Glucose Acutely Decreases pH of Secretory Granules in Mouse Pancreatic Islets

**MECHANISMS AND INFLUENCE ON INSULIN SECRETION**

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Glucose-induced insulin secretion requires a rise in β-cell cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)\(_c\)]\(_c\)) that triggers exocytosis and a mechanistically unexplained amplification of the action of [Ca\(^{2+}\)\(_c\)]\(_c\). Insulin granules are kept acidic by luminal pumping of protons with simultaneous Cl\(^{-}\) uptake to maintain electroneutrality. Experiments using patched, dialyzed β-cells prompted the suggestion that acute granule acidification by glucose underlies amplification of insulin secretion. However, others found glucose to increase granular pH in intact islets. In this study, we measured islet granular pH with Lysosensor DND-160, a fluorescent dye that permits ratiometric determination of pH < 6 in acidic compartments. Stimulation of mouse islets with glucose reversibly decreased granular pH by mechanisms that are dependent on metabolism and Cl\(^{-}\) ions but independent of changes in [Ca\(^{2+}\)\(_c\)]\(_c\) and protein kinase A or C activity. Granular pH was increased by concanamycin (blocker of the vesicular type H\(^{+}\)-ATPase) > methylamine (weak base) > Cl\(^{-}\) omission. Concanamycin and methylamine did not alter glucose-induced [Ca\(^{2+}\)\(_c\)]\(_c\) increase in islets but strongly inhibited the two phases of insulin secretion. Omission of Cl\(^{-}\) did not affect the first phase but decreased the second phase of both [Ca\(^{2+}\)\(_c\)]\(_c\) and insulin responses. Neither experimental condition affected the [Ca\(^{2+}\)\(_c\)]\(_c\) rise induced by 30 mM KCl, but the insulin responses were inhibited by concanamycin > methylamine and not affected by Cl\(^{-}\) omission. The amplification of insulin secretion by glucose was not suppressed. We conclude that an acidic granular pH is important for insulin secretion but that the acute further acidification produced by glucose is not essential for the amplification of secretion via the amplifying pathway.

The influence of glucose is paramount and exerted via two hierarchical signaling pathways, which both depend on glucose metabolism in β-cells (1). The triggering pathway involves changes in cytosolic adenine nucleotide concentration (2), closure of ATP-sensitive K\(^+\) ([K\(_{ATP}\)]\(_c\)) channels, membrane depolarization, Ca\(^{2+}\) influx through voltage-activated Ca\(^{2+}\) channels, and rise in the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_c\)]\(_c\)), which is the indispensable triggering signal for exocytosis of insulin granules (3–6). Simultaneously, glucose sets in motion an amplifying pathway that does not involve a further rise in [Ca\(^{2+}\)\(_c\)]\(_c\), but an augmentation of the Ca\(^{2+}\)\(_c\) efficacy on secretion (7–9). The mechanisms of the metabolic amplification are distinct from those by which hormones and neurotransmitters augment insulin secretion (1, 8). After exclusion of many potential mediators (10), three possible candidates are an increase in long chain acyl-CoAs with subsequent acylation of various proteins (11, 12), an increase in the NADPH/NADP\(^{+}\) ratio (13), and an increase in adenine nucleotides (2, 10, 14, 15). How these potential mediators could exert their effect has not been resolved, but an interesting hypothesis suggests that the increase in ATP/ADP ratio primes insulin granules to release by decreasing their pH (16).

The pH of insulin granules is acidic (17–20). It was estimated to be between 5 and 6 in preparations of isolated granules (19), and morphological approaches showed it to decrease from ~6.5 to ~5 with maturation of secretory vesicles in rat β-cells (20, 21). This acidification is thought to permit sequential action of pH-dependent prohormone convertases in the proteolytic processing of proinsulin (20, 22, 23) and to favor storage of insoluble Zn\(^{2+}\)-insulin hexamers (19).

The first suggestion that glucose promotes H\(^{+}\) uptake by insulin granules stemmed from observations of acridine orange accumulation in clusters of rat islet cells (18). More recently, attempts to measure changes of granular pH in real time have been reported. They were based on the expression of a pH-sensitive green fluorescent protein targeted to insulin granules (24) or on cell loading with a non-ratiometric fluorescent pH probe, Lysosensor DND-189 (16, 25). The results of these studies are contradictory. Glucose slightly increased pH of secretory vesicles in normal mouse islets (25) but acidified insulin granules to increase in islets but strongly inhibited the two phases of insulin secretion. Omission of Cl\(^{-}\) did not affect the first phase but decreased the second phase of both [Ca\(^{2+}\)\(_c\)]\(_c\) and insulin responses. Neither experimental condition affected the [Ca\(^{2+}\)\(_c\)]\(_c\) rise induced by 30 mM KCl, but the insulin responses were inhibited by concanamycin > methylamine and not affected by Cl\(^{-}\) omission. The amplification of insulin secretion by glucose was not suppressed. We conclude that an acidic granular pH is important for insulin secretion but that the acute further acidification produced by glucose is not essential for the amplification of secretion via the amplifying pathway.

Insulin secretion by pancreatic β-cells is finely regulated by the interaction of nutrients, hormones, and neurotransmitters.

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ules in RIN insulinoma cells (24). In mouse β-cells dialyzed in the whole-cell patch configuration, cytosolic ATP was found to promote granule acidification (16). From experiments using tolbutamide, inhibitors of the vesicular H^+-ATPase (v-H^+-ATPase), and blockers of Cl^− channels, the following model was proposed to explain granule acidification by glucose. By acting on a sulfonfonyurea-like protein in the granule membrane, ATP (formed from glucose metabolism) could promote Cl^− influx into the granule, thereby providing counterions for H^+ pumping by the v-H^+-ATPase (16).

In the present study, we have used Lysosensor DND-160, a fluorescent probe that, unlike Lysosensor DND-189 used previously, permits ratioing measurement of pH and therefore, decreases potential problems linked to differences in loading or redistribution between cellular compartments (26). This probe has previously been instrumental to measure stimulus-induced pH changes in lysosomes (27), in intracellular canaliculi of parietal gastric cells (28, 29), and in eosinophil granules (30). Our aims were to characterize the acute effects of glucose on granular pH in normal mouse islets, to establish the mechanisms of these effects, and to determine whether they are important for insulin secretion, in particular for its regulation via the amplifying pathway. To address the last question, the islets were subjected to experimental maneuvers expected to increase granular pH by distinct mechanisms.

**EXPERIMENTAL PROCEDURES**

**Solutions and Reagents—**The control medium was a bicarbonate-buffered solution containing 120 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl_2, 1.2 mM MgCl_2, 24 mM NaHCO_3, 10 mM glucose, and 1 mg/ml bovine serum albumin. It was gassed with O_2/CO_2 (94/6) to maintain a pH of 7.4. A similar solution was used as a control in the experiments with islets. The probe was dissolved (10 μM) in pyridine buffer of pH between 4.0 and 5.5 and excited (Exc.) at 340 or 380 nm. The emitted fluorescence was measured at 535 nm and expressed in arbitrary units (a.u.).

**Measurement of [Ca^{2+}]_c, pH_c, and NAD(P)H—**For [Ca^{2+}]_c measurements, islets were loaded with the Ca^{2+} indicator fura-PE3 (2 μM, 2 h at 37 °C) in 1 ml of control medium (except where otherwise specified). After loading, 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 with a CCD camera. From the ratio of fluorescence (at 340 and 380 nm), [Ca^{2+}]_c was calculated by comparison with a calibration curve. Details of the method have been reported elsewhere (4). For measurements of cytosolic pH (pH_c), islets were loaded with the pH indicator BCECF (0.5 μM, 2 h at 37 °C) in 1 ml of control medium (except where otherwise specified). They were examined in the same system as that used for Ca^{2+}, but the probe was excited at 440 and 490 nm, and emission was captured at 535 nm. pH_c was calculated by comparison with a calibration curve (37). For NAD(P)H measurements, islets were pretreated with 2 h at 37 °C in 1 ml of control medium (except
where otherwise specified) without probe. They were then examined with the same system as above and excited at 360 nm with recording of emitted fluorescence at 470 nm (4).

Characteristics and Localization of Lysosensor DND-160—An in vitro calibration curve was constructed by dissolving the probe in 50 mM pyridine buffer with a pH ranging from 4 to 5.5 (steps of 0.1 unit) and measuring the fluorescence emitted at 535 nm upon excitation at 340 and 380 nm. In intact cells, the probe is expected to accumulate in acidic compartments (26). To assess the intracellular localization of Lysosensor DND-160, secretory vesicles were labeled by expressing a green fluorescent protein fused within the C peptide of proinsulin (Ins-C-GFP) (38). The Ins-C-GFP virus was prepared and kindly provided by P. Drain (Pittsburgh, PA). Clusters of islet or MIN6 cells on coverslips were infected for 3 h in serum-free RPMI medium, with the virus at a multiplicity of infection of 10 and 2, respectively. After careful washing, they were cultured for 3 days in serum-containing medium. The cells attached to coverslips were then incubated for 2 h at 37 °C in 1 ml of control medium containing 2 μM Lysosensor DND-160. After quick rinsing, the preparations were examined on a Zeiss Axiosplan microscope equipped with a ×63/1.4 NA oil objective. The DND-160 fluorescence was excited at 365 nm, and the signal emitted above 400 nm was recorded by an Axiocam HRc camera (Zeiss). The GFP fluorescence was excited at 470 nm, and emission was recorded at 540 nm. Because superimposition of blue and green does not yield another color, the DND-160 signal was first digitized, and the blue fluorescence was converted in red. Merging red (converted DND-160) and green (Ins-C-GFP) images then permitted us to identify colocalization by the orange/yellow color.

A second approach was also used to measure the uptake of DND-160 in islet cells. Uninfected islet cell clusters were loaded with Lysosensor DND-160 as above. The analysis was then performed on 6–10 clusters of 10–20 cells each and then averaged for each sample. The preparations were excited as above, but the acquisition time by the camera was either preset or variable until the same preset amount of light was recorded, thereby permitting estimation of the amount of probe accumulated by the clusters.

Measurements of Granular pH in Islets—Intact islets were loaded with Lysosensor DND-160 (2 μM, 2 h, 37 °C) in 1 ml of control medium (except where otherwise specified). They were then examined with the same system as that used for [Ca²⁺], or pH, measurements. The preparation was excited at 340 and 380 nm, and emission was recorded at 535 nm. Successive images (ratios) were obtained over 0.74 s every 5.5 s. Results are presented as changes in fluorescence (ratio 340/380 nm).

Measurements of Insulin Secretion—Cultured islets were preincubated for 2 h at 37 °C in 1 ml of control medium (except where otherwise specified) without probe. Batches of 20 islets were then transferred into chambers of a perfusion system (39). The effluent medium was collected at 2-min
Characteristics of the pH-sensitive Probe Lysosensor DND-160—The fluorescence of Lysosensor DND-160 was measured in pyridine buffer. When pH was increased from 4.0 to 5.5, the emitted fluorescence (at 535 nm) remained fairly stable upon excitation at 340 nm but progressively decreased upon excitation at 380 nm (Fig. 1A). The calibration curve obtained by ratiocinating the 340/380 nm signals shows that pH changes can be measured between 4.5 and 5.5 (Fig. 1B). The 380–535 nm signal was very faint above pH 5.5 and not detectable above pH 6.0, indicating that Lysosensor DND-160 cannot report cytosolic pH changes.

The intensity of the signal increased with the probe concentration in buffer, but the 340/380 nm ratio was not affected (Fig. 1B, inset). This method of granular pH measurement is thus much more independent of loading differences or probe redistribution, which limit the reliability of non-ratiometric probes such as Lysosensor DND-189.

Localization of Lysosensor DND-160 in β-Cells—The localization of Lysosensor DND-160 in β-cells and MIN6 cells was determined by comparison with Ins-C-GFP, a reporter of insulin secretory granules (38). The two signals exhibited a similar localization in both β-cells (Fig. 2, A and B) and MIN6 cells (Fig. 2, C and D). The punctuated distribution of Lysosensor DND-160 (Fig. 2A, inset) was usually more difficult to evidence than that of the green Ins-C-GFP fluorescence, but the areas of high and low signal intensity were similar. Colocalization of Lysosensor DND-160-positive and Ins-C-GFP-positive granules was demonstrated by the orange/yellow color in merged images of a MIN6 cell (Fig. 2, E–H).

Since Lysosensor DND-160 accumulates in intracellular acid vesicles, the amount of dye taken up by β-cells is expected to decrease when either the pH of these vesicles increases or their number (volume) decreases. This was verified by measuring (excitation, 365 nm; emission, >400 nm) the intensity of Lysosensor DND-160 fluorescence in clusters of similar sizes. Fig. 3A shows the fluorescence of a control cluster captured by the camera after 0.6 s of recording. The heterogeneity of cell loading was not exceptional, some cells of a cluster being more or less strongly labeled than the majority (Fig. 3B). This may reflect superimposition of cells or true differences in dye accumulation. When loading with Lysosensor DND-160 was carried out in the presence of concanamycin, an inhibitor of the v-H+-ATPase (41), virtually no signal was detected when the recording time of the camera was preset to 0.6 s as for controls (Fig. 3C). With longer recording times (6 s), a diffuse signal could,
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however, be obtained that was equivalent in intensity to the control signal (Fig. 3D versus Fig. 3A). This is compatible with the dissipation of the pH gradient between acidic organelles and cytoplasm. The intensity of the fluorescence was then automatically measured by the camera in several clusters of a similar size in each preparation and normalized to the signal in control clusters loaded with Lysosensor DND-160 (Fig. 3G). The autofluorescence of non-loaded clusters was only ~10%, and the specific fluorescence of the probe was decreased by more than 80% by concanamycin and 30 mM NH₄Cl (used to alkalinize intracellular vesicles). Importantly, cluster degranulation (by overnight stimulation of insulin secretion with 0.01 μM PMA and 0.5 μM forskolin) also decreased the amount of accumulated probe (Fig. 3, E–G).

Similar experiments were then performed with intact islets loaded with Lysosensor DND-160 and examined (excitation, 340 nm; emission, 535 nm) with the system that will be used in subsequent experiments. The fluorescence intensity was not significantly affected by 30 min of treatment with 100 nM concanamycin but was decreased by 2 h of treatment with 20 or 100 nM concanamycin (Fig. 3H). A similar time- and concentration-dependent inhibition of fluorescence accumulation was observed with bafilomycin, another inhibitor of the v-H⁺-ATPase (not shown). Degranulation of the islets, by overnight stimulation of insulin secretion with forskolin and PMA as above, decreased the fluorescence intensity of Lysosensor DND-160 by 72% (Fig. 3H). Under these conditions, the insulin content of the islets was decreased by 88%, from 103 ± 4 to 13 ± 1 ng/islet (20 batches of 10 islets from three experiments). Both changes compare well since a fraction of the residual fluorescence is intrinsic to the islets and seen without dye loading (16% of total). Therefore, the fraction of Lysosensor DND-160 fluorescence originating from compartments other than insulin secretory granules is not a major one. Lysosomes, which also take up Lysosensor DND-160 (27), are not numerous in normal islet cells (16). The lysosomal volume is only 3–5% of that of secretory granules in mouse or rat β-cells (42, 43).

The above results show that Lysosensor DND-160 is largely concentrated in insulin secretory granules and validate its use to monitor changes in granular pH in situ. For all islets initially perfused with a control medium containing 1 mM glucose, the 340/380 ratio of Lysosensor DND-160 fluorescence averaged 1.32 ± 0.01 (n = 184). This corresponds to an estimated granular pH of ~5.00, a value that agrees with measurements based on other approaches (19, 20). However, because of possible shifts between calibration curves in vitro and in situ, the results will be presented as changes in fluorescence ratio. In all the following figures, a decrease in the 340/380 nm ratio reflects a decrease in granular pH.

Distinct Effects of Glucose on Cytosolic and Granular pH—As reported previously (37), islet pHc slowly declined when the medium contained only 1 mM glucose and rapidly increased, following a biphasic pattern, upon stimulation with high glucose (Fig. 4A). The changes in granular pH were strikingly different. In low glucose, granular pH slightly increased at least during the first 20 min of the experiments, whereas stimulation with 15 mM glucose caused a rapid, monophasic, and sustained decrease (Fig. 4B). A similar decrease was observed when the glucose concentration was changed from 3 to 15 mM, whereas 3 mM glucose had no significant effect (data not shown). Cytosolic and granular pH thus change in opposite directions upon glucose stimulation.

Mechanisms of Glucose Effects on Granular pH—The decrease in granular pH produced by 15 mM glucose was slowly reversible upon return to a low glucose medium (Fig. 5A). It was dependent on an increase in β-cell metabolism as shown by the rapid reversal by 5 mM azide, a mitochondrial poison (Fig. 5B), the occurrence of a similar decrease after the addition of the well metabolized ketoisocaproic acid (10 mM) to 1 mM glucose, and the lack of effect of the non-metabolized 3-O-methylglucose (15 mM) (data not shown). Blockade of insulin secretion by 1 μM clonidine, an α₂-adrenoceptor agonist, did not prevent glucose-induced acidification (Fig. 5C), which rules out the possibility that it is secondary to exocytosis of insulin granules. Diazoxyde, a KATP channel opener that prevents glucose-induced [Ca²⁺]c rise (4), did not affect granule acidification. Depolarizing β-cells and increasing [Ca²⁺]c by 30 mM KCl or by blocking KATP channels with 100 μM tolbutamide (4) had no effect on granular pH in low glucose and did not prevent the acidification by 15 mM glucose (Fig. 5, D–F). The acidification was also unaltered by the inhibition of the Ca²⁺-ATPases of the endoplasmic reticulum by 1 μM thapsigargin (data not shown). All these results indicate that the effects of glucose on granular pH are independent of changes in [Ca²⁺]c.

The acidification of insulin granules is attributed to a v-H⁺-ATPase. It was not possible to determine whether glucose...
metabolism activates this proton pump because the fluorescence was too faint after treatment of the islets with concanamycin or bafilomycin. The inward pumping of H\(^+\)/H\(_1\) is thought to be paralleled by an influx of Cl\(^-\)/H\(_2\) ions to maintain electroneutrality (16). Acute replacement of extracellular Cl\(^-\)/H\(_2\) by impermeant isethionate had little impact on granular pH in low glucose but attenuated the acidification produced by 15 mM glucose, as shown by the acceleration of pH decrease upon Cl\(^-\) readmission (Fig. 5G). When Cl\(^-\) ions were omitted from the medium 2 h before the start of the experiments (i.e. already during loading with Lysosensor DND-160), the initial granular pH was higher, and 15 mM glucose only produced a sluggish decrease that was markedly accelerated by reintroduction of Cl\(^-\) in the medium (Fig. 5H). It has been suggested that Cl\(^-\)
influx into granules occurs through ClC-3 channels regulated by a sulfonylurea receptor-like protein (16). Although SUR1 is present in the insulin granule membrane (44, 45), it is not involved in the acidification by glucose since the phenomenon was unaltered in islets from Sur1 knock-out mice (Fig. 5I). The possible effects of a blocker of Cl− channels, such as DIDS, could not be tested because of the fluorescence of the inhibitor itself (data not shown). Stimulation of the cAMP-protein kinase A pathway by activating cAMP formation with 1 µM forskolin (46) or stimulation of the phospholipase C-protein kinase C pathway by activating muscarinic receptors with 10 µM acetylcholine (47) had no effect on granular pH in low glucose and did not affect the acidification by 15 mM glucose (data not shown). Methylamine, a weak base that accumulates in and raises the pH of isolated insulin granules (19), is well taken up by islet cells (48). As shown by Fig. 5J, 2 mM methylamine rapidly and markedly increased granular pH in low glucose. This increase was completely reversible after withdrawal of methylamine but only partly counteracted by 15 mM glucose. Because LysoSensor DND-160 probably also accumulates in secretory granules of non-β-cells of the islets, we ascertained that the major effects observed above occurred in pseudo-islets exclusively composed of insulin-secreting MIN6 cells (36). As shown in Fig. 5K, 15 mM glucose decreased granular pH in MIN6 cells, and this effect was reversed by azide or methylamine.

In conclusion, our results show that glucose metabolism in β-cells leads to a Ca2+-independent acidification of insulin granules that probably results from the inward transport of H+ by a v-H+ -ATPase, with parallel influx of Cl− (or another anion). Overall, our results obtained in intact cells provide direct experimental support for a model previously based on experiments with dialyzed β-cells (16). They rule out the intervention of SUR1 in this acidification but do not detract from the proposal that the regulation is achieved by a related protein (49). We, however, disagree with one aspect of the model; in our hands, the acidification is not sensitive to diazoxide or tolbutamide. Because our measurements report an average pH, it remains uncertain

![Image of the graph showing effects of agents alkalinizing secretory granules on glucose-induced [Ca^2+], insulin secretion, and pH changes in mouse islets.](https://example.com/image6.png)
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whether the acute acidification occurs in all granules or only in a subgroup, such as immature granules with the highest basal pH. In this case, the phenomenon could facilitate proinsulin conversion by the pH-sensitive prohormone convertases (21–23).

Influence of Granular pH on Glucose-induced Insulin Secretion—Manipulating the composition of the cytosol in patched β-cells and measuring exocytosis as changes in membrane capacitance have prompted the suggestion that ATP-mediated acute acidification of insulin granules makes them release-competent (16). Testing whether the hypothesis is valid for glucose stimulation of intact β-cells is fraught with difficulties. It is indeed critical to verify that other important steps of stimulus-secretion coupling are not altered by the experimental conditions. We therefore used three approaches expected to reduce granular pH by different mechanisms: the inhibition of v-H+ -ATPase with concanamycin, direct alkalinization by a weak base, methylamine, and indirect inhibition of acidification by Cl− omission.

Islets pretreated with 100 nm bafilomycin or concanamycin, two inhibitors of the v-H+ -ATPase (41), accumulated only low amounts of Lysosensor DND-160, and no signal was measurable upon excitation at 380 nm. We estimate that granular pH is above 6.0 under these conditions. Bafilomycin has been reported not to affect immediate (<60 s) exocytosis of insulin granules as measured by amperometry in canine β-cells (50) but to inhibit insulin release from permeabilized INS-1 cells (51). In our hands, bafilomycin markedly inhibited glucose-induced insulin secretion by mouse islets but also impaired the glucose-induced [Ca2+]c rise (not shown), as in MIN6 cells (52). We therefore used concanamycin instead of bafilomycin. After concanamycin pretreatment, basal islet [Ca2+]c was slightly but consistently elevated for unexplained reasons (Fig. 6A). However, stimulation with high glucose was followed by typical (4) changes in [Ca2+]c including the initial small decrease, the first phase increase, and oscillations (masked by averaging) in the steady state (Fig. 6A). This normal [Ca2+]c response rules out major perturbations of glucose metabolism, a conclusion supported by the observation that glucose stimulation similarly increased NAD(P)H fluorescence in concanamycin-pretreated and control islets (by 46 ± 6% versus 50 ± 7%, n = 11). Despite a normal [Ca2+]c rise, glucose-induced insulin secretion was strongly inhibited during both phases (Fig. 6B). Concanamycin pretreatment augmented basal pHc in islets and attenuated, without changing the time course, the alkalinization produced by 15 mm glucose so that pHc was no longer different between test and control islets during steady state stimulation (Fig. 6C). Concanamycin, therefore, inhibits a distal step of stimulus-secretion coupling.

When 15 mm glucose and 2 mm methylamine were combined, granular pH was substantially higher than in the absence of methylamine (Fig. 5). Methylamine did not alter glucose-induced rise in islet NAD(P)H (by 55 ± 4% versus 56 ± 4% in controls), had no effect on basal or glucose-stimulated [Ca2+]c in islets (Fig. 6D), but strongly inhibited insulin secretion (Fig. 6E). Methylamine also slightly increased pHc in low glucose. Since the alkalinization in response to 15 mm glucose was of reduced amplitude, pHc was not different from that of control islets during steady state stimulation (Fig. 6F). Methylamine has previously been reported to inhibit glucose-stimulated insulin secretion without impairing metabolism (48, 53) or altering glucose-induced depolarization and electrical activity in β-cells (54). It was therefore proposed that methylamine interferes with a distal event of the secretory process (48, 54). Whether this interference involved the inhibition of transglutaminase was uncertain (48, 53). Our results showing a marked dissociation between glucose-induced [Ca2+]c rise and insulin secretion are in agreement with these earlier findings and now point to the rise in granular pH as the possible culprit.

We have already seen that omission of extracellular Cl− (to lower intracellular Cl−) increased granular pH and reduced the acidifying action of glucose. However, granular pH remained lower under these conditions than in the presence of methylamine or after concanamycin treatment (Fig. 5). Omission of Cl−
did not impair the increase in islet NAD(P)H produced by glucose (by 61 ± 2% versus 59 ± 2% in controls). In the Cl⁻-free solution, basal [Ca²⁺]ᵢ was unchanged, and the initial drop and first phase [Ca²⁺]ᵢ increase in response to 15 mM glucose were normal (Fig. 6G). The second phase of the [Ca²⁺]ᵢ rise was markedly inhibited, in agreement with the unexplained inhibition of electrical activity recorded under these conditions (55–57). The two phases of glucose-induced insulin secretion were also differentially affected; the first phase was unaltered, whereas the second phase was strongly inhibited (Fig. 6H). This surprising dissociation, observed previously in the perfused rat pancreas (58) and isolated rat islets (59), can now be explained by distinct [Ca²⁺]ᵢ changes during the two phases. From these experiments, we conclude that a moderate increase in granular pH and attenuation of the immediate acidification by glucose (Fig. 5H) do not impair first phase glucose-induced insulin secretion. This approach of Cl⁻ omission does not permit us to draw conclusions about granular pH and second phase insulin secretion because of the inhibition of the triggering Ca²⁺ signal. Moreover, an increase in cytosolic pH at both low and high glucose (Fig. 6I) may complicate the interpretation.

Role of Granular pH Changes in the Amplifying Pathway—The amplifying pathway of glucose-induced insulin secretion can be tested by depolarizing β-cells with KCl in the presence of diazoxide (1, 7). Under these conditions, glucose cannot act on KₐTP channels, and [Ca²⁺]ᵢ is similarly increased in 1 or 15 mM glucose (Fig. 7A). However, insulin secretion is much larger in high than in low glucose (Fig. 7B), a difference that corresponds to the amplification. Average [Ca²⁺]ᵢ, and average insulin secretion rate between 0 and 40 min are shown in Fig. 7, C and D, for control islets and test islets studied after pretreatment for 2 h with 100 nM concanamycin, in the presence of 2 mM methyla-
mine, or after Cl⁻ replacement with isethionate (Cl⁻-free). None of these experimental conditions affected KCl-induced [Ca²⁺]ᵢ, rise in 1 or 15 mM glucose (Fig. 7C), whereas the impact on secretion was variable. KCl-induced insulin secretion in low glucose was unaltered by Cl⁻ omission, not significantly decreased by methyla-
nine (Δ 20%, p > 0.05 by analysis of variance) and inhibited by concanamycin (Fig. 7D). In the presence of 15 mM glucose, the response was normal in the Cl⁻-free medium but inhibited by methyla-
nine or concanamycin. The amplifying effect of high glucose was 4.3 times in controls, 2.9 times after concanamycin, 2.7 times in the presence of methyla-
nine, and 3.9 times in the absence of Cl⁻. Overall, the secretory response to KCl decreased with the increase in granular pH, but the amplifi-
cation by glucose was not abolished. It was even unaltered in Cl⁻-free solution despite a marked attenuation of the change in granular pH.

Metabolic amplification is important mainly, although not exclusively (39), for the second phase of glucose-induced insu-
lin secretion (1, 5, 8, 9). Patch clamp studies have led to the proposal that the phenomenon reflects acidification-mediated recruitment of insulin granules into a releasable pool (16). Our study does not support the hypothesis. However, one should bear in mind that the necessity of granule recruitment is much higher in single patch-clamped β-cells than in intact islets, in which insulin secretion rates are considerably lower (39). It is possible that glucose-mediated acidification of insulin granules is implicated in the preferential release of newly formed insulin (60).

Conclusions—We show that an acidic granular pH is impor-
tant for Ca²⁺-induced insulin secretion. Thus, a large increase of granular pH by concanamycin or methyla-
nine (two agents acting by distinct mechanisms) was associated with a strong inhibition of immediate and sustained insulin secretion induced by glucose or KCl despite normal [Ca²⁺]ᵢ. However, no inhibition of secretion was observed when the increase in gran-
ular pH was moderate as during Cl⁻ omission, unless this maneuver also lowered [Ca²⁺]ᵢ (the second phase of glucose-
induced insulin secretion). We also show that glucose decreases granular pH in β-cells by a metabolism- and Cl⁻ dependent, Ca²⁺-independent mechanism, but our results do not support the hypothesis that this acute acidification is critical for the amplification of insulin secretion.

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