Intracellular pH Plays a Critical Role in Glucose-Induced Time-Dependent Potentiation of Insulin Release in Rat Islets

Subhadra C. Gunawardana and Geoffrey W.G. Sharp

The underlying mechanisms of glucose-induced time-dependent potentiation in the pancreatic β-cell are unknown. It had been widely accepted that extracellular Ca^{2+} is essential for this process. However, we consistently observed glucose-induced priming under stringent Ca^{2+}-free conditions, provided that the experiment was conducted in a HEPES-buffered medium as opposed to the bicarbonate (HCO_3^-)-buffered medium used in previous studies. The critical difference between these two buffering systems is that islets maintain a lower intracellular pH in the presence of HEPES. The addition of HEPES to a HCO_3^--buffered medium produced a dramatic decrease in the intracellular pH. If it is the lower intracellular pH in islets in a HEPES-buffered medium that is permissive for glucose-induced time-dependent potentiation (TDP), then experimental lowering of intracellular pH by other means should allow TDP to occur in a Ca^{2+}-free HCO_3^--buffered medium, where TDP normally does not occur. As expected, experimental acidification produced by dimethyl amiloride (DMA) allowed glucose to induce TDP in a Ca^{2+}-free HCO_3^--buffered medium. DMA also enhanced the priming normally present in HEPES-buffered media. Priming was also enhanced by transient acidification caused by acetate. Experimental alkalinization inhibited the development of priming. In the presence of Ca^{2+}, the magnitude of glucose-induced TDP was higher in a HEPES-buffered medium than in an HCO_3^--buffered medium. In summary, glucose-induced priming was consistently observed under conditions of low intracellular pH and was inhibited with increasing intracellular pH, irrespective of the presence of extracellular Ca^{2+}. These data indicate that glucose-induced TDP is critically dependent on intracellular pH. *Diabetes* 51: 105–113, 2002

From the Department of Molecular Medicine, College of Veterinary Medicine, Cornell University, Ithaca, New York.

Address correspondence and reprint requests to Geoffrey W.G. Sharp, Department of Molecular Medicine, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853. E-mail: gws2@cornell.edu.

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αKGDH, α-ketoglutarate dehydrogenase; carboxy-SNARF-AM, carboxy seminaphthorohodofluor-acetyloxymethyl ester; DMA, dimethyl amiloride; ICDH, isocitrate dehydrogenase; KRB, Krebs-Ringer bicarbonate; KRBH, Krebs-Ringer bicarbonate with HEPES; TDP, time-dependent potentiation.

Time-dependent potentiation (TDP), or "priming," of insulin release in the pancreatic β-cell was first observed in 1969 by Grodsky et al. (1). TDP can be defined as an enhancement of the insulin secretory response resulting from a previous exposure to a nutrient secretagogue such as glucose. The most remarkable feature of this time-dependent effect is that it does not require the continuous presence of glucose in the medium but rather acts through a memory induced in the stimulus-secretion pathway, which persists after the removal of glucose from the medium. The change induced in the β-cell by glucose enhances the secretory response to subsequently applied stimuli.

The underlying mechanisms of the induction and mediation of TDP are unknown. In an attempt to document the mechanisms of induction of TDP, several investigators have examined the requirement for extracellular Ca^{2+} for glucose-induced TDP. The early studies by Grill et al. (2) reported that the simple omission of Ca^{2+} from the priming medium was not sufficient to inhibit glucose-induced priming. However, subsequent studies by several groups (3–6) demonstrated that stringent removal of extracellular Ca^{2+} using EGTA abolishes priming completely, suggesting that Ca^{2+} plays a critical role in the induction of priming by glucose. In 1995, Taguchi et al. (7) investigated this in more detail, demonstrating that a concentration of extracellular Ca^{2+} as low as 58 μmol/l allows glucose to prime a subsequent secretory response, whereas further removal of Ca^{2+} using 0.5 mmol/l EGTA completely inhibits priming.

Contrary to the conclusions of the above-mentioned studies, we found that glucose induced strong priming under strict Ca^{2+}-free conditions to the same extent as it did in the presence of Ca^{2+} or better. This was observed when the islets were exposed to glucose in a HEPES-buffered medium during the priming period instead of a HCO_3^--buffered medium as in the previous studies. In seeking the crucial difference between the two buffers, we noted that islets maintain a lower intracellular pH in HEPES-buffered media than in HCO_3^--buffered media (8–11). Furthermore, other studies have reported that a lower intracellular pH in the β-cell favors nutrient-induced insulin release and related functions, whereas an increase of intracellular pH has an inhibitory effect (12–20). In this study, we confirmed that the presence of HEPES in the medium results in a significant decrease in intracellular pH

in islets. On the speculation that such a low intracellular pH may be permissive for glucose-induced priming, we examined the priming ability of glucose in a Ca^{2+}-free HCO_3^--buffered medium (where priming is not normally observed) under conditions of intracellular acidification using experimental agents such as dimethyl amiloride (DMA). Our results demonstrate that experimental lowering of intracellular pH unmasks glucose-induced priming in Ca^{2+}-free HCO_3^--buffered media and enhances priming in HEPES-buffered media with or without Ca^{2+}. Furthermore, glucose-induced priming consistently observed in HEPES-buffered media can be inhibited by experimental increase of intracellular pH. Thus, this study demonstrates that the priming ability of glucose is critically dependent on the intracellular pH of the β-cell.

RESEARCH DESIGN AND METHODS

Materials. Chemicals were purchased from the following companies: Fisher Scientific (Fair Lawn, NJ), Sigma (St. Louis, MO), Mallinkrodt Chemicals (Paris, KY), Molecular Probes (Eugene, OR), and Bio Rad (Hercules, CA). Radioactive insulin was purchased from New England Nuclear Life Science Products (Boston, MA), and cylinders of 95% O_2/5% CO_2 were purchased from Empire Airgas (Elmira, NY).

Isolation of islets. Male Wistar rats aged 2–3 months were obtained from Harlan (Indianapolis, IN). Pancreatic islets were isolated according to the collagenase digestion method described by Lacy and Kostianovsky (21), with slight modifications. The animals were fed ad libitum until they were killed.

Media. Krebs-Ringer bicarbonate (KRB) solution of pH 7.4 buffered with 10 mmol/l HEPES (KRBH) was used for islet isolation and for the major part of the incubations. A bicarbonate-buffered solution of pH 7.4 containing 25 mmol/l HCO_3^- and constantly gassed with 95% O_2 and 5% CO_2 (KRB) was used during the priming period where indicated to demonstrate the difference in glucose-induced priming in the two different buffer systems. The standard composition of KRBH solution was 128.8 mmol/l NaCl, 4.8 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 2.5 mmol/l $CaCl_2$, 5 mmol/l $NaHCO_3^-$, and 10 mmol/l HEPES. The composition of the KRB solution was similar except that it contained 25 mmol/l $NaHCO_3^-$ and no HEPES. All media were maintained at pH 7.4, except in the experiments in which the medium pH was raised to 8.3 to produce intracellular alkalinization. In the Ca^{2+}-free KRB or KRBH solutions used, Ca^{2+} was omitted from the medium, and 5 mmol/l EGTA was added to ensure stringent Ca^{2+}-free conditions. The basal glucose concentration used was 2.8 mmol/l, whereas the priming glucose concentration was 11.1 mmol/l in Ca^{2+}-free conditions and 16.7 mmol/l in the presence of Ca^{2+}. These are the optimal concentrations of glucose for the stimulation of insulin secretion in the absence and presence of Ca^{2+}, respectively (22). Stimulation of islets in the test period, as distinct from the priming period, was always done with 16.7 mmol/l glucose in the presence of Ca^{2+}. Bovine serum albumin (0.1%) was present in the medium during all incubations. To produce intracellular acidification, we added DMA to the KRB/KRBH solution to a final concentration of 40 μmol/l where indicated. Acetate (at a final concentration of 40 mmol/l) was used as an alternative acidifying agent. Intracellular alkalinization was produced, where indicated, by *1*) increasing the medium pH to 8.3, *2*) adding 5 mmol/l ammonium chloride to the medium, or *3*) substituting the Cl^- in the medium with $gluconate^-$. After adding the acidifying/alkalinizing agents to the media, the medium pH was readjusted to 7.4 (except when intracellular alkalinization was produced by increasing the medium pH). Similar media were used during the measurement of intracellular pH, except that the KRB media were not gassed and contained either 5 or 25 mmol/l HCO_3^- as indicated.

Static incubation method. Freshly isolated islets were preincubated for 30 min in basal KRBH solution with 2.8 mmol/l glucose. Islets were then subjected to a 45-min priming period, with or without Ca^{2+} as indicated. The concentration of glucose, the buffer system, and the presence of acidifying or alkalinizing agents during the priming period varied according to the experiment, as detailed in the text. After the priming period, the islets were washed and rested in basal KRBH for 20 min, followed by the test stimulation with 16.7 mmol/l glucose in KRBH for 20 min. At the end of this test period, samples were collected to measure insulin secretion and islet insulin content. In each static incubation experiment, three to five replicate batches of five size-matched islets were used for each priming condition. The number of times that each experiment was repeated is denoted by n in the figure legends.

Insulin measurement. Radioimmunoassay was used to measure the amount of insulin released during the final stimulation and the total content of insulin in the islets. Values are expressed as fractional release, as a percentage of the total content per 20 min. Absolute release rates ($pg \cdot islet^{-1} \cdot min^{-1}$ during the 20-min period) are presented in the figure legends.

Measurement of intracellular pH. Intracellular pH in islets was measured under various conditions using the pH-sensitive dye carboxy-SNARF-AM (carboxy seminaphthorhodofluor-acetyloxymethyl ester, or 126208-13-7 Spiro [7H-benzo[c]xanthene-7,1′(3H)-isobenzofuran]-ar′-carboxylic acid, 3-(acetyloxy)-10-(dimethylamino)-3′-oxo-,(acetyloxy)methyl ester), an agent that has been used successfully to measure intracellular pH in multicellular systems (23–26). As described by Muller-Borer et al. (23), this dye can be used for accurate quantitative measurement of intracellular pH in thick tissue specimens up to a depth of 300 μm. Even though emission fluorescence intensity may vary as a result of possible attenuation of emitted light in the deeper cell layers and/or possible differences in dye concentration among cells, the ratio between the two emission fluorescence intensities remains constantly proportionate to the pH (23), making SNARF-AM a suitable agent to measure pH in thick tissues. In our experiments, preferentially picked small islets of <100 μm in diameter were used to ensure uniform dye loading. Islets were loaded with carboxy-SNARF-AM at 37°C for 1 h, at a concentration of 5 μmol/l. Dye-loading in islets was monitored visually, and uniformly stained islets were picked for pH measurements. Using a Nikon Diaphot fluorescence microscope, the loaded islets were excited at 514 nm, and the emission fluorescence was measured at two different wavelengths, 580 and 640 nm. Intracellular pH was calculated from the ratio between the fluorescence at 580 and 640 nm. The calibration curve was derived using islets with their intracellular pH fixed at known values ranging from 6.0 to 8.0 in KRBH containing 100 mmol/l K^+ and 20 μmol/l nigericin. The pH of the media in which islets were maintained during the pH measurements and of the solutions added (e.g., HEPES, DMA) had been adjusted to 7.4. A representative recording for each experiment is shown in the figures. The number of such recordings done for each condition is denoted by n, and mean intracellular pH values from n recordings are indicated in the figure legends.

Statistical analysis Values were expressed as mean ± SE, and groups were compared using the paired t test.

RESULTS

In HEPES-buffered medium in the absence of Ca^{2+}, islets previously exposed to 11.1 mmol/l glucose produced higher insulin release in response to subsequent stimulation with 16.7 mmol/l glucose than the islets previously exposed to 2.8 mmol/l glucose. In other words, glucose induced strong TDP under stringent Ca^{2+}-free conditions (Fig. 1). Because this result was unexpected and contradicted the consensus in the literature, follow-up studies were performed to compare the ability of glucose to prime in the HEPES-buffered media (KRBH) and HCO_3^--buffered media (KRB).

When paired islets were pretreated with 11.1 mmol/l glucose in KRBH and KRB under strict Ca^{2+}-free conditions, TDP was observed in KRBH but not in KRB (Fig. 2). Thus, the ability of glucose to induce priming under Ca^{2+}-free conditions seems to be determined by the difference between HEPES and bicarbonate as buffer systems. As demonstrated later and described in the discussion, the presence of HEPES results in a lower intracellular pH in the islets than that maintained in HCO_3^--buffered media. This has been reported before in mouse islets (8–10). We demonstrated a similar pH-lowering effect of HEPES in rat islets by measuring intracellular pH in the presence and absence of HEPES. Intracellular pH measurements were performed in whole islets using the fluorescent dye carboxy SNARF-AM, an agent that has been used successfully to measure intracellular pH in multicellular systems (23–26). Islets loaded with 5 μmol/l SNARF-AM were maintained in KRBH (or other media as indicated), and the intracellular pH was recorded at 37°C during a 20-min period. The presence of HEPES in the

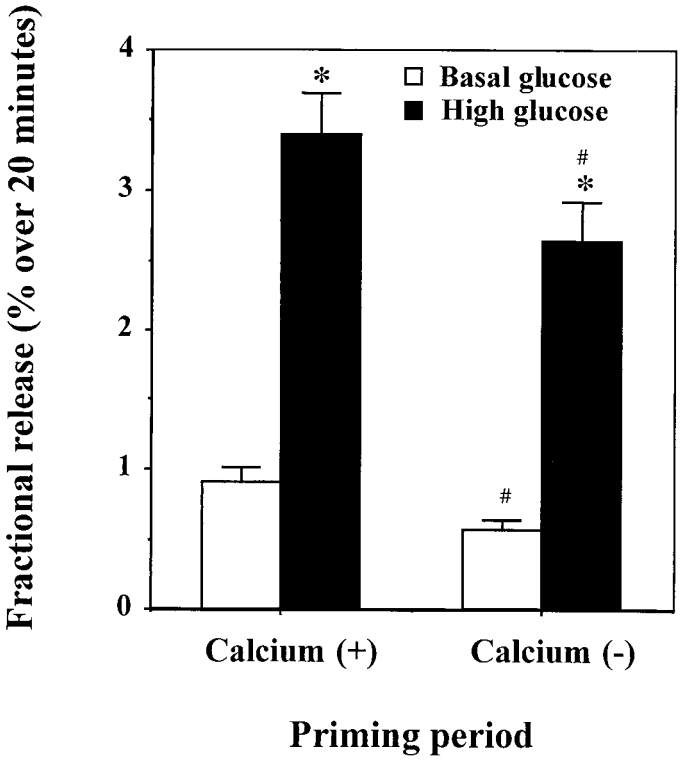

FIG. 1. Glucose induces priming under stringent Ca^{2+}-free conditions as well as in the presence of Ca^{2+}. The bars indicate the insulin release in response to 16.7 mmol/l glucose, in paired islets previously exposed to basal glucose (2.8 mmol/l) or high glucose (16.7 or 11.1 mmol/l, depending on the presence/absence of Ca^{2+}). The experiment was performed in KRBH medium. The 45-min period of initial exposure to glucose (priming) was carried out, as indicated, either in the presence of Ca^{2+} or under strict Ca^{2+}-free conditions with 5 mmol/l EGTA, using paired islets. After the priming period, islets were washed in Ca^{2+}-containing KRBH with 2.8 mmol/l glucose for 20 min. Islets were then challenged for 20 min with 16.7 mmol/l glucose in Ca^{2+}-containing KRBH, and samples were collected for insulin assay (n = 12); *P < 0.001 compared with the corresponding unprimed response; #P < 0.05 compared with each corresponding group in the presence of Ca^{2+}. Absolute secretion rates expressed as pg · islet^{-1} · min^{-1} (during 20 min) are as follows. In the presence of Ca^{2+}: unprimed islets (previously exposed to basal glucose), 19.7 ± 1.6; primed islets (previously exposed to high glucose), 68.2 ± 4.8. In the absence of Ca^{2+}: unprimed islets, 14.8 ± 2.2; primed islets, 75.8 ± 9.4.

medium uniformly resulted in a lower intracellular pH. The basal intracellular pH in islets maintained in regular KRBH solution (containing 10 mmol/l HEPES, 5 mmol/l HCO_3^-, and 2.8 mmol/l glucose) ranged from 7.0 to 7.2, with a mean value of 7.1 ± 0.02 (n = 60). In HEPES-free KRB with 5 mmol/l HCO_3^-, the basal pH was higher (7.4 ± 0.06; n = 25). When 10 mmol/l HEPES was added to HEPES-free KRB with 5 mmol/l HCO_3^-, the intracellular pH decreased to between 6.9 and 7.1, indicating the acidifying property of HEPES (Fig. 3*A*). A similar change was observed when 25 mmol/l HEPES was added to a HEPES-free KRB medium containing 25 mmol/l HCO_3^- (Fig. 3*B*). The acidifying effect of HEPES was similar in both the presence (Fig. 3*A* and *B*) and absence of Ca^{2+} (Fig. 3*C* and *D*). Previous studies in mouse islets (8,9) suggested that glucose-induced Ca^{2+} influx results in a decrease of intracellular pH, which is evident in the presence of HEPES, even though an earlier study reported no change in intracellular pH on removal of Ca^{2+} or when the glucose concentration was raised (10). In our hands, glucose did produce a decrease in islet pH but to a lesser degree (data not shown), possibly as a result of a difference in the intracellular buffering mechanisms between rat and mouse islets.

These results demonstrate that islets experience a significant decrease of intracellular pH in the presence of HEPES. As shown previously, glucose induces significant priming in the presence of HEPES even under stringent Ca^{2+}-free conditions, whereas no such priming is observed in the absence of HEPES (Fig. 2). To test whether it is the low intracellular pH that acts as the major permissive factor for glucose-induced priming, we experimentally modulated the intracellular pH in islets and determined its effects on priming. DMA, an inhibitor of the Na^+/H^+ exchanger in the β-cell, was selected as a suitable acidifying agent. The Na^+/H^+ exchanger is one of the major buffering mechanisms in the β-cell, which acts by extruding excess H^+ ions in exchange for medium Na^+. Inhibition of this exchanger by DMA results in accumulation of H^+ ions within the cell, producing a rapid decrease in intracellular pH. The ability of DMA to decrease intracellular pH in islets is demonstrated in Fig. 4. The addition of 40 μmol/l DMA to KRBH or KRB steadily decreased the islet intracellular pH in both the presence and absence of Ca^{2+}. In KRBH, the pH changed from 7.1 ± 0.03 to 6.9 ± 0.04 in the presence of Ca^{2+} (n = 21) and from 7. 1 ± 0.02 to 6.9 ± 0.03 (n = 12) in the absence of Ca^{2+}. In KRB containing 5 mmol/l HCO_3^-, the pH changed from 7.4 ±

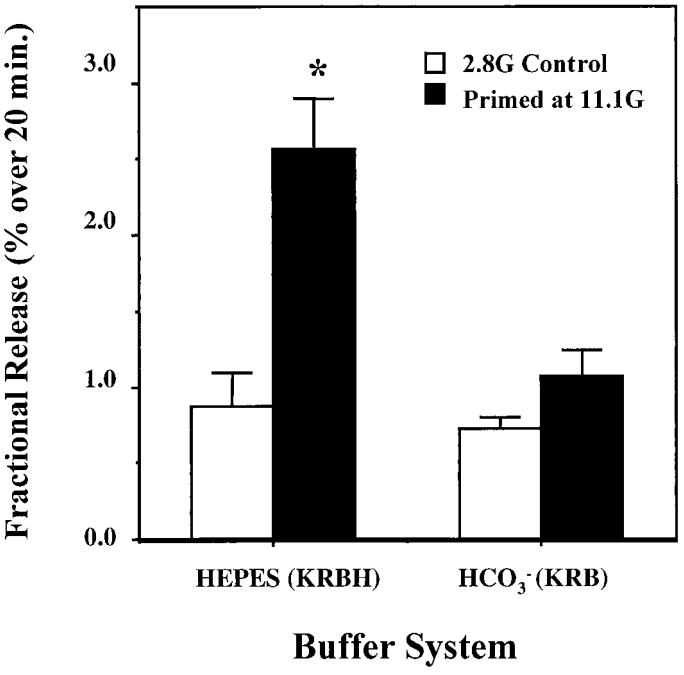

FIG. 2. Under Ca^{2+}-free conditions, glucose induces priming in KRBH but not in KRB. In the priming period, islets were exposed to 2.8 and 11.1 mmol/l glucose in either Ca^{2+}-free KRBH or Ca^{2+}-free KRB containing 5 mmol/l EGTA. After washing out the priming medium and incubating in Ca^{2+}-containing KRBH with 2.8 mmol/l glucose for 20 min, the islets were challenged with 16.7 mmol/l glucose in KRBH. Insulin release was measured during the 20-min stimulation period with 16.7 mmol/l glucose in the presence of Ca^{2+} (n = 4). G, mmol/l glucose in the priming period; *P < 0.001, compared with the unprimed response in KRBH and primed/unprimed responses in KRB. Absolute secretion rates, expressed as pg · islet^{-1} · min^{-1} (during 20 min), are as follows: unprimed islets in KRBH, 20.5 ± 8.7; primed islets in KRBH, 59.8 ± 11.3; unprimed islets in KRB, 15.9 ± 7.7; primed islets in KRB, 21.4 ± 2.6.

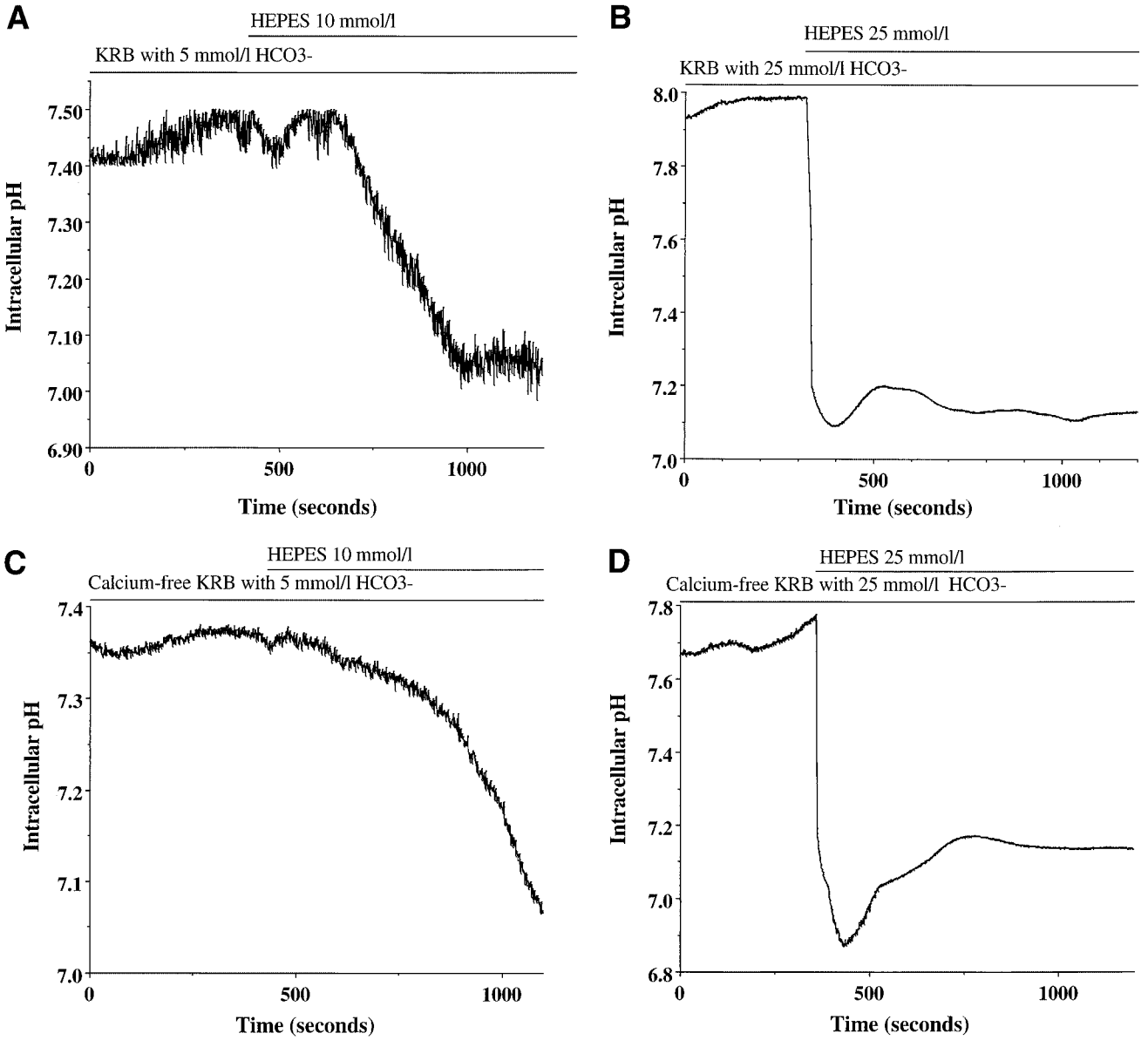

FIG. 3. Decrease in intracellular pH in islets brought about by the addition of HEPES to a HEPES-free medium. Intracellular pH was recorded from islets loaded with 5 μmol/l SNARF-AM. Islets were first placed in a HEPES-free KRB medium, and HEPES was added where indicated. The initial pH of both the KRB medium and the HEPES solution added had been adjusted to 7.4. The KRB medium had the regular composition described in RESEARCH DESIGN AND METHODS, with the variations indicated in each figure. A representative recording for each condition is shown. *A*: $n = 10$; mean initial pH = 7.4 ± 0.08; mean final pH after HEPES = 6.9 ± 0.08. *B*: $n = 3$; mean initial pH = 7.8 ± 0.17; mean final pH after HEPES = 7.0 ± 0.11. *C*: $n = 10$; mean initial pH = 7.4 ± 0.04; mean final pH after HEPES = 7.1 ± 0.06. *D*: $n = 8$; mean initial pH = 7.8 ± 0.08; mean final pH after HEPES = 7.1 ± 0.04.

0.04 to 7.2 ± 0.02 ($n = 6$). In Ca^{2+}-free KRB containing 25 mmol/l HCO_3^- (the medium in which glucose does not induce priming), the pH changed from 7.7 ± 0.06 to 7.2 ± 0.03 upon the addition of DMA ($n = 7$).

In previous studies (3–7) as well as ours, glucose failed to induce TDP in Ca^{2+}-free KRB (Fig. 2). However, when the intracellular pH in islets was decreased using 40 μmol/l DMA during the Ca^{2+}-free priming period, TDP was observed in KRB where it was formerly absent. A comparison of the effects of DMA on glucose-induced priming in Ca^{2+}-free KRBH and Ca^{2+}-free KRB is shown in Fig. 5. In islets primed with 11.1 mmol/l glucose, the presence of DMA produced a significant difference in the primed response, although no significant change was observed in unprimed islets maintained at basal glucose. The presence of DMA resulted in the development of glucose-induced TDP in Ca^{2+}-free KRB. In KRBH where glucose normally produces priming, the presence of DMA significantly enhanced the magnitude of the response (Fig. 5). Acetate, a weak acid that produces a reversible decrease in the intracellular pH, also enhanced the glucose-induced priming in KRBH (data not shown). Thus, a low intracellular pH is favorable for glucose-induced priming. Neither DMA nor acetate alone induced TDP (in the absence of a stimulatory concentration of glucose), indicating that this effect of intracellular pH on priming is dependent on the presence of a priming agent. In the presence of Ca^{2+} where glucose induces priming in both KRBH and KRB, the degree of

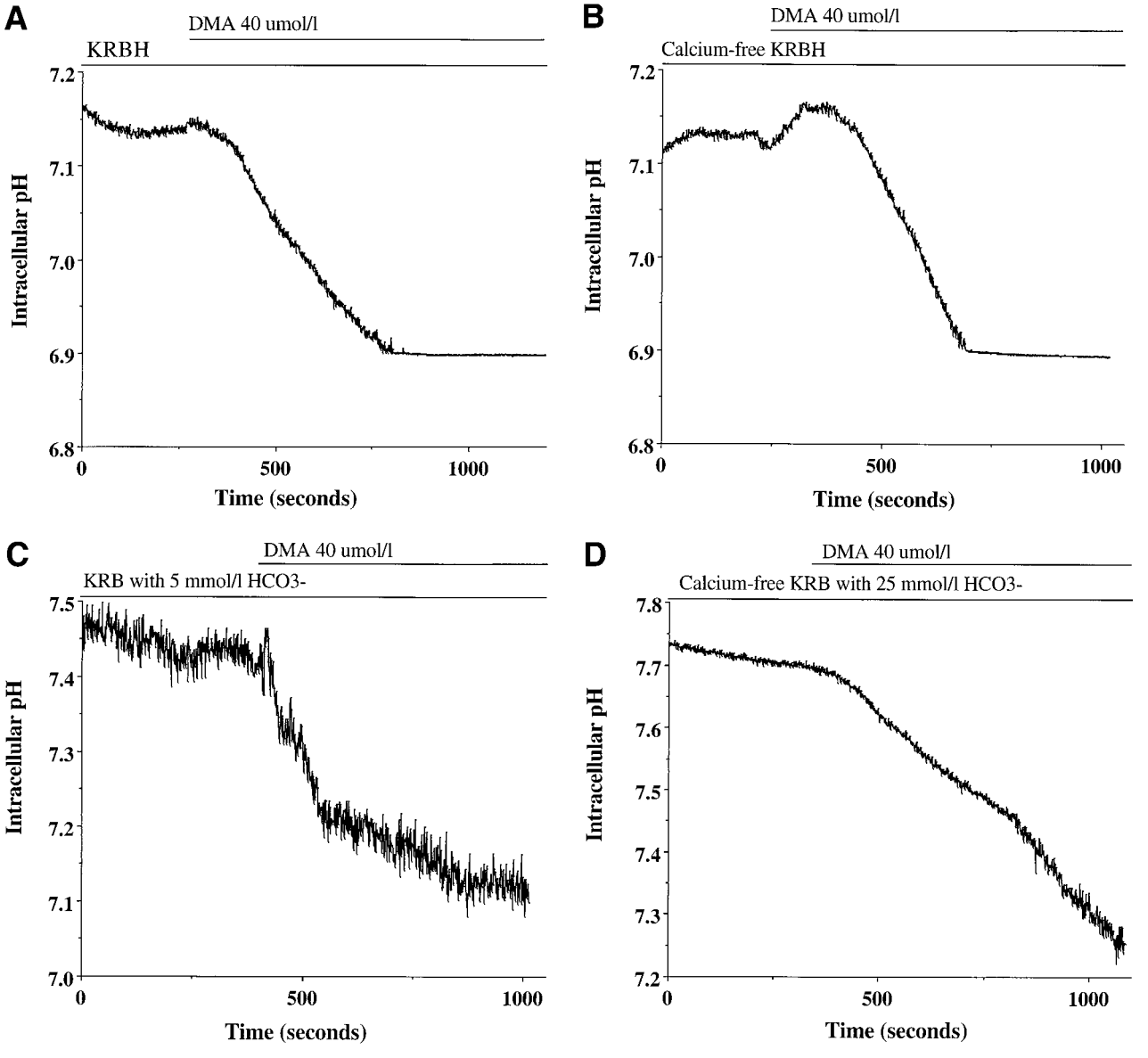

FIG. 4. Intracellular acidification brought about by DMA. Intracellular pH was recorded from islets loaded with 5 μmol/l SNARF-AM. Islets were first placed in regular KRBH or KRB with or without Ca^{2+} as indicated, and DMA was added where indicated, to a final concentration of 40 μmol/l. The initial pH of both the medium and the DMA solution added had been adjusted to 7.4. A representative recording for each condition is shown. *A*: n = 21; mean initial pH = 7.1 ± 0.03; mean final pH after DMA = 6.9 ± 0.04. *B*: n = 12; mean initial pH = 7. 1 ± 0.02; mean final pH after DMA = 6.9 ± 0.03. *C*: n = 6; mean initial pH = 7. 4 ± 0.04; mean final pH after DMA = 7.2 ± 0.02. *D*: n = 7; mean initial pH = 7. 7 ± 0.06; mean final pH after DMA = 7.2 ± 0.03.

TDP was higher in KRBH (Fig. 6), providing additional evidence of the favorable effect of low intracellular pH on priming.

These results clearly demonstrate the pH-lowering effect of HEPES and the ability of glucose to induce better priming in the presence of a low intracellular pH. In situations in which glucose induces strong priming, such as in KRBH (with or without Ca^{2+}) or KRB with DMA, the intracellular pH was maintained at or below 7.2. If a low intracellular pH is critical for glucose-induced priming, then an increase in the intracellular pH should inhibit the ability of glucose to induce TDP. To explore this possibility, we monitored glucose-induced priming in KRBH under conditions of intracellular alkalinization. During the priming period, the intracellular pH in islets was increased by raising the medium pH to 8.3. Increasing the medium pH to 8.3 resulted in a corresponding intracellular pH of 7.7 ± 0.04 (n = 7). Increasing the medium pH to 8.0, 8.1, or 8.2 did not produce significant changes in the intracellular pH, indicating the effective buffering capacity in the β-cell. As expected, glucose-induced priming was significantly inhibited when the intracellular pH was increased to 7.7 (Fig. 7). Alkalinization produced by other traditional methods such as the addition of 5 mmol/l NH_4Cl to the medium or the substitution of the medium Cl^- with $gluconate^-$ also produced significant inhibition of glucose-induced priming (data not shown).

DISCUSSION

The results of this study demonstrate that the induction of TDP by glucose is critically dependent on the intracellular

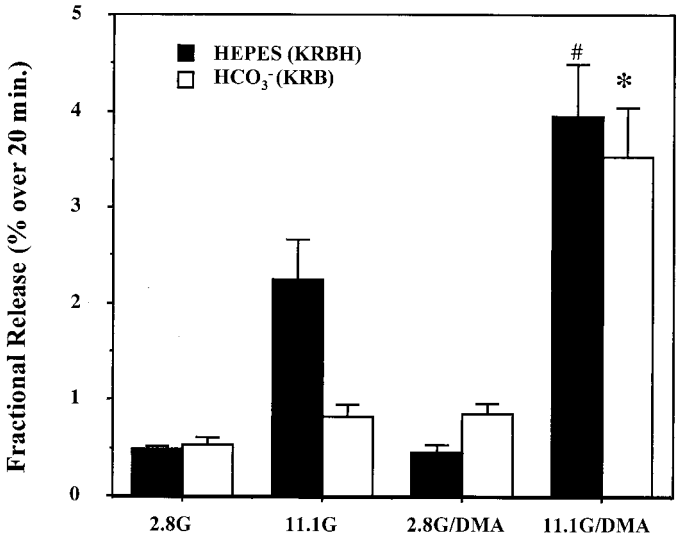

FIG. 5. Under Ca^{2+}-free conditions, intracellular acidification by DMA allows glucose-induced priming in KRB and enhances glucose-induced priming in KRBH. Priming was performed in the absence of Ca^{2+} with 5 mmol/l EGTA, in HEPES and HCO_3^--buffered media (KRBH and KRB). The rest of the experiment was carried out in Ca^{2+}-containing KRBH. During the priming period, islets were exposed to 2.8 and 11.1 mmol/l glucose, with or without 40 μmol/l DMA (n = 5). G, mmol/l glucose in the priming period; *P < 0.001, compared with the primed response in KRB; #P < 0.05 compared with the primed response in KRBH. Absolute secretion rates expressed as pg · islet^{-1} · min^{-1} (during 20 min) are as follows: unprimed islets in KRBH, 14.5 ± 2.6; unprimed islets in KRB, 12.5 ± 1.3; primed islets in KRBH, 56.8 ± 12.2; primed islets in KRB, 22.4 ± 3.1; unprimed islets in KRBH with 40 μmol/l DMA, 9.1 ± 1.0; unprimed islets in KRB with 40 μmol/l DMA, 13.7 ± 3.2; primed islets in KRBH with 40 μmol/l DMA, 76.9 ± 7.6; primed islets in KRB with 40 μmol/l DMA, 63.9 ± 8.8.

pH and not directly on the presence of extracellular Ca^{2+}. The first observation that eventually led to this conclusion was the ability of glucose to induce TDP under stringent Ca^{2+}-free conditions, in surprising contrast to the results of all previous studies on the subject. In previous investigations, conducted with rat islets in Ca^{2+}-free HCO_3^--buffered medium, the use of 0.5–1.0 mmol/l EGTA during the priming period was sufficient to completely abolish TDP (3–7). In our study, however, glucose consistently induced TDP in a Ca^{2+}-free HEPES-buffered medium, despite the presence of EGTA in as high a concentration as 5 mmol/l. The fold-change over the unprimed response was higher in the absence of Ca^{2+}, despite the possible attenuation of β-cell performance caused by a 45-min exposure to Ca^{2+}-free conditions.

An important difference between HEPES and HCO_3^- buffering systems is that HEPES buffer maintains a lower intracellular pH in the islets than does HCO_3^- buffer, as reported in previous studies (8–11). This effect is particularly evident in the presence of a stimulatory concentration of glucose. Shepherd and Henquin (8) demonstrated that the intracellular pH in mouse islets was 0.03 units lower in a HEPES-buffered medium than in an HCO_3^--buffered medium, and this was further decreased by 0.15 units when stimulated by high glucose. Similar results were reported in a previous study (10) in which the addition of HCO_3^- to a HEPES-buffered medium resulted in the increase of intracellular pH in mouse islets by 0.08–0.09 units. In rat islets, glucose-induced increase in intracellular pH was reversed by the absence of HCO_3^- (11). The proposed mechanism for this difference in intracellular pH between HEPES and HCO_3^--buffered media is as follows. The initial rise in intracellular pH caused by glucose as a result of the mitochondrial H^+ uptake during the oxidation of pyruvate (27) is later reversed by the influx of Ca^{2+}, which tends to lower the pH. The β-cell attempts to compensate for this fall in intracellular pH by activating the intracellular buffering systems, predominantly the Na^+/H^+ exchanger and the Cl^-/HCO_3^- exchanger. In a HEPES-buffered medium in which the concentration of HCO_3^- ions is significantly low, the buffering is carried out mainly by the Na^+/H^+ exchanger. In a HCO_3^--buffered medium, however, both exchangers are effective, resulting in overcompensation for the Ca^{2+}-induced acidification and stabilizing the intracellular pH at a higher point (8).

The acidifying effect of HEPES was confirmed in our study, in which the addition of HEPES to a HEPES-free HCO_3^- medium produced a dramatic decrease in the intracellular pH. The basal intracellular pH in our HEPES-buffered medium was 7.1 ± 0.02, significantly lower than that in an HCO_3^--buffered medium. Islets in HEPES-free KRB maintained an intracellular pH above 7.4, which was consistently decreased by the addition of HEPES. As in previous studies, we also noticed the tendency of high glucose to increase the intracellular pH in an HCO_3^--buffered medium and to decrease it in a HEPES-buffered medium, albeit to a lesser degree. The presence of Ca^{2+}, however, did not seem to produce any significant change in the intracellular pH of unstimulated or glucose-stimulated islets. The presence of HEPES uniformly produced a lower intracellular pH in islets, perhaps to a slightly

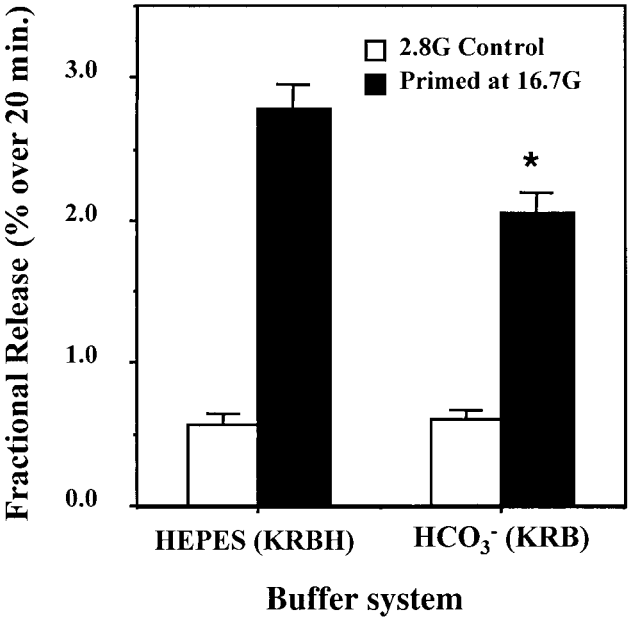

FIG. 6. In the presence of Ca^{2+}, the magnitude of glucose-induced priming is higher in KRBH than in KRB. The whole experiment was done in KRBH except for the priming period, in which two groups of islets were exposed to KRB; 2.5 mmol/l Ca^{2+} was present in the medium throughout the experiment (n = 3). G, mmol/l glucose in the priming period; *P < 0.05, compared with the primed response in HEPES-buffered medium. Absolute secretion rates expressed as pg · islet^{-1} · min^{-1} (during 20 min) are as follows: unprimed islets in KRBH, 12.3 ± 1.4; primed islets in KRBH, 65.7 ± 7.2; unprimed islets in KRB, 12.5 ± 1.4; primed islets in KRB: 49.7 ± 5.0.

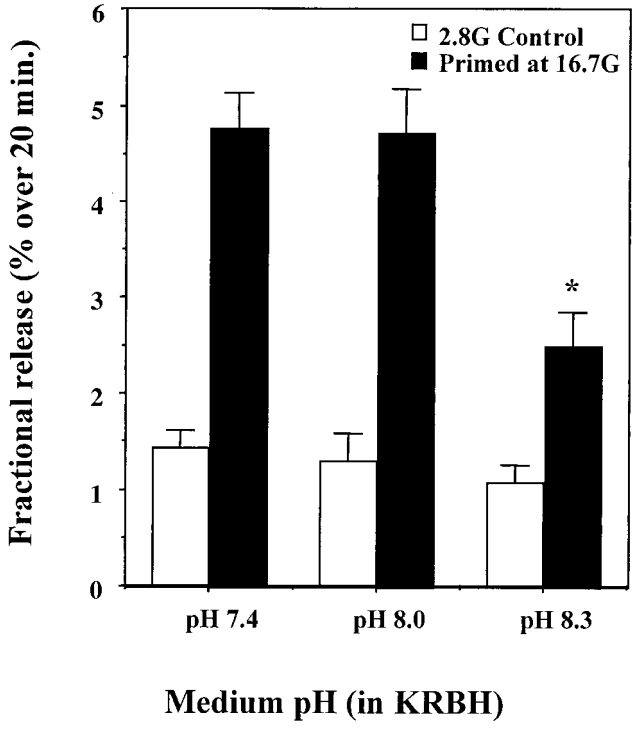

FIG. 7. Intracellular alkalinization produced by increasing the medium pH inhibits glucose-induced TDP. The experiments were conducted in Ca^{2+}-containing KRBH. During the priming period, the islets were exposed to basal and high glucose in Ca^{2+}-containing KRBH with the medium pH fixed at 7.4, 8.0, and 8.3 (n = 5). G, mmol/l glucose in the priming period; *P < 0.05 compared with the primed response at pH 7.4. Absolute secretion rates expressed as pg · islet^{-1} · min^{-1} (over 20 min) are as follows: unprimed islets at pH 7.4, 33.8 ± 3.5; primed islets at pH 7.4, 110 ± 6.7; unprimed islets at pH 8.0, 25.3 ± 5.1; primed islets at pH 8.0, 95.6 ± 8.1; unprimed islets at pH 8.3, 28.8 ± 4.2; primed islets at pH 8.3, 69.2 ± 6.4.

greater extent in the presence of Ca^{2+}. Because the acidifying effect of HEPES is consistently seen even in the absence of glucose stimulation, it is evident that HEPES lowers intracellular pH by mechanisms other than the inadequate compensation for glucose-induced acidification described in earlier studies (8,20). Strong evidence for such a pH-lowering property of HEPES comes from the fact that the addition of HEPES brings the intracellular pH down to 7.0–7.1 even in the presence of 25 mmol/l HCO_3^-, which would maintain a fully operative HCO_3^-/Cl^- exchanger (Fig. 3*B* and *D*). Hence, although an inoperative HCO_3^-/Cl^- exchanger contributes to the lower islet pH maintained in media buffered by HEPES alone, there must be another major mechanism whereby HEPES decreases intracellular pH independent of glucose-stimulation, medium Ca^{2+}, or HCO_3^-.

In view of this major difference between the two buffer systems, we considered the possibility that the lower intracellular pH in islets incubated in a HEPES-buffered medium may be permissive for the induction of TDP by glucose. If this hypothesis were true, then lowering the intracellular pH by experimental means in islets maintained in a HCO_3^--buffered medium should allow glucose to induce TDP. Hence, we investigated the priming ability of glucose in the presence of DMA, a powerful acidifying agent that decreases the intracellular pH by inhibiting the Na^+/H^+ exchanger. As shown in Fig. 4, DMA induced a persistent decrease in the intracellular pH in islets by 0.2 units or more, indicating this to be a suitable acidifying agent for this purpose. Glucose-induced priming is not normally evident in HCO_3^--buffered media in the absence of Ca^{2+}, as shown in several previous studies (3–7) as well as this one (Fig. 2). In the presence of DMA, however, priming was induced by 11.1 mmol/l glucose in a stringently Ca^{2+}-free HCO_3^--buffered medium containing 5 mmol/l EGTA (Fig. 5). In HEPES buffer in which glucose consistently induces priming regardless of the presence of extracellular Ca^{2+}, the magnitude of the primed response was significantly enhanced by the presence of DMA (Fig. 5) as well as by the acidification produced by acetate (data not shown). Furthermore, glucose-induced priming was consistently inhibited by intracellular alkalinization produced by increasing the medium pH (Fig. 7) or other methods in both the presence and absence of extracellular Ca^{2+}. All of these findings are strong evidence for the dependence of glucose-induced TDP on the intracellular pH and establish that glucose requires an appropriately low intracellular pH to induce TDP.

Under Ca^{2+}-free conditions, the critical intracellular pH below which glucose can induce TDP in rat islets is close to 7.2. In situations in which glucose can induce strong priming in the absence of Ca^{2+}, such as in KRBH or in KRB acidified by DMA, the islet intracellular pH is always maintained at or below 7.2 (equivalent to an H^+ concentration of 63 nmol/l). In the presence of Ca^{2+}, glucose can induce TDP in KRB as well as KRBH, as long as the islet intracellular pH is maintained at an appropriate level. When the intracellular pH is driven above 7.7 (equivalent to an H^+ concentration of 20 nmol/l) by experimental alkalinizing methods, the TDP normally present in KRBH is inhibited irrespective of the presence of Ca^{2+}. When the H^+ ion concentration in the cell is maintained above 63 nmol/l (i.e., at pH 7.2 and lower), TDP occurs irrespective of the presence of Ca^{2+}. Therefore, it can be concluded that a major factor that allows glucose to induce TDP is an adequate concentration of intracellular H^+ (at or higher than 63 nmol/l). In the range of H^+ concentration between 20 and 63 nmol/l (pH 7.7–7.2), however, Ca^{2+} plays a major role in priming. In a HEPES-free HCO_3^--buffered medium, when the islet H^+ concentration is maintained within the range of 20–63 nmol/l, glucose induces TDP in the presence of Ca^{2+} but not in its absence, albeit to a lesser degree than in the presence of HEPES. In this situation, the presence of Ca^{2+} overrides the deficit in H^+ ions. This brings us to the conclusion that depending on the circumstances, either a high intracellular H^+ concentration or normal intracellular Ca^{2+} concentrations are required for priming to occur. In other words, either one of the two cations is permissive for glucose-induced TDP and both may be acting on the same site(s). Furthermore, the combination of normal Ca^{2+} conditions and an experimentally increased H^+ concentration (e.g., in the presence of DMA) results in enhanced glucose-induced TDP. This suggests that they both can act simultaneously to optimize TDP.

In seeking the mechanism by which glucose induces TDP, it seems appropriate to look for reactions that are both pH- and Ca^{2+}-sensitive. It is widely known that certain mitochondrial enzymes are activated by Ca^{2+} and that energy and/or signal production after exposure to

stimulatory glucose concentrations would be impaired in the absence of extracellular Ca^{2+}. Among such enzymes are isocitrate dehydrogenase (ICDH) and α-ketoglutarate dehydrogenase (αKGDH). Although ICDH and αKGDH can act in the absence of Ca^{2+}, their activity is markedly enhanced by Ca^{2+}. Importantly, αKGDH is also activated by low pH (28–30), and glutamate dehydrogenase, another enzyme that enhances nutrient metabolism by providing anaplerotic input, is also sensitive to pH (31). Thus, one possibility for the results observed in this study is that Ca^{2+}- and pH-activated enzymes are responsible for the production of the signals that control TDP. Under normal physiological conditions, Ca^{2+} plays the major role in driving these reactions. Under nonphysiological conditions in the absence of Ca^{2+}, activation by low pH can take over the role of Ca^{2+} and provide a sufficient activating force to maintain the signals to TDP. Activation by both Ca^{2+} and H^+ leads to optimal TDP.

The importance of intracellular pH in the induction of priming by glucose is not surprising, considering all of the documented effects of intracellular pH on nutrient-induced acute insulin release. The influence of intracellular pH on insulin release has been the subject of a number of studies, the majority of the reports indicating that a lower intracellular pH favors increased insulin release. In studies on rat islets, Best et al. (14–16) used weak acids (acetate, propionate, and formate) and amiloride to decrease intracellular pH and NH_4Cl to increase it, while measuring intracellular pH with BCECF. The weak acids caused a rapid fall in the pH corresponding to 0.2 pH units, followed by a slow return to the basal value. Amiloride, an inhibitor of the Na^+/H^+ exchanger in the β-cell, produced a rapid fall in intracellular pH without recovery, and NH_4Cl induced a rise in pH of ~0.15 pH units. Nutrient-induced insulin release was observed to be enhanced with intracellular acidification, as were inositol lipid metabolism (14), reduction of $45Ca^{2+}$ and $86Rb^+$ outflow (15), and increase in intracellular Ca^{2+} (16). The opposite effects were observed on intracellular alkalinization with NH_4Cl. Similar results were reported in a number of other studies performed on mouse islets and insulinoma cell lines as well as on rat islets (12,13,17–20).

As demonstrated by these studies, nutrient-induced insulin release and related functions are favorably influenced by a decrease in intracellular pH. The data from our investigation show that priming, another important nutrient-induced effect in the β-cell, is also dependent on the intracellular pH. Work is currently under way to identify the specific pH-sensitive messengers involved in priming. Understanding the mechanisms of TDP is important in designing therapeutic approaches to type 2 diabetes. In certain patients who have diabetes and whose acute insulin secretory response is severely impaired, TDP remains intact and the impaired insulin response to glucose can be corrected by inducing TDP (32–34). In other patients in whom both the TDP and acute secretory response are impaired, correction of the defect in TDP may lead to correction of the acute secretory response. Hence, documenting the mechanisms of TDP could help in the design of new therapeutic approaches to type 2 diabetes, and the pH-sensitivity of TDP described in this study would be a useful tool in uncovering the underlying mechanisms.

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