Glucose stimulation raises the pH$_i$ of pancreatic β-cells, but the underlying mechanisms are not well understood. We have now investigated the acute effects of metabolizable (glucose and the mitochondrial substrate α-ketoisocaproic acid, KIC) and nonmetabolizable (high K$^+$ and the K-ATP channel blocker tolbutamide) insulin secretagogues on the pH$_i$ of pancreatic β-cells isolated from normal mice, as assessed by BCECF fluorescence from single cells or islets in the presence of external bicarbonate. The typical acute effect of glucose (22-30 mM) on the pH$_i$ was a fast alkalinization of approximately 0.11 unit, followed by a slower acidification. The relative expression of the alkalinizing and acidifying components was variable, with some cells and islets displaying a predominant alkalinization, others a predominant acidification, and others yet a mixed combination of the two. The initial alkalinization preceded the [Ca$^{2+}$]$_i$ rise associated with the activation of voltage-sensitive Ca$^{2+}$ channels. There was a significant overlap between the glucose-evoked [Ca$^{2+}$]$_i$, rise and the development of the secondary acidification. Depolarization with 30 mM K$^+$ and tolbutamide evoked pronounced [Ca$^{2+}$]$_i$ rises and concomitant cytosolic acidifications. Blocking glucose-induced Ca$^{2+}$ influx (with O Ca$^{2+}$, nifedipine, or the K-ATP channel agonist diazoxide) suppressed the secondary acidification while having variable effects (potentiation or slight attenuation) on the initial alkalinization. KIC exerted glucose-like effects on the pH$_i$ and [Ca$^{2+}$]$_i$, but the amplitude of the initial alkalinization was about twice as large for KIC relative to glucose. It is concluded that the acute effect of glucose on the pH$_i$ of pancreatic β-cells is biphasic. While the initial cytosolic alkalinization is an immediate consequence of the activation of H$^+$-consuming metabolic steps in the mitochondria, the secondary acidification appears to originate from enhanced Ca$^{2+}$ turnover in the cytoplasm. The degree of coupling between glucose metabolism and Ca$^{2+}$ influx as well as the relative efficacies of these processes determines whether the acute pH$_i$ response of a β-cell (or of a tightly coupled multicellular system such as an islet of Langerhans) is predominantly an alkalinization, an acidification, or a mixed proportion of the two.

Pancreatic β-cells are endocrine cells specialized in the synthesis and secretion of insulin. Physiological release of the hormone is the result of a complex sequence of biophysical and biochemical events, involving entry of glucose through the GLUT-2 transporter, metabolic degradation of glucose to yield ATP, inhibition of ATP-sensitive K$^+$ (K-ATP) channels following a rise in the cytosolic concentration of the nucleotide, membrane depolarization, activation of voltage-sensitive Ca$^{2+}$ channels, rises in the cytosolic free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$), protein phosphorylation, and subsequent steps leading to exocytosis (for reviews, see Refs. 1–3).

The study of the modulation of cellular function by cytosolic pH (pH$_i$) has drawn a great deal of interest in many different cell types including pancreatic β-cells. Experimental maneuvers thought to cause changes in the pH$_i$ of pancreatic β-cells have long been known to affect insulin secretion (4–10), leading to the concept that glucose-evoked pH$_i$ changes might exert feedback control over the release process (11–13). Several effector systems in the β-cell are either known or suspected to be pH-sensitive in the physiological range. For example, the key glycolytic enzyme phosphofructokinase is strongly pH-sensitive (acidification depresses the activity of the enzyme (14)). Furthermore, K-ATP channel activity in β-cells is exquisitely sensitive to pH$_i$ changes around the physiological levels (alkalinization above resting pH$_i$ enhances channel activity while acidification depresses it (15, 16)). This may in fact explain why glucose-induced electrical activity is so sensitive to the pH$_i$ changes imposed by the administration of weak acids and bases. Indeed, cytosolic acidification causes membrane hyperpolarization and inhibition of electrical activity while cytosolic acidification is thought to cause symmetric changes on these parameters (11, 17–19). Interestingly, in the absence of functional K-ATP channels (for example, in the presence of sulfonamides and high external Ca$^{2+}$ concentrations), the cytosolic acidification agent NH$_4$Cl evokes changes on bursting electrical activity which appear to be the mirror image of those evoked under regular conditions (20, 21), suggesting that pH$_i$ changes affect other ion channels besides K-ATP channels in pancreatic β-cells (for example, the L-type voltage-sensitive Ca$^{2+}$ channel; Ca$^{2+}$ currents are enhanced by cytosolic acidification in other cell types (22, 23)).

Although the activation of glucose metabolism has long been suspected to cause extensive changes in the pH$_i$ of pancreatic β-cells and of other insulin-secreting cells (24–26), it was not until pH$_i$ measurements could be carried out using intracellularly trapped fluorescent indicators (e.g. BCECF$^1$ (27)) that the
pattern of these changes started to be unravelled (9, 10, 13, 28, 29). Using bicarbonate-free solutions, Juntti-Berggren et al. (9) reported monophasic pH\textsubscript{i} rises in response to glucose, which they ascribed to the activation of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger. In a follow-up study (13, 30), the authors proposed a metabolic mechanism for the glucose-induced pH\textsubscript{i} rise in which pyruvate transport across the mitochondrial membrane and/or oxidation was assumed to play an essential role. In a very recent study using bicarbonate-containing solutions, Shepherd and Henquin (29) reported sustained pH\textsubscript{i} rises in response to glucose, which could be suppressed by DIDS, a blocker of the Na\textsuperscript{+}-dependent HCO\textsubscript{3}/Cl\textsuperscript{−} exchanger, and strongly attenuated (although in the intermediate-high glucose concentration range only) by replacing the external solution for a bicarbonate-free solution. These effects were interpreted assuming that, in the presence of bicarbonate and high glucose, the DIDS-sensitive exchanger overcompensates and opposes an acidifying tendency associated with Ca\textsuperscript{2+} influx and accumulation in the cytosol (29).

Using single cells and islets exposed to a physiological (bicarbonate-containing) buffer, we have now assessed the possibility that an acidifying mechanism linked to the stimulation of Ca\textsuperscript{2+} influx might contribute to the short-term effect of glucose on the cytosolic pH of pancreatic \( \beta \)-cells. Furthermore, we have compared the effects of glucose and KIC, a metabolic substrate that feeds directly the mitochondria, with the purpose of assessing the role of mitochondrial metabolism in the glucose-evoked pH\textsubscript{i} responses. The results indicate that the typical acute pH\textsubscript{i} response of mouse pancreatic \( \beta \)-cells to glucose is a transient rapid alkalinization, followed by a slower acidification. While the initial alkalinization is an immediate consequence of the activation of mitochondrial metabolism, the secondary acidification is probably linked to enhanced turnover (influx followed by active extrusion and/or accumulation in organelles) of Ca\textsuperscript{2+} in the cytoplasm.

**EXPERIMENTAL PROCEDURES**

**Islet Isolation and Culture—**Islet isolation and culture were carried out essentially as described previously (31), with some modifications (32). Briefly, 3- to 6-month-old female albino mice (Charles River Breeding Laboratories) weighing 28-40 g were killed by cervical dislocation, and the islets were isolated by collagenase (type P, Boehringer Mannheim) digestion of the pancreas. For culture, the islets were transferred into sterile RPMI 1640-based medium supplemented with 10% fetal calf serum and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin), pH adjusted to 7.2 with NaOH to give 7.4 in the medium. Islets were typically incubated in the original culture medium supplemented with 4 μM fura-2/AM or 1 μM BCECF/AM for 45 min at 37°C. Previous studies involving isolated cells were carried out using the original cell-containing coverslips as bottoms for the perfusion chamber. The temperature and flow rate in the chamber were 37°C and approximately 2 ml/min, respectively. The perfusion system used in the experiments and the respective temperature control stages have been described previously in detail (33). The [Ca\textsuperscript{2+}]\textsubscript{i} and the pH\textsubscript{i} were recorded from single cells or islets using a dual excitation microfluorescence system (Deltascan, Photon Technology International, Princeton, NJ), essentially as described previously for single islets and single chromaffin cells (20, 32–34). Briefly, single cells or islets were alternately excited at 340/380 nm (for [Ca\textsuperscript{2+}]\textsubscript{i} measurements) or at 440/500 nm (for pH\textsubscript{i} measurements) via two monochromators. The fluorescence was detected by a photomultiplier after passing through a band pass interference filter centered at 510 ([Ca\textsuperscript{2+}]\textsubscript{i} measurements) or 535 nm (pH\textsubscript{i} measurements). The data were automatically corrected for background fluorescence and acquired at 10 Hz by a 365×16 MHz computer. The measuring field was routinely centered on the cell or islet of interest by means of a rectangular diaphragm placed on the emission path. Cells which have been double-labeled with fura-2 and BCECF (for combined [Ca\textsuperscript{2+}]\textsubscript{i} and pH\textsubscript{i} measurements) and which were stimulated for the reaction were captured by the use of more than two different excitation wavelengths at a time. Inner filter effects (resulting in faint fura-2 fluorescence) did not allow combined [Ca\textsuperscript{2+}]\textsubscript{i} and pH\textsubscript{i} measurements to be carried out from single islets. In control experiments, the pH\textsubscript{i} proved to be extremely sensitive to medium pH and to the flow rate of the perfusion solution. While the medium pH was strictly and permanently monitored (and corrected by CO\textsubscript{2} gassing if necessary) prior to perfusion, regular checks were carried out to make sure that the flow rate did not change significantly upon changing the solutions.

**Calibration of Fluorescence Signals in Terms of pH\textsubscript{i} and [Ca\textsuperscript{2+}]\textsubscript{i}—**The fluorescence ratio \( F_{500/440} \) was converted into pH\textsubscript{i} values using an in vitro calibration procedure, as follows. Fluorescence excitation spectra were recorded from droplets of MOPS-buffered BCECF (1 μM) solutions, under conditions equivalent to those used for the recording of islet fluorescence. The data were automatically corrected for background fluorescence and acquired at 10 Hz by a 365×16 MHz computer. The measuring field was routinely centered on the cell or islet of interest by means of a rectangular diaphragm placed on the emission path. Cells which have been double-labeled with fura-2 and BCECF (for combined [Ca\textsuperscript{2+}]\textsubscript{i} and pH\textsubscript{i} measurements) and which were stimulated for the reaction were captured by the use of more than two different excitation wavelengths at a time. Inner filter effects (resulting in faint fura-2 fluorescence) did not allow combined [Ca\textsuperscript{2+}]\textsubscript{i} and pH\textsubscript{i} measurements to be carried out from single islets. In control experiments, the pH\textsubscript{i} proved to be extremely sensitive to medium pH and to the flow rate of the perfusion solution. While the medium pH was strictly and permanently monitored (and corrected by CO\textsubscript{2} gassing if necessary) prior to perfusion, regular checks were carried out to make sure that the flow rate did not change significantly upon changing the solutions.

**Materials—**Fura-2/AM, fura-2 (free acid), BCECF/AM, and BCECF (free acid) were from Molecular Probes. Antibiotics and RPMI 1640 medium were from Biological Industries (Beth Haemek, Israel). Experiments were also performed with other chemicals and reagents.

**Data Analysis—**All results (text and figures) were expressed as mean ± S.D. The plots in the figures were generated using commercially available software (Sigma Plot, Jandel Scientific).
Fig. 1. Single islet pHi and [Ca\textsuperscript{2+}] responses to glucose stimulation. A, representative examples of single islet pHi responses to 22 mM glucose (22G), as monitored by BCECF fluorescence. B, representative examples of single islet [Ca\textsuperscript{2+}] responses to 22 mM glucose, as monitored by fura-2 fluorescence. Islets 5-8 in B are different from islets 1-4 in A. The shadowed areas denote the periods of stimulation with high glucose. Basal glucose concentration was 3 mM.

RESULTS

Effects of Glucose on the pHi and [Ca\textsuperscript{2+}]. Recorded from Single Islets—We have measured the intracellular pH from single mouse islets of Langerhans using the fluorescent probe BCECF. The average resting pHi in the presence of 3 mM glucose was 7.01 ± 0.07 (± S.D., n = 49 islets). The effects of raising glucose concentration from 3 to 22 mM on the pHi are depicted in Fig. 1A. The typical pHi response was an initial alkalinization of approximately 0.11 unit, followed by a slower acidifying phase (e.g., islets 2 and 3 in Fig. 1A). This pattern was representative of 69% of the islets examined (34 out of 49 islets). The glucose-evoked pHi responses were highly variable from islet to islet, with some islets (21%, i.e. 11 out of 49 islets; e.g. islet 1) actually displaying a pure alkalinizing phase while others (8%, i.e. 4 out of 49 islets; e.g. islet 4) displayed a predominant acidifying phase with residual signs of alkalinization. It is also noteworthy that islets displaying a multiphasic pHi response to glucose exhibited a mixed proportion of the alkalinizing and acidifying components. For example, while in some islets (e.g. islet 2 in Fig. 1A) the pHi remained close to or distinctly above the resting level after 4-6 min exposures to high glucose, in other islets (e.g. islet 3) the pHi dropped to near-baseline levels or even below baseline during continued stimulation with high glucose.

The maximal amplitude of the alkalinizing phase (difference between peak and basal pHi), measured from experiments displaying either multiphasic pHi responses (initial alkalinization, followed by a pronounced acidifying phase), a pure alkalinizing phase, or a predominant acidifying phase with residual alkalinization in response to 22 mM glucose, was 0.11 ± 0.05 (n = 26 islets)\textsuperscript{2}; in these experiments, the alkalinizing phase occurred 31 ± 10 s after the beginning of the stimulus. The time-to-peak of the initial alkalinization, measured from experiments displaying either multiphasic pHi responses or a predominant acidifying phase, averaged 85 ± 30 s (n = 20 islets).

Thus, while in most of the islets examined, the pHi was slowly and monophasically recovered to baseline levels following the decrease in glucose concentration from 22 to 3 mM (e.g. islet 1 in Fig. 1A), in other cases, the pHi transiently overshot the levels reached during stimulation before it finally declined to baseline (e.g. islets 2 and 3).

Using separate islets labeled with the Ca\textsuperscript{2+} indicator fura-2, we have characterized the effects of high glucose on the [Ca\textsuperscript{2+}] with the main aim of assessing the physiological responsiveness of the islet pool used for the pHi experiments. Examples of typical responses are shown in Fig. 1B. Raising glucose concentration from 3 to 22 mM typically resulted in a drop of [Ca\textsuperscript{2+}] below baseline lasting 1-3 min (average 88 ± 24 s, n = 12 islets). This was followed by a pronounced [Ca\textsuperscript{2+}] rise, after which the [Ca\textsuperscript{2+}] either remained elevated (albeit with oscillations, e.g. islets 5 and 8 in Fig. 1B) or declined to lower levels (e.g. islets 6 and 7) during continued stimulation with high glucose. In these experiments, the time-to-peak and the maximal amplitude of the [Ca\textsuperscript{2+}] responses were 270 ± 58 s and 133 ± 68 nM (n = 12 islets), respectively. Removal of the high glucose stimulus was often accompanied by a transient accentuation of the oscillatory behavior.

\textsuperscript{2}The computation of accurate response amplitude, time lag, and time-to-peak values required single islet experiments where the basal BCECF fluorescence ratio did not undergo any evident spontaneous drift. This was, however, not the case for a fraction of the overall pool of valid pHi experiments. Indeed, there were occasions when the basal pHi was found to be drifting, albeit not at such a high rate that it would compromise the whole experiment. Moreover, in several other experiments, the pHi was recorded from glucose-stimulated islets that had been subjected to a previous glucose challenge (after an appropriate recovery time in 3 mM glucose). While these experiments have been taken into account for the calculation of average resting pHi and for qualitative assessment of pHi response patterns, they were not used for the computation of amplitude, time lag, or time-to-peak values since these parameters are expected to be critically sensitive to glucose priming (see below). Thus, the estimation of these parameters was made from a smaller pool (n = 20 or 26 islets) of valid pHi experiments than that used for qualitative assessment and for the estimation of average resting pHi (n = 49 islets).
Effects of Glucose on the pHi and [Ca2+]i—Cells—The pHi and the [Ca2+]i, experiments depicted in Fig. 1 suggest that maximal alkalinization might occur at a time when the [Ca2+]i remains low or even below baseline in response to the glucose challenge. Since, owing to inner filter effects (see "Experimental Procedures"), the pHi and the [Ca2+]i, cannot be recorded from the same islet, we have double-labeled isolated β-cells with BCECF and fura-2, aiming at investigating the relationship between glucose-evoked pHi and [Ca2+]i, changes at the single cell level.

Due to the specificity of the available microfluorescence detection system, the pHi and the [Ca2+]i recordings were carried out in sequence, rather than simultaneously. In the following experiments, the cells were typically subjected to 30 mM glucose for 4–6 min in the [Ca2+]i recording mode, allowed to recover for at least 11 min in basal (2 mM) glucose, and finally subjected to an identical high glucose pulse in the pHi recording mode. Identification of the monitored cells as β-cells relied on the specific responsiveness of the latter to blockers of the K-ATP channel, e.g., the sulfonylurea tolbutamide (36).

Thus, we have routinely stimulated the cells with 250 μM tolbutamide at the beginning of each experiment and considered a pronounced [Ca2+]i response to the sulfonylurea as evidence that the monitored cell was indeed a β-cell (see Fig. 4B for a typical response).

Fig. 2A (traces labeled with a single asterisk) shows that most β-cells responded to the high glucose pulse with a small and transient fall in [Ca2+]i, which was followed by a pronounced rise toward a level several hundreds of nanomolars above baseline. It is also apparent that both the onset and the time course of the glucose-evoked [Ca2+]i rises were highly variable from cell to cell. These characteristics are similar to what has been described previously for rat and ob/ob mouse β-cells (37, 38). Interestingly, a small fraction of the β-cells examined did not display any [Ca2+]i rise in response to high glucose, as depicted by cell 4 in Fig. 2A.

The pHi traces in Fig. 2A were made with a heavier line and further labeled with double asterisks for easier identification in the figure. In the presence of 2 mM glucose, the average resting pHi of BCECF- and fura-2-labeled cells was 7.04 ± 0.06 (n = 14 cells). Similarly to isolated islets, the typical pHi response of single β-cells to glucose was an initial alkalinization of approximately 0.15 unit, followed by a slower acidifying phase (e.g., cells 1, 2, and 3 in Fig. 2A). This pattern was representative of 58% (23 out of 39) of the cells examined. Furthermore, while some cells (34%, i.e., 13 out of 39; e.g., cell 4 in Fig. 2A) displayed a pure alkalinizing phase, others (8%, i.e., 3 out of 39; not shown in Fig. 2A) displayed a predominant acidifying phase with residual signs of alkalinization. The glucose-evoked effects on β-cell pHi were often slowly reversible, usually resulting in poor recovery within 4–6 min after withdrawing the high glucose stimulus, as evidenced by cells 2 and 3 in Fig. 2A.

Visual inspection of the relationship between the glucose-evoked [Ca2+]i and pHi transients indicates that the initial pHi rise occurred at a time when the [Ca2+]i remained at near-basal levels following glucose stimulation (Fig. 2A, cells 1, 2, and 3; see also average data from several single cell experiments in Fig. 2B). Indeed, the pHi and the [Ca2+]i, started to rise 18 ± 7 s and 97 ± 28 s (n = 14 cells) after the delivery of the high glucose pulse, respectively. Furthermore, the time required to raise the pHi and the [Ca2+]i, to half the respective maximal levels averaged 38 ± 9 s and 209 ± 67 s, respectively. It is also apparent from Fig. 2A (cells 2 and 3) and Fig. 2B that there is a significant overlap between the secondary acidifying phase of the multiphasic pHi response to glucose and the rising phase of the [Ca2+]i transient, suggesting that the acidifying response might be a consequence of Ca2+ influx and of the associated [Ca2+]i rise (but see the priming data of Fig. 3 and

![Fig. 2](image-url)

**Fig. 2.** Combined pHi and [Ca2+]i recordings from single β-cells. A, representative examples of single cell pHi and [Ca2+]i responses to 30 mM glucose, as monitored fluorometrically from BCECF- and fura-2-loaded β-cells. The shadowed areas denote the periods of stimulation with high glucose. Basal glucose concentration was 2 mM. The cells were stimulated twice with identical glucose pulses, first in the [Ca2+]i- (lighter traces denoted by single asterisks) and then in the pHi-recording mode (heavier traces denoted by double asterisks). The cells were allowed to rest in 2 mM glucose for 11–17 min (14, 15, 12, and 11 min for the experiments depicted by cells 1 through 4, respectively) between stimulations. B, average pHi (heavier trace denoted by a double asterisk) and [Ca2+]i (lighter trace denoted by a single asterisk) responses to 30 mM glucose. The data were pooled from single cell experiments such as those depicted in A. Vertical bars represent ± S.D. of 14 single cell measurements.
the corresponding discussion). This hypothesis would be consistent with the response displayed by cell 4 in Fig. 2A, which is illustrative of β-cells that failed to produce a Ca\(^{2+}\) response to high glucose and did not exhibit the secondary acidifying phase.

Closer inspection of Fig. 2A indicates, however, that in some cells (e.g. cell 3 and, albeit to a lesser extent, cell 2) the onset of the Ca\(^{2+}\) rise lags a few tens of seconds behind the onset of the acidification. Accordingly, the beginning of the average Ca\(^{2+}\) rise does not match exactly the beginning of the average pH\(_i\) fall (Fig. 2B). One possibility to interpret this apparent discrepancy is that the cells might respond faster to glucose once primed by a previous stimulation, as previously acknowledged (39–41). In order to assess this hypothesis, we have carried out experiments whereby the cells were subjected to the same double stimulation protocol while recording the Ca\(^{2+}\). Under these conditions, the operation of a priming mechanism would be expected to enhance the Ca\(^{2+}\) response to the second high glucose challenge at earlier time points. Fig. 3 shows that this was indeed the case in most experiments. On average, the lag time between the first and the second Ca\(^{2+}\) rise was 49 ± 21 s (n = 5 cells). It is important to note that, in these experiments, the cells were allowed to rest for 12–18 min (average 14.5 ± 3 min, n = 5 cells) between the high glucose pulses. The corresponding time for the combined Ca\(^{2+}\)/pH\(_i\) experiments depicted in Fig. 2 was 11–17 min (average 13.5 ± 2.3 min, n = 14).

Effects of Enhancing and Suppressing Ca\(^{2+}\) Influx on the pH\(_i\)—The hypothesis that the acidifying phase of the pH\(_i\) response might be secondary to Ca\(^{2+}\) influx was further tested using two alternative strategies. One of them is depicted in Fig. 4 (A and B), where two different experimental maneuvers known to depolarize the cells and to activate voltage-sensitive Ca\(^{2+}\) channels (i.e. challenging the cells with high K\(^+\) and with the sulphonylurea blocker of the K-ATP channel, tolbutamide) are shown to raise the Ca\(^{2+}\); while concomitantly decreasing the pH\(_i\) (average peak Ca\(^{2+}\) and pH\(_i\) changes evoked by 30 mM K\(^+\): 335 ± 184 nm and 0.05 ± 0.01, respectively; average peak Ca\(^{2+}\) and pH\(_i\) changes evoked by 250 μM tolbutamide: 333 ± 42 nm and 0.11 ± 0.02, respectively; n = 3 cells). Interestingly, in these experiments, the high K\(^+\)-evoked pH\(_i\) fall exhibited a significant tendency to decline during stimulation, in contrast with the tolbutamide-induced pH\(_i\) decrease, which proceeded at an almost constant rate throughout.

The effects of tolbutamide on Ca\(^{2+}\) and pH\(_i\) were also investigated in islets that have been loaded separately with fura-2 and BCECF, as depicted in Fig. 4C. In the later experiments, the Ca\(^{2+}\) started to rise 27 ± 9 s (range 13–38 s, n = 5 islets) after stimulation with tolbutamide; the pH\(_i\) started to fall 36 ± 26 s (range 13–61 s, n = 4 islets) after stimulation. It is therefore possible to infer that the pH\(_i\) fall either occurs simultaneously with or is subsequent to the Ca\(^{2+}\) rise, in essential agreement with the single cell data.

The second strategy (depicted in Fig. 5) consisted in comparing the glucose-evoked pH\(_i\) responses in the presence and absence of conditions known to suppress Ca\(^{2+}\) influx through voltage-sensitive Ca\(^{2+}\) channels (the major modality of Ca\(^{2+}\) influx involved in glucose stimulation of β-cells (2)). These conditions were the removal of Ca\(^{2+}\) from the extracellular medium (1), the use of the dihydropyridine nifedipine (a blocker of L-type Ca\(^{2+}\) channels (42)), and the use of the hyperpolarizing agent and K-ATP channel agonist, diazoxide (43, 44).

For the experiments depicted in Fig. 3, single BCECF-loaded islets were stimulated by raising glucose concentration from 3 to 22 mM. The major consequence of exposing the islets to Ca\(^{2+}\)-free solutions, nifedipine, or diazoxide was the suppression of the acidifying phase of the multiphasic pH\(_i\) response to glucose. It is also noteworthy that the alkalinizing response was slower in the presence of any of these agents. Furthermore, the steady-state pH\(_i\) recorded in the absence of Ca\(^{2+}\) or in the presence of nifedipine or diazoxide was often (e.g. islets 2 and 3 in Fig. 4), albeit not always (e.g. islet 1), higher than the peak pH\(_i\) recorded in control.

Effect of KIC on the pH\(_i\) and Ca\(^{2+}\). Recorded from Single β-Cells—In the following experiments, we have used KIC, a metabolic substrate that feeds directly the mitochondria, as a probe to assess the possible involvement of mitochondrial metabolism in the pH\(_i\) response of β-cells to glucose. KIC is an endogenous substrate of the Krebs’ cycle arising from the oxidative deamination of leucine. When applied externally, KIC permeates the plasmalemma and the mitochondrial membrane, thereby providing direct activation of the Krebs cycle and enhancing ATP production (45, 46); the subsequent rise in the cytosolic ATP/ADP ratio is the proposed mechanism by which KIC inhibits K-ATP channels, stimulates the electrical activity, and elicits insulin release in a manner entirely similar to glucose (47). In this study, stimulation of the cells was provided by adding 30 mM KIC to the perifusion solution for specified periods; the pH\(_i\) and the Ca\(^{2+}\) were recorded from single cells which have been double-labeled with BCECF and fura-2.

Fig. 6A shows examples of single β-cell [Ca\(^{2+}\)] responses to KIC. These effects are rather similar to the homologous responses to 30 mM glucose, as can be seen by comparing the average KIC responses depicted in Fig. 6B with the average glucose responses depicted in Fig. 2B. It is also noteworthy that the pH\(_i\) responses of individual β-cells to KIC are heterogenous, with some cells displaying a predominant alkalinization and others a marked acidifying phase following an initial alkalin-
Multiphasic Action of Glucose on \( \beta \)-Cell \( \text{pH}_i \)

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**Fig. 4.** Relationship between depolarization-evoked \( \text{pH}_i \) and \([\text{Ca}^{2+}]_i\), transients in pancreatic \( \beta \)-cells. A and B, representative examples of single cell \( \text{pH}_i \), and \([\text{Ca}^{2+}]_i\) responses to 30 mM KCl (A) and 250 \( \mu \)M tolbutamide (B), as monitored fluorometrically from BCECF- and fura-2-loaded \( \beta \)-cells. Each cell was stimulated twice with identical high K\(^+\) or tolbutamide pulses, first in the \([\text{Ca}^{2+}]_i\) (lighter traces denoted by single asterisks) and then in the \( \text{pH}_i \) recording mode (heavier traces denoted by double asterisks). Each experiment is representative of three similar experiments. C. representative examples of single islet \([\text{Ca}^{2+}]_i\), (islet 1) and \( \text{pH}_i \), (islet 2) responses to 500 \( \mu \)M tolbutamide. Each experiment is representative of five similar experiments. The shadowed areas denote the periods of stimulation with high K\(^+\) or tolbutamide. Glucose concentration in the perfusion medium was 2 mM throughout.

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**DISCUSSION**

Using physiologically buffered solutions and single BCECF-loaded islets and isolated \( \beta \)-cells from normal mice as a model system to investigate the effects of glucose on cytosolic \( \text{pH}_i \), we found that the typical acute \( \text{pH}_i \) response to high (22–30 mM) concentrations of the hexose has two distinct phases: a fast alkalinizing phase peaking at approximately 90 s and a slower acidifying phase responsible for bringing the \( \text{pH}_i \) to near-basal levels in approximately 4 min of continued stimulation. The presence of the secondary acidifying component has not been reported consistently by other groups. Using monolayers of pancreatic \( \beta \)-cells isolated from obese hyperglycemic (ob/ob) mice and suspensions of clonal insulin-secreting (HIT) cells exposed to bicarbonate-free solutions, Juntti-Berggren et al. (9) reported monophasic \( \text{pH}_i \) rises in response to 8–20 mM glucose. Glucose-evoked alkalinizations of approximately 0.16 unit had already been reported using ob/ob mouse islets and a detection procedure based on the redistribution of \( ^{14}\text{C} \)-labeled 5,5-dimethyl-2,4-oxazolidinedione (26), but the only data available from this study refers to the 7th min of stimulation with 20 mM glucose. More recently, Shepherd and Henquin (29) reported essentially sustained \( \text{pH}_i \) rises in single pancreatic islets isolated from normal (NMRI) mice, after long-term exposures to 7–30 mM glucose in the presence of bicarbonate. It should also be emphasized that multiphasic \( \text{pH}_i \) responses resembling those described in our work are already apparent from some of the single cell experiments reported in a previous study (30).

There has been debate in the literature concerning the origin of the acute glucose-evoked alkalinization in pancreatic \( \beta \)-cells. The inhibitor of mitochondrial pyruvate transport, 3-hydroxycytocinnamate, has been reported to prevent glucose-induced alkalinization, leading to the concept that the \( \text{pH}_i \) rise can either be accounted for by pyruvate transport across the mitochondrial membrane or by its subsequent oxidation (13, 30). This result is consistent with the finding that the glycolytic substrate dihydroxyacetone increased the \( \text{pH}_i \) in a glucose-like manner (29). We have characterized the effect of the mitochondrial substrate KIC on the \( \text{pH}_i \) aiming at clarifying the role of mitochondrial metabolism in the glucose-evoked effect on \( \text{pH}_i \). The fact that KIC had glucose-like effects on the \( \text{pH}_i \) indicates that the initial alkalinization induced by the hexose is primarily the result of one or several H\(^+\)-consuming steps in the mitochondria. It is noteworthy that KIC exceeded glucose in alkalinizing capacity, as revealed by the larger magnitude of the initial alkalinization and by the longer duration of the overall \( \text{pH}_i \) rise (Fig. 2B versus Fig. 6B). This may be interpreted taking into account that formation of pyruvate is associated with a net production of protons (48).

Busa and Nuccitelli (48) proposed that the stimulation of the aerobic metabolism of glucose occurs at the expenses of a mas-
sive net consumption of protons, the consequence of which would be a marked initial rise in pH. However, the initial alkalinization may, at a later stage, be compensated for by the cytosolic accumulation of protons arising from ATP hydrolysis, so that in the steady-state little or no net change in pH may actually occur (48). Pancreatic β-cells might be expected to fit in the model proposed by Busa and Nuccitelli (48). This is because 1) in β-cells the aerobic transformation of glucose largely exceeds that of anaerobic glycolysis (49); 2) even assuming that pyruvate oxidation by mitochondria may not be the major catabolic route for glucose in β-cells and that most of the reducing equivalents are brought into mitochondria through the operation of the glycerol phosphate shuttle (50–52), a net consumption of protons would still be expected to take place due to the combined oxidation of cytosolic NADH and electron transfer along the respiratory chain; and 3) in stimulated β-cells, hydrolysis of cytosolic ATP is expected to proceed at a high rate, as a consequence of enhanced Ca\(^{2+}\)-ATPase activity (required for active Ca\(^{2+}\) extrusion and sequestration by organelles) and protein phosphorylation (53).

In various cell types, the pH set-point of the Na\(^+\)/H\(^+\) antiporter is raised following activation of protein kinase C with phorbol esters, a process that might lead to pronounced increases in the pH\(_i\) (54–56). The prediction of an elevated pH\(_i\) applies especially to bicarbonate-free conditions, since in the presence of the anion there might be conflicting consequences of the activation of the Na\(^+/\)H\(^+\) antiporter and of the Na\(^+\)-independent HCO\(_3^-\)/Cl\(^-\) exchanger (57). The original finding that EIPA (a blocker of the Na\(^+\)/H\(^+\) antiporter) suppressed the glucose-evoked alkalinization recorded in the absence of bicarbonate appeared to lend credit to an essential role for the antiporter, but the fact that down-regulating protein kinase C failed to affect the pH\(_i\) rise was taken as an argument that protein kinase C-supported phosphorylation was not involved (9). Nonetheless, the negative results obtained by Shepherd and Henquin (29) (dimethyl amiloride had virtually no effect on the glucose-evoked pH\(_i\) rise recorded in the presence of bicarbonate) strongly opposes the hypothesis that activation of the Na\(^+\)/H\(^+\) antiporter might mediate the glucose-evoked alkalinization recorded under physiological conditions. We propose the following explanation to account for the variable effects of amiloride derivatives on the glucose-evoked alkalinization. In resting cells, the pH\(_i\) is far from equilibrium due to sustained operation of the Na\(^+\)/H\(^+\) antiporter and of the Na\(^+\)-dependent HCO\(_3^-\)/Cl\(^-\) exchanger. Blockade of the Na\(^+\)/H\(^+\) antiporter would then be expected to lead to cytosolic acidification, but the actual extent of this pH\(_i\) fall depends critically on the availability of the second exchanger to rescue the cells from the acid load. Since the key glycolytic enzyme phosphofructokinase is thought to be markedly inhibited by modest physiological acidifications (14), large pH\(_i\) falls (such as those that occur in cells exposed to Na\(^+\)/H\(^+\) antiporter blockers in bicarbonate-free solutions) have the potential to inhibit glucose metabolism downstream phosphofructokinase, resulting in the suppression or pronounced inhibition of the associated pH\(_i\) rise.

Since we report that the average glucose-evoked alkalinization is essentially over in approximately 4 min, our data are in apparent contradiction with the study by Shepherd and Henquin (29), which was carried out under comparable experimental conditions (normal mouse islets and bicarbonate-containing solutions). It should be mentioned, however, that the sustained pH\(_i\) rise reported by these authors was apparently preceded by a transient increase lasting approximately 2.5 min. Thus, our study concentrates on the acute effect of glucose on pH\(_i\), whereas the latter authors address fundamentally the long-term actions of the hexose. Implicit in the model proposed by Shepherd and Henquin (29) to account for a sustained pH\(_i\) rise is the possibility that the pH set-point of the Na\(^+\)-dependent HCO\(_3^-\)/Cl\(^-\) exchanger undergoes a positive shift following stimulation with high glucose concentrations, similar to what has been reported previously to occur in other cell types in response to growth factors (57). The fact that the pH\(_i\) rise observed immediately after glucose stimulation is transient would then imply that the alteration in the set-point of the exchanger has a slow time course and only becomes noticeable by the end of several minutes of continued stimulation.

It is well known that the secretory response of pancreatic β-cells to glucose may be enhanced by a previous challenge with glucose or other secretagogues, in a phenomenon known as priming, memory, or time-dependent potentiation (59, 60). Although the detailed mechanism for this potentiation is not well understood, some authors have reported evidence for the occurrence of priming at the level of glucose-evoked Ca\(^{2+}\).
changes (41). In agreement with previous reports (39–41), our experiments (Fig. 3) show that pretreatment of pancreatic β-cells with 30 mM glucose 12–18 min in advance to a second glucose stimulus accelerates the latter response by an average time of 49 s. Since the combined [Ca\(^{2+}\)]/pH\(_{i}\) experiments depicted in Fig. 2 have been carried out using a double stimulation protocol similar to that of Fig. 3 (with the [Ca\(^{2+}\)] and pH\(_{i}\) measurements relating to the first and second glucose pulses, respectively), the operation of a priming mechanism may be critical to establish an accurate relationship between the glucose stimulus. Thus, even taking into account the possibility of priming, our results indicate that the initial alkalinization was significantly decreased by any of these agents. This may reflect the Ca\(^{2+}\) concentration rises in the mitochondrial matrix are important for the optimization of metabolic reaction rates.

We have also demonstrated that β-cells exposed to physiologically buffered solutions undergo pronounced cytosolic acidifications in response to glucose or KIC stimulation. We propose that this secondary acidifying phase is specifically associated with the stimulation of Ca\(^{2+}\) influx and may be considered a consequence of enhanced Ca\(^{2+}\) turnover in the cytosol. This is because: 1) in the combined [Ca\(^{2+}\)]/pH\(_{i}\) experiments (single β-cells), the pH\(_{i}\) decrease concomitantly with the [Ca\(^{2+}\)] rises after correction for priming (as seen above the predicted lower limit for the beginning of the acidification is 106 s, in essential agreement with the start of the [Ca\(^{2+}\)] rise); 2) the pH\(_{i}\) did not decrease in cells lacking a measurable Ca\(^{2+}\) influx in response to glucose; 3) specific removal of Ca\(^{2+}\) influx (with 0 Ca\(^{2+}\), nifedipine, and diazoxide) suppressed the secondary pH\(_{i}\) decrease. The conclusion that the stimulation of Ca\(^{2+}\) influx leads to an acidification of the cytosol is also supported by the high K\(^+\) and tolbutamide experiments, where more direct depolarization of the cells was shown to acidify the cytosol.

![A](image1.png), B](image2.png)

**Fig. 6. Effect of the mitochondrial substrate α-ketoisocaproic acid (KIC) on the pH\(_{i}\) and [Ca\(^{2+}\)]. of single β-cells.** A, representative examples of single cell pH\(_{i}\) and [Ca\(^{2+}\)] responses to 30 mM KIC, as monitored fluorometrically from BCECF- and fura-2-loaded β-cells. The shadowed areas denote the periods of stimulation with KIC (no glucose present). The cells were stimulated twice with identical KIC pulses, first in the [Ca\(^{2+}\)] (lighter traces denoted by single asterisks) and then in the pH\(_{i}\)-recording mode (heavier traces denoted by double asterisks). The pH\(_{i}\) and the [Ca\(^{2+}\)] traces in each panel have been superimposed for clarity. 

B, average pH\(_{i}\) (heavier trace denoted by a double asterisk) and [Ca\(^{2+}\)] (lighter trace denoted by a single asterisk) responses to 30 mM KIC. The data were pooled from single cell experiments such as those depicted in A. Vertical bars represent ± S.D. of 4 single cell measurements.

2 The islets used for the [Ca\(^{2+}\)] and pH\(_{i}\) experiments depicted in Fig. 1 have been subjected to single high glucose pulses, implying that there is no priming in these experiments. The alkalinization phase in these experiments reached its maximum level by an average time of 85 s; raising glucose concentration resulted in a drop of [Ca\(^{2+}\)] below baseline lasting an average time of 88 s. In pancreatic β-cells, this drop in [Ca\(^{2+}\)] is attributed to the stimulation of the Ca\(^{2+}\) buffering power by glucose (64, 65); a second phase ensues, where voltage-sensitive Ca\(^{2+}\) channels become activated and the [Ca\(^{2+}\)] rises (66). Thus, the single islet data indicate that, on average, the [Ca\(^{2+}\)] starts to rise at 88 s and the pH\(_{i}\) to fall at 85 s after stimulation, thus reinforcing the view that the time course of the pH\(_{i}\) fall is essentially compatible with that of the [Ca\(^{2+}\)] rise.
cytosol in essential agreement with data reported by other authors (28, 67). The concept that the stimulation of Ca$^{2+}$ influx might cause the acidification of the cytosol was recently put forward by Shepherd and Henquin (29) to explain the finding that removal of Ca$^{2+}$ or exposure to diazoxide strongly enhanced the pH$_i$ response recorded in bicarbonate-free medium. However, the authors reported no effect of the Ca$^{2+}$ influx suppressors on the glucose-evoked alkalinization recorded in the presence of bicarbonate.

Our experiments do not address the mechanism by which the stimulation of Ca$^{2+}$ influx leads to cytosolic acidifications in pancreatic $\beta$-cells. Since cytosolic acidification appears to be a natural consequence of enhanced ATP hydrolysis (48) and a significant fraction of the ATP yielded by glucose oxidation is likely to be utilized by Ca$^{2+}$-ATPases, the secondary acidifying component that we found in glucose-stimulated $\beta$-cells may reflect primarily H$^+$ accumulation associated with active Ca$^{2+}$ transport out of the cells and into internal stores. Alternatively, Ca$^{2+}$ accumulated intracellularly may displace protons from binding sites in the cytosol and/or exchange for protons when it is incorporated in organelles (e.g. endoplasmic reticulum), as proposed for other cell types (68–70).

In conclusion, we have shown that acute glucose stimulation of pancreatic $\beta$-cells evokes a multiphasic pH$_i$ response consisting of an initial alkalinization and a secondary acidification. Underlying these two phases are essentially distinct but interlinked mechanisms. While the initial alkalinization is linked to the activation of H$^+$-consuming metabolic steps in the mitochondria, the secondary acidification is probably linked to enhanced Ca$^{2+}$ turnover in the cytosol (Ca$^{2+}$ influx and Ca$^{2+}$ extrusion/sequestration).

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