glibenclamide response, Fig. 6B presents images from an additional three of the eight experiments showing the time-dependent loss of insulin granules labeled by Ins-C-emGFP in response to glibenclamide. Importantly, the images indicate that the glibenclamide stimulus does not elicit any sudden massive loss of insulin granules. Rather, gradual, time-dependent loss of fluorescent granules was observed from the β-cells in all eight experiments. The results exclude the possibility that glibenclamide stimulates complete degranulation from a minority of β-cells with little or no effect on the majority of cells.

**Glibenclamide-stimulated release depends on calcium.** Glibenclamide-stimulated release might also share with glucose-stimulated release the property of calcium dependence. Alternatively, glibenclamide might be working by a calcium-independent, nonphysiological pathway, altogether distinct from what happens during GSIS. To further compare the stimuli, we studied the extracellular calcium dependence of glibenclamide-stimulated release. Extracellular calcium was omitted, and the calcium chelator EGTA was added at 1 mM to KRBB. The 1.6 mM Mg²⁺ as a divalent normally present in KRBB should suffice for maintenance of the β-cell membrane. Figure 7 shows the results of three experiments where glibenclamide-stimulated release failed to proceed without free calcium. Insulin secretion returned once free calcium was restored in the secretory buffer, indicating that the islets were competent for release. Therefore, glibenclamide and high extracellular potassium are insufficient for insulin secretion in the absence of calcium.

**DISCUSSION**

Under conditions of sustained high-potassium depolarization and elevated β-cell calcium, we found that glibenclamide stimulated a second-phase release of insulin that mimicked second-phase release of insulin by high glucose. Insulin secretion and calcium levels were each measured in intact islet β-cells by using distinct and direct methods. Together with the identification of KATP channels at insulin granules (21, 47, 50), the results provide evidence for a second, granule-localized...
K\text{ATP} channel-dependent pathway underlying insulin secretion that can be stimulated by either glibenclamide or high-glucose metabolism. The results expand on previous experiments indicating that, at high glucose concentrations, calcium is not the sole controlling parameter for insulin secretion (36, 19, 20, 40) and further implicate ATP and ADP signals as candidate coupling factors (13, 14, 30, 38).

Previous support for granule-localized K\text{ATP} channels include observations that high-affinity sulfonylurea receptors cosegregated with insulin secretory granules through sucrose gradient purification (7, 34). These reports also show that glibenclamide localized to insulin dense core granules, as revealed by immunoelectron microscopy, and cross-linked to 140-kDa granule receptor proteins. More recent findings have demonstrated that the major site for K\text{ATP} channels, which comprise the 140-kDa SUR1 and the 43-kDa Kir6.2 subunits, reside on insulin secretory granules (21, 47, 50). The evidence reported included localization of fluorescent glibenclamides to insulin granules and not to other intracellular membranous organelles or the \(\beta\)-cell plasmalemma. Together with the functional evidence reported presently, the observations suggest models in which granule K\text{ATP} channels functionally couple high-glucose metabolism, or glibenclamide stimulation, to speeding the resupply of calcium-releasable secretory granules at sites for exocytic release.

Fig. 6. Single \(\beta\)-cells stimulated by glibenclamide show incremental time-dependent loss of insulin granule fluorescence. \(A\): response to high-K\text{"} stimulation. \(Top\) row of images are from an experiment showing the relatively minor decays in cellular fluorescence immediately after the high-K\text{"} stimulation and before glibenclamide addition (\(n = 8\)). Dashed lines in the first and last images highlight a region of general cellular fluorescence loss. Boxes with arrowheads indicate single fluorescent granule loss. \(Bottom\) row of images are the boxed regions from the row above enlarged to better show single fluorescent granule release. \(B-D\): responses to glibenclamide stimulation. Images in each row are shown from each of 3 experiments in which glibenclamide was used as the second stimulus. Each row shows a \(\beta\)-cell with fluorescently labeled granules at 0 (\(left\)), 200 (\(middle\)), and 400 s (\(right\)) after the glibenclamide stimulation. All \(\beta\)-cells responded similarly with an overall net loss in fluorescently labeled granules (\(n = 8\)). In no case did the glibenclamide stimulus elicit a sudden dramatic loss of granules. Note that for the stationary granules in these experiments, fluorescence intensity typically decremented by 0.05, which was comparable with the decrement in unstimulated whole cell fluorescence. All optical \(z\) planes are immediately proximal to the coverslip to maximize the ability to observe granules that potentially could release. Images were viewed using a Plan Apo \(\times 60\) oil, NA 1.4, objective, Fluoview 300 confocal microscope.

Fig. 7. Calcium dependence of glibenclamide stimulation of insulin secretory rate beyond that elicited by elevated KCl. Parallel perifusions were performed in EGTA-0 \(\text{Ca}^{2+}\) KRB with 7.5 mM glucose. At 10 min, KCl was stepped from 4.8 to 30 mM in each perifusion for another 10 min. After this, the perifusions were treated differently. To one perifusion at 20–45 min, 4 \(\mu\)M glibenclamide was added to the buffer with 30 mM KCl (solid line). To the other perifusion at 20–45 min, nothing was added (dashed line). Next, to both perifusions at 45 min, 3.5 mM CaCl\(_2\) was added. ELISA was used to determine insulin content in the fractions of the collected secretion medium, as indicated. At the end of the perifusions, \(\text{Ca}^{2+}\) was added back to demonstrate that the islets were otherwise secretion competent. Statistical comparison of the glibenclamide vs. mock addition control perifusions showed no significant difference (\(P > 0.1\)) during any of the basal or stimulus periods with or without extracellular free \(\text{Ca}^{2+}\).
Mouse $K_{\text{ATP}}$ channel knockout models (15, 32, 44) and an additional knockdown model (31), but not another knockout model (33, 42), are consistent with a granule-localized $K_{\text{ATP}}$ channel-dependent pathway. In $K_{\text{ATP}}$ and SUR1 knockout models, isolated islets show elevated $\beta$-cell calcium levels in low glucose (2.8 mM), yet in high glucose (16.7 mM), they show neither changes in the high calcium levels nor significant GSIS (15, 32, 44). In the knockdown model, in which $K_{\text{ATP}}$ expression was disrupted by hammerhead ribozymes, high glucose also failed to stimulate second-phase insulin release despite chronically elevated calcium (31). Another SUR1 knockout model, however, in earlier studies showed islets with dramatically slowed insulin secretory responses to glucose regulation (42), whereas more recent results showed surprisingly normalized GSIS (33). In this model, either the $K_{\text{ATP}}$ channel is dispensable for normal GSIS or compensatory factors have come into play (33).

An intracellular role for sulfonlureas has been previously suggested based on in vitro results showing that intracellular injection of tolbutamide into whole cell clamped $\beta$-cells increased its cell capacitance as a measure of insulin secretion (4, 18). Intracellular injection of ADP was also shown to block the $\beta$-cell capacitance increase. The results reported presently extend these findings to intact islets and show glibenclamide-stimulated secretion associated with second-phase measured directly by insulin assay. In the confocal measurements reported presently, the overall decrease in membrane fluorescence observed indicates that any stimulated rate of granule cargo arrival at the membrane is less than the rates of the exocytic release and endocytic internalization. The sustained stimulated secretory rate observed by insulin assay, together with the decrease in membrane cargo fluorescence, is consistent with a stimulated arrival rate, but one that is more than offset by the other two departure rates.

The actions of glibenclamide on insulin secretion reported presently are consistent with models in which decreased ADP and increased ATP binding to the granule-localized $K_{\text{ATP}}$ channels contributes to insulin granule trafficking or priming mechanisms resupplying calcium-releasable granules for second-phase release. Thus two $K_{\text{ATP}}$ channel-dependent pathways regulating insulin secretion can be distinguished at least in part by their plasmamemal and granule locations in the $\beta$-cell. Our results in no way indicate that adenine nucleotides are exclusive signals from high-glucose metabolism that govern second-phase insulin secretion (28). In the case of adenine nucleotides, we speculate their binding to granule $K_{\text{ATP}}$ channels might regulate priming of the granules to a calcium-releasable state by an ionic mechanism (5, 45), stimulate trafficking to release sites, or enhance interactions there with exocytic and endocytic proteins (11, 17, 29, 35). Further investigation of these regulatory pathways will be important to more fully understand the role of $K_{\text{ATP}}$ channels in the treatment of diabetic subjects (8, 22, 39, 49).

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