"Glucose-induced cytosolic pH changes in beta-cells and insulin secretion are not causally related: studies in islets lacking the Na+/H+ exchange R NHE1."

Stiernet, Patrick ; Nenquin, Myriam ; Moulin, Pierre ; Jonas, Jean-Christophe ; Henquin, Jean-Claude

ABSTRACT

The contribution of Na(+)/H(+) exchange (achieved by NHE proteins) to the regulation of beta-cell cytosolic pH(c), and the role of pH(c) changes in glucose-induced insulin secretion are disputed and were examined here. Using real-time PCR, we identified plasmalemmal NHE1 and intracellular NHE7 as the two most abundant NHE isoforms in mouse islets. We, therefore, compared insulin secretion, cytosolic free Ca(2+) ([Ca(2+)](c)) and pH(c) in islets from normal mice and mice bearing an inactivating mutation of NHE1 (Slc9A1-swe/swe). The experiments were performed in HCO(-)(3)/CO(2) or HEPES/NaOH buffers. PCR and functional approaches showed that NHE1 mutant islets do not express compensatory pH-regulating mechanisms. NHE1 played a greater role than HCO(-)(3)-dependent mechanisms in the correction of an acidification imposed by a pulse of NH(4)Cl. In contrast, basal pH(c) (in low glucose) and the alkalinization produced by high glucose were independent of NHE1. Dimethylamiloride, a classic...

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Glucose-induced Cytosolic pH Changes in β-Cells and Insulin Secretion Are Not Causally Related

STUDIES IN ISLETS LACKING THE NA⁺/H⁺ EXCHANGER NHE1

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The contribution of Na⁺/H⁺ exchange (achieved by NHE proteins) to the regulation of β-cell cytosolic pH, and the role of pH changes in glucose-induced insulin secretion are disputed and were examined here. Using real-time PCR, we identified plasmalemmal NHE1 and intracellular NHE7 as the two most abundant NHE isoforms in mouse islets. We, therefore, compared insulin secretion, cytosolic free Ca²⁺ ([Ca²⁺]c) and pHc in islets from normal mice and mice bearing an inactivating mutation of NHE1 (Slc9A1-swe/swe). The experiments were performed in HCO₃⁻/CO₂ or HEPES/NaOH buffers. PCR and functional approaches showed that NHE1 mutant islets do not express compensatory pH-regulating mechanisms. NHE1 played a greater role than HCO₃⁻ in stimulus-secretion coupling. The changes in [Ca²⁺]c further (5, 8–10).

Glucose induces changes in β-cell cytosolic pH (pHc), the mechanisms and role of which in stimulus-secretion coupling are still debated. Except for two studies reporting no effect of glucose on pHc in mouse β-cells (11) or an acidification in rat islets (12), there is a large consensus that high glucose increases pHc in mouse islets (13–17), rat islets (18, 19) and insulin-secreting cell lines (20, 21). In contrast, there is no agreement on the mechanisms implicated in this alkalinization. Activation of unidentified HCO₃⁻/Cl⁻ exchangers (15), Na⁺/H⁺ exchangers (14, 19, 20) or both (18) has been suggested using pharmacologically based experiments.

Normal glucose homeostasis requires precise regulation of insulin secretion, a complex process that pancreatic β-cells achieve through changes in their metabolism (1–4). Current models ascribe the control of insulin secretion by glucose to two hierarchical signaling pathways (5). The triggering pathway involves closure of ATP-sensitive K⁺ channels (Kir₆.2), membrane depolarization, Ca²⁺ influx through voltage-dependent Ca²⁺ channels, and a rise in the cytosolic Ca²⁺ concentration ([Ca²⁺]c), which is the indispensable triggering signal for exocytosis of insulin granules (4–7). Simultaneously, a still incompletely understood amplifying pathway is activated, which augments the amount of released insulin without increasing [Ca²⁺]c further (5, 8–10).

Glucose induces changes in β-cell cytosolic pH (pHc), the mechanisms and role of which in stimulus-secretion coupling are still debated. Except for two studies reporting no effect of glucose on pHc in mouse β-cells (11) or an acidification in rat islets (12), there is a large consensus that high glucose increases pHc in mouse islets (13–17), rat islets (18, 19) and insulin-secreting cell lines (20, 21). In contrast, there is no agreement on the mechanisms implicated in this alkalinization. Activation of unidentified HCO₃⁻/Cl⁻ exchangers (15), Na⁺/H⁺ exchangers (14, 19, 20) or both (18) has been suggested using pharmacologically based experiments.

Even more confusing is the issue of a possible role of these pHc changes in insulin secretion. Studies in which changes in β-cell pHc were imposed by manipulation of pH and ionic composition of the extracellular medium or by exposure to weak bases or acids have led to the contradictory proposals that alkalinization augments (14, 22) or inhibits (23) insulin secretion, and that acidification increases it (24–26). Other studies, relying on amiloride derivatives to inhibit Na⁺/H⁺ exchange (27, 28) and cause β-cell acidification, also concluded that low pHc is beneficial to glucose-induced insulin secretion (19, 24–26). Glucose-induced priming of insulin secretion (i.e. augmentation of the response to a second stimulation), which is thought to be mediated by the amplifying pathway (29), has been linked to β-cell acidification (26, 30). These proposals are at odds with the fact that glucose increases islet pHc and with the correlative evidence that only those fuels which increase β-cell pHc amplify insulin secretion (31). It is thus unclear whether a Na⁺/H⁺ exchanger is important for glucose-induced pHc changes in β-cells, and...
whether these changes play a role in the stimulation of insulin secretion, through one or both pathways. These were the questions addressed in the present study.

To date, ten isoforms of Na+/H+ exchangers (NHE1 to NHE10), encoded by the Slc9 family of genes, have been identified (32–34). We recently showed that NHE1 is strongly expressed in rat islets and is present in mouse islets (35). Here, we first compared the expression of NHE isoforms in mouse islets. Having found that NHE1 is, by far, the most abundant among the plasma membrane isoforms, we next studied Slc9A1swe/swe mice (swe: slow wave epilepsy) mutants to test the role of Na+/H+ exchange in β-cell pHc regulation under basal conditions and glucose stimulation. Finally, we measured pHc, [Ca2+]c and insulin secretion in control and NHE1 mutant islets perifused with HCO3
-
containing and HCO3
-
free solutions to assess the possible role of pHc in glucose stimulation of insulin secretion through the triggering and amplifying pathways.

EXPERIMENTAL PROCEDURES

Solutions and Reagents—A bicarbonate-buffered medium (HCO3
-
medium) and a HEPES-buffered medium (HEPES medium) were used. The HCO3
-
medium contained 120 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl2, 1.2 mM MgCl2, 24 mM NaHCO3, and 1 mg/ml bovine serum albumin and was gassed with O2/CO2 (94:6%) to maintain pH at 7.4. The HEPES medium contained 135 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl2, 1.2 mM MgCl2, 10 mM HEPES, and 1 mg/ml bovine serum albumin and was gassed with O2. Its pH was adjusted to 7.4 with NaOH. When the concentration of KCl was increased to 30 mM, that of NaCl was decreased accordingly. Diazoxide was a gift from Schering-Plough, Brussels. Fura PE3-AM and BCECF-AM were obtained from Molecular Probes. Cariporide (50 mM) and DIDS (150 mM) were pre-
pared in Me2SO, and aliquots were added to test solutions. The solvent alone had no effects.

Animals—C57BL/6.SIL+/swe (swe: slow wave epilepsy) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). These heterozygous mice were mated, and genotyping of the pups was performed as previously described (36). Homozygous NHE1 mutant mice (Slc9A1swe/swe, NHE1 mutant) and wild-type littermates (Slc9A1+/+, control) were studied at 7–9 weeks of age.

Preparations—Islets were aseptically isolated by collagenase digestion of the pancreas of NHE1 mutant and control mice (37). After hand selection, the islets were cultured for about 18 h at 37 °C in RPMI 1640 medium containing 10 mM glucose, 10% heat-inactivated fetal bovine serum, 100 international units/ml penicillin and 100 μg/ml streptomycin.

RNA Extraction and Real-time Quantitative PCR—Total RNA was extracted, quantified, and reverse-transcribed into cDNA as previously described (38) using random hexamers and 200 units of M-MLV Reverse Transcriptase RNase H– Point Mutant (Promega, Madison, WI). The sense and antisense primers were chosen in the coding region of gene mRNA sequences, and their specificity was checked by BLAST search in the GenBank™ data bank (Table 1). Real-time PCR was performed with the iCycler iQ Real-Time PCR detection system (Bio-Rad) using the fluorescent dye SYBR Green I to monitor DNA amplification. The reaction was performed in duplicate, in a 25-μl reaction volume containing cDNA (2–20 ng of total RNA equivalents), 300 nM primers (Table 1), 12.5 μl iQ superMIX (Bio-Rad), and water to volume. TATA box-binding protein (TBP) was used as a reporter gene. Under these conditions, PCR efficiencies for amplification of NHE isoforms and TBP were similar. For all NHE isoforms, real-time PCR was performed in parallel in islets and control tissues mRNA (Table 1), with positive amplification in controls. After amplification, the specificity of PCR products was checked by agarose gel electrophoresis and analysis of the melting curve. The threshold cycle (Ct) was determined using iCycler iQ software 3.0. After

**TABLE 1**

Sequence of oligonucleotide primers and control tissues used for real-time PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Size of product (bp)</th>
<th>Control tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBP</td>
<td>Sense: 5′-ACCCTTCACGATTGACTCTAG-3′</td>
<td>157</td>
<td></td>
</tr>
</tbody>
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correction of the Cts for differences in cDNA input in the PCR, the ΔCt values were calculated for each sample in each NHE isoform as follows: ΔCtNHEX = Ct(NHEX) - Ct(TBP). The NHEx over TBP mRNA ratios can be estimated by the formula 2-ΔCt (39).

Measurements of Islet [Ca2+]i and pH—For [Ca2+]i measurements, islets were loaded with Fura-PE3 (2 μM, 2 h at 37 °C) in 1 ml HCO3 - or HEPES medium, depending on the medium used during the subsequent experiment. After loading, the islets were transferred into a chamber mounted on the stage of a microscope and maintained at 37 °C. The Fura-PE3 probe was excited at 340 and 380 nm, and emission was captured at 510 nm with a CCD camera. From the ratio of fluorescence (at 340 and 380 nm), [Ca2+]i was calculated by comparison with a calibration curve. Details of the method have been reported elsewhere (40). For measurements of pH, islets were loaded with BCECF (0.5 μM, 2 h at 37 °C) in 1 ml of HCO3 - or HEPES medium. They were then examined in the same system as that used for Ca2+, but the probe was excited at 440 and 490 nm and emission was captured at 535 nm. The pH was calculated from an in situ calibration curve constructed from the ratio values obtained by perfusing loaded islets with solutions of different pH (between 6 and 7.5) containing 10 μg/ml nigerin. The medium had the following composition: 136 mM KCl, 4 mM NaCl, 5 mM MgCl2, 5 mM glucose, and 20 mM HEPES.

Measurements of Insulin Secretion and Islet or Pancreas Insulin Content—Batches of 20 cultured islets were transferred into chambers of a perifusion system (41). The effluent was collected at 42-s or 2-min intervals and saved for measurement of insulin by radioimmunoassay using rat insulin as a standard and ethanol to precipitate bound insulin (42). A small volume of the NaHCO3 or neutralized HEPES solution was used for Ca2+ extraction and measurement. The insulin content of the whole pancreas of NHE1 mutant and control mice was determined after acid-ethanol precipitation and measurement. The insulin content of the whole pancreas of NHE1 mutant and control mice was determined after extraction by homogenization and sonication of the tissue in acid ethanol.

Morphological Studies—The pancreas of control and NHE1 mutant mice was fixed in formalin for 24 h and embedded in paraffin. Glucagon cells were immunostained as described elsewhere (43) using a mouse monoclonal antibody (GLU-001, NovoBioMabs, Bagsvaerd, Denmark). Stained pancreas slides were digitized through a Zeiss microscope coupled to a DAGE-MTI CCD camera (Michigan City, IN). Fields containing islets (5–11 per slide) were captured through a ×10 objective (0.727 × 0.758 μm/pixel). Images were analyzed with a Zeiss KS400 imaging system. Islets were delineated by the user, and glucagon cells were identified by their gray level.

Presentation of Results—Results are presented as means ± S.E. for the indicated number of animals or experiments. The statistical significance of differences between control and NHE1 mutant mice was evaluated by unpaired Student’s t test. The effect of various drugs on insulin secretion was assessed by analysis of variance followed by a Dunnett’s test for multiple comparisons with controls.

RESULTS

Identification of NHE Isoforms Expressed in Mouse Islets—We used real-time PCR to compare the expression of the 10 known isoforms of NHE in control mouse islets (Fig. 1). The amount of mRNA for each isoform was reported to that of TBP. Among the isoforms present in the plasma membrane (NHE1 to NHE5 and NHE10) (32, 34), NHE1 was the most largely expressed. Although primers and conditions of PCR amplification used for the 10 isoforms were different, a cautious comparison indicates that NHE1 expression is about 50-fold higher than NHE5, 450-fold higher than NHE2 and 1800-fold higher than NHE4. No NHE3 was detected even after an amplification of 50 cycles. Of the organelar isoforms of NHE (NHE6 to NHE9) (32, 33), NHE7 was strongly expressed, ~50-fold more than the other three.

Because NHE1 was, by far, the most abundant plasma membrane isoform in normal islets, mice with an inactivating mutation of NHE1 (36) could be a useful model to study the role of the Na+/H+ exchanger in insulin secretion. As expected, no NHE1 was detected in NHE1 mutant mouse islets, which expressed the other isoforms as in controls, with only two exceptions. The plasma membrane isoforms NHE2 and 4 were more expressed in NHE1 mutant than control islets, but this expression remained very low compared with that of NHE1 in control islets, and lower than that of NHE5 in both types of islets (Fig. 1).

Characteristics of Control and NHE1 Mutant Mice—As previously reported, NHE1 mutant mice (Slc9a1swe/swe) exhibited ataxia in the hind limbs and seizures, and grew more slowly than controls (36). Although many died of their neurological problems a few weeks after weaning, we found it possible to
Control islets are shown by a HCO_3^− removed as indicated. DMA (40 μM) prolonged the lifespan of the mice by providing food and water close to the floor of the cages. The in vitro experiments using isolated islets were performed with control and NHE1 mutant mice of a mean age of 54 days (range 45–63). Table 2 compares NHE1 mutant and age-matched control mice. The body weight of NHE1 mutant mice was ∼30% smaller. Their blood glucose was not different, but their plasma insulin was lower. The insulin content of the pancreas was lower, but not different from controls when expressed relative to the mass of pancreas or to body weight, indicating that there is no specific impact of the lack of NHE1 on β-cell development. Microscopic examination of the pancreas of NHE1 mutant mice showed that the morphological organization of the islets was normal, with a regular to oval shape and peripheral localization of glucagon cells. However, the average diameter of the islets was smaller (142 ± 7 μm, n = 63) than in control mice (193 ± 8 μm, n = 76, p < 0.007), which may explain the 33% lower insulin content of the NHE1 mutant islets that we used for in vitro experiments (Table 2).

Role of NHE1 Exchanger in Islet pH_c Regulation—Previous studies, using pharmacological tools, have suggested that pH_c regulation in β-cells involves unidentified HCO_3^−/Cl^− and Na^+/?H^+ exchangers (14, 15, 18, 44). Basal pH_c (in 3 mM glucose) measured during the first 5 min of the experiments was not different in NHE1 mutant islets (7.03 ± 0.01, n = 175) and control islets (7.06 ± 0.01, n = 203) in HCO_3^- medium, but it was lower (p < 0.001) in NHE1 mutant islets (6.78 ± 0.01, n = 117) than control islets (7.02 ± 0.01, n = 164) in HEPES medium. It should be noted, however, that basal pH_c decreased with time and did so more markedly in HEPES than HCO_3^− medium. Lower pH_c values would thus be obtained at later times of the experiments, without affecting the comparison between NHE1 mutant and control islets.

To ascertain that the lack of NHE1 was not compensated for by other pH_c-regulating mechanisms, we compared the ability of control and NHE1 mutant islets to correct an intracellular acidification produced by a prepulse of NH_4Cl (Fig. 2). The islets were perfused with a HCO_3^- medium in which both HCO_3^- dependent mechanisms and Na^+/?H^+ exchangers can function, or with a HEPES medium in which only Na^+/?H^+ exchangers can operate. In the HCO_3^- medium, the correction of the acidification was rapid in control islets and delayed in NHE1 mutant islets (Fig. 2A). Dimethylamiloride (DMA), an inhibitor of Na^+/?H^+ exchangers (27, 28), had no effect on the correction of pH_c in NHE1 mutant islets but impaired the correction in control islets, as did the mutation of NHE1 in test islets (Fig. 2B). In contrast, DIDS, an inhibitor of HCO_3^-/Cl^- exchangers (45, 46), blocked the pH_c correction in NHE1 mutant islets but had only little effect in control islets (Fig. 2C) because of the operation of Na^+/?H^+ exchange. Thus, the simultaneous addition of DIDS and DMA completely prevented pH_c correction in control islets (Fig. 2D) as did DIDS alone in NHE1 mutant islets (Fig. 2C). In HEPES medium, when HCO_3^- dependent pH regulation is inoperative, the correction of acidification by control islets was slightly slower than in the HCO_3^- medium, and no correction occurred in NHE1 mutant islets (Fig. 2E). That the correction observed in control islets is mediated by the Na^+/?H^+ exchange is shown by its suppression with DMA (Fig. 2F). EIPA and cariporide, two other inhibitors of Na^+/?H^+ exchanges, showed a similar effect.

### Table 2

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controls</th>
<th>NHE1 mutant</th>
</tr>
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<tbody>
<tr>
<td><strong>Age (days)</strong></td>
<td>54 ± 0.6 (62)*</td>
<td>54 ± 0.6 (63)*</td>
</tr>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>19.1 ± 0.4 (62)</td>
<td>13.7 ± 0.3 (63)*</td>
</tr>
<tr>
<td><strong>Blood glucose (mM)</strong></td>
<td>8.9 ± 0.2 (62)</td>
<td>8.5 ± 0.2 (63)</td>
</tr>
<tr>
<td><strong>Plasma insulin (ng/ml)</strong></td>
<td>1.3 ± 0.1 (45)</td>
<td>0.7 ± 0.1 (47)*</td>
</tr>
<tr>
<td><strong>Pancreatic weight (mg)</strong></td>
<td>182 ± 5 (7)</td>
<td>120 ± 4 (8)*</td>
</tr>
<tr>
<td><strong>Pancreatic insulin (μg)</strong></td>
<td>92.0 ± 7.0 (7)</td>
<td>55.1 ± 3.8 (8)*</td>
</tr>
<tr>
<td><strong>Pancreatic insulin (μg/g)</strong></td>
<td>0.48 ± 0.03 (7)</td>
<td>0.47 ± 0.03 (8)</td>
</tr>
<tr>
<td><strong>Islet insulin (μg/islet)</strong></td>
<td>4.5 ± 0.3 (7)</td>
<td>4.0 ± 0.2 (8)</td>
</tr>
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</tr>
</tbody>
</table>

*Values are means ± S.E. for (n) animals or measurements. 
*p < 0.001 by Student’s t test.

### Figure 2

**Role of NHE1-exchanger in islet pH_c regulation.** The NH_4Cl prepulse technique was used to study how islet cells correct the cytosolic acidification following removal of NH_4Cl from the extracellular medium. Control islets are shown by thin lines and NHE1 mutant islets by thick lines. The experiments were performed in a HCO_3^- medium (A–D) or a HEPES medium (E–F) containing 3 mM glucose (G3), with NH_4Cl (20 mM) added and removed as indicated. DMA (40 μM), or DIDS (200 μM) were added for 10 min (B, C, F). In D, both drugs were added together for 10 min before DIDS was omitted while DMA remained present for another 10 min. Values are means ± S.E. for 31–58 islets (from 5 to 10 preparations).
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exchange (27) produced similar effects to DMA in controls and were ineffective in NHE1 mutant islets (data not shown). Overall, these results confirm that both Na⁺/H⁺ and HCO₃⁻-dependent mechanisms contribute to the regulation of pHᵢ in normal β-cells (15), show that no mechanism has compensated for the lack of NHE1 in mutant islets, and indicate that Na⁺/H⁺ exchange is more important than HCO₃⁻-dependent mechanisms for correction of an imposed acidification.

Effects of Na⁺/H⁺ Exchange Blockers on Insulin Secretion—Because DMA and the genetic loss of NHE1 produced similar effects on pHᵢ, one could argue that the pharmacological approach is adequate to study the impact of Na⁺/H⁺ exchange and pHᵢ on β-cell secretory function. The following experiments show that this is not the case. When control islets were perfused with a HCO₃⁻ medium containing 15 mM glucose, DMA produced a small, rapid, and reversible decrease in pHᵢ in ~50% (19/36) of the islets (Fig. 3A) and a consistent, delayed, irreversible increase in insulin secretion (Fig. 3B). As anticipated, DMA did not affect pHᵢ in NHE1 mutant islets (Fig. 3A) but, most unexpectedly, increased insulin secretion as in controls (Fig. 3B). In a second series of experiments, we compared the effects of 3 inhibitors of Na⁺/H⁺ exchange. Although they had virtually no effect on pHᵢ under these conditions (data not shown), DMA and EIPA markedly potentiated glucose-induced insulin secretion (p < 0.01), while the trend observed with cariporide did not reach statistical significance. Again the effect of the three drugs was similar in control and NHE1 mutant islets (Fig. 3C). Taken together, these results indicate that the increase in insulin secretion produced by these inhibitors is unrelated to a change in pHᵢ but is caused by an action of the drugs on a target other than the Na⁺/H⁺ exchanger. DMA and related substances cannot reliably be used to evaluate the influence of pHᵢ on insulin secretion.

Influence of pHᵢ on Glucose-induced Insulin Secretion—In HCO₃⁻ medium, pHᵢ of control islets decreased slowly when the glucose concentration remained low (3 mM), and increased when the glucose concentration was raised to 15 mM (Fig. 4A) (15). The stimulation by high glucose also produced typical changes in [Ca²⁺]ᵢ, characterized by a small initial decrease below basal values, followed by a sharp peak and oscillations during the second phase (Fig. 4B). Glucose also induced a biphasic increase in insulin secretion (Fig. 4C). In HEPES medium, basal pHᵢ decreased more rapidly and no increase occurred upon stimulation by high glucose (Fig. 4A). In contrast, the increases in [Ca²⁺]ᵢ and insulin secretion were similar to those in HCO₃⁻ medium, with only a small lag in the first phase (Fig. 4, B and C).

When NHE1 mutant islets were perfused with a HCO₃⁻ medium, basal pHᵢ slightly decreased in 3 mM glucose and increased in response to 15 mM glucose (Fig. 4D). This shows that NHE1 is not responsible for the alkalinization induced by high glucose. Glucose-induced [Ca²⁺]ᵢ and insulin secretion changes in NHE1 mutant islets followed a similar biphasic pattern as in control islets (Fig. 4, E and F). In HEPES medium, basal pHᵢ was lower than in HCO₃⁻ medium, and high glucose produced an additional marked decrease to reach a very low pH of 6.3 (Fig. 4D). This decrease in pHᵢ is due to Ca²⁺ influx as shown by its abrogation by diazoxide or chelation of extracellular CaCl₂ with EGTA (data not shown) (15). In HEPES medium, the increase in [Ca²⁺]ᵢ produced by glucose was not only delayed, but strongly inhibited, with only small and irreg-
ular oscillations during the second phase (Fig. 4E). There was, however, no evidence for desynchronization of these \([Ca^{2+}]_c\) oscillations between different regions of the islets (data not shown). Paradoxically, glucose-induced insulin secretion by NHE1 mutant islets was similar in HEPES and HCO\(_3\) media during the 50 min of stimulation (Fig. 4F). However, when the period of glucose stimulation in HEPES medium was longer, the insulin secretory rate steadily decreased in NHE1 mutant islets (data not shown).

Fig. 4, G–I show integration of the responses during the last 20 min of the experiments. During glucose stimulation in HCO\(_3\) medium, \(pH_c\) was similar but \([Ca^{2+}]_c\) was slightly higher \((p < 0.05)\) in NHE1 mutant than control islets, which can account for the trend to a higher insulin secretory response. The interpretation of the experiments performed in HEPES medium is complicated by the interference that low \(pH_c\) can cause with \([Ca^{2+}]_c\) measurements based on EGTA-derived probes such as Fura-PE3 (47, 48). If the measured values are corrected according to Batlle et al. (48), \([Ca^{2+}]_c\) is increased by 13% in controls (to 266 nM) and by 36% in NHE1 mutant islets (to 253 nM). In the latter case, no difference persists between \([Ca^{2+}]_c\) in NHE1 mutant islets stimulated by glucose in HCO\(_3\) and HEPES media, which is compatible with the similar secretory response despite the marked difference in \(pH_c\) (Fig. 4, G–I).

Overall, the substantial differences in \(pH_c\) did not have an impact on insulin secretion.

Influence of \(pH_c\) on the Amplifying Pathway of Insulin Secretion—The amplifying pathway of glucose-induced insulin secretion can be investigated by holding K\(_{ATP}\) channels open with diazoxide and depolarizing \(\beta\)-cells with 30 mM KCl in the presence of low or high glucose (8). Fig. 5, A, C, and E show the results obtained with control islets perfused with a HCO\(_3\) medium. \(pH_c\) was slightly higher in 15 than 1 mM glucose and barely affected by 30 mM KCl (Fig. 5A). \([Ca^{2+}]_c\) increased similarly in 1 and 15 mM glucose, following a biphasic pattern with a sharp first peak and a flat second phase (Fig. 5C). Although \([Ca^{2+}]_c\) was similar at both glucose concentrations, insulin secretion was ~3-fold larger at high than low glucose (Fig. 5E), which corresponds to the amplifying action of glu-
Insulin secretion was much larger in 15 mM glucose (Fig. 5F). A similar amplification of insulin secretion by glucose (2.6- versus 3.2-fold) thus occurred in the two buffers although $pH_c$ was markedly different.

**DISCUSSION**

The aims of our study were to determine the contribution of $Na^+/H^+$ exchange to $pH_c$ regulation in pancreatic islet cells under basal conditions and during glucose stimulation, and to assess the debated role of glucose-induced $pH_c$ changes for the stimulation of insulin secretion.

**NHE1 and Islet Cell $pH_c$ Regulation**—$Na^+/H^+$ exchange is achieved by the NHE membrane proteins, encoded by the family of *Slc9A* genes. Out of the ten known isoforms of NHE (32–34), only NHE3 was not detected at the mRNA level in mouse islets. The two most abundantly expressed isoforms were the ubiquitous NHE1 and NHE7. It is possible that some of the other, weakly expressed, isoforms are restricted to non-endocrine cells (e.g. endothelial cells) of the islets. In other cell types, NHE7 is intracellular and mainly catalyzes $K^+/H^+$ exchange (49). Its function in islet cells is unknown but, by analogy, a role in the intravesicular processing of polypeptide precursors is plausible (33). NHE1 (encoded by *Slc9A1*) was the most expressed among the isoforms associated with the plasma membrane (NHE1-NHE5 and NHE10). Using radioactive RT-PCR, we recently showed that NHE1 is strongly expressed, whereas NHE2 and NHE5 are weakly expressed, and NHE3 and NHE4 are not detected in rat islets (35).

*Slc9A1*<sup>swe/swe</sup> mutant mice bear a spontaneous point mutation, which introduces an aberrant stop codon within the coding region, and do not produce the NHE1 protein (36). We confirm (35) that the corresponding mRNA is absent from their islets and show that no significant compensatory expression of another isoform occurred. Our observations that DMA had no effect on $pH_c$ in islets from mouse.
NHE1 mutant mice established that these islet cells really lack functionally significant Na\(^+\)/H\(^+\) exchanger in their plasma membrane.

Under basal conditions (3 mM glucose), the lack of NHE1 did not affect islet pH\(_i\) in HCO\(_3^-\) medium but resulted in a marked lowering of pH\(_i\) in HEPES medium. Basal pH\(_i\) also rapidly decreased in control islets perfused with a HEPES medium, despite the presence of NHE1. This shows that HCO\(_3^-\)-dependent mechanisms are more important than Na\(^+\)/H\(^+\) exchange for basal pH\(_i\) regulation in islet cells. In contrast, NHE1 significantly contributed to normal pH\(_i\) restoration after an acid load (NH\(_4\)Cl prepulse technique). This contribution was evidenced by the slower increase in pH\(_i\) in NHE1 mutant than control islets in HCO\(_3^-\) medium, and by the ability of control islets, in contrast to NHE1 mutant islets, to correct pH\(_i\) in HEPES medium. HCO\(_3^-\)-dependent mechanisms proved less important for this correction of an acid pH\(_i\), as shown by the smaller impact of DIDS or HCO\(_3^-\) emission than of NHE1 inhibition by DMA in control islets. However, when NHE1 is lacking or inhibited, these mechanisms ensure a slow pH\(_i\) correction.

Upon stimulation by high glucose, pH\(_i\) similarly increased in NHE1 mutant and control islets. These observations conclusively establish that β-cell alkalinization by glucose is not mediated by Na\(^+\)/H\(^+\) exchange as sometimes proposed (14, 19, 20, 44). The failure of glucose to increase islet pH\(_i\) in HEPES medium points to the implication of a HCO\(_3^-\)-dependent mechanism, presumably of a HCO\(_3^-\)/Cl\(^-\) exchanger because this alkalinization is also inhibited by DMA (15). However, the exact mechanism, in particular the identity of the implicated exchangers, remains to be determined. Out of the numerous HCO\(_3^-\)/Cl\(^-\) exchangers (50), only one Na\(^+\)-dependent isoform (SLC4A10) has been identified in islet cells (51), but the presence of other isoforms has not been excluded. An intervention of NHE1 was detectable when glucose stimulation occurred in HEPES medium. Under these conditions, high glucose produced a major decrease in pH\(_i\) in NHE1 mutant islets but only had a marginal acidifying effect in control islets. These results and those of the experiments based on the NH\(_4\)Cl prepulse, therefore, indicate that Na\(^+\)/H\(^+\) exchange is mainly involved in a protection of islet cells against marked cytosolic acidification.

**NHE1 and Insulin Secretion**—Blockers of NHE1, such as amiloride and DMA (27, 28) have previously been used to study various facets of β-cell function, in addition to pH\(_i\) regulation. They have been reported to decrease ⁸⁶Rb\(^+\) efflux from preloaded islets (an indication of K\(^+\) channel closure) (19), to increase glucose-induced electrical activity in β-cells (24), and to augment insulin secretion (15, 19, 20, 26, 30). These effects were attributed to blockade of Na\(^+\)/H\(^+\) exchange, leading to cytosolic acidification and, eventually, closure of K\(_{\text{ATP}}\) channels either directly (19, 25) or via improved glucose metabolism (26). However, the decrease in pH\(_i\) by DMA, which is admitted significantly in HEPES medium, is minimal in HCO\(_3^-\) medium. Moreover, we show here that DMA and EIPA also increased insulin secretion from NHE1 mutant islets without changing pH\(_i\). In fact, studies of excised patches of cardiomyocyte (52) or β-cell (53) membranes have shown that micromolar concentrations of amiloride and derivatives can directly inhibit K\(_{\text{ATP}}\) channels by interacting with the pore. Our results do not rule out the possibility that changes in pH\(_i\) can influence K\(_{\text{ATP}}\) channels in β-cells (54, 55), but preclude the use of amiloride derivatives to test the role of Na\(^+\)/H\(^+\) exchange itself and of pH\(_i\) changes in insulin secretion. We therefore used two other approaches: the NHE1 mutant islets and the direct comparison of insulin secretion and pH\(_i\) changes in HCO\(_3^-\) and HEPES media.

Compared with control islets, glucose-induced insulin secretion was not impaired in NHE1 mutant islets. The Na\(^+\)/H\(^+\) exchanger is thus dispensable for stimulus-secretion coupling at least when the experiments are performed in a physiological HCO\(_3^-\) medium.

**β-Cell pH\(_i\) and Insulin Secretion**—Most proposals that changes in β-cell pH\(_i\) participate in stimulus-secretion coupling are based on experiments testing islets or cell lines in artificial HCO\(_3^-\)-free medium and imposing large pH\(_i\) changes by drugs (14, 19, 20, 23, 26, 30), by acidifying or alkalinizing agents (14, 22, 26, 30, 56), or by modifications of extracellular pH (22, 26, 30, 56). These large, forced excursions of pH\(_i\) (above 7.2 and below 6.8) may influence the secretory process, but the underlying mechanisms are unlikely to be physiologically relevant. It has been suggested that the optimal β-cell pH\(_i\) for nutrient-induced insulin secretion ranges between 6.4–6.8 (26). Only one of our experiments might suggest that low pH\(_i\) is favorable for insulin secretion. In NHE1 mutant islets, glucose-induced insulin secretion was similar in HEPES medium (islet pH\(_i\) ~ 6.3) and HCO\(_3^-\) medium (islet pH\(_i\) ~ 7.0) although [Ca\(^{2+}\)]\(_c\) was significantly lower in HEPES medium, and thus seemingly having a greater efficacy on exocytosis at lower pH\(_i\). However, this difference in [Ca\(^{2+}\)]\(_c\) is probably apparent only, and attributable to the interference of low pH with the measurement of [Ca\(^{2+}\)]\(_c\) (47, 48). When this confounding factor was taken into account, no beneficial influence of low pH\(_i\) on glucose-induced insulin secretion was detectable. β-Cell pH\(_i\) admittely decreases upon stimulation in HEPES medium, and even becomes very low when Na\(^+\)/H\(^+\) exchange is pharmacologically inhibited under these conditions. However, this situation is completely artificial, glucose increasing β-cell pH\(_i\) in physiological HCO\(_3^-\) medium.

An increase in islet pH\(_i\) has also been implicated in stimulus-secretion coupling and suggested to augment (14, 22) or impair (23, 30) glucose-induced insulin secretion. Our results show that the rise in pH\(_i\) that high glucose produces in HCO\(_3^-\) medium is not important for the secretory response. Thus, the absence of such an increase in pH\(_i\) in HEPES medium was not accompanied by a significant alteration of glucose-induced insulin secretion. Previous observations that all metabolized agents capable of increasing insulin secretion through the amplifying pathway were also causing an increase in islet pH\(_i\) led us to propose that β-cell alkalinization might be implicated in the amplifying process (31). The present results do not support the hypothesis. Thus, the experiments in which [Ca\(^{2+}\)]\(_c\) was increased and clamped by depolarizing the islets with KCl in the presence of diazoxide convincingly showed that the amplification of insulin secretion by high glucose is independent of pH\(_i\) or changes in pH\(_i\).

**Conclusions**—We have shown that NHE1 (plasma membrane isoform) and NHE7 (intracellular isoform) are the most
expressed Na$^+$/H$^+$ exchangers in mouse islets. NHE1 contributes to correction of an imposed acidification of $\beta$-cells, but is not implicated in the increase in pH$_c$ produced by high glucose. This alkalinization is mediated by HCO$_3^-$-dependent mechanisms but is not causally related to insulin secretion. Overall our study provides evidence against the hypotheses that either a decrease or an increase in pH$_c$ is important for glucose-induced insulin secretion via the triggering or the amplifying pathways.

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