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Multiphasic Action of Glucose and α-Ketoisocaproic Acid on the Cytosolic pH of Pancreatic β-Cells

EVIDENCE FOR AN ACIDIFICATION PATHWAY LINKED TO THE STIMULATION OF Ca^{2+} INFLUX*

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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 0 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 alkalinization 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 sulfonylureas and high external Ca^{2+} concentrations), the cytosolic alkalinizing agent NH_4Cl 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 alkalinization 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

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[1] The abbreviations used are: BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; BCECF/AM, acetoxymethyl ester of BCECF; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; fura-2/AM, acetoxymethyl

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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$_i$ rises in response to glucose, which they ascribed to the activation of the Na^+/H^+ exchanger. In a follow-up study (13, 30), the authors proposed a metabolic mechanism for the glucose-induced pH$_i$ 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$_i$ rises in response to glucose, which could be suppressed by DIDS, a blocker of the Na^+-dependent HCO_3^-/Cl^- 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^{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^{2+} influx might contribute to the short-term effect of glucose on the cytosolic pH of pancreatic β-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$_i$ responses. The results indicate that the typical acute pH$_i$ response of mouse pancreatic β-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^{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 dislodgement, 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 incubator.

Cell Preparation and Culture—Collagenase-isolated mouse islets (700–1200 islets from 6 pancreata) were hand-picked and transferred into an Eppendorf tube containing Ca^{2+}- and Mg^{2+}-free medium supplemented with 1 mM EGTA (in mM: 112.6 NaCl, 4.7 KCl, 1.2 Na_2SO_4, 20 $NaHCO_3$, 1.2 NaH_2PO_4, 16 HEPES, pH 7.4). The islets were washed twice in this medium, allowed to incubate for 10–15 min, and finally dissociated into cells by trypsinization with 0.05% trypsin, as described previously (31). Cell viability, as assessed by trypan blue exclusion, was typically higher than 90%. The dispersed cells were finally resuspended in RPMI 1640-based medium, aliquoted into glass coverslips which had been coated previously with 100 μg/ml poly-L-lysine (Sigma) and stored in Petri dishes at 37 °C in a 5% CO_2, 95% air incubator. The cells were allowed to attach to the coverslips for about 2–3 h, after which fresh culture medium was added to the Petri dishes.

Solutions—The standard salt solution used in the microfluorescence experiments had the following composition (mM): 125 NaCl, 5 KCl, 25 $NaHCO_3$, 2.56 $CaCl_2$, 1.1 $MgCl_2$. This solution was supplemented with different concentrations of glucose or KIC, as required, and constantly gassed with 95% O_2/5% CO_2 for a final pH of 7.4. The solutions used to calibrate fura-2 fluorescence in terms of $[Ca^{2+}]_i$ were as described previously (32, 33). The solutions used to calibrate BCECF fluorescence in terms of pH$_i$ had the following composition (mM): 100 KCl, 10 NaCl, 1 $MgCl_2$, and 10 MOPS (pH adjusted to different values with KOH or HCl).

Dye Loading and Microfluorometry—Cells attached to coverslips were washed in physiological salt solution supplemented with 1% bovine serum albumin. The cells were then loaded with 4 μM fura-2/AM and/or 2 μM BCECF/AM for 60 min at 37 °C in this medium. Groups of 4–8 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. The loaded islets were then transferred into a small (34 μl) fast perifusion chamber placed on the stage of an inverted epifluorescence microscope (Nikon Diaphot). The bottom of the chamber was coated previously with poly-L-lysine to facilitate islet attachment, which was usually achieved in less than 10 min under stationary conditions. Experiments involving isolated cells were carried out using the original cell-containing coverslips as bottoms for the perifusion chamber. The temperature and flow rate in the chamber were 37 °C and approximately 2 ml/min, respectively. The perifusion system used in the experiments and the respective temperature control stages have been described previously in detail (33). The $[Ca^{2+}]_i$ and the pH$_i$ 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 $[Ca^{2+}]_i$ measurements) or at 440/500 nm (for pH$_i$ measurements) via two monochromators. The fluorescence was detected by a photomultiplier after passing through a band pass interference filter centered at 510 ($[Ca^{2+}]_i$ measurements) or 535 nm (pH$_i$ measurements). The data were automatically corrected for background fluorescence and acquired at 10 Hz by a 386SX/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 $[Ca^{2+}]_i$ and pH$_i$ measurements) were stimulated twice with glucose or KIC, first in the $[Ca^{2+}]_i$- and then in the pH$_i$-recording mode. This was because the available beam splitting equipment did not allow the use of more than two different excitations wavelengths at a time. Inner filter effects (resulting in faint fura-2 fluorescence) did not allow combined $[Ca^{2+}]_i$ and pH$_i$ measurements to be carried out from single islets. In control experiments, the pH$_i$ proved to be extremely sensitive to medium pH and to the flow rate of the perifusion solution. While the medium pH was strictly and permanently monitored (and corrected by forced CO_2 gassing if necessary) prior to perifusion, 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$_i$ and $[Ca^{2+}]_i$—The fluorescence ratio F_{500}/F_{440} was converted into pH$_i$ 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 intracellular dye fluorescence. A calibration curve of F_{500}/F_{440} *versus* pH was obtained after applying the latter procedure to different calibration solutions in the pH range 6.5–7.8. The calibration data were fitted to a linear function, and the respective fitting parameters used to read the pH$_i$ values off the calibration curve. For identical monochromator slits, the BCECF fluorescence recorded from single cells at 440 nm was typically very low compared to that at 500 nm, resulting in noisy F_{500}/F_{440} (or calibrated pH$_i$) records. In the single cell experiments we have minimized this noise by increasing the exit slit of the excitation monochromator yielding the F_{440} signal, as required. This strategy was, however, not feasible in the case of the single islet experiments, presumably owing to differential inner filter effects at the two excitation wavelengths. To reduce the noise associated with the ratiometric fluorescence recording from single islets, we have resorted to replacing the F_{440} data for a computer-generated polynomial function with a virtually identical time course. The fluorescence ratio F_{340}/F_{380} was converted into $[Ca^{2+}]_i$ values using the calibration equation of Grynkiewicz *et al.* (35), as described previously by our laboratory (32–34).

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 Haemeck, Israel). Except where otherwise indicated, all other chemicals were from Sigma.

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).

ester of fura-2; K-ATP channel, ATP-sensitive K^+ channel; KIC, α-ketoisocaproic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; pH$_i$, cytosolic pH; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid.

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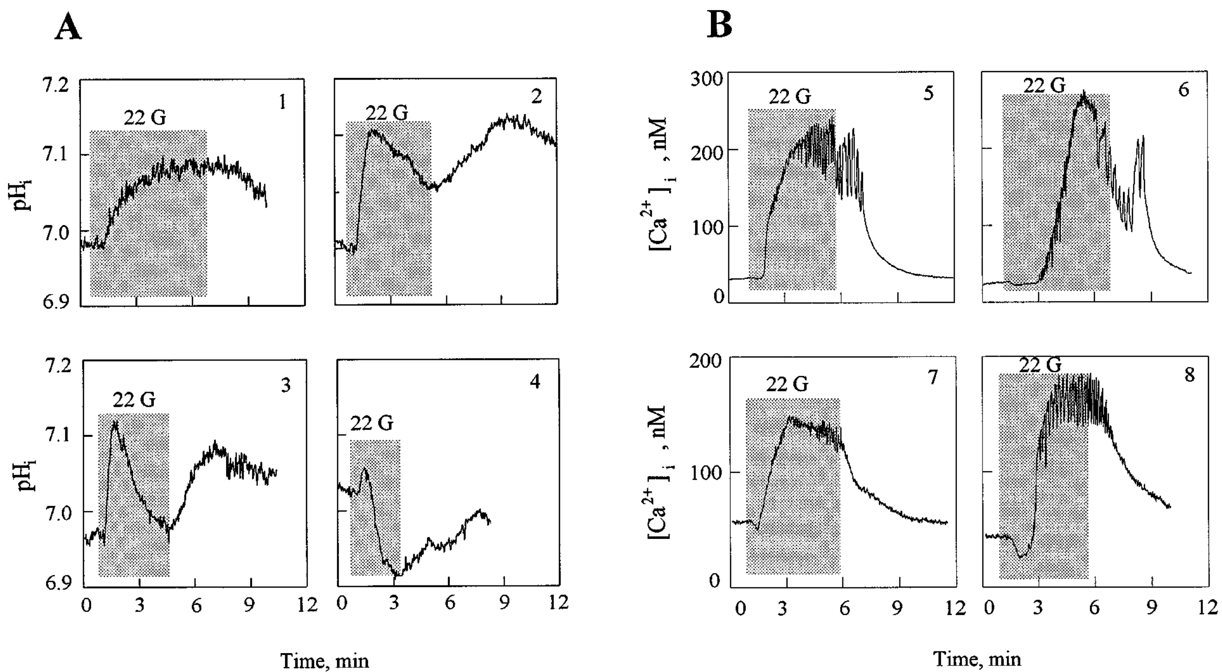

FIG. 1. **Single islet pH$_i$ and [Ca^{2+}]$_i$ responses to glucose stimulation.** *A*, representative examples of single islet pH$_i$ responses to 22 mM glucose (*22G*), as monitored by BCECF fluorescence. *B*, representative examples of single islet [Ca^{2+}]$_i$ responses to 22 mM glucose, as monitored 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 pH$_i$ and [Ca^{2+}]$_i$ Recorded from Single Islets—We have measured the intracellular pH from single mouse islets of Langerhans using the fluorescent probe BCECF. The average resting pH$_i$ 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 pH$_i$ are depicted in Fig. 1*A*. The typical pH$_i$ response was an initial alkalinization of approximately 0.11 unit, followed by a slower acidifying phase (*e.g.*. islets 2 and 3 in Fig. 1*A*). This pattern was representative of 69% of the islets examined (34 out of 49 islets). The glucose-evoked pH$_i$ 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 pH$_i$ 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. 1*A*) the pH$_i$ 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 pH$_i$ 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 pH$_i$), measured from experiments displaying either multiphasic pH$_i$ 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)[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 pH$_i$ responses or a predominant acidifying phase, averaged 85 ± 30 s (n = 20 islets).

The islets displayed a heterogenous off response to glucose. Thus, while in most of the islets examined, the pH$_i$ 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. 1*A*), in other cases, the pH$_i$ 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^{2+} indicator fura-2, we have characterized the effects of high glucose on the [Ca^{2+}]$_i$ with the main aim of assessing the physiological responsiveness of the islet pool used for the pH$_i$ experiments. Examples of typical responses are shown in Fig. 1*B*. Raising glucose concentration from 3 to 22 mM typically resulted in a drop of [Ca^{2+}]$_i$ below baseline lasting 1–3 min (average 88 ± 24 s, n = 12 islets). This was followed by a pronounced [Ca^{2+}]$_i$ rise, after which the [Ca^{2+}]$_i$ either remained elevated (albeit with oscillations, *e.g.* islets 5 and 8 in Fig. 1*B*) 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^{2+}]$_i$ 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.

[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 pH$_i$ experiments. Indeed, there were occasions when the basal pH$_i$ 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 pH$_i$ 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 pH$_i$ and for qualitative assessment of pH$_i$ 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 pH$_i$ experiments than that used for qualitative assessment and for the estimation of average resting pH$_i$ (n = 49 islets).

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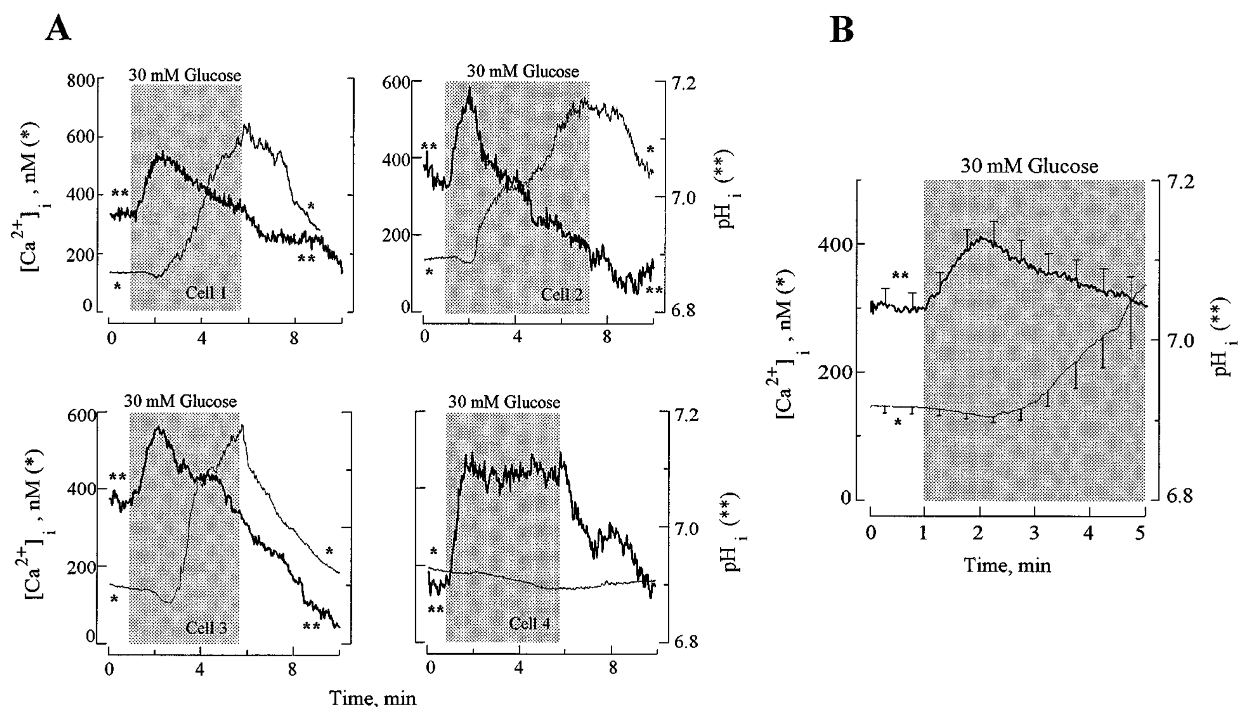

FIG. 2. **Combined pH$_i$ and [Ca^{2+}]$_i$ recordings from single β-cells.** *A*, representative examples of single cell pH$_i$ and [Ca^{2+}]$_i$ responses to 30 mM glucose, as monitored fluorometrically from BCECF- and fura-2-loaded β-cells. The *shadowed areas* denote the periods of stimulation with high glucose. Basal glucose concentration was 2 mM. The cells were stimulated twice with identical glucose pulses, first in the [Ca^{2+}]$_i$- (lighter traces denoted by *single asterisks*) and then in the pH$_i$-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 pH$_i$ (heavier trace denoted by a *double asterisk*) and [Ca^{2+}]$_i$ (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.

Effects of Glucose on the pH$_i$ and [Ca^{2+}]$_i$ Recorded from Single β-Cells—The pH$_i$ and the [Ca^{2+}]$_i$ experiments depicted in Fig. 1 suggest that maximal alkalinization might occur at a time when the [Ca^{2+}]$_i$ remains low or even below baseline in response to the glucose challenge. Since, owing to inner filter effects (see "Experimental Procedures"), the pH$_i$ and the [Ca^{2+}]$_i$ cannot be recorded from the same islets, we have double-labeled isolated β-cells with BCECF and fura-2, aiming at investigating the relationship between glucose-evoked pH$_i$ and [Ca^{2+}]$_i$ changes at the single cell level.

Due to the specificity of the available microfluorescence detection system, the pH$_i$ and the [Ca^{2+}]$_i$ recordings were carried out in sequence, rather than simultaneously. In the following experiments, the cells were typically subjected to 30 mM glucose pulses for 4–6 min in the [Ca^{2+}]$_i$ 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 pH$_i$ 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 [Ca^{2+}]$_i$ response to the sulfonylurea as evidence that the monitored cell was indeed a β-cell (see Fig. 4*B* for a typical response).

Fig. 2*A* (traces labeled with a *single asterisk*) shows that most β-cells responded to the high glucose pulse with a small and transient fall in [Ca^{2+}]$_i$, 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 [Ca^{2+}]$_i$ 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 [Ca^{2+}]$_i$ rises in response to high glucose, as depicted by cell 4 in Fig. 2*A*.

The pH$_i$ traces in Fig. 2*A* 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 pH$_i$ of BCECF- and fura-2-labeled cells was 7.04 ± 0.06 (n = 14 cells). Similarly to isolated islets, the typical pH$_i$ 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. 2*A*). 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. 2*A*) displayed a pure alkalinizing phase, others (8%, *i.e.* 3 out of 39; not shown in Fig. 2*A*) displayed a predominant acidifying phase with residual signs of alkalinization. The glucose-evoked effects on β-cell pH$_i$ 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. 2*A*.

Visual inspection of the relationship between the glucose-evoked [Ca^{2+}]$_i$ and pH$_i$ transients indicates that the initial pH$_i$ rise occurred at a time when the [Ca^{2+}]$_i$ remained at near-basal levels following glucose stimulation (Fig. 2*A*, cells 1, 2, and 3; see also average data from several single cell experiments in Fig. 2*B*). Indeed, the pH$_i$ and the [Ca^{2+}]$_i$ 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 pH$_i$ and the [Ca^{2+}]$_i$ to half the respective maximal levels averaged 38 ± 9 s and 209 ± 67 s, respectively. It is also apparent from Fig. 2*A* (cells 2 and 3) and Fig. 2*B* that there is a significant overlap between the secondary acidifying phase of the multiphasic pH$_i$ response to glucose and the rising phase of the [Ca^{2+}]$_i$ transient, suggesting that the acidifying response might be a consequence of Ca^{2+} influx and of the associated [Ca^{2+}]$_i$ rise (but see the priming data of Fig. 3 and

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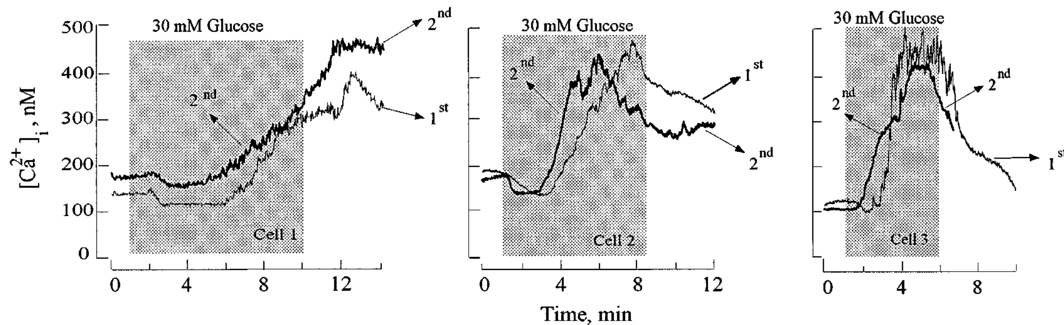

FIG. 3. **Priming effect of glucose on [Ca^{2+}]$_i$.** The cells were stimulated twice with 30 mM glucose in the [Ca^{2+}]$_i$-recording mode, in order to assess the priming effect of the first glucose challenge. The solution switching protocol was similar to that used in the experiments depicted in Fig. 2. The cells were allowed to rest in 2 mM glucose for 12–18 min (18, 16, and 12 min for the experiments depicted by *cells 1* through *3*, respectively) between stimulations. The *shadowed areas* denote the periods of stimulation with high glucose. Basal glucose concentration was 2 mM.

the corresponding discussion). This hypothesis would be consistent with the response displayed by cell 4 in Fig. 2*A*, which is illustrative of β-cells that failed to produce a [Ca^{2+}]$_i$ response to high glucose and did not exhibit the secondary acidifying phase.

Closer inspection of Fig. 2*A* indicates, however, that in some cells (*e.g.* cell 3 and, albeit to a lesser extent, cell 2) the onset of the [Ca^{2+}]$_i$ rise lags a few tens of seconds behind the onset of the acidification. Accordingly, the beginning of the average [Ca^{2+}]$_i$ rise does not match exactly the beginning of the average pH$_i$ fall (Fig. 2*B*). 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+}]$_i$. Under these conditions, the operation of a priming mechanism would be expected to enhance the [Ca^{2+}]$_i$ 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+}]$_i$ rises 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 sulfonylurea blocker of the K-ATP channel, tolbutamide) are shown to raise the [Ca^{2+}]$_i$ while concomitantly decreasing the pH$_i$ (average peak [Ca^{2+}]$_i$ and pH$_i$ changes evoked by 30 mM K$^+$: 335 ± 184 nM and 0.05 ± 0.01, respectively; average peak [Ca^{2+}]$_i$ 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+}]$_i$ and pH$_i$ were also investigated in islets that have been loaded separately with fura-2 and BCECF, as depicted in Fig. 4*C*. In the later experiments, the [Ca^{2+}]$_i$ 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+}]$_i$ 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. 5, 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+}]$_i$ 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+}]$_i$ were recorded from single cells which have been double-labeled with BCECF and fura-2.

Fig. 6*A* shows examples of single β-cell [Ca^{2+}]$_i$ 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. 6*B* with the average glucose responses depicted in Fig. 2*B*. 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-

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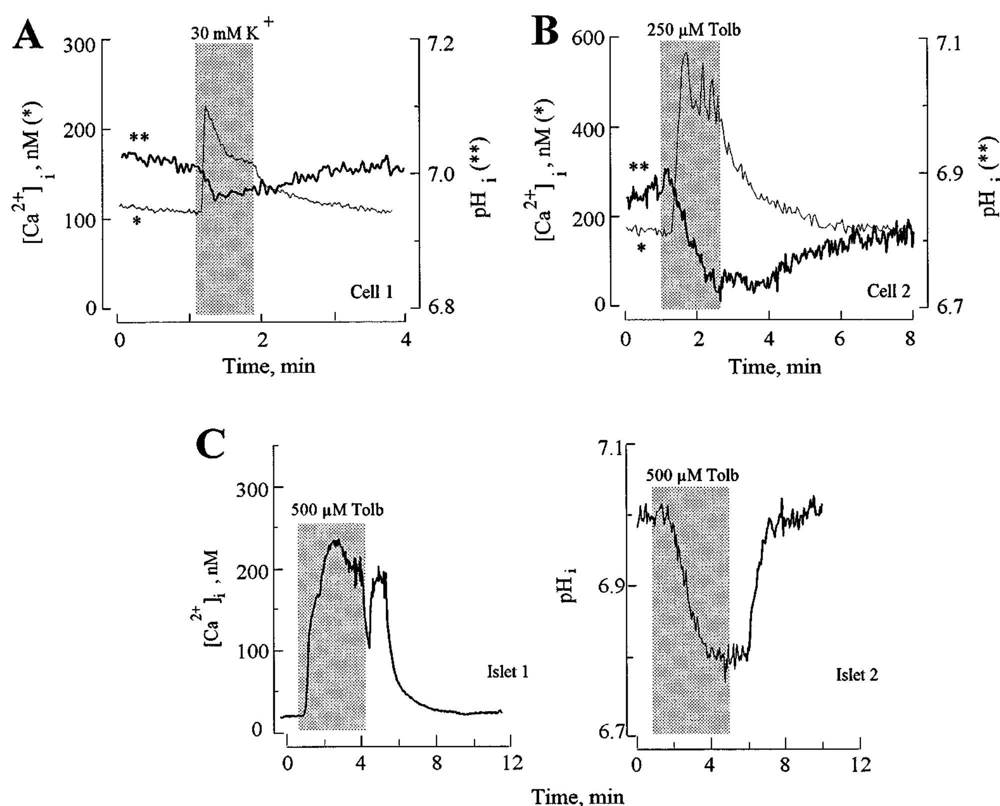

FIG. 4. **Relationship between depolarization-evoked pH_i and $[Ca^{2+}]_i$ transients in pancreatic β-cells.** *A* and *B*, representative examples of single cell pH_i and $[Ca^{2+}]_i$ responses to 30 mM KCl (*A*) and 250 µM tolbutamide (*Tolb*; *B*), as monitored fluorometrically from BCECF- and fura-2-loaded β-cells. Each cell was stimulated twice with identical high K^+ or tolbutamide pulses, first in the $[Ca^{2+}]_i$- (lighter traces denoted by *single asterisks*) and then in the pH_i-recording mode (heavier traces denoted by *double asterisks*). Each experiment is representative of three similar experiments. *C*, representative examples of single islet $[Ca^{2+}]_i$ (*islet 1*) and pH_i (*islet 2*) responses to 500 µM 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 perifusion medium was 2 mM throughout.

ization. Thus, the effects of KIC on pH_i are also qualitatively similar to the homologous responses to glucose (this again may be better assessed by comparing the average responses depicted in Figs. 2*B* and 6*B*). Notice however that, on average, the maximal amplitude of the KIC-evoked initial alkalinization is about twice as large as that of the glucose-evoked response. Yet another difference is that, in contrast to the situation found with glucose, we have been unable to find examples of cells with predominant acidifying responses (and concomitant slight alkalinizations) to KIC.

DISCUSSION

Using physiologically buffered solutions and single BCECF-loaded islets and isolated β-cells from normal mice as a model system to investigate the effects of glucose on cytosolic pH, we found that the typical acute 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 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 β-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 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}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 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 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 β-cells. The inhibitor of mitochondrial pyruvate transport, 3-hydroxy-cyanocinnamate, has been reported to prevent glucose-induced alkalinization, leading to the concept that the 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 pH_i in a glucose-like manner (29). We have characterized the effect of the mitochondrial substrate KIC on the pH_i aiming at clarifying the role of mitochondrial metabolism in the glucose-evoked effect on pH_i. The fact that KIC had glucose-like effects on the 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 pH_i rise (Fig. 2*B* *versus* Fig. 6*B*). 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-

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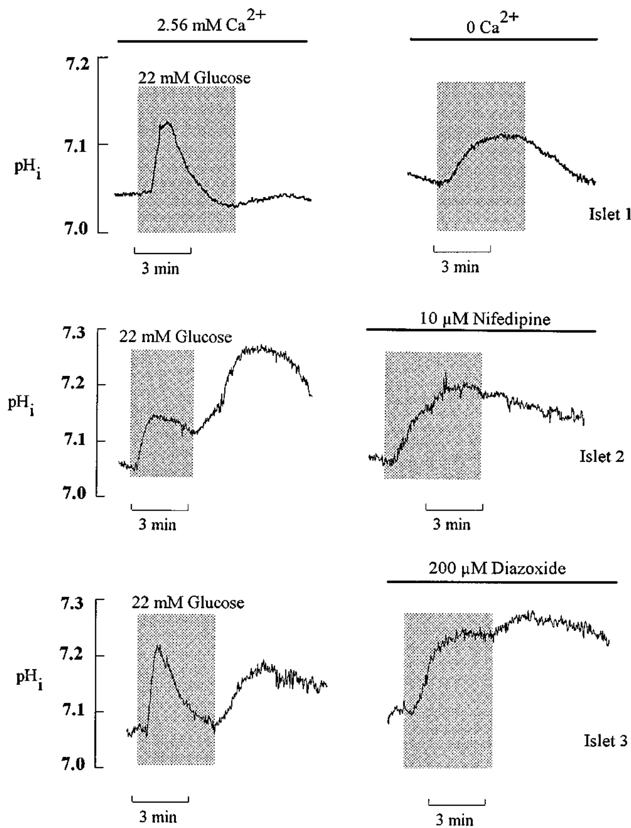

FIG. 5. **Modulation of glucose-evoked pH_i responses by the suppression of Ca^{2+} influx.** Individual BCECF-loaded islets were sequentially stimulated with 22 mM glucose, first in the absence (*left*) and later in the presence (*right*) of the Ca^{2+} influx suppressor (from *top* to *bottom*: exposure to a Ca^{2+}-free solution, the L-type Ca^{2+} channel blocker nifedipine, and the K-ATP channel agonist diazoxide). The *shadowed areas* denote the periods of stimulation with high glucose. Glucose concentration in the perifusion medium was 3 mM throughout. Each experiment is representative of three similar experiments.

sive net consumption of protons, the consequence of which would be a marked initial rise in pH_i. 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_i 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.[3] 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,[4] 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 $[Ca^{2+}]_i$

[3] Exposing β-cells to EIPA in the absence of bicarbonate causes cytosolic acidifications of at least 0.2 pH unit (9), while the tendency in the presence of the anion is for dimethyl amiloride causing marginal (approximately 0.03 pH unit (29)) acidifications. This resembles the effect of dimethyl amiloride on the pH_i of glomerulosa cells (58).

[4] At substimulatory glucose concentrations, the steady-state pH_i rise is largely insensitive to either DIDS or removal of bicarbonate (29), implying that the putative alteration in the pH set-point of the exchanger has a strict glucose dependence and suggesting that the glucose-evoked pH_i rise observed at high glucose concentrations is also partially mediated by an exchanger-independent mechanism (*i.e.* that it is partially the result of the activation of H^+-consuming metabolic steps, as proposed for the short-term pH_i rises described in our study).

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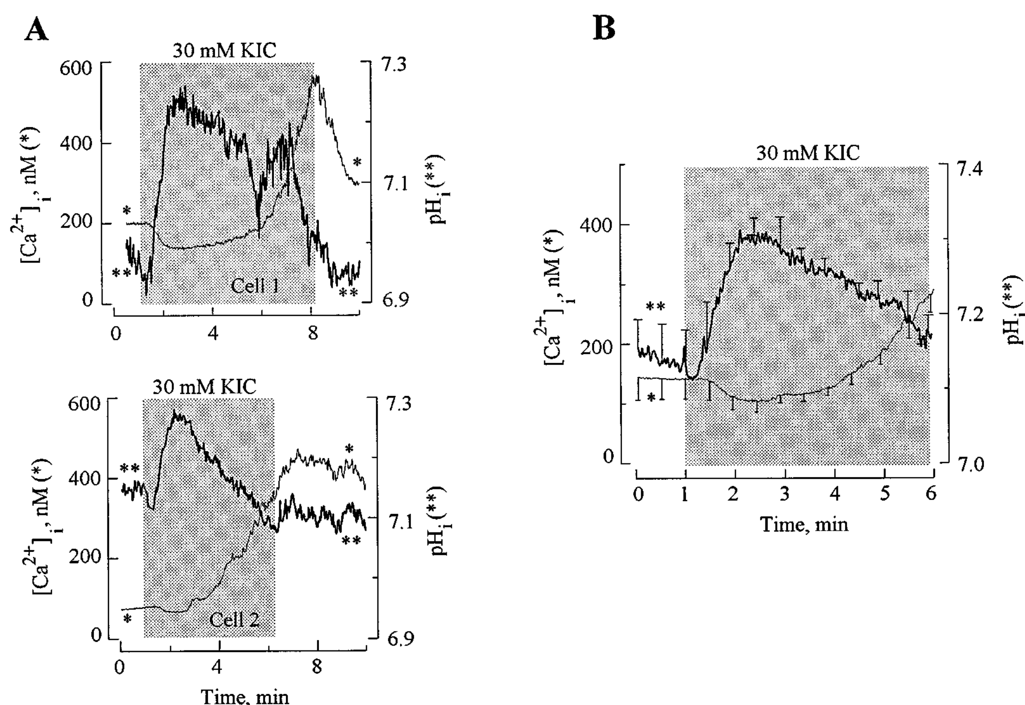

FIG. 6. **Effect of the mitochondrial substrate α-ketoisocaproic acid (KIC) on the pH_i and $[Ca^{2+}]_i$ of single β-cells.** *A*, representative examples of single cell pH_i and $[Ca^{2+}]_i$ responses to 30 mM KIC, as monitored fluorometrically from BCECF- and fura-2-loaded β-cells. The *shadowed areas* denote the periods of stimulation with KIC (no glucose present). The cells were stimulated twice with identical KIC pulses, first in the $[Ca^{2+}]_i$- (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 $[Ca^{2+}]_i$ traces in each panel have been superimposed for clarity. *B*, average pH_i (heavier trace denoted by a *double asterisk*) and $[Ca^{2+}]_i$ (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.

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 $[Ca^{2+}]_i/pH_i$ experiments depicted in Fig. 2 have been carried out using a double stimulation protocol similar to that of Fig. 3 (with the $[Ca^{2+}]_i$ 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-evoked $[Ca^{2+}]_i$ and pH_i changes. For example, Fig. 2*B* indicates that the average pH_i rise peaked 57 s after the delivery of the high glucose pulse. Assuming that the priming mechanism affects glucose metabolism (61) and the associated alkalinization, the theoretical pH_i transient (*i.e.* the pH_i response to the first glucose challenge should this have been recorded) may need to be offset by approximately 49 s compared to the actual (recorded) response, implying that the peak of the theoretical response may occur 106 s after the delivery of high glucose.

Analysis of the single cell experiments depicted in Fig. 2 indicates that the $[Ca^{2+}]_i$ started to rise, on average, 97 s after the glucose stimulus. Thus, even taking into account the possibility of priming, our results indicate that the initial alkalinization occurs at near-basal $[Ca^{2+}]_i$ levels. Since the $[Ca^{2+}]_i$ rise reflects primarily Ca^{2+} influx through voltage-sensitive Ca^{2+} channels (32, 62), this indicates, in agreement with earlier reports (13, 30), that the initial alkalinization is not a consequence of Ca^{2+} influx. This conclusion is further supported by the data in Fig. 5, which show that the alkalinizing phase remained essentially intact when the islets were stimulated with glucose in the presence of various agents known to suppress voltage-sensitive Ca^{2+} influx (*i.e.* 0 Ca^{2+}, nifedipine, and diazoxide). Interestingly, the rate of the initial alkalinization was significantly decreased by any of these agents. This may reflect the Ca^{2+} dependence of specific mitochondrial enzymes (*e.g.* dehydrogenases (63)) and suggests that 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 $[Ca^{2+}]_i/pH_i$ experiments (single β-cells), the pH_i decreased concomitantly with the $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ rise)[5]; 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

[5] The islets used for the $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ below baseline lasting an average time of 88 s. In pancreatic β-cells, this drop in $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ rises (66). Thus, the single islet data indicate that, on average, the $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ rise.

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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 β-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 β-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 β-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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