Chloride Channels Regulate HIT Cell Volume but Cannot Fully Account for Swelling-Induced Insulin Secretion

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Insulin-secreting pancreatic islet β-cells possess anion-permeable Cl^- channels ($I_{Cl,islet}$) that are swelling-activated, but the role of these channels in the cells is unclear. The Cl^- channel blockers 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and niflumic acid were evaluated for their ability to inhibit $I_{Cl,islet}$ in clonal β-cells (HIT cells). Both drugs blocked the channel, but the blockade due to niflumic acid was less voltage-dependent than the blockade due to DIDS. HIT cell volume initially increased in hypotonic solution and was followed by a regulatory volume decrease (RVD). The addition of niflumic acid and, to a lesser extent, DIDS to the hypotonic solution potentiated swelling and blocked the RVD. In isotonic solution, niflumic acid produced swelling, suggesting that islet Cl^- channels are activated under basal conditions. The channel blockers glyburide, gadolinium, or tetraethylammonium-Cl did not alter hypotonic-induced swelling or volume regulation. The Na/K/2Cl transport blocker furosemide produced cell shrinkage in isotonic solution and blocked cell swelling normally induced by hypotonic solution. Perifused HIT cells secreted insulin when challenged with hypotonic solutions. However, this could not be completely attributed to $I_{Cl,islet}$-mediated depolarization, because secretion persisted even when Cl^- channels were fully blocked. To test whether blocker-resistant secretion occurred via a distal pathway, distal secretion was isolated using 50 mmol/l potassium and diazoxide. Under these conditions, glucose-dependent secretion was blunted, but hypotonically induced secretion persisted, even with Cl^- channel blockers present. These results suggest that β-cell swelling stimulates insulin secretion primarily via a distal $I_{Cl,islet}$-independent mechanism, as has been proposed for K_{ATP}-independent glucose- and sulfonylurea-stimulated insulin secretion. Reverse transcriptase–polymerase chain reaction of HIT cell mRNA identified a CLC-3 transcript in HIT cells. In other systems, CLC-3 is believed to mediate swelling-induced outwardly rectifying Cl^- channels. This suggests that the proximal effects of swelling to regulate cell volume may be mediated by CLC-3 or a closely related Cl^- channel. *Diabetes* 50: 992–1003, 2001

Whereas much is known about the respective roles of cations and membrane cation channels in islet function (1), by comparison, there is little detailed information available concerning the physiological role of anions or anion-handling mechanisms in islets. This is true even though it has been known for some time that islets have glucose-sensitive anion fluxes (2) and that anion substitution significantly modifies insulin secretion (2,3). In addition, anion channel blockers have been shown to strongly modulate glucose-induced islet electrical activity (4). Most of these earlier studies of the effects of anions on islet physiology presumed that anion transporters or pumps, rather than anion channels, were the predominant anion transport mechanisms of islet cells.

The discovery of a novel anion-selective channel in pancreatic islet β-cells by Kinard and Satin (5) and Best et al. (6) suggests that some of the previously observed effects of anions on islet function may be mediated by an anion-permeable ion channel. This anion-permeable pancreatic islet β-cell Cl^- channel ($I_{Cl,islet}$) is an outwardly rectifying Cl^- channel that is activated by cell swelling (induced by the application of hypotonic solution) or by a rise in intracellular cAMP (5). $I_{Cl,islet}$ is inhibited by the well-known Cl^- channel blocker 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) or by 100 μmol/l glyburide (5). The channel may also be under metabolic control, either by virtue of its sensitivity to intracellular ATP (5,6) or because of β-cell swelling associated with increased glucose metabolism (7).

Due to the relatively depolarized reversal potential of $I_{Cl,islet}$ (–30 mV) (5), its activation under physiological conditions would be expected to depolarize β-cells by producing a net inward current mediated by a net efflux of anions (5,6). Thus, $I_{Cl,islet}$ activation after increased glucose metabolism would lead to β-cell depolarization, the activation of voltage-activated Ca^{2+} channels, increased Ca^{2+} influx, and enhanced Ca^{2+}-dependent insulin secretion. Activation of $I_{Cl,islet}$ might thus account in part for the depolarizing actions of islet stimulators as diverse as cAMP (8–13), hypotonic solutions (14), or glucose itself (15). These effects could synergize with the more well-known effects of glucose metabolism to depolarize β-cells and stimulate insulin release by closing membrane K_{ATP}

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4-AP, 4-aminopyridine; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentrations; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; $I_{Cl,islet}$, anion-permeable pancreatic islet β-cell Cl^- channel; PCR, polymerase chain reaction; RT, reverse transcriptase; RVD, regulatory volume decrease; RVI, regulatory volume increase; TEA, tetraethylammonium-Cl; TTX, tetrodotoxin.

channels (1,16,17). Because it is known that hypotonic solutions stimulate a transient phase of insulin secretion in rat islets (18), it has been hypothesized that cell swelling releases insulin by activating swelling-activated Cl^- channels, thus depolarizing the islets (6,14). A similar mechanism has been suggested by Moser et al. (19) to account for hypotonic-induced catecholamine secretion from rat adrenal chromaffin cells.

We had several objectives in the present study. First, we examined the pharmacological sensitivity of $I_{Cl,islet}$ to the channel blockers DIDS and niflumic acid to identify a useful Cl^- channel blocker that could be used to probe Cl^- channel function in physiological studies. Second, we examined the effect of blocking Cl^- channels on HIT cell volume regulation to test the hypothesis that islet Cl^- channels play a role in this process and to contrast their possible participation with that of the furosemide-sensitive Na/K/2Cl cotransporter (20) or cation channels. Third, we examined the hypothesis that $I_{Cl,islet}$ activation mediates swelling-induced insulin secretion; we did this by testing whether the two different Cl^- channel blockers inhibited hypotonically induced insulin release from perifused HIT cells. A standard protocol was then used to evaluate the hypothesis that swelling might increase secretion by directly affecting distal steps in insulin granule exocytosis. Finally, as a first step toward ultimately establishing the molecular identity of $I_{Cl,islet}$, reverse transcriptase (RT)–polymerase chain reaction (PCR) was used to determine whether mRNA for CLC-3 was present in HIT cells, because this Cl^- channel isoform is a strong candidate for the outwardly rectifying swelling-activated Cl^- channel of other tissues (21–26).

RESEARCH DESIGN AND METHODS

Cell culture. Insulin-secreting HIT-T15 cells were cultured in Ham's F-12 medium and passaged weekly using trypsin-EDTA, as previously described (27,28). HIT cells from passages 50–70 were seeded at a density of 5×10^4 cells/ml onto glass cover slips placed in 35-mm petri plates. Culture plates were kept at 37°C in an air/CO_2 incubator, and the cells were fed every 2–3 days.

Electrophysiology and solutions. HIT cells were placed in a recording chamber affixed to the stage of an inverted microscope (IMT-2 or IX50; Olympus, Tokyo). The whole-cell patch-clamp technique (29) was used to measure Cl^- current, as previously described (5). Cells were voltage-clamped to −65 mV. The recording chamber was continuously superfused with an external solution that contained the following (in mmol/l): 115 NaCl, 3 $CaCl_2$, 5 CsCl, 0.2 $CdCl_2$, 10 tetraethylammonium-Cl (TEA), 1 $MgCl_2$, 5×10^{-4} tetrodotoxin (TTX), 10 HEPES, and 11.1 glucose, pH 7.2 (274 ± 5 mOsm). Electrodes were filled with a solution containing (in mmol/l): 114 Cs-aspartate, 10 CsCl, 2 Mg_2ATP, 20 HEPES, 1 EGTA, and 10 4-aminopyridine (4-AP), pH 7.2 (269 ± 5 mOsm). $CdCl_2$ was added to the external solution to block Ca^{2+} current. Na^+ currents were blocked by TTX, and K^+ currents were blocked by internal ATP, 4-AP, replacement of all internal K^+ with Cs^+, and external TEA^+. These solutions isolated Cl^- current from other known β-cell currents (5). Hypotonic solution (222 ± 3 mOsm) was identical to the control solution, except that [NaCl] was reduced by 25%, from 115 to 86.3 mmol/l, which reduced the osmolarity by 20% (0.8T). All chemicals were obtained from Sigma (St. Louis, MO). Cl^- channel blockers were dissolved in DMSO and made fresh daily; after dilution, the final concentration of DMSO vehicle in the solutions was 0.1%.

Electrodes were fabricated from borosilicate tubing using a Sutter horizontal puller (P-97; Sutter Instruments, Novato, CA). Standard tight-seal whole-cell patch-clamp was used to record Cl^- current (29) with an Axopatch-1D patch-clamp amplifier (Axon Instruments, Foster City, CA). All electrophysiological and volume experiments were carried out at room temperature (20–22°C). Pipette and seal resistances ranged from 5 to 20 MΩ and 2 to 20 GΩ, respectively. Standard techniques were used to compensate for pipette series resistance. The liquid junction potential between the low Cl^-–Cs-aspartate internal solution and the high Cl^- external solution was +8 mV. The potentials shown in the figures were not corrected for this offset.

Estimations of HIT cell volume. Cell volume was monitored periodically by measuring HIT cell diameter with an eyepiece reticule at 600× magnification using phase microscopy (average HIT cell diameter was 22.9 ± 0.8 μm, n = 10) (5). Measurements of HIT cell diameter were used to estimate normalized cell volumes, using the following:

$$\text{Volume of sphere} = 1/6\pi d^3 \ (d = \text{diameter}).$$

Data analysis. Data acquisition and analysis were carried out using a Macintosh Quadra 800 or G3 computer (Apple Computer, Cupertino, CA), a 16-bit 200-kHz hardware interface (Instrutech, Elmont, NY), IgorPro 3.0 software (Wavemetrics, Lake Oswego, OR), and Pulse Control software (30). Membrane currents, activated by computer-generated current-voltage (I-V) or monitoring test pulses, were filtered at 2 kHz and digitized at 5 kHz. Pulse-evoked currents were analyzed as described by Satin and Cook (28), except that data were not baseline- or leak-subtracted. Only single isolated cells were chosen for study to avoid possible electrical complications of cell–cell coupling (28). Student's t test or analysis of variance was used to determine significant differences in the data.

Insulin secretion. HIT cells were seeded at a density of 2.5×10^5 cells/ml in two 75-cm^2 flasks and harvested 5 days later. Flasks were rinsed with 5 ml phosphate-buffered saline containing 100 mg/dl glucose and placed in an incubator at 37°C for 60 min. Next, the cells were dislodged with mechanical pipetting, centrifuged, and resuspended in 4.5 ml Krebs-Ringer bicarbonate buffer containing (in g/l): 5.76 NaCl, 0.37 KCl, 0.17 KH_2PO_4, 0.30 $MgSO_4$-7 H_2O, 2.18 $NaHCO_3$, 0.39 $CaCl_2$, pH 7.4, and 0.1% bovine serum albumin with 0 glucose. Columns containing polyacrylamide gel beads (Bio Gel P-2; Bio Rad, Richmond, CA) were used as a support matrix for HIT cells, and the columns were perifused at 1 ml/min for 2 h to determine insulin release patterns. Cells were layered on top of the gel bed just before the start of perifusion. For the experiments designed to isolate distal secretion, 50 mmol/l [KCl] and 100 μmol/l diazoxide were used to persistently open K_{ATP} channels and depolarize the cells (31). The hypotonic solutions used to trigger insulin release did not affect HIT cell viability.

Standard procedures were used for insulin radioimmunoassay (32). Insulin release was expressed as the percentage fractional release for each experimental condition, calculated as [(the amount of insulin collected from incubation buffer) (insulin collected from incubation buffer + insulin content of cell layer)] × 100%. Details of HIT cell perifusion, including the sensitivity of these cells to glucose, are described by Fujimoto and Teague (33).

Molecular biologynone. Oligonucleotide primers were designed to be homologous to published guinea pig (26) and rat (25) CLC-3 chloride channel sequences. The 5′ primer used corresponded to bp 4–24 of the guinea pig sequence (ACA ATG ACA AAT GGA GGC AGC) (26). The 3′ primer corresponded to bp 576–596 on the opposite strand of guinea pig CLC-3 (CCT CTG ATG ATG AAT CCA CTC) (26). These were used in the PCR to generate a 593-bp fragment in HIT cell mRNA. HIT cell mRNA was prepared using standard methodologies (Trizol Reagent LTI, Gaithersburg, MD). Total HIT cell mRNA was reverse-transcribed and amplified for CLC-3 cDNA using an RNA PCR kit (PE Express, Foster City, CA). Total mRNA (1 μg) was added to a tube containing reverse transcription mixture (1 mmol/l $MnCl_2$, 50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.3, 0.2 mmol/l dGTP, 0.2 mmol/l dATP, 0.2 mmol/l dTTP, 0.2 mmol/l dCTP, 1 U/μl placental RNAase inhibitor, 2.5 U/μl Moloney murine leukemia virus RT, and 2.5 μmol/l 3′ primer) in a final volume of 20 μl. Reverse transcription was carried out at 60°C for 5 min. The entire reverse transcription mixture was diluted into 100 μl with $MnCl_2$, KCl, and Tris-HCl, pH 8.3, added to final concentrations of 2, 50, and 10 mmol/l, respectively. Next, the 5′ primer (0.15 μmol/l, final) was added. The thermocycler cycles used were 2 min at 95°C for 1 cycle, 1 min at 95°C, and 3 min at 60°C for 30 cycles, then 7 min at 60°C for 1 cycle. As a negative control, we assessed whether genomic DNA possibly contaminated the RNA. Reverse transcription was thus performed using the 5′ primer for the reverse transcription step (which would transcribe DNA but not the appropriate RNA), followed by PCR with the 3′ primer. As a positive control, interleukin-1α mRNA was amplified using primers supplied in the kit to produce a 308-bp cDNA product. The PCR products were then gel-purified and cloned into the pGEM T-Easy Cloning vector (Promega, Madison, WI). The resulting samples were manually sequenced via the dideoxy method (US Biochemical, Cleveland, OH), and confirmed by sequencing both strands (Retrogen, San Diego, CA).

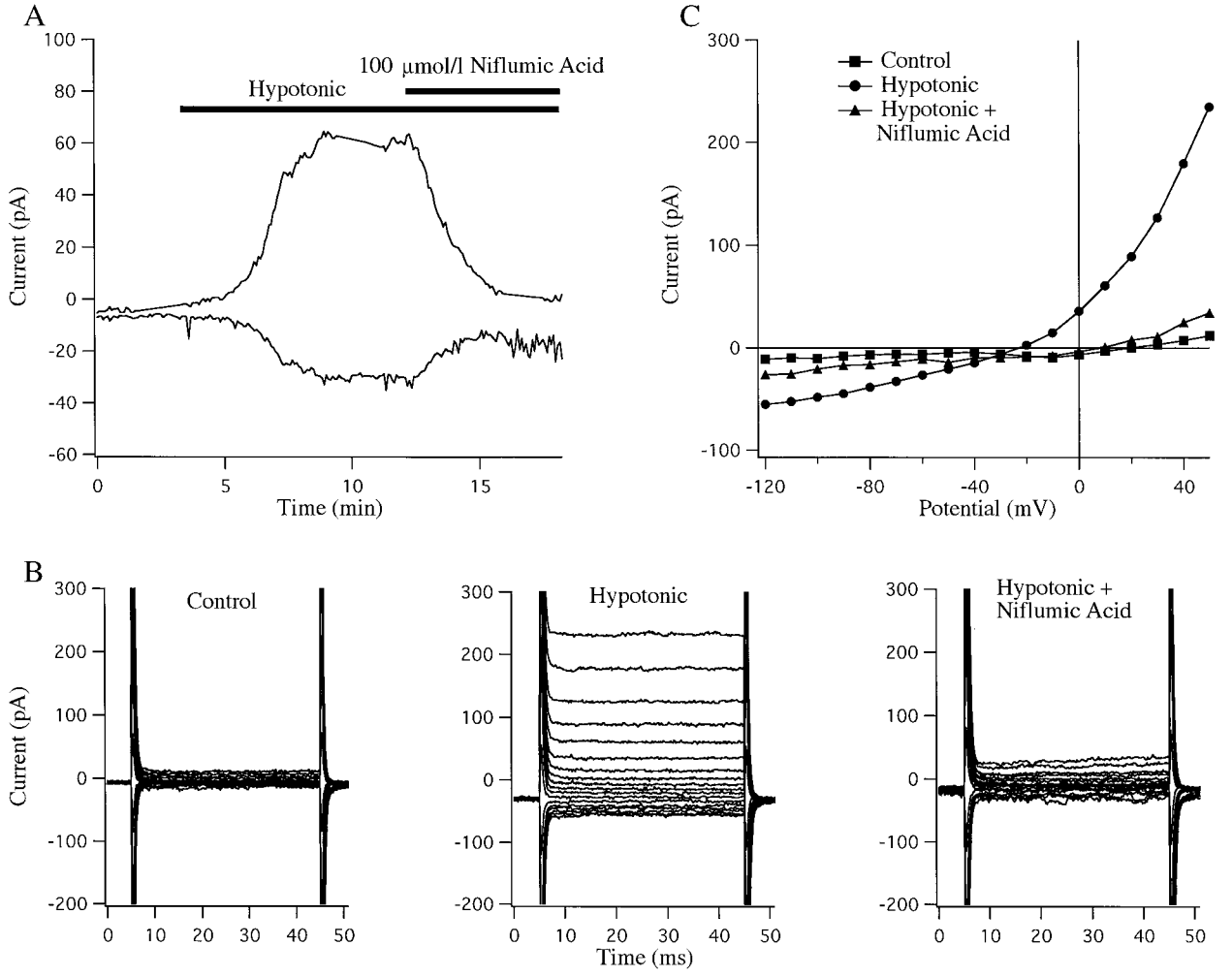

FIG. 1. Swelling-activated Cl^- current in a representative HIT cell. *A*: Time course showing activation of $I_{Cl,islet}$ by hypotonic solution (0.8T) and inhibition by the Cl^- channel blocker niflumic acid (100 μmol/l). $I_{Cl,islet}$ was measured at +10 mV (top trace) and −65 mV (bottom trace). *B*: Current traces obtained in the above solutions during 40-ms voltage pulses applied in +10-mV increments from −120 to +50 mV. *C*: Corresponding current-voltage relationship for peak currents shown in *B*. Hypotonic conditions activated an outwardly rectifying current that reversed near –30 mV.

RESULTS

Niflumic acid blocks $I_{Cl,islet}$ in HIT cells. We have previously shown that the Cl^- channel blocker DIDS blocks $I_{Cl,islet}$ in HIT cells (5), and we thus hypothesized that niflumic acid, another Cl^- channel blocker (21,34), would also be effective. It has been proposed that niflumic acid is a better probe than DIDS for assessing the physiological role of volume-regulated Cl^- channels in tissues such as heart because its block is less voltage-dependent (35).

With Na^+, K^+, and Ca^{2+} channels blocked, only small baseline currents were observed in voltage-clamped HIT cells under isotonic conditions. Application of an external solution that was 20% hypotonic to isotonic control saline resulted in a progressive increase in cell volume and a concomitant increase in inward currents (at −65 mV) and outward currents (at +10 mV) due to $I_{Cl,islet}$ activation (Fig. 1*A*) (5). The application of 100 μmol/l niflumic acid to the hypotonic solution promptly decreased $I_{Cl,islet}$ at both test voltages (Fig. 1*A*). Figure 1*B* shows representative current traces elicited by 40-ms voltage pulses that surveyed membrane potentials ranging from –120 to +50 mV, in +10-mV increments. Only a small nearly linear background current was visible under isotonic conditions (Fig. 1*B*) (5). However, after exposure to hypotonic solution, inward and outward currents mediated by $I_{Cl,islet}$ were clearly activated (Fig. 1*B*). Hypotonically activated current was relatively time-independent over the voltage range studied, and showed no evidence of inactivation during these brief voltage pulses (5). As shown in the figure, both pulse and holding currents were suppressed by 100 μmol/l niflumic acid.

Figure 1*C* shows the current-voltage (I-V) relationships obtained in isotonic solution, hypotonic solution, and hypotonic solution that also contained niflumic acid. Under basal isotonic conditions, there was a small inward current at −120 mV (mean current was −26.0 ± 7.9 pA, n = 10) and an outward current at +50 mV (+78.4 ± 16.0 pA, n = 10). After application of hypotonic solution, peak current increased to −90.9 ± 73.1 pA (n = 5) at −120 mV and to +592.0 ± 104.1 pA (n = 5) at +50 mV. The mean reversal potential of the hypotonically induced current was −32 ± 2.4 mV, after correction for the liquid junction potential effect. The addition of 100 μmol/l niflumic decreased peak inward

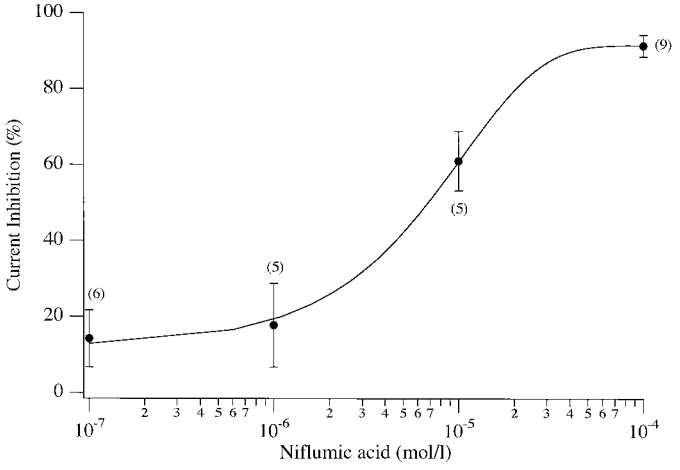

FIG. 2. A dose-response curve plotting percent inhibition of hypotonically activated currents elicited at +10 mV by niflumic acid. The IC_{50} obtained from this curve was 7 μmol/l.

current to −34.2 ± 4.8 pA (n = 5) at –120 mV and outward current to +69.0 ± 20.1 pA (n = 5) at +50 mV, which was close to the original baseline level.

The blockade of $I_{Cl,islet}$ by niflumic acid (21,34) was dose-dependent (Fig. 2). $I_{Cl,islet}$ in this case was measured at +10 mV in hypotonic solution, and then different doses of niflumic acid were added to the hypotonic solution. The IC_{50} obtained from curve-fitting these data was 7 μmol/l for niflumic acid. At a dose of 100 μmol/l, niflumic acid blocked 94% of $I_{Cl,islet}$. A dose-response relationship was not obtained for DIDS because we had difficulty obtaining measurable blockade at doses <100 μmol/l. However, for comparison, we previously showed that 100 μmol/l DIDS blocks ~64% of $I_{Cl,islet}$ at +10 mV (5).

Membrane potential significantly affected $I_{Cl,islet}$ blockade due to DIDS or niflumic acid. Thus, at +50 mV, 100 μmol/l DIDS blocked 73 ± 3.3% of peak Cl current, and 100 μmol/l niflumic acid blocked 79.6 ± 6.3%. This difference was not significant ($P > 0.05$). However, at −120 mV, DIDS blocked only 19.88 ± 6.86%, whereas niflumic acid blocked 54 ± 7.56% of the peak Cl current ($P < 0.05$). Both drugs blocked significantly more current at +50 mV than at −120 mV. Thus, the DIDS block of $I_{Cl,islet}$ appeared to be more sensitive to membrane voltage than the blockade associated with niflumic acid. Because the physiological voltage range of β-cells is approximately –70 to –10 mV, niflumic acid would appear to be a more appropriate probe for determining the physiological roles of $I_{Cl,islet}$.

$I_{Cl,islet}$ is involved in the regulation of HIT cell volume. The volumes of individual HIT cells were estimated by measuring HIT cell diameter and then calculating spherical cell volumes (see RESEARCH DESIGN AND METHODS) (5). When individual HIT cells remained in isotonic solution, their normalized volumes remained relatively constant (Fig. 3*A*). However, after the introduction of hypotonic (0.8T) solution, cell volume progressively increased over several minutes (by 14.2%, for the data shown in Fig. 3*A*), followed by a spontaneous regulatory volume decrease (RVD). RVDs have been observed previously after the application of hypotonic solutions to rat β-cells (36,37), RINm5F cells (38), and mouse β-cells (39). When the hypotonic solution also contained 100 μmol/l niflumic acid, the RVD was largely blocked, and cell volume increased significantly by an additional 29.5% over that observed when hypotonic solution alone was applied ($P < 0.05$) (Fig. 3*A*). Hypotonically induced swelling could be reversed by washing the cells with isotonic control solution.

In a separate set of experiments (Fig. 3*B*), the addition of hypotonic solution was again observed to cause an increase in cell volume (29.8% in this case) followed by an RVD, similar to the data shown in Fig. 3*A*. The addition of 100 μmol/l DIDS to the hypotonic solution again fully blocked the RVD and potentiated cell swelling, although the potentiation due to DIDS was smaller than that seen with niflumic acid and was not statistically significant. Thus, the addition of either niflumic acid or DIDS to hypotonic solution blocked the RVD and, in the case of niflumic acid, significantly potentiated cell swelling. $I_{Cl,islet}$ block would be expected to increase water entry into the cell during osmotic shock if Cl^- channel opening normally supports a net efflux of anions to restore cell volume (see DISCUSSION). As shown in Fig. 3*C*, the addition of niflumic acid under isotonic conditions resulted in transient cell swelling (~11.2%), suggesting that Cl^- channels help regulate HIT cell volume even in the absence of an osmotic challenge (e.g., under isotonic conditions).

To rule out time-dependent changes in the ability of HIT cells to respond to a second osmotic challenge, we subjected the cells to two identical osmotic challenges using drug-free hypotonic solutions. As shown in Fig. 3*D*, HIT cells swelled to the same extent after a second exposure to the same hypotonic solution ($P > 0.05$). Thus, the changes in cell swelling that we observed in other experiments in which drugs were present during the second exposure cannot be accounted for by time-dependent loss of osmotic responsiveness, such as that which might occur due to an irreversible loss of intracellular solutes.

The role of cation channels and the Na/K/2Cl cotransporter in HIT cell volume regulation. In previous studies of cell volume regulation in other systems, the activation of cation channels after cell swelling was often found to accompany anion channel activation (40). In our studies, the addition of glyburide (at 100 μmol/l) to hypotonic solution had little or no effect on cell swelling compared with hypotonic solution alone (15.8 vs. 20.4%) and did not inhibit the RVD (Fig. 3*E*). At this dose, glyburide completely blocks K_{ATP} channel current (rev. in 1,17), and inhibits Cl current by 44% (5). This suggests that K_{ATP} does not participate in HIT cell volume regulation and that the partial blockade of islet Cl channels may not be sufficient to affect cell volume regulation. Similarly, the addition of gadolinium (Fig. 3*F*), which blocks stretch-activated cation channels (41), or TEA (Fig. 3*G*), which blocks a wide spectrum of K^+ channels in islet cells (42), failed to either potentiate cell swelling or inhibit the cell RVD.

We next examined the contribution of the Na/K/2Cl cotransporter to HIT cell volume regulation. Furosemide, which blocks the Na/K/2Cl cotransporter of islets (43–45)

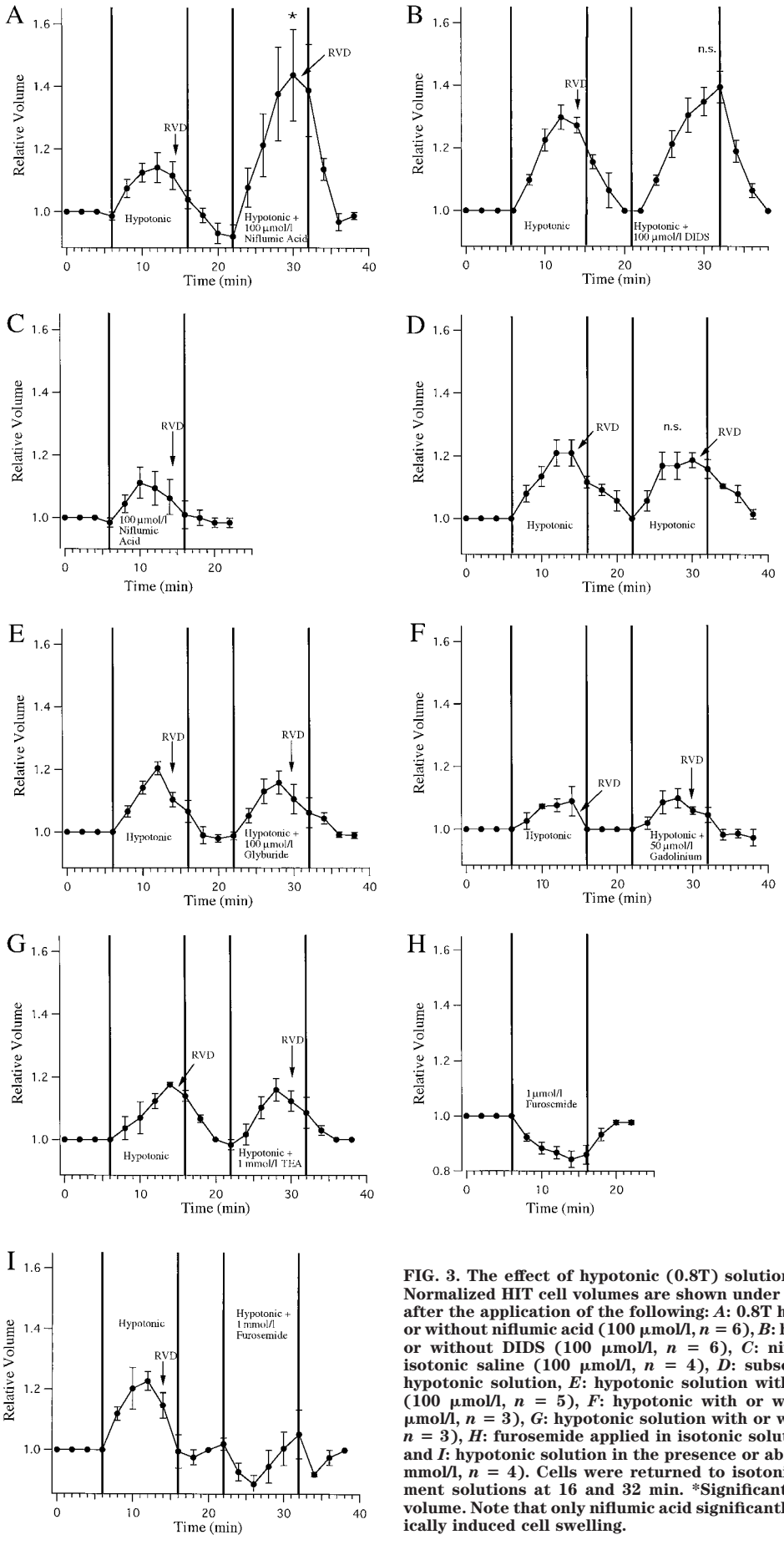

FIG. 3. The effect of hypotonic (0.8T) solutions on HIT cell volume. Normalized HIT cell volumes are shown under control conditions and after the application of the following: *A*: 0.8T hypotonic solution with or without niflumic acid (100 μmol/l, $n = 6$), *B*: hypotonic solution with or without DIDS (100 μmol/l, $n = 6$), *C*: niflumic acid applied in isotonic saline (100 μmol/l, $n = 4$), *D*: subsequent applications of hypotonic solution, *E*: hypotonic solution with or without glyburide (100 μmol/l, $n = 5$), *F*: hypotonic with or without gadolinium (50 μmol/l, $n = 3$), *G*: hypotonic solution with or without TEA (1 mmol/l, $n = 3$), *H*: furosemide applied in isotonic solution (1 mmol/l, $n = 3$), and *I*: hypotonic solution in the presence or absence of furosemide (1 mmol/l, $n = 4$). Cells were returned to isotonic solution after treatment solutions at 16 and 32 min. *Significant increase in peak cell volume. Note that only niflumic acid significantly potentiated hypotonically induced cell swelling.

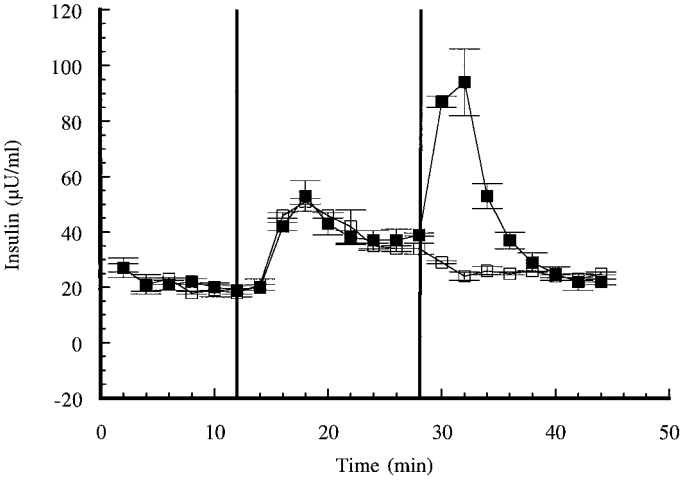

FIG. 4. Glucose and hypotonic solution trigger insulin release from perifused HIT cells. Insulin secretion was measured from cells perifused with the following: *first panel*: 0 glucose (0–12 min), *second panel*: 5 mg/dl glucose (12–28 min), and *third panel*: 5 ml/dl glucose in the presence of isotonic solution (□, $n = 2$) or 0.75T hypotonic solution (■, 28–44 min; $n = 2$).

but does not block islet Cl^- channels (T.A.K., L.S.S., unpublished data), did not mimic the actions of DIDS or niflumic acid on HIT cell volume regulation. As shown in Fig. 3*H*, the application of 1 mmol/l furosemide in isotonic solution reversibly decreased cell volume by ~15.7%, suggesting that basal transporter activity mediates a tonic swelling influence, probably due to a net uptake of ions (see DISCUSSION). Furthermore, the addition of furosemide to hypotonic solution disrupted normal islet cell volume regulation (Fig. 3*I*). These results suggest that cell volume regulation in insulin-secreting HIT cells is primarily mediated by volume-sensitive Cl^- channels rather than stretch-activated cation channels, or K^+ channels, and that Cl^- transporter blockade has very different effects from Cl^- channel blockade.

Cl^- channels and swelling-induced insulin secretion. It has been known for some time that exposing islets to hypotonic solutions stimulates insulin secretion (7,18). This would be expected if cell swelling activates $I_{Cl,islet}$, producing cell depolarization, an increase in Ca^{2+} channel opening, and an increase in β-cell intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), resulting in increased insulin granule exocytosis. A similar model has been suggested to account for hypotonically induced catecholamine release in chromaffin cells (19). To test this hypothesis in β-cells, we applied hypotonic solutions to perifused HIT cells to activate $I_{Cl,islet}$ and used a standard radioimmunoassay protocol to assay secreted insulin (Fig. 4). After maintaining steady baseline insulin release in 0 glucose, glucose was increased to 5 mg/dl, which transiently increased secretion (Fig. 4). Subsequent exposure of the cells to a hypotonic (0.75T in this case) solution caused an additional transient increase in secretion. Hypotonicity was achieved by decreasing [NaCl]. However, the mannitol added to the reduced Na^+ solution to restore tonicity fully blocked the hypotonically induced secretion. This verifies that the secretion was attributable to cell swelling and not Na^+ removal (data not shown).

As shown in Fig. 5*A*, the fractional insulin release from perifused HIT cells increased above basal levels after the application of 5 mg/dl glucose and was biphasic. After this characteristic response to glucose, fractional insulin secretion transiently increased after the introduction of hypotonic solution (Fig. 5*B*). Both DIDS and niflumic acid were found to reduce glucose-stimulated secretion under isotonic conditions (compare Fig. 5*C* and *D* with Fig. 5*A* and *B*), although this reduction was not statistically significant. The addition of DIDS significantly decreased hypotonically induced insulin secretion by 56% ($P < 0.05$) (compare Fig. 5*C* with Fig. 5*B*). In a previous study, 100 μmol/l DIDS was found to block 67% of hypotonically induced insulin release from rat islets (38). In contrast, hypotonically induced secretion was unaffected by 100 μmol/l niflumic acid, even though niflumic acid was found to be a more effective blocker of $I_{Cl,islet}$ ($P < 0.05$) (compare Fig. 5*D* with Fig. 5*B*). Although it may seem paradoxical that DIDS blocked more of the hypotonically induced secretion despite it being a less efficacious blocker, the increased cell swelling due to niflumic acid (Fig. 3*F*) may have compensated for the decrease in proximal depolarization expected for niflumic acid. Because neither DIDS nor niflumic acid completely inhibited swelling-induced secretion, it appears that the activation of $I_{Cl,islet}$ may not fully account for the insulin secretion that followed cell swelling. We thus hypothesized that a component of swelling-induced secretion may occur via distal steps in the insulin secretory pathway, which would explain why hypotonic solution could still evoke significant secretion despite the blockade of plasmalemmal Cl^- channels.

To test this, we perifused HIT cells with a solution containing 50 mmol/l KCl, 100 μmol/l diazoxide, and either 0 or 10 mg/dl glucose. The inclusion of diazoxide, which opens K_{ATP} channels, and high K^+ levels would be expected to persistently depolarize the HIT cells (31,46). Previous studies have shown that islets still exhibit glucose-stimulated (31) and sulfonylurea-stimulated (47–49) insulin release under these conditions, suggesting that these secretagogues can stimulate insulin secretion by interacting with a distal K_{ATP}-independent pathway.

As shown in Fig. 6*A*, under persistent depolarizing conditions (with V_{max} calculated to be −27 mV using the Nernst equation and assuming the intracellular K^+ concentration is 140 mmol/l), basal HIT cell secretion was elevated even in 0 glucose (compare with Fig. 5), and the subsequent addition of 10 mg/dl glucose produced slow and blunted increases in secretion, presumably occurring via distal pathways. However, a striking phase of transient secretion could still be evoked under these conditions by exposing the cells to hypotonic saline (0.75T in this case), even when 100 μmol/l DIDS or 100 μmol/l niflumic acid was also present. Percent fractional insulin release reached very high levels under these conditions, and peak insulin secretion in response to hypotonic solution (even with niflumic acid present) was significantly higher than secretion elicited by glucose under isotonic conditions (compare the second panel with the third panel in Fig. 6*A* and *C*) ($P < 0.05$). Interestingly, unlike the slow glucose-dependent secre-

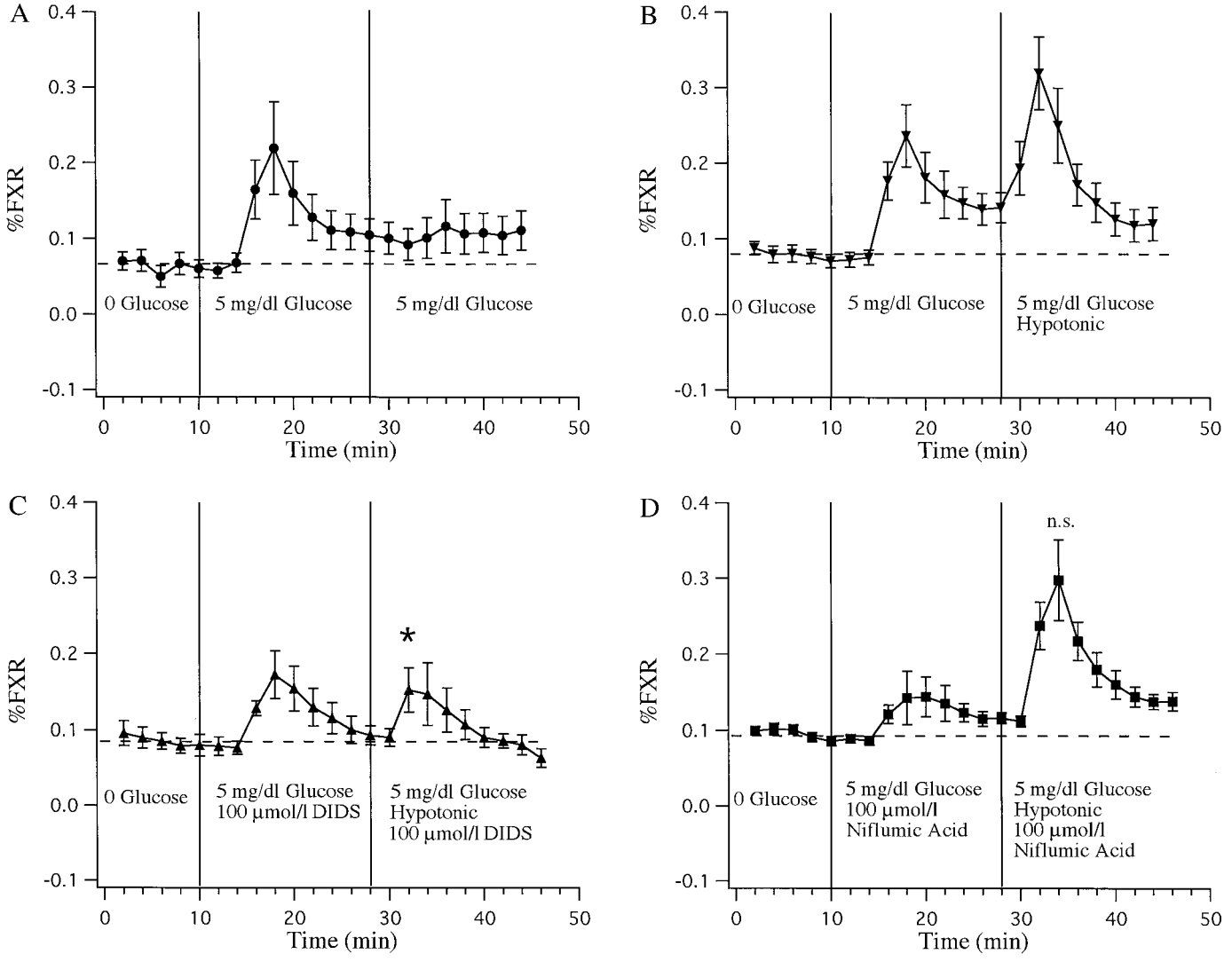

FIG. 5. Time course of percent fractional insulin release (%FXR) obtained under various conditions using perifused HIT cells. *A*: Increasing glucose from 0 to 5 mg/dl induced biphasic insulin secretion. *B*: The application of hypotonic solution (0.75T) in the continued presence of glucose triggered a transient phase of secretion. *C*: The application of 100 µmol/l DIDS significantly reduced, but did not abolish, hypotonically induced secretion (P* < 0.05, *C* vs. *B*. *D*: The application of niflumic acid did not affect hypotonically induced insulin secretion (*P* > 0.05, *D* vs. *B*). The results shown are the means from *n* = 4; error bars denote SEs.**

tion observed when high potassium and diazoxide were present under isotonic conditions, hypotonically evoked secretion with diazoxide and high potassium was brisk, transient, and of larger amplitude, reaching a peak of 0.6–0.8% release within 6 min of changing solutions. The addition of mannitol to maintain tonicity also blocked this hypotonic-induced distal secretion (data not shown). These results thus support the hypothesis that a component of hypotonically induced secretion likely occurs via a distal pathway not involving K_{ATP} channels or membrane depolarization, as has been proposed for glucose (31) and sulfonylureas (47).

Message for the CLC-3 Cl^- channel is present in HIT cells. A number of different chloride channels of the CLC family have been cloned following the cloning of CLC-0 from *Torpedo electroplax* (50). To determine which molecular isoform of chloride channel might mediate $I_{Cl,islet}$ in HIT cells, RT-PCR was carried out after the extraction of total HIT cell mRNA. Oligonucleotide primers were designed to amplify a published sequence from the NH_2-terminal of the CLC-3 Cl^- channel (26). Analysis of HIT cell cDNA after RT-PCR revealed a band at 593 bp, the predicted size of the CLC-3 transcript (Fig. 7, *lane 2*). In this figure, *lane 1* indicates the DNA ladder, and *lanes 3* and *4* were negative and positive controls, respectively. DNA sequencing revealed that this HIT cell PCR product closely matched CLC-3, using a BLAST search (51). The sequence of the HIT cell PCR product is shown in Fig. 8. Sequence analysis reveals 91% nucleic acid identity with the human CLC3 cDNA (90% amino acid identity), 93% nucleic acid identity with mouse, and 90% (90% amino acid) identity with the guinea pig (52).

DISCUSSION

Although niflumic acid and DIDS both blocked $I_{Cl,islet}$, the blockade produced by niflumic acid displayed less voltage dependence. Hypotonic solutions (0.75–0.8T) produced measurable HIT cell swelling, followed by a small RVD. The effect of niflumic acid on cell swelling added

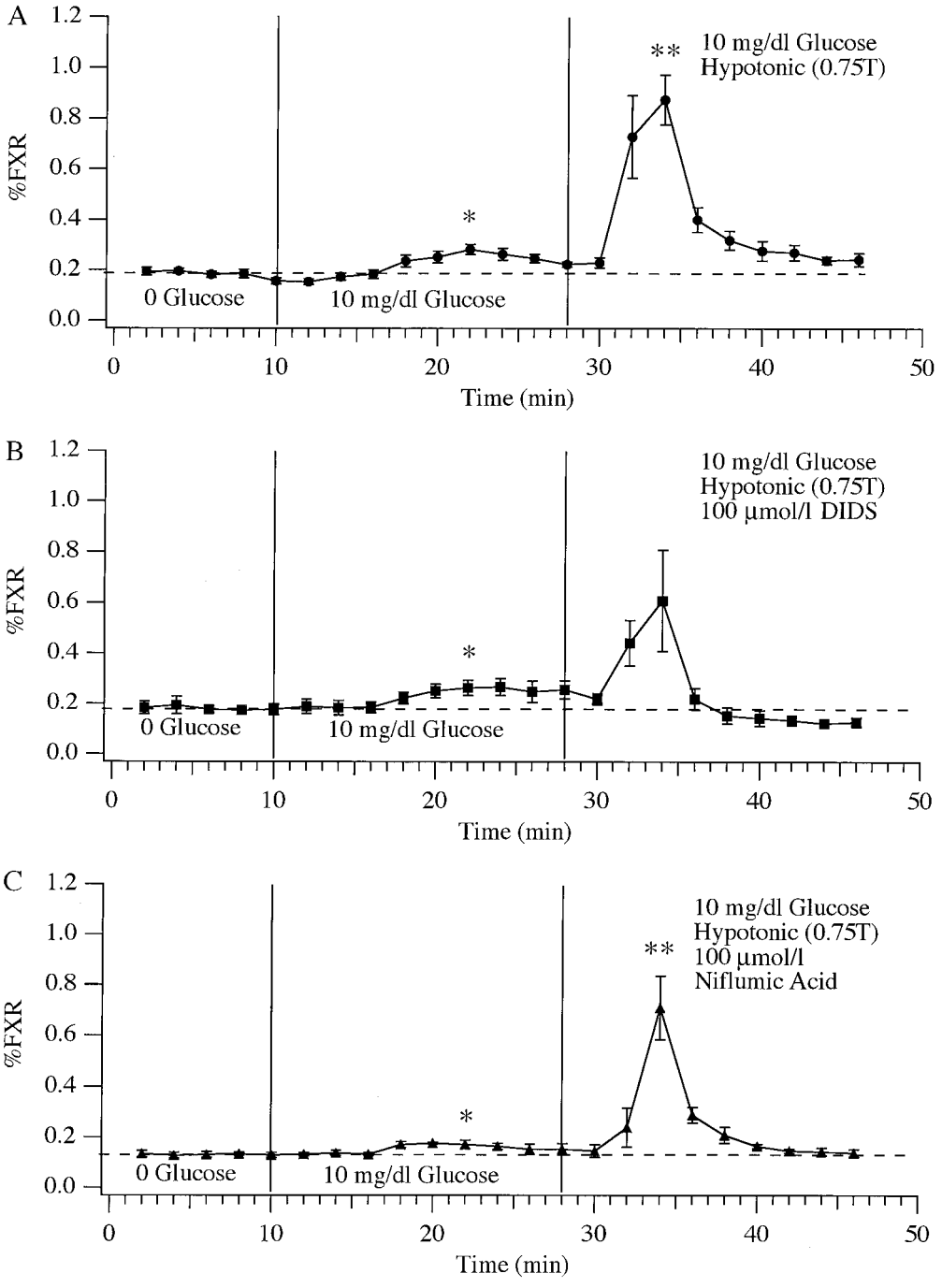

FIG. 6. The elimination of hypotonically induced membrane depolarization does not block hypotonically induced insulin secretion. Solutions contained 50 mmol/l potassium and 100 μmol/l diazoxide. Persistent depolarization due to the application of high potassium and diazoxide blunted glucose-dependent secretion, whereas hypotonically induced secretion was maintained or even enhanced by these conditions. Fractional insulin secretion slowly increased after an elevation in glucose from 0 to 10 mg/dl, whereas the subsequent application of hypotonic solutions (0.8T) provoked a transient phase of secretion (*A*) which remained despite the presence of either DIDS (*B*) or niflumic acid (*C*). Hypotonically induced secretion was significantly greater than in isotonic solution. The results shown are means of four different perifusion columns, taken from two different batches of HIT cells; the error bars denote SE. *$P < 0.05$; **$P < 0.05$.

to the effect of hypotonicity and blocked the RVD, consistent with the hypothesis that proximal Cl^- channels participate in β-cell volume regulation in HIT cells. HIT cell swelling was next found to stimulate a transient phase of insulin secretion, as expected from earlier studies. However, although niflumic acid or DIDS slightly reduced glucose-dependent insulin secretion under isotonic conditions, hypotonically triggered secretion persisted despite Cl^- channel blockade. This suggests that swelling likely stimulated secretion through a more distal mechanism that was independent of plasmalemmal Cl^- channels. Furthermore, hypotonically induced secretion persisted under conditions that isolated distal secretory mechanisms, ruling out the participation of proximal Cl channels in this process.

In light of these new results, we present a novel model for [Cl^-]-dependent volume regulation in islet cells and an alternate interpretation to account for the stimula-

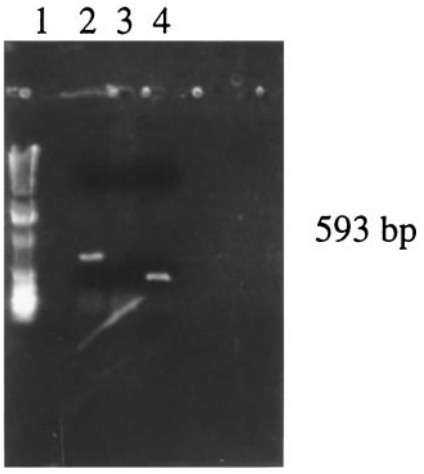

FIG. 7. RT-PCR of HIT cell RNA revealed a CLC-3 transcript. *Lane 1* shows the DNA ladder, and *lane 2* shows amplified HIT cell cDNA, with a band evident at 593 bp. *Lane 3* shows a negative control to rule out contamination by genomic DNA. As a positive control, IL-1 mRNA was amplified, resulting in a 308-bp product shown in *lane 4*.

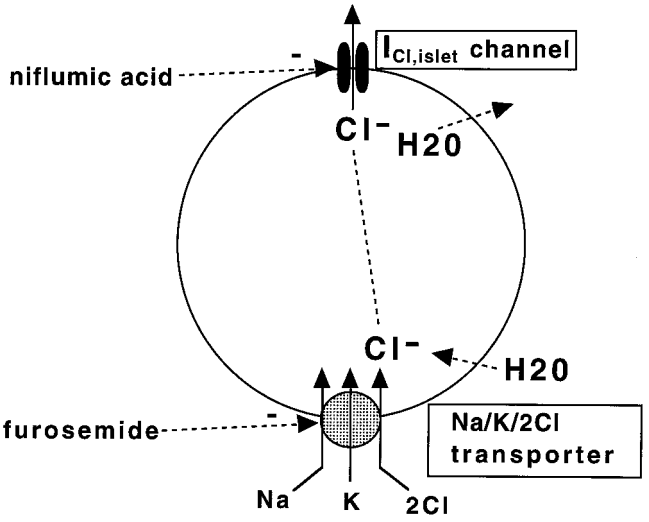

FIG. 9. A model of anion and volume regulation in β-cells. $I_{Cl,islet}$ activation results in the net efflux of Cl^-, followed by the efflux of water, causing a decrease in cell volume. Cl^- efflux is shown to be blockable by niflumic acid. In contrast, Na/K/2Cl transporter activity mediates Cl^- influx, followed by water influx, producing tonic cell swelling. Furosemide is shown as a blocker of this process. The balance between the two pathways is proposed to result in β-cell volume under basal isotonic and anisotropic conditions.

tory action of hypotonic solutions on insulin secretion (Fig. 9). In this model, Cl^- or other anions can leave the cell under basal conditions by passing through the $I_{Cl,islet}$ channel. Water leaves after net Cl^- efflux, producing a tonic shrinking influence. Because a small niflumic acid–blockable inward Cl^- current is observed in isotonic solution (Fig. 1), this implies that the net electrodiffusion of anions from the cell can occur via this pathway. Cl^- channel blockade by niflumic acid decreases this Cl^- efflux, concomitantly decreasing water efflux, resulting in HIT cell swelling. This hypothesis is consistent with our observation that niflumic acid applied to cells under isotonic conditions results in cell swelling. Cl^- is also known to enter islet cells via a Na/K/2Cl transporter (43), which would be expected to cause tonic swelling because of water entering with these ions. Because the transporter is blocked by furosemide (20), furosemide applied under isotonic conditions would be expected to produce cell shrinkage, which is indeed what was observed. Interestingly, furosemide does not block Cl^- channels, which would be consistent with our finding that the chloride channel blocker niflumic acid and furosemide had different effects. However, we cannot completely rule out other actions of niflumic acid on anion transporters (53). Although we expected that cation channels would be involved in the regulation of HIT cell volume, in part to maintain electroneutrality (54), we did not observe any measurable alterations in cell volume regulation after the application of inhibitors of voltage-gated K^+ channels, K_{ATP} channels, or stretch-activated cation channels. Although these studies are limited by the strictly pharmacological approach we used, the results suggest that electrical balance is likely maintained through other, as yet unidentified, ionic mechanisms in islet β-cells.

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1    CAATGACAAATGGAGGCAGCATTAATAGCTCTACACACTTGCTGGATCTTTTGGATGAAC
1      M  T  N  G  G  S  I  N  S  S  T  H  L  L  D  L  L  D  E  P

61   CGATCCCAGGTGTTGGTACATACGATGATTTCCATACTATTGACTGGGTTCGAGAGAAAT
21     I  P  G  V  G  T  Y  D  D  F  H  T  I  D  W  V  R  E  K  C

121  GTAAAGACAGAGAAAGGCACAGACGTATCAACAGTAAAAAAAAAGAGTCAGCATGGGAAA
41     K  D  R  E  R  H  R  R  I  N  S  K  K  K  E  S  A  W  E  M

181  TGACAAAAAGTCTGTATGATGCCTGGTCAGGATGGCTGGTAGTGACACTGACAGGACTGG
61     T  K  S  L  Y  D  A  W  S  G  W  L  V  V  T  L  T  G  L  A

241  CTTCAGGGGCACTAGCTGGATTGATAGACATCGCTGCTGACTGGATGACTGACCTAAAGG
81     S  G  A  L  A  G  L  I  D  I  A  A  D  W  M  T  D  L  K  E

301  AGGGCATTTGCCTCAGTGCATTGTGGTACAACCATGAGCAGTGTTGTTGGGGGTCTAATG
101    G  I  C  L  S  A  L  W  Y  N  H  E  Q  C  C  W  G  S  N  E

361  AAACAACATTTGAAGAGAGGGATAAATGTCCACAGTGGAAAACATGGGCAGAGTTAATCA
121    T  T  F  E  E  R  D  K  C  P  Q  W  K  T  W  A  E  L  I  I

421  TAGGTCAAGCAGAGGGTCCGGGATCTTATATCATGAACTACATAATGTACATCTTTTGGG
141    G  Q  A  E  G  P  G  S  Y  I  M  N  Y  I  M  Y  I  F  W  A

481  CCCTGAGTTTTGCCTTTCTTGCAGTTTCTTTGGTGAAAGTATTTGCTCCATATGCCTGTG
161    L  S  F  A  F  L  A  V  S  L  V  K  V  F  A  P  Y  A  C  G

541  GCTCTGGAATTCCAGAGATTAAAACTATTTTGAGTGGATTCATCATCAGAGGA 593
181    S  G  I  P  E  I  K  T  I  L  S  G  F  I  I  R  G   197
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FIG. 8. Sequence of the hamster CLC-3 cDNA. The cDNA sequence and predicted amino acid sequence of the HIT CLC-3 cDNA are shown.

In other cell systems, a primary role exists for volume-activated outwardly rectifying Cl^- channels in cell volume regulation (6,22–24,36,55–59). Thus, in response to swelling, Cl^- channels mediate the net efflux of anions, which in turn promotes an RVD (60–62). In β-cells, cell swelling produced by lowered extracellular osmolarity has been reported to be followed by an RVD, which is mediated by the loop of Henle-type Na/K/2Cl cotransporter because the RVD is inhibited by furosemide (39). In our hands, however, furosemide disrupted cell volume regulation rather than blocking the RVD per se. Miley et al. (7) observed a regulatory volume increase (RVI) in rat pancreatic β-cells after the application of a hypertonic solution that initially decreased cell volume; this RVI was blocked by DIDS. These findings suggest an important role for Cl^- transport mechanisms in islet cell volume regulation, although the exact role of these individual pathways is still not clear. Our results suggest an important role of $I_{Cl,islet}$ in RVD mediation in intact HIT cells. Thus, we suggest that these channels play a

selective role in β-cell volume regulation, ionic homeostasis, and the control of at least a component of proximal insulin secretion.

In rat β-cells, concentrations of glucose that stimulate insulin secretion have been reported to increase β-cell volume, suggesting that a volume-sensitive mechanism could contribute to glucose-dependent insulin secretion (7). Both our results and those of Miley et al. (7) showed that increasing cell volume provoked transient insulin release from HIT and rat pancreatic β-cells; Blackard et al. (18) found similar results in intact rat islets. Because hypotonic solution activates $I_{Cl,islet}$ after increased cell swelling, our original hypothesis (5) was that $I_{Cl,islet}$-mediated membrane depolarization, which would occur as the cells swelled, induced insulin secretion via the depolarization-dependent influx of Ca^{2+} (1). Our present results, however, do not support this hypothesis, because neither DIDS nor niflumic acid could fully inhibit hypotonic-induced secretion, despite significant Cl^- channel blockade. In addition, we show that hypotonic-induced secretion persists under conditions in which HIT cells are persistently depolarized (e.g., diazoxide and high K^+). Thus, our results suggest that hypotonic solution triggers insulin secretion primarily at sites that are distal to the membrane signaling (e.g., ionic) events of the β-cell stimulus secretion coupling cascade.

It is known that vesicle docking, fusion, and the formation of a fusion pore precede the expansion of the granule matrix and the subsequent exocytosis of secretory products in several cell types (63,64). It is plausible that increased water flux into the cell and/or water flux across the membranes of a docked pool of granules could promote insulin granule exocytosis, perhaps even at a constant level of $[Ca^{2+}]_i$. This would be consistent with our finding that secretion was stimulated by hypotonic solution even after the HIT cells were persistently depolarized by the combination of high KCl and diazoxide, conditions known to isolate a distal secretory pathway in islets (31,46,65). It is interesting that in the original observations of Blackard et al. (18), hypotonic conditions in rat islets were found to stimulate the release of not only insulin, but also glucagon, somatostatin, and other hormones. This suggests that rather than targeting ionic or proximal signaling pathways specific to the pancreatic β-cell, cell swelling may target more generic mechanisms not strictly limited to β-cells. Because it is likely that the distal exocytotic steps used by different cell types share similar molecular mechanisms, swelling-induced vesicular release in this case might be expected to result in the release of many islet hormones if it occurred through a common pathway.

The first voltage-gated Cl^- channel to be cloned was CLC-0 (66). This led to the discovery of the CLC superfamily, which includes CLC-0, CLC-1, CLC-2, CLC-3, CLCK1, CLCK2, and other channels (25,66–70). Although there was early evidence to support the hypothesis that protein-mediating nucleotide-sensitive chloride current (P_{Cln}) was the ubiquitous outwardly rectifying volume-regulated Cl^- channel (71), it is now believed that CLC-3, which is present in brain, kidney, and heart, among other tissues (25), is the likely molecular counterpart to volume-stimulated Cl^- channels (72). RT-PCR of HIT cell mRNA, using oligonucleotide primers based on the published CLC-3 sequence, isolated a 593-bp transcript that was >90% homologous to guinea pig, rat, mouse, or human CLC-3. These data suggest that CLC-3 or a CLC-3 variant may be the molecular counterpart of $I_{Cl,islet}$ in HIT cells. Whereas it is clear that the CLC-3 message is present in HIT cells, the biophysical characteristics of $I_{Cl,islet}$ are not completely consistent with the known properties of CLC-3. Thus, although CLC-3 and $I_{Cl,islet}$ are both outwardly rectifying Cl^- channels that are blocked by niflumic acid, DIDS, and NPPB (26), cyclic AMP activates $I_{Cl,islet}$ (5) but inhibits CLC-3 (73). Furthermore, the anion selectivity sequences of the two channels are different, with $I_{Cl,islet}$ having the halide sequence $Br^- > Cl^- > I^-$ (5,6), whereas CLC-3 has the sequence $I^- > Br^- > Cl^-$ (74). Interestingly, $I_{Cl,islet}$ shares its unique halide selectivity sequence with the cystic fibrosis transmembrane conductance regulator (75). This suggests that another channel, possibly a close relative of CLC-3, mediates $I_{Cl,islet}$ in HIT cells. It should be noted that mutating a single site within the CLC-3 pore is sufficient to alter the permeability sequence of CLC-3 (26). To the best of our knowledge, ours is the first report of CLC-3 being present in an insulin-secreting preparation. Although there are some discrepancies between the properties of CLC-3 and $I_{Cl,islet}$, it is conceivable that a close relative of CLC-3 or a CLC-3 splice variant may underlie the functional islet anion channel. Alternatively, an altogether different protein may mediate $I_{Cl,islet}$, with CLC-3 subserving some other, as yet undefined, role in β-cells.

As stated above, CLC-3 may be involved in proximal Cl^- channel activity in response to cell swelling in β-cells, resulting in Ca^{2+} influx and a component of insulin secretion attributable to this signaling pathway. However, swelling may also have an impact on exocytosis at a more distal site that is independent of proximal ionic events, as has been proposed recently for glucose (31,46,65) and sulfonylureas (47). These distal actions may result from the direct facilitation of granule fusion or release by increased water flux. Alternatively, swelling could release intracellular Ca^{2+} from endoplasmic reticulum stores, as has been reported in other systems (76,77). Because hypotonic stimuli have also been linked to the activation of several intracellular second messenger cascades, including activation of phospholipase C or tyrosine kinase, we cannot rule out the possibility that these mechanisms link cell swelling to distal granule release (76).

Interestingly, Barg et al. (78) recently suggested that sulfonylureas work distally in β-cells by binding SUR1 receptors present in the membranes of the insulin secretory granules (78). In this hypothesis, sulfonylurea binding to SUR1 opens granule membrane Cl^- channels, promoting Cl^- and water flux into the granule. The resultant granule swelling in turn induces granule exocytosis. This hypothesis is consistent with their finding that the distal actions of glyburide appear to be blocked by DIDS. We have previously provided evidence to suggest that sulfonylureas indeed can activate Cl^- channels on the surface membrane of the β-cell (5), although the

ability of glyburide to activate $I_{Cl,islet}$ may not be shared by all the sulfonylureas as a group (79).

It is attractive to speculate that water flux into the β-cell after the application of hypotonic solution could trigger granule fusion and exocytosis through the same distal mechanism proposed for the sulfonylureas. Rorsman and colleagues hypothesize that granule swelling, requires the opening of DIDS-blockable granule Cl^- channels and concomitant anion influx, granular swelling, and exocytosis. However, in our experiments, our use of a hypotonic stimulus to release insulin might more directly cause granule swelling and thus would be less sensitive to Cl^- channel blockers. Indeed, the application of DIDS or niflumic acid to the high K^+/diazoxide solutions used in our study did not abolish distal insulin secretion. If glucose metabolism activates $I_{Cl,islet}$ by causing β-cell swelling and, concomitantly, plasma membrane depolarization (7), DIDS or niflumic acid should interfere with glucose-induced secretion through this pathway, as we found. It remains to be determined specifically how CLC-3 may be involved in these proximal and distal steps in the β-cell stimulus-secretion coupling pathway and whether alterations in these complex steps play a role in the reduced glucose-dependent insulin secretion that is known to be a part of type 2 diabetes.

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