Stimulation of Insulin Secretion by Denatonium, One of the Most Bitter-Tasting Substances Known

Susanne G. Straub, Jennifer Mulvaney-Musa, Hiroki Yajima, Gregory A. Weiland, and Geoffrey W.G. Sharp

Denatonium, one of the most bitter-tasting substances known, stimulated insulin secretion in clonal HIT-T15 β-cells and rat pancreatic islets. Stimulation of release began promptly after exposure of the β-cells to denatonium, reached peak rates after 4–5 min, and then declined to near basal values after 20–30 min. In islets, no effect was observed at 2.8 mmol/l glucose, whereas a marked stimulation was observed at 8.3 mmol/l glucose. No stimulation occurred in the absence of extracellular Ca^{2+} or in the presence of the Ca^{2+}-channel blocker nitrendipine. Stimulated release was inhibited by α_2-adrenergic agonists. Denatonium had no direct effect on voltage-gated calcium channels or on cyclic AMP levels. There was no evidence for the activation of gustducin or transducin in the β-cell. The results indicate that denatonium stimulates insulin secretion by decreasing KATP channel activity, depolarizing the β-cell, and increasing Ca^{2+} influx. Denatonium did not displace glybenclamide from its binding sites on the sulfonylurea receptor (SUR). Strikingly, it increased glybenclamide binding by decreasing the K_d. It is concluded that denatonium, which interacts with K^+ channels in taste cells, most likely binds to and blocks Kir6.2. A consequence of this is a conformational change in SUR to increase the SUR/glybenclamide binding affinity. *Diabetes* 52: 356–364, 2003

Multiple signal transduction mechanisms in taste cells detect sweet, sour, bitter, salty, and umami tastes and involve both receptor-mediated second messenger pathways and direct interactions with ion channels (1–3). Four mechanisms have been identified for signal transduction by bitter compounds. Two mechanisms result from bitter substances interacting with receptors of the T2R/TRB family and activation of gustducin (4–10). In one, the α-subunit of gustducin activates cyclic nucleotide phosphodiesterase activity to decrease cyclic nucleotide monophosphate (cyclic NMP) levels and de-inhibit the activity of a cyclic NMP-inhibited cation channel leading to depolarization and increased $[Ca^{2+}]_i$ (11,12). In the second, the βγ-subunits of gustducin (probably β1γ13 and/or β3γ13) activate PLCβ2 and increase IP_3 and DAG levels (13,14). IP_3 increases $[Ca^{2+}]_i$ via IP_3R3 activation (14–16). A third mechanism involves a direct interaction and inhibition of K^+ channels resulting in depolarization and increased $[Ca^{2+}]_i$ (17). The fourth known mechanism is the direct gating of nonselective cation channels and depolarization, again leading to increased $[Ca^{2+}]_i$ (18–20). In all cases, increased $[Ca^{2+}]_i$ leads to increased neurotransmitter release and activation of the afferent gustatory nerves that transmit the bitter signal to the brain. There is evidence for the activation of multiple second messenger pathways in individual taste cells (4). There is also evidence that some bitter stimuli activate only subpopulations of bitter-sensitive taste cells (4–6). Also, individual taste cells can respond selectively to different bitter substances (7). However, there is still much that is unknown about individual taste cell regulation.

Stimulation of taste cell membranes with denatonium activates not only gustducin but also transducin (9), with which it shares high sequence homology (21). Because transducin mRNA is reported to be present in pancreatic islets at approximately one-fifth the level of that in the retina (22), we were interested to determine the effects of denatonium on insulin secretion in β-cells as a tool to study the potential involvement of gustducin or transducin in stimulus-secretion coupling. The experiments described here were performed on clonal HIT-T15 cells and rat pancreatic islets.

RESEARCH DESIGN AND METHODS

Cell culture. Clonal HIT-T15 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin in an atmosphere of 95% air and 5% CO_2. Cells of passage numbers 72–85 were used for the experiments.

Islet isolation. Pancreatic islets from Sprague Dawley rats were isolated using the collagenase digestion technique of Lacy and Kostianovsky (23).

Insulin secretion. Static incubation measurements of insulin release by HIT-T15 cells were performed in Krebs-Ringer-HEPES bicarbonate buffer (KRBH) of the following composition (in mmol/l): 129 NaCl, 5 $NaHCO_3$, 4.8 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 10 HEPES, and 1 $CaCl_2$ at pH 7.4, with 0.1% BSA and the indicated glucose concentrations. Preincubation and test incubation periods were 30 min each. For dynamic measurements under perifusion conditions, $\sim 2 \times 10^6$ HIT-T15 cells on glass coverslips were placed in 700-μl chambers and perifused with KRBH buffer at a flow rate of 1 ml/min. A preperifusion for 50 min was carried out to equilibrate the secretion rates before exposure of the cells to control and test conditions. Samples were collected at 1- or 2-min intervals and kept at −20°C before radioimmunoassay with a charcoal separation method. For the measurement of insulin release by rat islets under static incubation conditions, the KRBH contained 2.5 mmol/l $CaCl_2$. The islets were subjected to a 60-min preincubation before being exposed to fresh buffer solution containing the test agents for an additional 60 min. Samples of the incubation medium were collected and stored at −20°C before radioimmunoassay.

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Received for publication 8 April 2002 and accepted in revised form 17 October 2002.

PMSF, phenylmethylsulfonyl fluoride; SUR, sulfonylurea receptor.

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Cyclic AMP determination. After a 30-min preincubation period in KRBH buffer, HIT-T15 cells were exposed to the test agents for 5 min and the incubation was stopped by the addition of 0.25 ml of 6% trichloroacetic acid. The cells were scraped free, transferred to Eppendorf tubes, and centrifuged at 2,500*g* for 10 min at 4°C. The cell pellet was solubilized in 0.1 mmol/l NaOH, and protein content was assessed using the Bradford assay. The supernatant was used for determination of the cyclic AMP content by radioimmunoassay after ether extraction of the trichloroacetic acid.

Electrophysiology. Cell membrane potentials and currents were measured under current clamp or voltage clamp conditions using either whole-cell or perforated-patch configurations (24,25). All recordings were made at room temperature. HIT-T15 cells plated on 18-mm circular glass coverslips were placed in a recording chamber mounted on an inverted microscope. Whole-cell recordings were performed within 2–4 days after the cells were plated. Patch-clamp recordings were done on cells that were not in contact with other cells to avoid possible cell-cell coupling artifacts. Patch-clamp electrodes were made from borosilicate glass capillaries (Garner Glass Company) using a two-stage puller (Narishighe). Electrodes were adjusted by firepolishing to obtain a tip resistance of ~2–4 megohms. Amphotericin B was added to the intracellular solution from a stock solution to obtain a final concentration of 200 μg/ml. Recording electrodes were tip-filled with clean intracellular solution and then back-filled with intracellular solution containing 200 μg/ml Amphotericin B. The electrode tip resistance was monitored by applying a 1-mV square pulse of 10 ms at 10 Hz. Once a high resistance seal (2–10 gigohms) was formed between the recording pipette and the cell, the access resistance was continuously monitored until it reached values <30 megohms. Capacitance was monitored before, during, and after each experiment. All voltage clamp protocols were generated and currents were recorded using an EPC-7 amplifier (List Electronic) and pClamp 5.51 acquisition system (Axon Instruments). The extracellular solution was exchanged continuously during recordings to add/washout drugs. Bath perfusion was performed by exchanging the content of the 18-mm recording chamber with extracellular solutions at a rate of ~2 ml/min using a gravity flow system. Drugs were added in the extracellular solution.

Solutions for the electrophysiological studies. For recording currents through voltage-gated calcium channels, the solutions were (in mmol/l) as follows: external: 20 $CaCl_2$, 110 NaCl, 5 CsCl, and 10 HEPES (pH 7.4 with CsOH); and internal: 10 CsCl, 90 $CsSO_4$, 10 EGTA, 1 $CaCl_2$, and 10 HEPES (pH 7.2 with CsOH). Currents were recorded from cells stepped for 200 ms from a holding potential of −60 mV to test potentials between −50 and 40 mV at 10-mV increments.

For recording KATP currents, the solutions were (in mmol/l) as follows: external: 140 KCl, 2 $CaCl_2$, 1.1 $MgCl_2$, and 10 HEPES (pH 7.4 with NaOH) and internal: 70 K_2SO_4, 10 KCl, 10 NaCl, 2 $MgCl_2$, and 10 HEPES (pH 7.2). Currents were evoked by stepping from a −60 mV holding potential to membrane potentials ranging from −130 mV to −50 mV in 10-mV increments for 200 ms.

Western blot analysis. Membrane and cytosol fractions were prepared from HIT-T15 cells. Briefly, cells were harvested and homogenized with a Dounce-glass homogenizer in a buffer containing 10 mmol/l Tris (pH 7.5), 10% (vol/vol) glycerol, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 10 μg/μl aprotinin, 10 μg/μl leupeptin, 10 μg/μl pepstatin A, and 1 mmol/l phenylmethylsulfonyl fluoride (PMSF). Unbroken cells were removed by centrifugation at 1,500*g* for 10 min. After a 30-min centrifugation at 96,000*g* at 4°C, the supernatant was collected as the cytosol fraction and the pellet was solubilized in the above buffer supplemented with 1% Triton X-100 and used as the membrane fraction. Cytosol and membrane samples were mixed with 5× Laemmli sample buffer, boiled for 5 min, and resolved on a 14% gel by SDS-PAGE. After transfer to a polyvinylidene fluoride membrane and blocking in a 5% BSA solution, Western blot analysis was performed using a gustducin or transducin antibody and visualizing the immunoreactive bands by enhanced chemiluminescence (Amersham).

Trypsin digestion assay for activated transducin. The method was similar to those described previously (11,26). Membranes of HIT-T15 cells (prepared as above) were resuspended in DEAE buffer (in mmol/l: 150 NaCl, 25 Tris, 1 $MgCl_2$, 5 DTT, and 1 CHAPS at pH 7.5 with 100 μmol/l GDP and 1 μmol/l GTPγS) and then incubated with the heterotrimeric form of rod transducin GαGDP*βγ (a gift of Drs. Richard Cerione and Jon Erikson) plus denatonium and other taste substances for 1 h at 30°C. AlF_4^- used as a positive control was prepared using 30 μmol/l $AlCl_3$ and 10 mmol/l NaF. Subsequently, trypsin digestions (1:25 wt/wt trypsin to total protein) were performed for different periods of time (0, 30, 60, and 90 min) at room temperature and stopped by the addition of soybean trypsin inhibitor at a 10-fold excess over trypsin. Samples were boiled for 5 min in SDS sample buffer and subjected to SDS-PAGE and immunoblotting as described.

[^{3}H]Glybenclamide binding assay. HIT-T15 cells were collected from confluent 175-cm^2 flasks and washed twice with ice-cold PBS. The cells were scraped off into 10 ml of the PBS. The cells were pelleted and resuspended in 20 volumes of 5 mmol/l Tris-HCl (pH 8.0) at 4°C containing 0.1 mmol/l PMSF. They were incubated on ice for 40 min and then homogenized with 15–20 strokes in a Dounce glass homogenizer. The homogenate was centrifuged for 10 min at 900*g*, and the supernatant was collected and centrifuged at 96,000*g* for 30 min. The supernatant was discarded, and the pellets from the second centrifugation were immediately frozen in liquid nitrogen and stored at −80°C until used.

The method was similar to that used by Hu et al. (27). HIT-T15 cell membranes were suspended in 20 mmol/l MOPS (pH 7.4) containing 0.1 mmol/l PMSF and rehomogenized. The protein concentration of the membrane preparation was determined using the Biorad DC protein assay with BSA as the standard. The "competition" assay was performed with 1.0 nmol/l [^{3}H]glybenclamide, denatonium over the concentration range of 0, 0.01–3 mmol/l, and membranes at a protein concentration of 100–200 μg/ml at room temperature for 2 h. Nonspecific binding was determined in the presence of 2 μmol/l unlabeled glybenclamide. The assay was performed in 50 mmol/l MOPS (pH 7.4), 0.1 mmol/l $CaCl_2$ (buffer A), in a total volume of 1 ml/tube. All assays were performed in duplicate. Binding was terminated by rapid filtration through Whatman GF/F 25-mm-diameter glass microfiber filters (presoaked in buffer A) followed by five washes with 5 ml of cold 0.1 mol/l NaCl. The filters were placed in 10 ml of Formula 989 scintillation fluid (Packard, Meriden, CT), and radioactivity was determined after overnight incubation by liquid scintillation spectrometry. Saturation binding assays were performed under the same conditions with [^{3}H]glybenclamide at concentrations from 0.1 to 10 nmol/l in the presence or the absence of 1 mmol/l denatonium. Nonspecific binding was determined in the presence of 2 μmol/l unlabeled glybenclamide. K_d and Bmax values were determined by nonlinear curve fitting of the saturation data using Kaleidagraph (Synergy Software).

Reagents. Denatonium benzoate, amphotericin B, glybenclamide, DIDS, and clonidine were obtained from Sigma (St. Louis, MO). ^{125}I-insulin, ^{3}H-glybenclamide, and the ^{125}I-cyclic AMP radioimmunoassay kit were obtained from DuPont/NEN (Boston, MA). The rabbit polyclonal antigustducin and antitransducin antibodies were from Santa Cruz Biotech (Santa Cruz, CA).

Statistical analysis. Results are presented as mean ± SE. Statistical analysis was by Student's *t* test for paired and unpaired data as appropriate, except for the results in Fig. 9*A*, which was by one-way ANOVA with pairwise comparison by Dunnett's method.

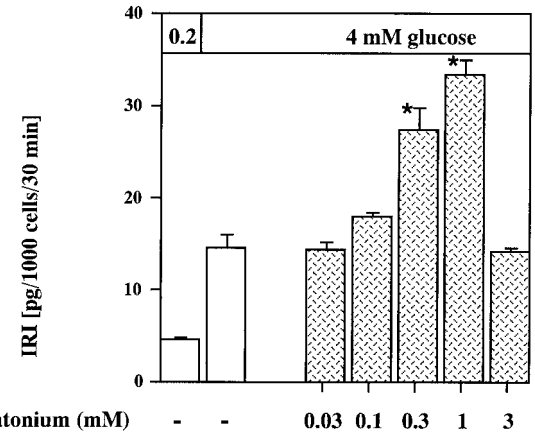

FIG. 1. Concentration-response characteristics for the effect of denatonium to stimulate insulin secretion in HIT-T15 cells. Denatonium was tested at concentrations from 0.03 to 3 mmol/l under conditions of static incubation (n = 4). *P < 0.01 vs. 4 mmol/l glucose.

RESULTS

Concentration-response characteristics for the actions of denatonium on insulin release in HIT-T15 cells. The effect of denatonium was first examined in the presence of 0.2 mmol/l glucose. Only small stimulatory responses were observed to denatonium at concentrations of 0.3 and 1 mmol/l (data not shown). In the presence of 4 mmol/l glucose, denatonium had a marked stimulatory effect on secretion. Concentration-response characteristics for the action of denatonium on insulin secretion in the HIT-T15 cell are shown in Fig. 1. Experiments were

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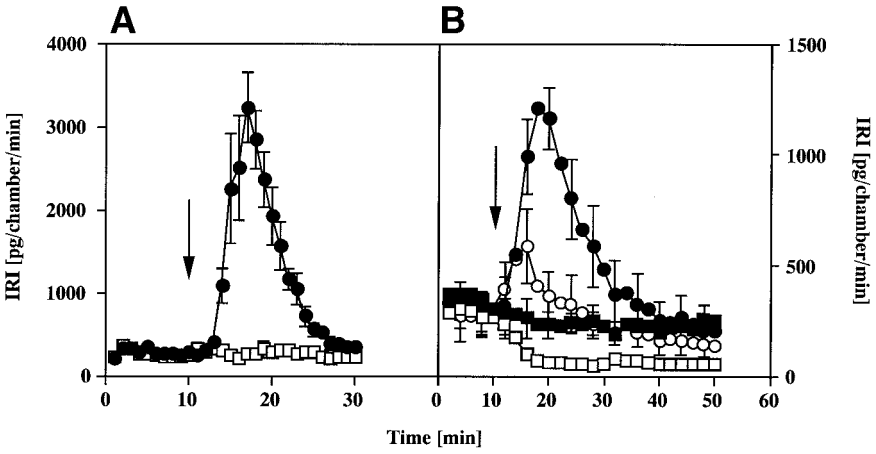

FIG. 2. *A*: **The effect of 1 mmol/l denatonium (solid circles) and lack of effect of 1 mmol/l sodium benzoate (□) in the presence of 4 mmol/l glucose on HIT-T15 cells under perifusion conditions.** *B*: **The effect of 10 μmol/l clonidine (□) on insulin secretion stimulated by 4 mmol/l glucose (■) and the effect of clonidine (○) on the insulin secretion stimulated by glucose plus 1 mmol/l denatonium (●).** $n = 4$.

carried out under static incubation conditions for 30 min. The rate of insulin release in the presence of 4 mmol/l glucose was 3.2-fold that of the basal release rate at 0.2 mmol/l glucose. Denatonium at a concentration of 30 μmol/l, in the presence of 4 mmol/l glucose, did not affect the rate of insulin release. However, in the range between 100 μmol/l and 1 mmol/l, it stimulated insulin secretion ($P < 0.01$) in a concentration-dependent manner with the maximal response occurring at 1 mmol/l. Denatonium at 3 mmol/l had no effect on the secretory response. Denatonium exerted its maximal effect at 1 mmol/l and more than doubled the rate of insulin secretion.

Temporal profile of the stimulation of insulin release by denatonium. Insulin secretion was examined under perifusion conditions in the presence of 4 mmol/l glucose to assess the magnitude and duration of the stimulatory responses. After addition of 1 mmol/l denatonium, the rate of insulin release increased rapidly and reached the peak response after 8 min (Fig. 2*A*). This maximum response reflects a 13-fold stimulation over the basal rate. Secretion rates declined thereafter to reach near-basal values after ~20 min. Also shown in Fig. 2*A* is the lack of effect of 1 mmol/l benzoic acid studied as a control for the benzoate component of the denatonium salt. In the next series of experiments, we determined the effect of the partial α_2-agonist clonidine on insulin release stimulated by 4 mmol/l glucose and on the potentiation of 4 mmol/l glucose-stimulated insulin secretion by 1 mmol/l denatonium. As can be seen from the data in Fig. 2*B*, clonidine added at minute 10 inhibited both glucose-stimulated insulin secretion and the denatonium-induced potentiation of release. Thus the stimulation of release by denatonium is exerted via the normal physiological mechanisms of exocytosis.

The effect of denatonium on rat islets. In the presence of 2.8 mmol/l glucose, concentrations of denatonium up to 1 mmol/l had no effect on the release of insulin (2.8 mmol/l glucose = 564 ± 97 pg · islet^{-1} · 60 min^{-1} vs. 2.8 + 1 mmol/l denatonium = 570 ± 294 pg · islet^{-1} · 60 min^{-1}; $P > 0.49$, $n = 4$). However, in the presence of 8.3 mmol/l glucose, denatonium stimulated insulin secretion. From the results shown in Fig. 3, it can be seen that 8.3 mmol/l glucose caused an approximately sevenfold increase in insulin release. Denatonium at 100 and 300 μmol/l potentiated the rate of insulin secretion as a result of 8.3 mmol/l glucose in a concentration-dependent manner ($P < 0.03$ and $P < 0.002$, respectively). Denatonium at 1 mmol/l potentiated insulin secretion ($P < 0.02$) but gave a smaller response than 300 μmol/l. Denatonium at 3 mmol/l had no stimulatory effect and significantly inhibited the secretion as a result of 8.3 mmol/l glucose ($P < 0.05$).

The role of Ca^{2+} in the stimulation of insulin release by denatonium. The ability of denatonium to stimulate insulin release in the absence of extracellular Ca^{2+} and in the presence of nitrendipine was studied. Both the absence of extracellular Ca^{2+} and the addition of nitrendipine in the presence of extracellular Ca^{2+} inhibited both 4 mmol/l glucose-stimulated release ($P < 0.02$) and denatonium-potentiated insulin release ($P < 0.001$). The results suggest that the action of denatonium is associated with Ca^{2+} entry via L-type voltage-dependent Ca^{2+} channels. The results are shown in Fig. 4.

Effect of denatonium on cyclic AMP levels in HIT-T15 cells. To determine whether denatonium was enhancing the rate of insulin secretion by an action to elevate intracellular cyclic AMP levels, we measured the cyclic AMP content of HIT-T15 cells in the absence and presence of 1 mmol/l denatonium (the maximally effective concentration). No change of cyclic AMP was detected. The control value in the presence of 4 mmol/l glucose was 22 ± 1 pmol/mg protein, and after 5 min exposure to 1 mmol/l denatonium (at which time insulin secretion is maximally stimulated), the value was 24 ± 1 pmol/mg protein ($P >$

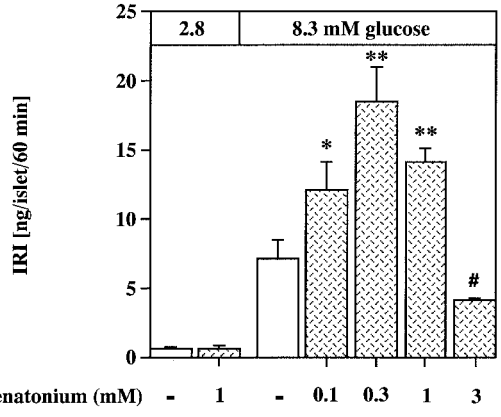

FIG. 3. Concentration-response characteristics for the effect of denatonium to stimulate insulin secretion by isolated rat pancreatic islets. Denatonium was tested at concentrations from 0.1 to 3 mmol/l under conditions of static incubation ($n = 5$). *$P < 0.03$; **$P < 0.002$; #$P < 0.05$ (all vs. 8.3 mmol/l glucose alone).

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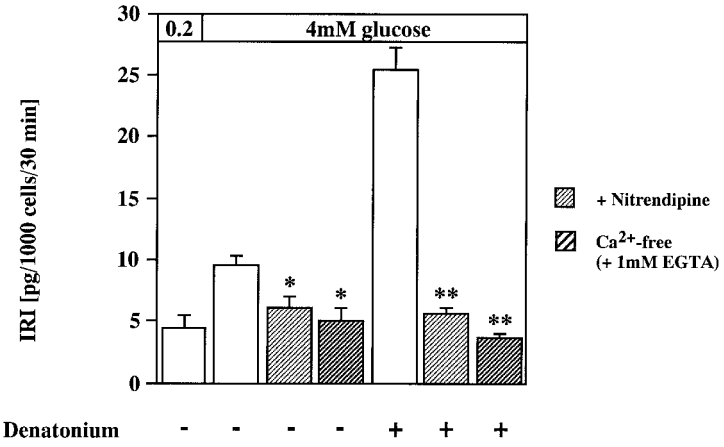

FIG. 4. The lack of effect of denatonium to stimulate insulin release in the absence of extracellular Ca^{2+} or in the presence of 10 μmol/l nitrendipine. The experiments were performed on HIT-T15 cells in the presence of 4 mmol/l glucose. Both glucose- and denatonium-stimulated release were inhibited by the absence of extracellular Ca^{2+} and by nitrendipine in the presence of extracellular Ca^{2+} ($n = 4$). *$P < 0.02$ vs. 4 mmol/l glucose; **$P < 0.001$ vs. 4 mmol/l glucose + denatonium.

0.18 versus control, $n = 4$). As a positive control for the assay, the cyclic AMP content of the cells after a 5-min exposure to 300 nmol/l forskolin was 364 ± 21 pmol/mg protein ($P < 0.001$ vs. control, $n = 4$).

Effect of denatonium on membrane potential. Denatonium induced a glucose-dependent depolarization and increase in membrane potential fluctuations. Initial experiments examined changes in HIT-T15 cell membrane potential and electrical activity after exposure to denatonium at two different glucose concentrations. The resting potential of cells, measured after a 15- to 30-min exposure to 0.2 mmol/l glucose, was −62.2 ± 6 mV ($n = 6$). Application of 500 μmol/l denatonium caused a slight depolarization (−55.7 ± 5 mV, $n = 5$), which was rapidly reversible after washout of the denatonium. Generally, no action potentials developed after denatonium application to cells maintained in 0.2 mmol/l glucose. However, application of 500 μmol/l denatonium to cells maintained in 4 mmol/l glucose resulted in a substantial depolarization (13.5 ± 7 mV, $n = 10$) and an increase in membrane activity that was often accompanied by generation of multiple action potentials. The denatonium-stimulated depolarization and increase in electrical activity was quickly reversible after washing of the cell. Figure 5 shows membrane potential changes from a representative single cell exposed to denatonium at glucose concentrations of 0.2 and 4 mmol/l. Subsequent experiments were performed to investigate the underlying ionic mechanisms that contribute to the denatonium-induced glucose-dependent depolarization and increase in membrane activity in HIT-T15 cells.

Effect of denatonium on the voltage-gated Ca^{2+} channel. The data presented thus far demonstrate that the stimulation of insulin secretion by denatonium is associated with depolarization and requires the influx of extracellular calcium. The denatonium-induced depolarization would certainly contribute to the opening of voltage-gated calcium channels. However, it is also conceivable that denatonium could have a direct effect on voltage-gated calcium channels via a biochemical modification that increases the frequency of channel openings. It has been shown in RINm5F cells, for example, that glyceraldehyde increases the open probability and duration of calcium channel activity (28). We used whole-cell patch-clamp conditions to perform a direct examination of the effects of denatonium on current flow through voltage-gated calcium channels at three different glucose concentrations. Calcium was used as the charge carrier, and the peak current was observed at 10 mV. The peak voltage-activated calcium current in control cells was −155 ± 64 pA ($n = 20$ cells). In the presence of 500 μmol/l denatonium, the peak current was −159 ± 53 pA ($n = 20$ cells). In these experiments designed to look exclusively at current flow through voltage-gated calcium channels, there was no observable difference in the peak current at glucose concentrations of 0.2, 4, and 10 mmol/l (data not shown). There was also no shift in the voltage dependence

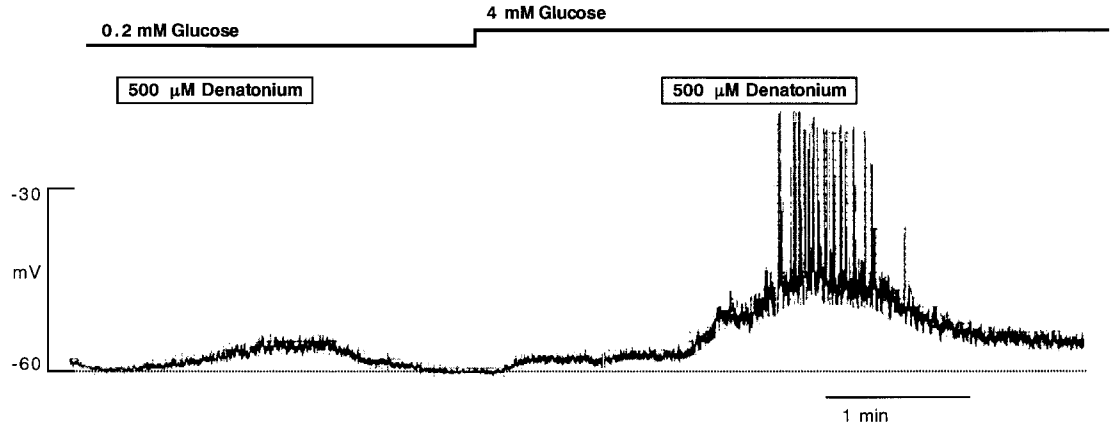

FIG. 5. The effect of denatonium on the membrane potential of an isolated HIT-T15 cell using the whole-cell perforated-patch technique. The cell was equilibrated in 0.2 mmol/l glucose for ~15 min, and 500 μmol/l denatonium was added to the bath for the period indicated by the rectangle. The cell was then exposed to 4 mmol/l glucose as indicated by the upper line and to denatonium for the period indicated by the rectangle.

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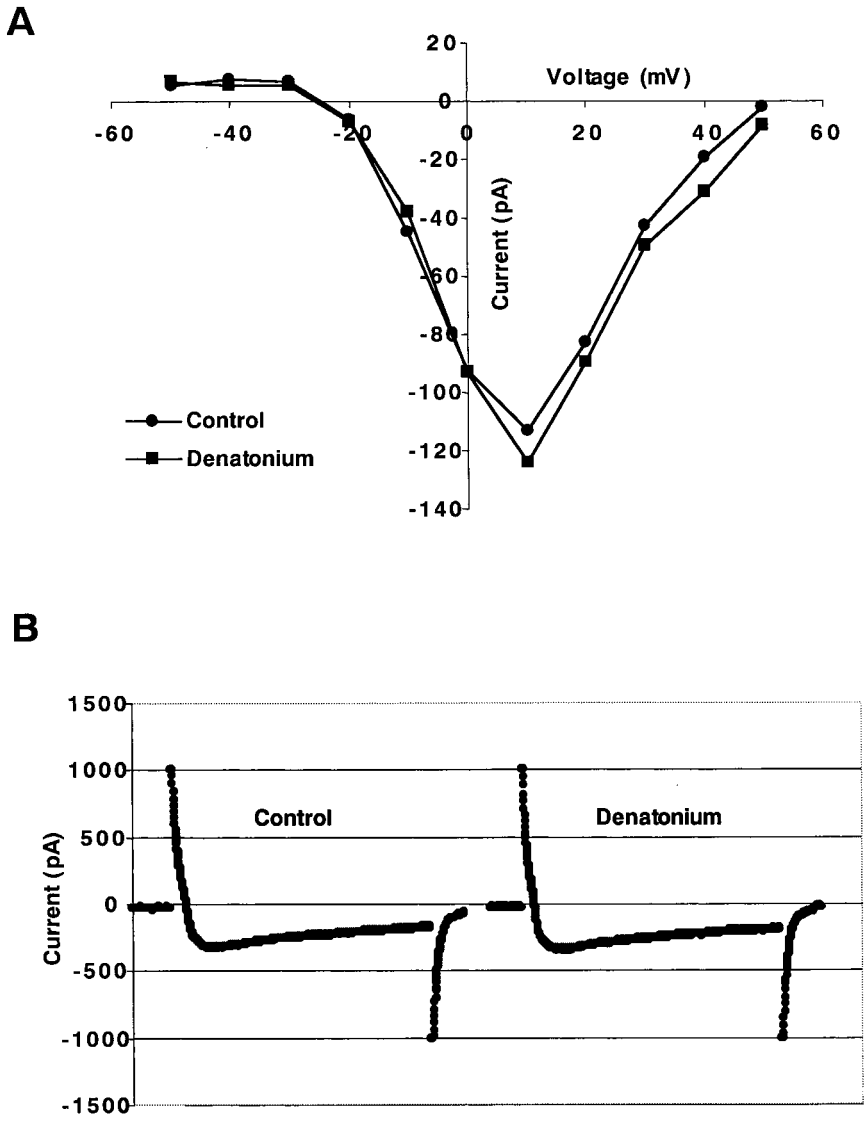

FIG. 6. Voltage-gated calcium currents are not directly influenced by denatonium. *A*: Current-voltage relationship for peak currents from a representative HIT-T15 cell held at −60 mV and stepped from −50 mV to 50 mV at 10-mV increments. *B*: Time course showing activation of voltage-gated calcium current in a representative HIT-T15 cell held at −60 mV and stepped to 10 mV in control solution and after the application of 500 μmol/l denatonium. Solutions contained 4 mmol/l glucose.

of current activation in the presence of denatonium. Figure 6*A* shows the current-voltage relationships from a representative cell in control solution and in the presence of denatonium. Figure 6*B* contains data from a representative cell in 4 mmol/l glucose showing the time course of the voltage-gated calcium current in the absence and presence of 500 μmol/l denatonium. Taken together, these data indicate that denatonium has no direct effect on the voltage-gated calcium channels.

Effect of denatonium on the ATP-sensitive KATP channel. A likely target for the depolarizing action of denatonium on membrane potential in the β-cell is the regulation of potassium permeability. Glucose itself depolarizes β-cells by closing the KATP channel. We investigated whether denatonium influenced the whole-cell potassium current in the presence of three different glucose concentrations. Whole-cell KATP channel current was isolated using solutions designed to shift the reversal potential for potassium to a positive potential to isolate the current from voltage-activated potassium currents and to make the KATP current larger. Cells were held at −60 mV and stepped for 200 ms to potentials between −110 mV and 50 mV at 10-mV increments. Measurements of peak KATP current, observed when cells were stepped from −60 mV to −90 mV, were obtained by averaging data points from this 200-ms step. To determine the percentage of current reduction, we compared peak KATP currents with the peak control currents obtained from the same cell before drug application. Addition of 500 μmol/l denatonium reduced the whole-cell KATP channel current at all three glucose concentrations (Fig. 7). As was found for the denatonium-induced depolarization, there seemed to be a glucose dependence to the reduction of the peak current. In low glucose (0.2 mmol/l), the peak KATP channel current was reduced by ~30%; in 4 mmol/l glucose, the reduction was 50%, whereas in 10 mmol/l glucose, denatonium reduced the peak current by nearly 60% when compared with control currents recorded before application of denatonium (Fig. 7*B*). However, one can argue also that the absolute change in peak current was not significantly different at any glucose concentration. For confirming that denatonium was affecting the KATP channel current and not that of other K^+ channels, cells were pretreated with 1 μmol/l glybenclamide, a specific blocker of the KATP channel, before the application of denatonium. Glybenclamide reduced the peak KATP channel

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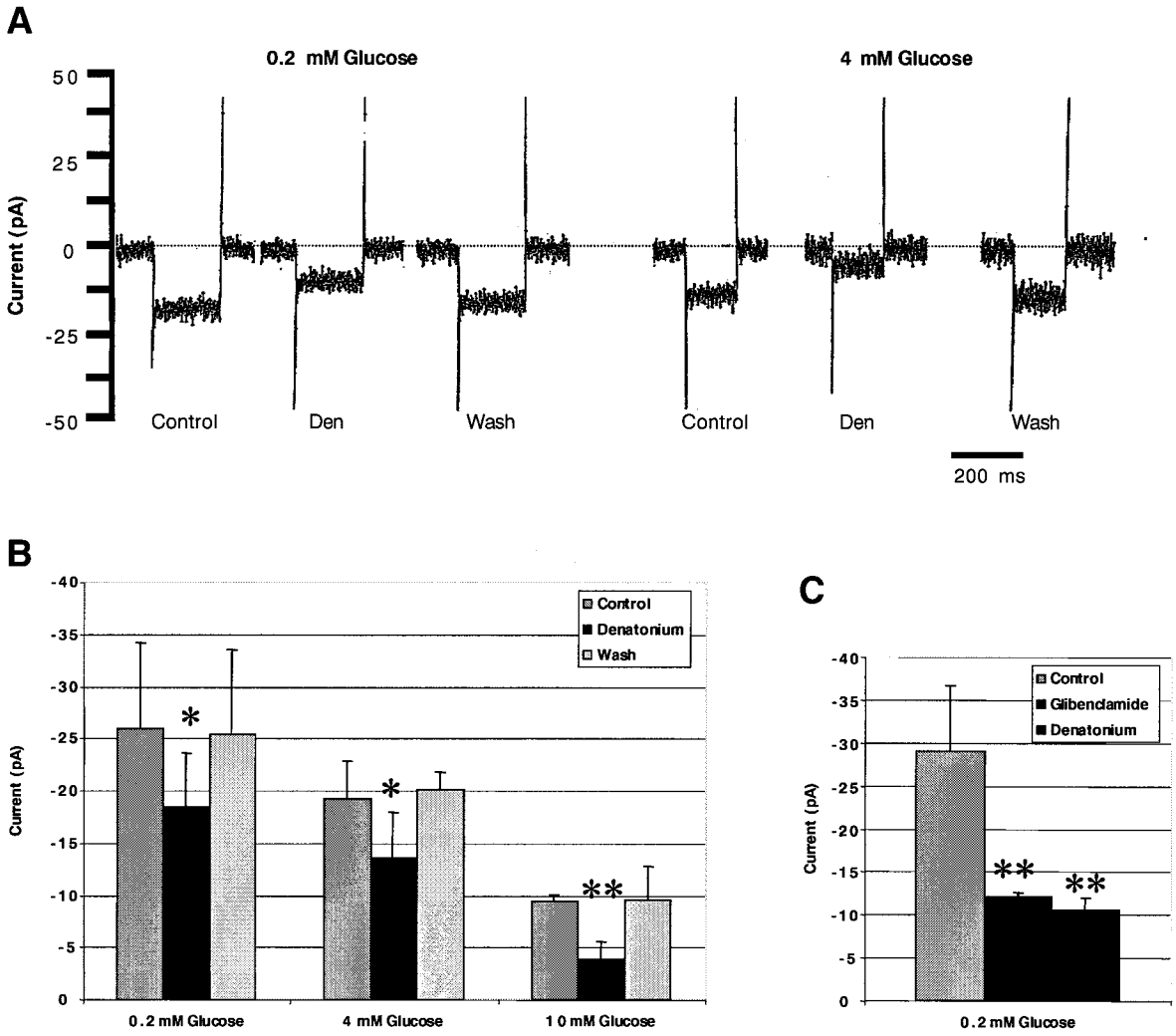

FIG. 7. Denatonium reduces current flow through KATP channels in HIT-T15 cells. *A*: Effect of denatonium on KATP currents in the presence of 0.2 and 4 mmol/l glucose. *B*: Summary of the effect of denatonium on KATP currents in solutions containing 0.2 mmol/l (n = 5 cells), 4 mmol/l (n = 6 cells), or 10 mmol/l (n = 5 cells) glucose. Each point represents the peak current ± SD from cells held at −60 mV and stepped to −90 mV. *P = 0.03, **P < 0.01. *C*: Summary of the effect of denatonium on KATP currents in cells pretreated with 1 μmol/l glibenclamide. Solutions contained 0.2 mmol/l glucose (n = 5 cells). Each point represents the peak current from cells held at −60 mV and stepped to −90 mV ± SE. **P < 0.01.

current by approximately the same magnitude as that observed when 10 mmol/l glucose and denatonium were applied together, and denatonium did not significantly reduce the current further (Fig. 7*C*). These data show that denatonium, like glucose, influences the glybenclamide-sensitive KATP channel and seems to have no major effect on other K^+ channels.

Transducin breakdown assay and Western blot analyses. Using an in vitro assay (11,26), we examined the potential involvement of gustatory G proteins in the stimulus-secretion coupling by denatonium in HIT-T15 cells. This assay takes advantage of conformational differences in the GDP and GTP bound states of transducin or gustducin. Nonactivated transducin (i.e., in the GDP-bound form) is broken down by trypsin digestion to a 23-kDa fragment, whereas transducin in the activated GTP-bound form would yield a higher molecular weight fragment of 32 kDa. The results of one such experiment are shown in the immunoblot in Fig. 8. With the exception of *lanes 1* and *2*, all lanes represent incubations for 1 h followed by a 60-min digestion with trypsin. *Lane 1* is the marker lane with Gt alone (39 kDa); *lane 2* is the 0-min control (Gt + reaction mixture for 1 h followed by the simultaneous addition of trypsin and soybean trypsin inhibitor); lanes *3*, *6*, and *9* are 60-min control lanes (no denatonium in the reaction mixture); *lanes 4* and *7* tested the effect of 1 mmol/l denatonium; *lanes 5* and *8* tested the effect of 3 mmol/l denatonium; and *lane 10* was the positive control with AlF_4^-. The proteolysis of the 39-kDa rod transducin (shown in *lanes 1* and *2*) to a 23-kDa band was similar for all of the conditions shown in *lanes 3–9*, i.e., both control and denatonium-treated conditions. Thus denatonium, at concentrations of either 1 mmol/l (which maximally stimulates insulin secretion) or 3 mmol/l, failed to increase the amount of the 32-kDa fragment. In contrast, as shown in *lane 10*, activation of Gt by AlF_4 resulted in the almost complete proteolysis of Gt to the 32-kDa fragment. In numerous such experiments (a total of 24 in which responses to denatonium and the additional tastants saccharin and quinine were studied), only the inclusion of AlF_4^- in the incubation medium yielded the 32-kDa fragment. Thus, induction of the secretory response by denatonium seems not to include the activation of a gustatory G protein in the HIT-T15 cell line.

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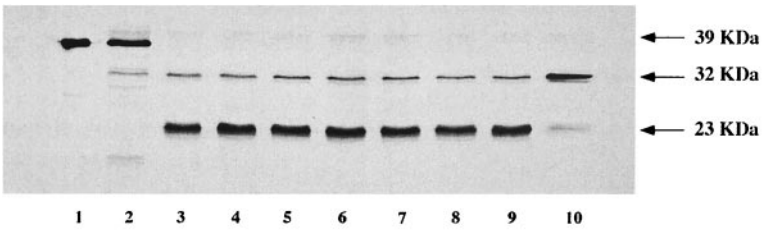

FIG. 8. Denatonium treatment of HIT-T15 cell membranes fails to activate transducin (Gt) in the trypsin proteolysis assay. With the exception of *lanes 1* and *2*, the lanes represent incubations of HIT-T15 cell membranes with Gt for 1 h followed by a 60-min digestion with trypsin. *Lane 1* is the marker lane with Gt alone (39 kDa). *Lane 2* is the 0-min control (Gt reaction mixture for 1 h followed by the simultaneous addition of trypsin and soybean trypsin inhibitor). *Lanes 3, 6,* and *9* are 60-min control lanes without denatonium in the Gt reaction mixture). *Lanes 4* and *7* tested the effect of 1 mmol/l denatonium, and *lanes 5* and *8* tested the effect of 3 mmol/l denatonium. *Lane 10* was the positive control with AlF_4^-. The proteolysis of the 39-kDa Gt (shown in *lanes 1* and *2*) to a 23-kDa band was similar for all of the conditions shown in *lanes 3–9*, i.e., both control and denatonium-treated conditions. In *lane 10*, in contrast, activation of Gt by AlF_4^- resulted in the almost complete proteolysis of Gt to the 32-kDa fragment.

Effect of denatonium on [^{3}H]glybenclamide binding to HIT-T15 cell membranes. ^{3}H-glybenclamide binding to HIT-T15 cell membranes was studied to determine whether denatonium was acting like a sulfonylurea in its action to close KATP channels or whether it had some other unrelated action. Two possibilities were obvious. As there is some structural similarity between denatonium and agonists such as nateglinide and meglitinide (and related hypoglycemic benzoic acid derivatives), it could produce its effect by binding to the same site on the sulfonylurea receptor (SUR) as do these compounds, in which case it would be expected to displace [^{3}H]glybenclamide from its binding site. The alternative possibility is that denatonium interacts directly with the K^+ channel (Kir6.2) in the β-cell in the same way that it interacts with K^+ channels in taste cells. In this case, it might have no effect on [^{3}H]glybenclamide binding or it might cause a conformational change in SUR, which could increase or decrease the amount of [^{3}H]glybenclamide bound. The results showed that denatonium increased [^{3}H]glybenclamide binding in a concentration-dependent manner (Fig. 9*A*). With 1 nmol/l [^{3}H]glybenclamide, 0.3 mmol/l denatonium increased radioligand binding by 8% ($P < 0.05$), and both 1 and 3 mmol/l denatonium increased the binding by 17% ($P < 0.001$ for both; $n = 8$). As found previously (27), [^{3}H]glybenclamide bound specifically to a single class of binding sites on the HIT-T15 cell membranes with a K_d value of 0.37 ± 0.05 nmol/l and a Bmax value of 0.56 ± 0.05 pmol/mg protein (Fig. 9*B*). Saturation analysis revealed that this was due to an increase in the affinity of [^{3}H]glybenclamide for its binding site on the SUR. In the presence of 1 mmol/l denatonium, the K_d for [^{3}H]glybenclamide binding decreased 30 ± 6% to 0.25 ± 0.03 nmol/l ($P < 0.02$; $n = 7$). The increase in binding reflected only an increase in affinity because there was no significant change in Bmax ($P > 0.5$). When the 1 mmol/l denatonium data from the experiments in Fig. 9*A* (where 1 nmol/l [^{3}H]glybenclamide was used) were combined with the 1 nmol/l [^{3}H]glybenclamide data in Fig. 9*B* (where 1 mmol/l denatonium was used), the result was unequivocal. Denatonium at 1 mmol/l increased the binding of 1 nmol/l [^{3}H]glybenclamide by 18 ± 2.5% ($P < 0.0001$, $n = 15$).

The increased affinity of glybenclamide binding induced by denatonium demonstrates that denatonium does not interact with the glybenclamide binding site on the SUR. Possible mechanisms include that denatonium binds to a separate site on the SUR or that it interacts directly with the KATP channels in a similar manner to its interaction with K^+ channels in taste cells. Furthermore, this interaction with Kir6.2 induces a conformational change in the SUR that is responsible for the increased affinity.

DISCUSSION

The results of these studies demonstrate a concentration- and glucose-dependent stimulation of insulin secretion in response to denatonium that is due to depolarization of the β-cell and increased Ca^{2+} entry. The action of denatonium to reduce KATP channel activity would be the primary cause of the depolarization. Denatonium does not seem to have any major effect on K^+ channels other than the KATP channel or any direct effect on the L-type Ca^{2+} channel. However, part of the strong depolarizing effect of denatonium in the presence of glucose will be explained by an indirect action on L-type Ca^{2+} channels. Certainly, in the presence of denatonium, voltage-gated Ca^{2+} channels are activated as a result of the membrane depolarization caused by the inhibition of KATP channels. Therefore, the additional KATP channel-related depolarization observed in the presence of both denatonium and glucose would trigger the opening of more voltage-activated Ca^{2+} channels purely as a result of increased membrane depolarization. The inhibition of the KATP channel with no apparent effect on other K^+ channels and the lack of a direct effect on voltage-gated Ca^{2+} channels in the β-cell illustrate the selectivity and specificity of the effects of denatonium.

The detailed mechanism by which denatonium selectively affects the KATP channels remains to be understood. It is possible that denatonium interacts with the sulfonylurea receptor that is an essential component of the functional KATP channel (29,30). Denatonium has some structural similarity to nateglinide, meglitinide, and other recently introduced blood glucose–lowering compounds that also act on the KATP channel and that displace glybenclamide from its binding site on SUR (27,31–34). However, denatonium did not displace glybenclamide from its binding site on the SUR. Instead, it actually increased [^{3}H]glybenclamide binding by an increase in affinity. Thus, the structural similarity of denatonium to nateglinide and meglitinide is not such that it can bind to the same site. Two possible explanations come to mind: (*1*) denatonium is binding to a site on the SUR that is distinct from the glybenclamide binding site but that still induces channel closure; and (*2*) denatonium acts directly on the channel, closes it, and induces a conformational change in SUR. Evidence in favor of this is that denatonium is known to act directly on K^+ channels in taste cells, including delayed rectifier and Ca^{2+}-activated K^+ channels

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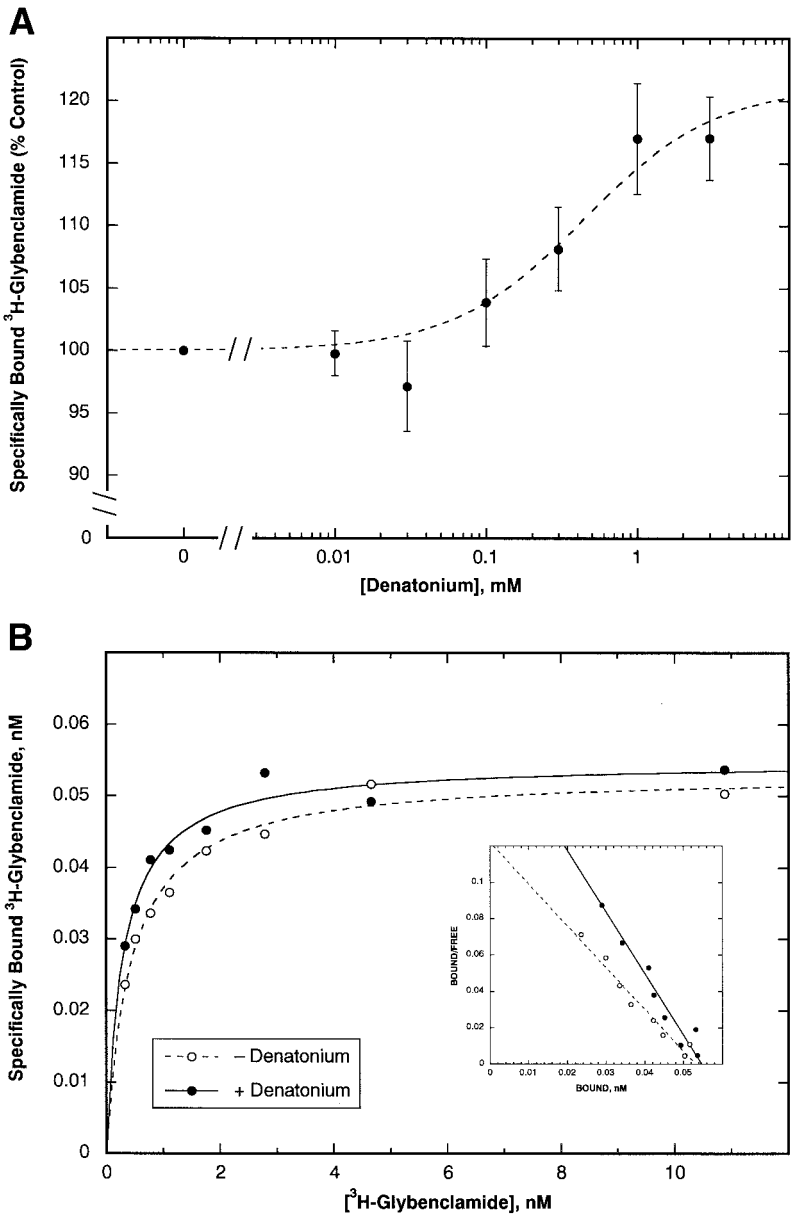

FIG. 9. The effects of denatonium on the binding of [^{3}H]glybenclamide to HIT cell membranes. *A*: Effects of denatonium at concentrations from 0.01 to 3 mmol/l on the binding of 1 nmol/l [^{3}H]glybenclamide. *B*: Amounts of [^{3}H]glybenclamide bound (at concentrations from 0.3 to 11 nmol/l) in the absence and presence of 1 mmol/l denatonium. A Scatchard analysis of the data is shown in the inset.

(17). In view of this, an action of denatonium on the Kir6.2 channel that affects the conformation of SUR would suffice to produce the observed effects. The nature of the observed glucose dependence for the action of denatonium to stimulate insulin secretion is not known but is similar to that seen with the sulfonylureas at low glucose concentrations, e.g., 2.8 mmol/l glucose for rat islets. The presence or absence of 5 mmol/l ATP in the binding assays did not influence the effect of denatonium to increase the K_d for glybenclamide (data not shown), indicating a lack of ATP dependence to the binding and to the conformational change required to affect the SUR. Also, denatonium inhibits the KATP current in the presence of low glucose concentrations. Thus, the glucose dependence lies at some point in stimulus-secretion coupling distal to membrane depolarization.

A question that arises from these observations is their relationship to the responses of taste receptor cells to denatonium and whether the signal transduction mechanisms are similar. An obvious similarity is that a K^+ channel is involved, although denatonium has not been shown previously to affect a KATP channel in taste cells. This may be because the effects of bitter substances on KATP channels in taste cells have not been investigated yet, or more likely because this channel is not expressed in taste cells.

In summary, the β-cell effects of denatonium are not mediated by T2R/TRB receptors and activation of either gustducin or transducin. The known effects of agonist-T2R/TRB interactions are gustducin α-subunit activation of cyclic NPDE and βγ-subunit activation of PLCβ2, neither of which has been associated with inhibition of K^+ channels. In this connection, there were no changes in intracellular cyclic AMP levels that might reflect increased cyclic NPDE activity or of any mobilization of intracellular Ca^{2+} to reflect PLC activation. Finally, using the trypsin digestion assay, we found no evidence that transducin was activated by denatonium that would have implicated an interaction with T2R/TRB receptors. In view of all of the available data, we conclude that denatonium most likely

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stimulates insulin secretion by an action on the Kir6.2 channel and depolarization of the β-cell. This raises the possibility that structure-function studies on denatonium could give rise to other compounds with greater potency and specificity for the KATP channel that could be used orally as insulin secretagogues.

ACKNOWLEDGMENTS

This work was supported by a Mentor-Based Postdoctoral Fellowship from the American Diabetes Association and grants DK-42063, DK-54243, and DK-59813 from the National Institutes of Health (to G.W.G.S.).

We are grateful to Dr. Rick Cerione and Dr. Jon Ericson for advice on gustducin/transducin signaling pathways and the trypsin-digestion assay; Dr. Leslie Satin for advice on the electrophysiological studies; and Dr. Troitza Bratanova-Tochkova for tissue culture of the clonal HIT-T15 cells.

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