Effects of Glucose and Amino Acids on Free ADP in βHC9 Insulin-Secreting Cells

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Stimulation of insulin release by glucose is widely thought to be coupled to a decrease in the activity of ATP-sensitive K^+ channels (K_{ATP} channels) that is caused by a decreased concentration of free ADP. To date, most other investigators have reported only on total cellular ADP concentrations, even though only a small fraction of all ADP is free and only the free ADP affects K_{ATP} channels. We tested the hypothesis that amino acids elicit insulin release via a decrease in the activity of K_{ATP} channels owing to a decrease in the level of free ADP. We estimated the concentration of free ADP in βHC9 hyperplastic insulin-secreting cells based on the cell diameter and on luminometric measurements of ATP, phosphocreatine, and total creatine. The concentration of free ADP fell exponentially as the concentration of glucose increased. A physiological mixture of amino acids greatly stimulated insulin release at 0–30 mmol/l glucose but affected the concentration of free ADP only to a minor degree and significantly so only at ≤2 mmol/l glucose. In the presence of 2-deoxyglucose and NaN_3, amino acids were unable to stimulate insulin release. When K_{ATP} channels were held open with diazoxide (and the plasma membrane partially depolarized with high extracellular KCl), amino acids still stimulated insulin release. We conclude that amino acid–induced insulin release depends on two components: a yet-unknown amino acid sensor and K_{ATP} channels, which serve to attenuate hormone release when cellular energy stores are low. We propose that glucose-induced insulin release may be regulated similarly by two components: glucokinase and K_{ATP} channels. *Diabetes* 50:291–300, 2001

According to the most widely accepted hypothesis, glucose induces insulin release as follows. Glucose rapidly equilibrates across the plasma membrane and is phosphorylated with the help of glucokinase, which determines flux through glycolysis (1). Pyruvate from glycolysis enters the citric acid cycle and determines ATP production from oxidative phosphorylation. As a result, the concentrations of ATP and free ADP reflect the concentration of blood glucose. The plasma membrane contains ATP-sensitive K^+ channels (K_{ATP} channels). ATP inhibits the channels, and ADP relieves this inhibition (2). In the absence of glucose, K_{ATP} channels are quite active and dictate the membrane potential. In the presence of glucose, initially, the balance between K^+ flux through K_{ATP} channels and ionic fluxes through yet unknown "leak conductances" determines the membrane potential (2). Once the membrane potential is more positive than about –40 mV, Ca^{2+} channels open intermittently, allowing the influx of extracellular Ca^{2+} (2). As intracellular Ca^{2+} rises, the exocytotic machinery is activated, which moves secretory granules containing insulin to the plasma membrane surface. In partial support of the above hypothesis, patients with a mutant glucokinase of abnormal affinity to glucose have an abnormal set point for glucose homeostasis (3–5), and other patients with K_{ATP} channels with abnormally low affinity for ADP fail to turn off insulin release properly at low concentrations of blood glucose (6,7).

Previous investigators generally limited their studies to measuring total cellular ADP, though most of the ADP is bound to proteins and only the free ADP regulates the activity of K_{ATP} channels. Ghosh et al. (8) were the first to estimate free ADP in B-cell–rich rat pancreatic islet cores based on measurements of ATP, phosphocreatine, and total creatine. With a background of 4 mmol/l amino acid, increasing glucose from 4 to 8 mmol/l led to a decrease of free ADP from ~44 to ~31 µmol/l. These microhistochemical measurements were based on enzymatic cycling and fluorescence detection of nicotinamide adenine dinucleotides. We recently devised methods to incubate cells, extract them, and determine their content of ATP, phosphocreatine, and creatine with luminescence detection, which simplifies the analytical work (9). Here, we expand the observations of Ghosh et al. (8) to cover a whole range of glucose concentrations with and without amino acids. We used clonal βHC9 cells, which derive from hyperplastic islet cells of transgenic mice that express the SV40 T-antigen under the control of an insulin promoter (10). The glucose sensitivity of these cells is close to normal, as the maximal activity of glucokinase is ~10 times that of hexokinase (10,11). We found that increasing concentrations of glucose are associated with an exponential decline of the concentration of free ADP, while the concentration of ATP remained nearly constant. The addition of amino acids did not affect the concentration of ATP or free ADP, provided that ≥2 mmol/l glucose was present. Nevertheless, amino acids greatly increased the rate of insulin release. The results reported here lead us to conclude that B-cells have an amino acid sensor, which is separate from K_{ATP} channels.

Evidence is accumulating that glucose, besides its effects on the concentrations of ATP and ADP, also regulates insulin release in other ways. Thus, Gembal et al. (12) reported that

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Received for publication 23 February 2000 and accepted in revised form 11 October 2000.

GLP, glucagon-like peptide; IBMX, isobutyl methyl xanthine; K_{ATP} channel, ATP-sensitive K^+ channel.

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glucose stimulates insulin release even when K_{ATP} channels are held open pharmacologically (with diazoxide) and the plasma membrane is partially depolarized (with increased extracellular KCl). One possible explanation is that glucokinase not only paces glycolysis and thus cellular ATP production, but also acts as a signaling molecule by itself. Glucokinase may be suited for this purpose because it changes conformation on binding glucose and relaxes only slowly from this conformation (13).

Little is known about the mechanisms by which B-cells sense amino acids. Recently, it became clear that patients with a mutant glutamate dehydrogenase of decreased affinity for the inhibitor GTP release excessive amounts of insulin after a protein meal, leading to pronounced hypoglycemia (14). This finding has raised interest in glutamate dehydrogenase as a possible amino acid sensor. Glutamine (a precursor to glutamate that is efficiently taken up into B-cells) alone is not a stimulus for insulin release (15,16). Although leucine alone stimulates insulin release, the combination of leucine (an allosteric activator of glutamate dehydrogenase) and glutamine is a far stronger stimulus of insulin release than leucine alone (15,16). Through an unknown mechanism, antecedent hyperglycemia decreases the secretory response of B-cells to glutamine plus leucine (17). Glucose-induced insulin release is amplified by arginine or lysine alone (18–20). Thereby, on a molar basis, arginine is about equipotent with a physiological mixture of amino acids (18). The stimulation of insulin release by arginine and lysine is popularly attributed to membrane depolarization due to uptake of charged species (20).

Originally, it was thought that among islet cells only B-cells contain K_{ATP} channels. Evidence is accumulating that non-B islet cells contain K_{ATP} channels, as well (21–26). This may call for a reevaluation of the role that has been ascribed to K_{ATP} channels in B-cells. Based on our data, we propose that the function of K_{ATP} channels is mainly one of shaping the stimulus-response curve in the hypoglycemic range and of preventing cells with a low energy level from secreting insulin.

RESEARCH DESIGN AND METHODS

Materials. With the following exceptions, all reagents were obtained from Sigma Chemical (St. Louis, MO): NaF from Fluka Chemical (St. Louis, MO); DNase, hexokinase, and creatine kinase from Boehringer/Roche Diagnostics (Indianapolis, IN); fetal bovine serum and iron-supplemented calf serum ("cosmic calf serum") from HyClone (Logan, UT); glucagon-like peptide (GLP)-1 (7–37) [CP95,253] as a gift from Pfizer (Groton, CT).

Equipment. We used a Berthold model LB9501 photon-counting luminometer (Wallac, Gaithersburg, MD) and a centrifuge with temperature indicator (Hettich Universal 16R, Tuttlingen, Germany) and either a low-speed swing-out rotor or a high-speed fixed angle rotor.

Culture of βHC9 hyperplastic islet–derived cells. Clonal βHC9 insulin-secreting cells that derive from hyperplastic mouse islets (10) were obtained from the cell repository of the Diabetes Research Center at the University of Pennsylvania, with permission of Dr. Douglas Hanahan (Department of Biochemistry and Biophysics, University of California, San Francisco, CA). The cells were grown in Dulbecco's modified Eagle's medium (27,28) in the presence of 25 mmol/l glucose, 10^5 U/l penicillin, 0.1 g/l streptomycin, 3 mmol/l creatine, 10% fetal bovine serum, and 5% iron-supplemented calf serum in a humidified atmosphere of 5% CO_2 in air at 37°C.

Preparation of βHC9 hyperplastic islet–derived cells. On the day of the experiment, the cultured cells covered 50–80% of the surface of the flask. They were harvested with trypsin, acclimated to buffer of reduced bicarbonate content (i.e., 124 mmol/l NaCl, 5.4 mmol/l KCl, 1.8 mmol/l $CaCl_2$, 0.8 mmol/l $MgSO_4$, 1 mmol/l NaH_2PO_4, 2.8 mmol/l glucose, 14.3 mmol/l $NaHCO_3$, and 10 mmol/l HEPES, gassed with 5% CO_2 in oxygen and pH adjusted to 7.3 with NaOH) with added DNase (17 µg/ml) for 1 h, spun through 33% Percoll, filtered through 60 µm nylon mesh, and resuspended. The cell density was determined by use of a counting chamber. A known number of cells was then washed with 140 mmol/l NaCl, 5.6 mmol/l KCl, 2.6 mmol/l $CaCl_2$, 1.2 mmol/l $MgCl_2$, 2% radioimmunoassay-grade bovine serum albumin, and 10 mmol/l HEPES-NaOH, pH 7.4, and maintained in this buffer at room temperature for 0.2–1.1 h.

Creatine kinase activity in βHC9 cells. βHC9 cells were suspended to 16,000 cells/µl in 0.1 mol/l K-phosphate, pH 7.2, 0.5 mmol/l dithiothreitol, and 2.5 mmol/l EDTA. Triton X-100 was added to a 2 mg/ml final concentration, and the cells were placed on ice. Creatine kinase activity was measured 0.25–3 h later at a 10- to 50-fold dilution by following the increase in absorbance at 340 nm using the following assay medium: 100 mmol/l imidazole acetate, 2 mmol/l EDTA, 10 mmol/l $MgCl_2$, 2 mmol/l ADP, 5 mmol/l AMP, 10 µmol/l P^1,P^5 di(adenosine-5′)pentaphosphate, 20 mmol/l glucose, 2 mmol/l NADP, 0.5 mmol/l dithiothreitol, 3.5 U/ml hexokinase, 2.3 U/ml glucose 6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*), pH 6.75 (slightly modified from 29). The net creatine kinase activity was calculated from the phosphocreatine (30 mmol/l)-induced increase in NADPH production.

Incubation and extraction of βHC9 cells. In 0.5-ml conical polypropylene centrifuge tubes, in a total volume of 250 µl, 90,000 cells were incubated in various media for 20 min at 37°C; mixing was achieved by repeated use of a pipetter. When added, amino acids were present at the following concentrations (in mmol/l; total = 15): Ala, 1.62; Arg, 0.69; Asp, 0.15; citrulline, 0.35; Glu, 0.45; Gln, 1.85; Gly, 1.11; His, 0.29; Ile, 0.35; Leu, 0.60; Lys, 1.37; Met, 0.18; Orn, 0.26; Phe, 0.31; Pro, 1.30; Ser, 2.11; Thr, 1.00; Trp, 0.28; Val, 0.75. After the incubation, the cells were pelleted at 24,000*g* (30 s run time, including acceleration but excluding deceleration; total of 20 s at >16,000*g*) and 36–38°C. We believe that the pelleted cells were not anoxic, because the pellet was just barely visible to the naked eye and must therefore have been very thin, thus allowing adequate access of oxygen to the cells. Within 2–3.5 min of the start of the centrifugation, we removed a portion of the supernatant for the determination of insulin and aspirated the remainder of the supernatant with a 21-gauge needle connected to a vacuum line. The pelleted cells were immediately extracted by addition of 30 µl of 0.1 mol/l NaOH/0.5 mmol/l EDTA and incubation in a water bath at 60°C for 20 min. The supernatants and cell extracts were stored frozen at –20°C.

Radioimmunoassays for insulin. Insulin was assayed against a rat insulin standard (Lilly, Indianapolis, IN) using a guinea pig anti-bovine insulin antiserum (ICN Biomedicals, Costa Mesa, CA; #65-101) and receptor-grade, monoiodinated pork insulin (DuPont/NEN, Boston, MA). After a 20-h incubation at room temperature, free and bound insulin were separated with dextran-coated charcoal.

Assays of ATP, phosphocreatine, and total creatine. Assays of ATP, phosphocreatine, and total creatine were described in detail in a recent publication (9). In brief, ATP was measured based on the light emission of the luciferase-catalyzed ATP-dependent oxidation of luciferin. Phosphocreatine was measured after destruction of endogenous ATP by converting it to ATP with exogenous ADP and creatine kinase. Total creatine was measured like phosphocreatine after all creatine had been converted to phosphocreatine with exogenous ATP and creatine kinase.

Cell volume. Cells were photographed under phase contrast illumination. From the photographs, the diameters of the cells were determined relative to a micrometer scale. With this procedure, human red blood cells in 154 mmol/l NaCl had an apparent diameter of 8.0 ± 0.1 µm (mean ± SE; expected: 7.5 ± 0.3), whereas βHC9 insulin-secreting cells had an apparent diameter of 10.9 ± 1.8 µm (mean ± SD, n = 348). We assumed that the recently trypsinized cells were perfect spheres; this seemed reasonable because no floating aspheric cells were visible microscopically, whereas the flattened shape of settling red blood cells could easily be observed. The average volume of the insulin-secreting cells was therefore estimated at 0.75 ± 0.02 pl (mean ± SE, n as above), and the water space was assumed to be 80% of this volume.

Calculations. We assumed that the intracellular concentration of free Mg^{2+} is similar to liver, i.e., 1 mmol/l (30); this is substantiated by studies of Gylfe (31) with mag-fura-2-loaded *ob/ob* mouse islet cells. Like Lawson and Veech (30), we further assumed that the cytosolic pH is 7.2. For these conditions at 38°C, the apparent equilibrium constant for the creatine kinase–catalyzed reaction ADP + phosphocreatine ↔ ATP + creatine is 104.7 according to Lawson and Veech (30) and 114.9 according to Golding et al. (32); we used an intermediate value of 110.

RESULTS

Evidence for creatine kinase activity in βHC9 insulin-secreting cells.

βHC9 cells were maintained at room temperature in fuel-free medium for 10–25 min. Then, they were diluted into either similar fuel-free medium, medium with 2 mmol/l glucose, or medium with 5 mmol/l 2-deoxyglu-

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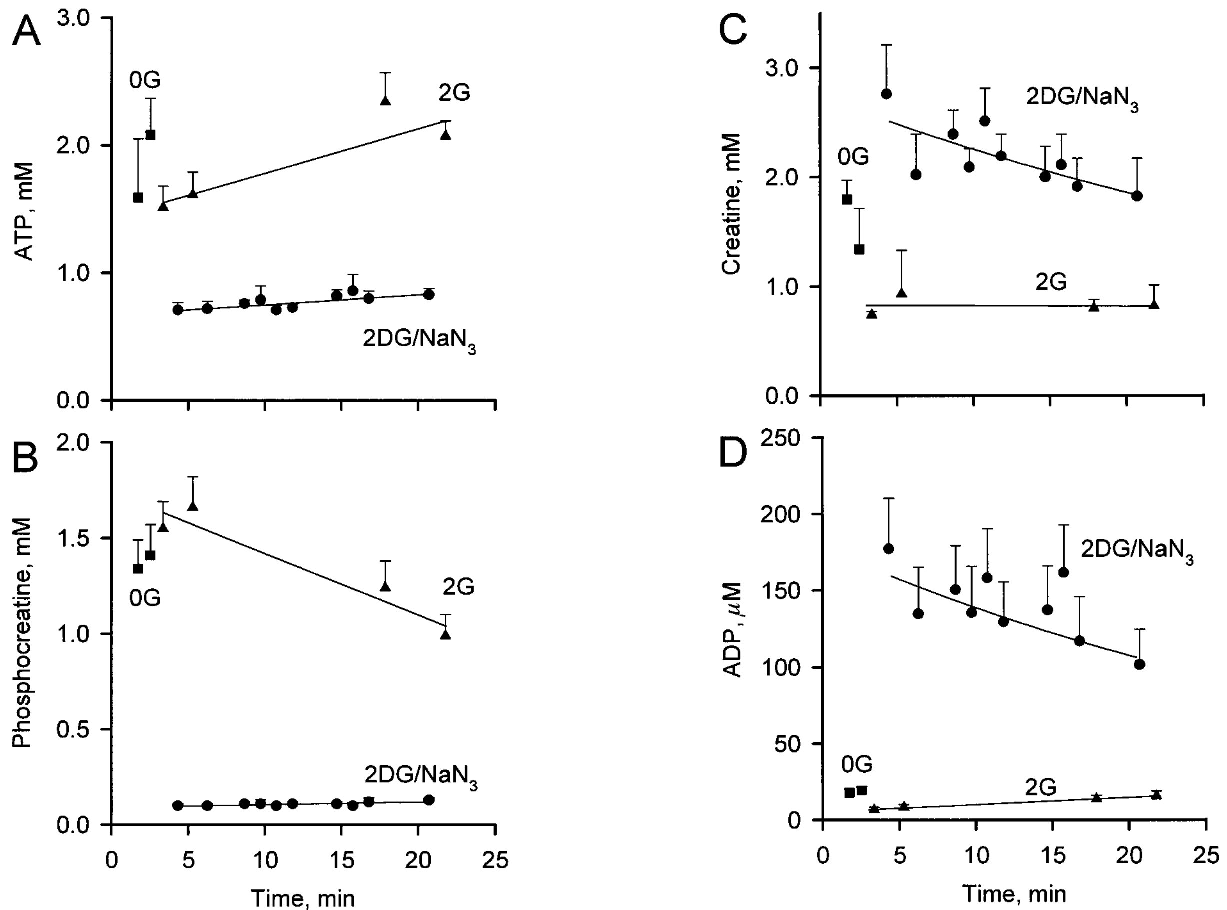

FIG. 1. Demonstration of creatine kinase activity in βHC9 insulin-secreting cells. Cells were cultured in 3 mmol/l creatine to increase phosphocreatine and creatine content. Before start of experiment, cells were maintained in fuel-free medium at room temperature for 10–25 min. At time 0, cells were diluted into medium to achieve the following conditions: 2 mmol/l glucose (2G), no fuel (0G), or 5 mmol/l each of 2-deoxyglucose and sodium azide (2DG/NaN_3). Cells were extracted with NaOH + EDTA, and endogenous enzymes were heat inactivated. ATP (*A*) in extracts was determined luminometrically with luciferin and luciferase. Phosphocreatine (*B*) was also determined luminometrically, but only after destruction of endogenous ATP, followed by conversion of phosphocreatine to ATP. Creatine (*C*) was determined luminometrically after conversion to phosphocreatine, then using essentially the same procedure as for phosphocreatine. Concentration of free ADP (*D*) was calculated based on measurements of ATP, phosphocreatine, and creatine, and with the following assumptions: equilibrium of the reaction ATP + creatine → ADP + phosphocreatine, intracellular pH of 7.2, intracellular free Mg^{2+} of 1 mmol/l, and an apparent equilibrium constant of 110. Means ± SE of four experiments.

cose and 5 mmol/l sodium azide, and incubated at 37°C. 2-Deoxyglucose acts chiefly as a phosphate trap, and azide inhibits electron transport at the level of cytochrome oxidase. As expected, in the presence of 2-deoxyglucose and sodium azide, the total cellular ATP content dropped quickly (within <4 min; Fig. 1*A*). Because the concentration of phosphocreatine also dropped rapidly (Fig. 1*B*), creatine kinase must be maintaining the following reaction near equilibrium (within the observed time frame): ADP + phosphocreatine + H^+↔ ATP + creatine.

The simultaneous decrease in phosphocreatine content and increase in ATP content in cells incubated in 2 mmol/l glucose suggests that βHC9 cells might lose some of their creatine during incubation. This was clearly evident in cells incubated with 2-deoxyglucose and NaN_3 (Fig. 1*C*). The total creatine and phosphocreatine inside the cells did not differ between incubation conditions, but it decreased at a rate of 2%/min (not shown). Nevertheless, estimates of the concentration of free ADP appeared reasonable throughout this period (Fig. 1*D*). With 2 mmol/l glucose, βHC9 cells may not produce enough ATP, hence the gradual, time-dependent increase in the concentration of free ADP; conversely, cells poisoned with 2-deoxyglucose and NaN_3 may gradually lower their concentration of free ADP by degrading ADP and increasing the level of phosphate intracellularly. Finally, because the rate of cellular creatine loss is many-fold smaller than the rate of the 2-deoxyglucose + NaN_3–induced decrease in cellular phosphocreatine, we expect the leakage to affect the steady-state concentrations of ADP only to a minor degree.

As a control for the experiments shown in Fig. 1, we also measured the activity of creatine kinase in Triton X-100 solubilized βHC9 cells. At 27°C, it amounted to 97 ± 18 U/ml cell volume (mean V_{max} ± SE of three preparations); at 37°C, the maximal velocity is expected to be about two times higher than at 27°C (29). This activity is comparable to the activity of creatine kinase in heart muscle (33,34). In single experiments, the K_m values for phosphocreatine and ADP were 1.7 ± 0.1 and 0.14 ± 0.01 mmol/l, respectively (one experiment each; least squares estimates to Michaelis Menten equation

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for measurements at seven or eight different substrate concentrations each in the presence of a saturating concentration of the other substrate, ± asymptotic SE). Assuming that the kinetics are first order in ADP, it can be shown that the concentration of free ADP will be within 2% of the equilibrium value after only 0.2 s (2.0 s for phosphocreatine with the same assumptions). This calculation suggests that near-equilibrium of the reaction is indeed reached well within the time frame of our incubations (20 min usually).

Time dependence of basal and stimulated insulin release from βHC9 cells. βHC9 cells were incubated at 37°C for 4–58 min, either without any fuel or with a combination of 20 mmol/l glucose, 15 mmol/l amino acids (physiological mixture), and 1 nmol/l GLP-1(7–37) (to enhance insulin release via an elevated concentration of cAMP). This cocktail elicits cells to release insulin at a high rate that can easily be distinguished from basal release. With glucose, amino acids, and GLP-1, insulin release was linear with time and amounted to 0.96 fg insulin · min^{-1} · $cell^{-1}$ (mean of two experiments). In the absence of any stimulus, insulin release increased minimally with time (0.04 fg insulin · min^{-1} · $cell^{-1}$, mean of two experiments). Hence, constitutive insulin release occurred only at a very low rate, and we ascribe the constant level of insulin present in the supernatant (1.9 µg/l or 5.3 fg/cell) to the handling (pipetting) of the cells. The intercept of the linear regressions for stimulated and unstimulated insulin release was at 4.3 min. This intercept most likely reflects the aggregate lag time for warming the cells and for glucose and amino acids to initiate hormone release. In subsequent experiments, cells were pipetted every 45 s for incubation at 37°C; the slight increase in insulin in the stock solution of cells due to constitutive release during storage at room temperature was estimated from the insulin seen in samples incubated with diazoxide at both the beginning and end of these pipettings. All data were corrected for this small increase. Furthermore, for convenience of incubation and centrifugation, samples were incubated for slightly different times (18.5–20.8 min). Measured insulin in the supernatant was linearly adjusted for the duration of the incubation, taking into account a 4.3-min lag time and a basal amount of insulin similar to 85% of that seen with 2-deoxyglucose + NaN_3 after a 20-min incubation (these numbers are based on the experiments discussed above). These corrections to the raw data amounted to 4% on average (range 0–9%).

Effect of glucose and amino acids on insulin release from βHC9 cells. βHC9 cells were incubated in media containing various concentrations of glucose. Amino acids were added in relatively high concentration (15 mmol/l total), because initial experiments with a lower, more physiological concentration (5 mmol/l) failed to reveal effects on cellular adenine nucleotide levels. We boosted insulin release with 1 nmol/l GLP-1(7–37), so that glucose-induced insulin release became more easily measurable above background. Control media contained 5 mmol/l 2-deoxyglucose plus 5 mmol/l sodium azide, or 2 mmol/l glucose plus 0.25 mmol/l diazoxide (a K_{ATP} channel opener). Incubation conditions were randomized between experiments. As is evident from Fig. 2, glucose half-maximally induced insulin release at a near-physiological concentration of ~7 mmol/l. The presence of 15 mmol/l amino acids led to a much larger increase in insulin release than did glucose alone (probability of identical release with and without amino acids is <0.001 at each concentration of glucose; Wilcoxon's signed-rank tests). Amino acid–induced insulin release was half-maximal at ~2 mmol/l glucose.

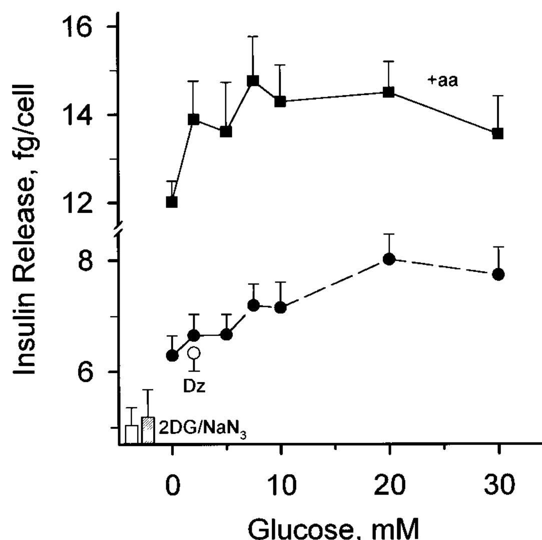

FIG. 2. Glucose- and amino acid–induced insulin release from βHC9 cells. Cells were cultured as described in Fig. 1. Cells were incubated for 20 min with 1 nmol/l GLP-1(7–37) in the absence (●) or presence (■) of 15 mmol/l amino acids (+aa) and with glucose as shown. Insulin was determined by radioimmunoassay. ○, incubation in the presence of 0.25 mmol/l diazoxide (Dz; no amino acids present). Bars reflect insulin release in the presence of 5 mmol/l 2-deoxyglucose (2DG) and 5 mmol/l NaN_3 (□, no amino acids; ▨, with 15 mmol/l amino acids). Means ± SE of seven experiments with two runs each are shown. Note the break in the *y*-axis and associated slight change in scale. According to Wilcoxon's signed-rank tests, *P* < 0.001 for identical release with and without amino acids, except for the presence of 2-deoxyglucose and NaN_3, in which case *P* = 0.8. In absence of amino acids, probability that release at ≥7 mmol/l glucose is identical to that at 0 mmol/l glucose is <0.01, and probability that release at 7.5, 20, or 30 mmol/l glucose is identical to that at 5 mmol/l glucose is <0.04.

In the absence of GLP-1(7–37), both glucose-induced and amino acid–induced insulin release amounted to only ~35% of the release in the presence of GLP-1(7–37) (Fig. 2 vs. controls in Fig. 8; other experiments, which are not shown). In the absence of GLP-1(7–37), it was difficult to assess the glucose dependence of insulin release. For the same reason, others chose to boost insulin release with the phosphodiesterase inhibitor isobutyl methyl xanthine (IBMX). In our hands, in the presence of 0.1 mmol/l IBMX, insulin release in response to either glucose or amino acids was ~25% greater than in the presence of 1 nmol/l GLP-1(7–37). Nonetheless, increasing GLP-1(7–37) from 1 to 10 nmol/l did not increase glucose- and amino acid–induced insulin release further. However, the addition of IBMX (0.1 mmol/l) to media containing 1 nmol/l GLP-1(7–37) did lead to a further ~2.2-fold potentiation of glucose- and amino acid–induced insulin release. We used GLP-1(7–37) alone in the belief that it would be associated with no or few side effects.

Effect of glucose and amino acids on ATP concentrations in βHC9 cells. We determined the concentration of ATP in alkaline extracts of the βHC9 cells that were used for the studies of insulin release reported above [hence, all these data pertain to cells incubated in the presence of 1 nmol/l GLP-1(7–37)]. As shown in Fig. 3, the presence of glucose alone (0–10 mmol/l) was associated with a small increase in ATP content ($P \leq 0.02$ for lack of linear correlation of means). No

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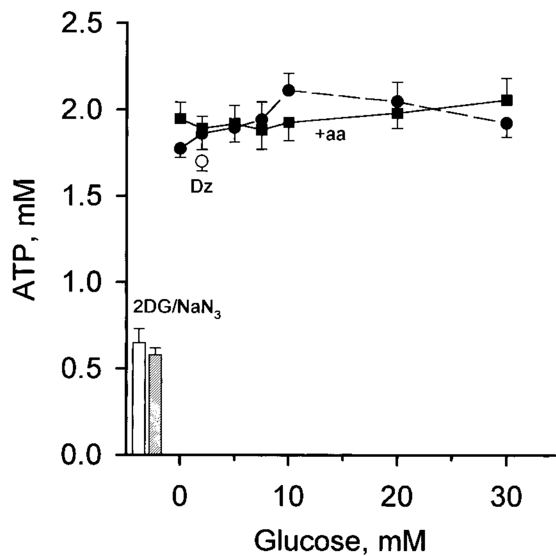

FIG. 3. Effect of glucose and amino acids on ATP content of βHC9 cells. Same experiments as in Fig. 2. ● and ○, no amino acids; ■, amino acids present. Same analytical procedures as in Fig. 1. Based on measurements of cell diameter, intracellular water space was assumed to be 0.6 pl/cell. Mean ± SE of seven experiments with two runs each. ATP content in 10 mmol/l glucose was unlikely to be identical with ATP content in 0, 2, or 5 mmol/l glucose (no amino acids present; $P < 0.05$; Wilcoxon's signed-rank tests). Introduction of amino acids had no statistically significant effect, except at 0 and 10 mmol/l glucose ($P < 0.04$).

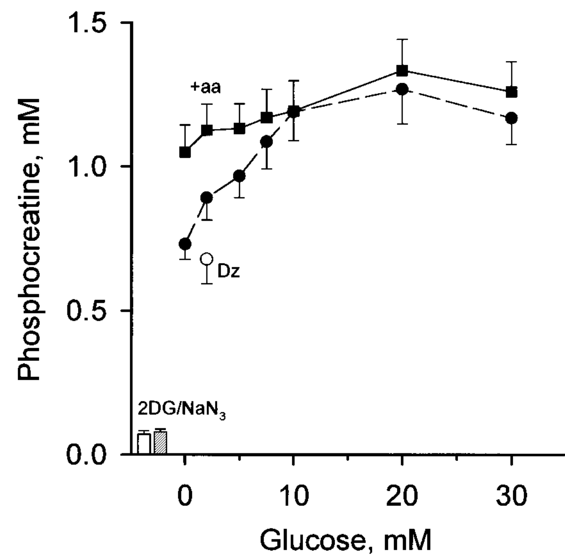

FIG. 4. Effect of glucose and amino acids on phosphocreatine content of βHC9 cells. Same cells and experiments as in Figs. 2 and 3. ● and ○, no amino acids; ■, amino acids present (+aa). Same analytical procedures as in Fig. 1. Mean ± SE of seven experiments with two runs each. Wilcoxon's signed-rank tests suggest the following significant differences: glucose increases phosphocreatine content ($P < 0.03$ for 7.5 vs. 5 mmol/l, $P < 0.003$ for 10–30 mmol/l vs. 5 mmol/l glucose); at 0–5 mmol/l glucose, introduction of amino acids increases phosphocreatine content ($P < 0.003$). Otherwise, probabilities that amino acids have no effect are >5%.

such correlation was observed in the presence of a mixture of amino acids (15 mmol/l; $P = 0.7$). In general, amino acids did not affect the ATP content (except at 0 and 10 mmol/l glucose, $P < 0.04$ for no effect; Wilcoxon's signed-rank tests). At 10 and 20 mmol/l glucose (without amino acids), the ATP content was significantly greater than at 5 mmol/l glucose ($P < 0.05$). However, in the presence of amino acids, at 7.5–30 mmol/l glucose the ATP content did not differ significantly from that at 5 mmol/l glucose.

Effect of glucose and amino acids on phosphocreatine concentrations in βHC9 cells. Figure 4 shows the phosphocreatine data for the experiments shown in Figs. 2 and 3. The cellular phosphocreatine content showed a greater dependence on the concentration of glucose than ATP did (compare Figs. 3 and 4). Between 0 and 10 mmol/l glucose, the concentrations of glucose and phosphocreatine were correlated (probability for lack of linear correlation of means: $P < 0.002$ in the absence and $P < 0.02$ in the presence of amino acids). At ≥7.5 mmol/l glucose, the phosphocreatine content significantly differed from that at 5 mmol/l glucose ($P < 0.03$ for 7.5 mmol/l, $P < 0.003$ for 10–30 mmol/l glucose; Wilcoxon's signed-rank tests). Furthermore, at 0, 2, and 5 mmol/l glucose, the presence of amino acids was associated with a marked increase in phosphocreatine content ($P < 0.003$ for no effect). At ≥7.5 mmol/l glucose, amino acids had no statistically significant effect on phosphocreatine content ($P > 0.05$).

Effect of glucose and amino acids on creatine concentrations in βHC9 cells. Figure 5 shows the creatine content of the cell extracts to which Figs. 3 and 4 referred. Amino acids had no significant effect on the creatine content ($P > 0.1$), except at 10 mmol/l glucose ($P < 0.01$; Wilcoxon's signed-rank tests), which we think is accidental.

Effect of glucose and amino acids on concentrations of free ADP in βHC9 cells. Based on the concentrations of ATP, phosphocreatine, and creatine shown in Figs. 3–5, we estimated the concentration of free ADP in βHC9 cells (Fig. 6). The estimates are based on equilibrium being established for the reaction ADP + phosphocreatine → ATP + creatine, as shown in Fig. 1. As described in RESEARCH DESIGN AND METHODS, we assumed that the intracellular pH is 7.2, that the intracellular concentration of free Mg^{2+} is 1 mmol/l, and that the equilibrium constant is 110. Figure 6 shows means ± SE of 14 different extracts for each condition. As the concentration of glucose increased from 0 to 30 mmol/l, the concentration of free ADP fell exponentially and in highly statistically significant fashion ($P < 0.001$ for 2–30 mmol/l vs. 0 mmol/l glucose; $P < 0.02$ for 10–30 mmol/l vs. 5 mmol/l glucose; in presence of amino acids, $P < 0.002$ for 2–30 mmol/l vs. 0 mmol/l glucose and $P < 0.05$ for 30 mmol/l vs. 5 mmol/l glucose; Wilcoxon's signed-rank tests). Only at 0 and 2 mmol/l glucose did amino acids lead to a significant decrease in the concentration of free ADP ($P < 0.005$). Likewise, in the presence of 2-deoxyglucose and sodium azide, amino acids led to a significant decrease in the concentration of free ADP ($P < 0.05$). The amino acid–induced decrease in free ADP at 0 and 2 mmol/l glucose together with the amino acid–induced increase in ATP at 0 mmol/l glucose and in phosphocreatine at 0, 2, and 5 mmol/l glucose provide a tentative explanation for the lower glucose sensitivity of amino acid–induced insulin release compared with glucose-induced insulin release (Fig. 2). Thus, in the hypoglycemic range, amino acids serve as substrates for energy metabolism, which leads to a distortion of the relationship between glucose, ATP, and free ADP. At ≥10 mmol/l glucose, the effect of

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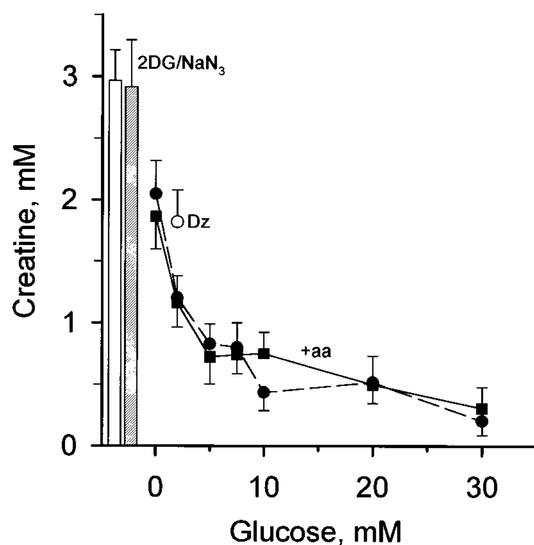

FIG. 5. Effect of glucose and amino acids on creatine content of βHC9 cells. Same experiments as in Figs. 2–4. ● and ○, no amino acids; ■, amino acids present (+aa). Creatine content was calculated from separate measurements of creatine + phosphocreatine and from phosphocreatine alone (Fig. 4). Creatine + phosphocreatine was measured luminometrically after converting creatine to phosphocreatine, destroying ATP and converting phosphocreatine to ATP. Means ± SE of seven experiments with two runs each. As the concentration of glucose increases, the concentration of creatine drops significantly (P < 0.001 for 2–30 vs. 0 mmol/l glucose in both absence and presence of amino acids; P < 0.05 for 10–30 vs. 5 mmol/l glucose in absence of amino acids; P < 0.05 for 30 vs. 5 mmol/l glucose in presence of amino acids; Wilcoxon's signed-rank tests). Amino acids have no significant effect, except at 10 mmol/l glucose (P < 0.01), which is thought to be due to chance.

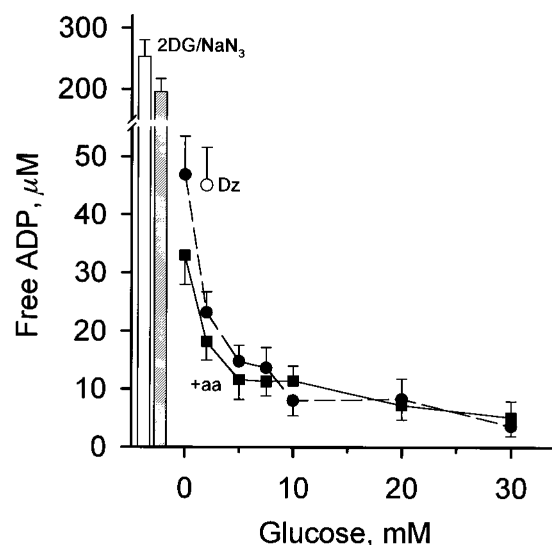

FIG. 6. Effect of glucose and amino acids on concentration of free ADP in βHC9 cells. Same experiments as in Figs. 2–5. ● and ○, no amino acids; ■, amino acids present (+aa). Concentration of free ADP was calculated as described in Fig. 1. Concentration of glucose correlates with decrease in concentration of free ADP (P < 0.001 for no difference between 2–30 and 0 mmol/l glucose; P < 0.02 for no difference between 10–30 and 5 mmol/l glucose; P < 0.002 for 2–30 vs. 0 mmol/l glucose in presence of amino acids; P < 0.05 for 30 vs. 5 mmol/l glucose in presence of amino acids; Wilcoxon's signed-rank tests). Amino acids had a significant effect only at 0 and 2 mmol/l glucose (P < 0.005) and in the presence of 2-deoxyglucose (2DG) + NaN_3 (P < 0.05).

amino acids on the concentrations of ATP and free ADP was trivial, and the large amino acid–induced increase in insulin release must therefore be attributed to another factor.

Correlation between insulin release and concentrations of ATP and free ADP. In Fig. 7, we show the correlation of our measurements of insulin release and our estimates for the intracellular concentrations of ATP and free ADP. Insulin release increased as the concentration of ATP increased and the concentration of free ADP decreased. For ATP ≥1.9 mmol/l and free ADP ≤15 µmol/l, amino acids clearly induced a pronounced increase in insulin release that was not accompanied by any change in the concentration of ATP or free ADP. The inset in Fig. 7 illustrates the data for glucose-induced insulin release in greater detail. It is obvious that free ADP is well suited for a role in attenuating insulin release during hypoglycemia. Indeed, patients with K_{ATP} channels of decreased sensitivity to ADP fail to diminish insulin release during hypoglycemia (7). The relative glucose-induced changes in the concentration of ATP are much smaller than the concomitant relative changes in the concentration of free ADP.

Effect of a physiological mixture of amino acids on insulin release in the presence of diazoxide + KCl. It is well known that glucose stimulates insulin release even when K_{ATP} channels are held open pharmacologically with diazoxide, provided that the B-cells are partially depolarized with a high concentration of extracellular K^+ (12). Whether the same holds true for amino acid–induced insulin release was not known. The data shown above suggested that amino acids stimulate insulin release independent, in part, of the cellular energy charge and hence independent of K_{ATP} channels. Therefore, we expected amino acids to stimulate insulin release even in the presence of both diazoxide and an elevated concentration of KCl. As is evident from Fig. 8, this was indeed the case at both 0 and 20 mmol/l glucose (P = 0.02 and 0.05, respectively; n = 4; paired Student's t test). Note that these experiments were performed in the absence of GLP-1(7–37), and glucose itself therefore had only a minor stimulatory effect on insulin release. Remarkably, amino acids stimulated insulin release even though the introduction of diazoxide and KCl led to a marked decrease in cellular ATP content (Fig. 9) and a slight increase in free ADP when 20 mmol/l glucose was present (Fig. 10). In the absence of glucose, the introduction of diazoxide + KCl led to a large increase in the concentration of free ADP, but this was reversed by the addition of amino acids (Fig. 10).

DISCUSSION

We show that at 7.5–30 mmol/l glucose, a mixture of amino acids (15 mmol/l total concentration) greatly increases insulin release without significantly affecting the concentrations of ATP and free ADP. Only at 0 mmol/l glucose did amino acids significantly increase the concentration of ATP, and only at 0–2 mmol/l glucose did they significantly decrease the concentration of free ADP. By contrast, in a meeting abstract, Wroblewski et al. (35) had previously indicated that in the absence of glucose, amino acids do not affect the level of free ADP in β-TC3 insulinoma cells; their estimates were based on nuclear magnetic resonance measurements of ATP and phosphocreatine. We also used 2-deoxyglucose and sodium azide to decrease greatly the

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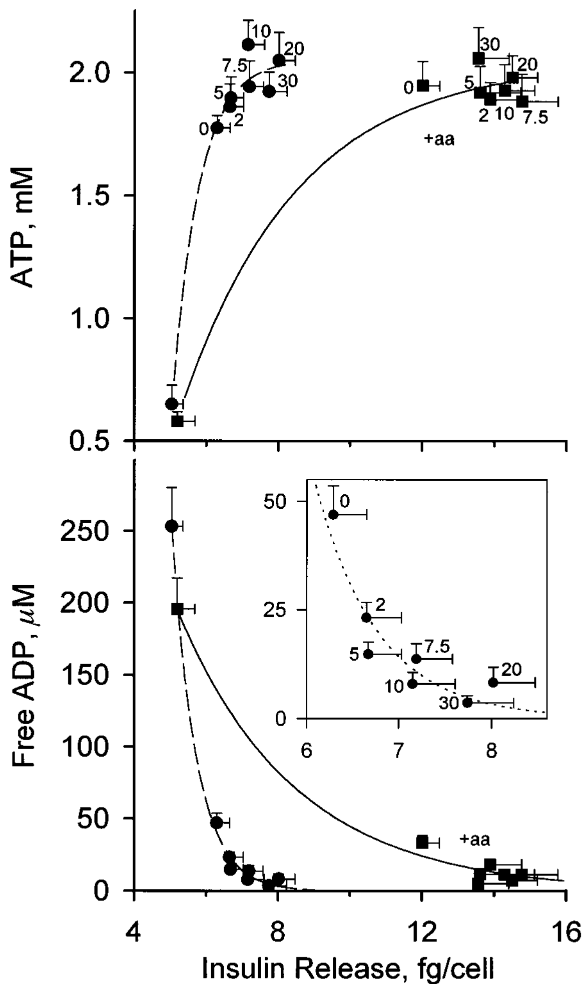

FIG. 7. Correlation of glucose- and amino acid–induced insulin release with concentration of ATP and free ADP in βHC9 cells. Correlation is based on data shown in Figs. 2, 3, and 6. ●, no amino acids; ■, amino acids present (+aa). Means ± SE of 14 extracts for each condition. Exponential regression lines were fitted to data using lowest ATP and highest free ADP (obtained in presence of 2-deoxyglucose and NaN_3), respectively, as fixed points. Numbers next to symbols indicate concentration of glucose. *Inset:* Detail of correlation for glucose-induced insulin release.

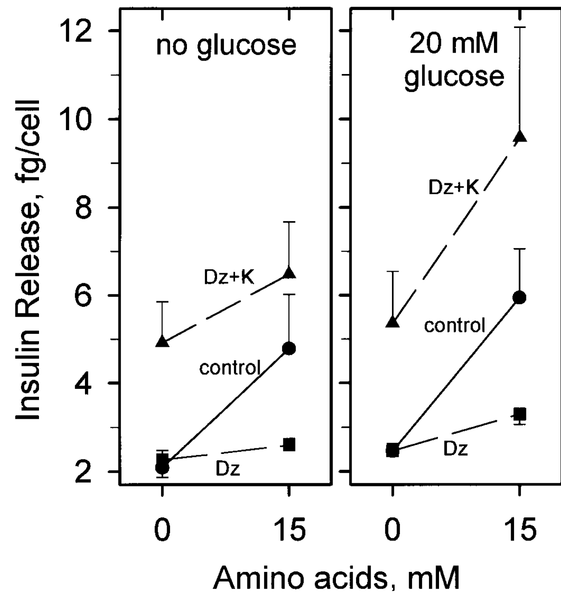

FIG. 8. Effect of a physiological mixture of amino acids on insulin release in the presence of diazoxide + KCl. βHC9 cells were incubated for 20 min at various concentrations of glucose and amino acids (control) or under the same conditions with the addition of 0.25 mmol/l diazoxide (Dz) or of 0.25 mmol/l diazoxide + 54 mmol/l KCl (Dz+K). Means ± SE of four experiments are shown. Amino acids have a significant stimulatory effect in controls at 20 mmol/l glucose (P = 0.04) and in the presence of diazoxide + KCl at 0 mmol/l glucose (P = 0.02) and 20 mmol/l glucose (P = 0.05; Student's t test for paired data).

concentration of ATP and increase the concentration of free ADP. Under these conditions, amino acids no longer stimulated insulin release. Finally, we show that amino acids stimulate insulin release even when K_{ATP} channels are held open with diazoxide and the plasma membrane is partially depolarized with extracellular KCl. We conclude that a yet-unknown amino acid sensor and K_{ATP} channels together regulate amino acid–induced insulin release. If the amino acid sensor senses amino acids, a signal is given for insulin release. This signal has to pass the K_{ATP} channel checkpoint. If the cell is well energized, i.e., when K_{ATP} channels have low activity, the B-cells release insulin. If the cell is poorly energized (as at a low concentration of glucose or in the presence of 2-deoxyglucose and NaN_3), K_{ATP} channels are more active, polarize the plasma membrane, and thus prevent Ca^{2+} influx and insulin release. A thorough inspection of Fig. 5 in Bolea et al. (36) reveals that in islet B-cells, a fixed frequency of electrical spikes is associated with greater insulin release when amino acids are present than when they are absent. Hence, it is conceivable that the amino acid sensor acts on a pathway beyond Ca^{2+} influx.

During a 20-min incubation, βHC9 cells released up to ~8 fg insulin per cell in response to amino acids plus glucose but <2 fg insulin per cell in response to glucose alone (Fig. 2)—i.e., the addition of amino acids boosted glucose-induced insulin release up to about fivefold. By comparison, perfused rat pancreases release only about three times more insulin when 15 mmol/l of a mixture of amino acids is added together with 5 mmol/l glucose (18). Furthermore, in the absence of glucose, βHC9 cells showed very appreciable insulin release in response to amino acids (~70% of the release seen at 20 mmol/l glucose), whereas in the perfused rat pancreas amino acid–induced insulin release is minimal (<5% of the release at 20 mmol/l glucose) in the absence of glucose (18). Exposure of βHC9 cells to GLP-1 is unlikely to be responsible for this difference. Thus, as can be seen from Fig. 8 (controls; no GLP present), amino acids alone increased insulin release, though statistical significance was not reached with the small number of experiments (P = 0.12; Student's t test, n = 4). When duplicate runs (not shown) for each daily experiment were included, amino acids did significantly stimulate insulin release (P = 0.01 for no effect; Wilcoxon's signed-rank test).

Though our data on ATP and free ADP was obtained with βHC9 hyperplastic insulin-secreting islet cells, it compares favorably with data on normal islet cells, at least for the few instances in which conditions were similar. Thus, Ghosh et al. (8) reported that microdissected B-cell–rich islet cores from in vitro perfused rat pancreases show a 30% decrease in free

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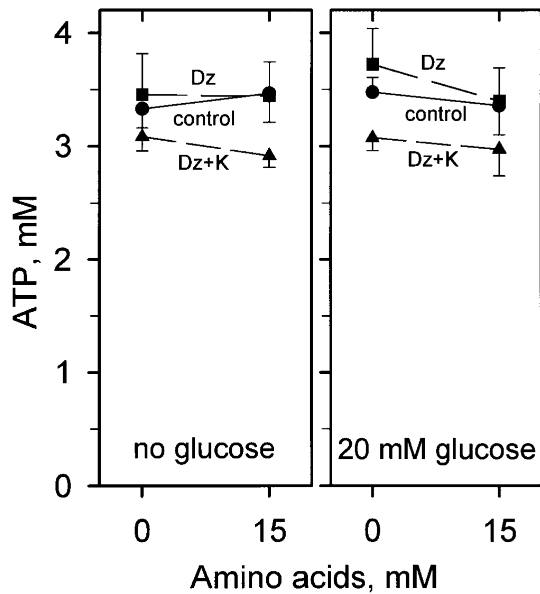

FIG. 9. Effect of a physiological mixture of amino acids in the presence of diazoxide + KCl (Dz+K) on ATP content of βHC9 cells. Same experiments as in Fig. 8. ATP was determined as described in Fig. 1. Means ± SE of four experiments are shown.

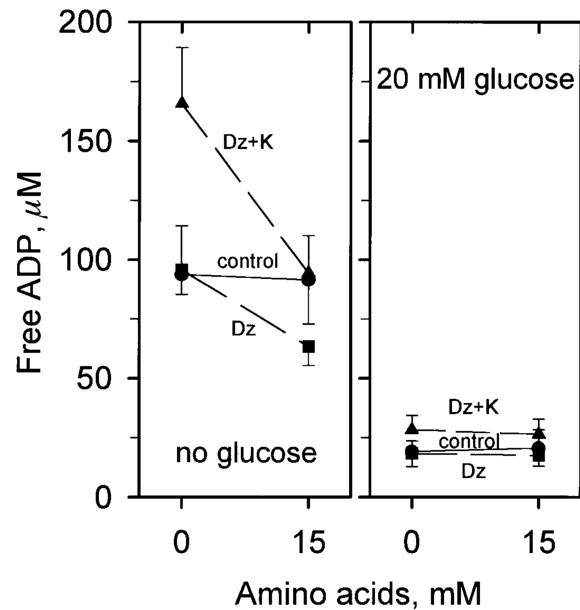

FIG. 10. Effect of a physiological mixture of amino acids in the presence of diazoxide + KCl (Dz+K) on the concentration of free ADP in βHC9 cells. Same experiments as in Figs. 8 and 9. Phosphocreatine and creatine were determined as described in Figs. 1 and 5. Free ADP was calculated as described in Fig. 6. Means ± SE of four experiments are shown. Glucose induced a significant decrease in free ADP in controls (P = 0.001) and in the presence of diazoxide + KCl (P = 0.03). Amino acids induced a significant decrease in free ADP in the presence of diazoxide + KCl when glucose was absent (P = 0.02)

ADP as glucose is increased from 4 to 8 mmol/l on a background of 4 mmol/l amino acids. By comparison, we report here a ~50% decrease in free ADP in βHC9 cells as glucose is increased from 2 to 7.5 mmol/l on a background of 15 mmol/l amino acids (Fig. 6). Detimary et al. (37) determined the total (not free) ADP content of flow-cytometrically sorted rat B-cells at 1, 10, and 20 mmol/l glucose. At 10 mmol/l glucose, the ATP content was ~110% higher and the ADP content ~55% lower than at 1 mmol/l glucose (our interpolated numbers are ~+16% and ~–75%). Between 10 and 20 mmol/l glucose, Detimary et al. (37), like us, found no significant change in ATP or ADP content. Furthermore, in good agreement with our data on the glucose dependence of the concentration of free ADP, B-cells of normal (but not heterozygous or homozygous glucokinase knockout) mice show an exponential decrease in K_{ATP}-channel current, whereby the current is half-maximal at ~3 mmol/l glucose (38).

Recently, several investigators measured the light output from live, luciferase-expressing islet and insulinoma cells and then estimated the concentration of ATP. Unexpectedly, luciferase expressed in these cells had a many-fold higher K_m for ATP than does purified luciferase (39). The data of Maechler et al. (40) on partially fuel-depleted INS-1 insulinoma cells suggest that the concentration of ATP is ~7.9 mmol/l in 2.8 mmol/l glucose and is ~9.7 mmol/l in 12.8 mmol/l glucose (based on linearity of luciferase light output). Köhler et al. (41), using mouse islets, observed an ~10% increase in luminescence upon increasing glucose from 3 to 20 mmol/l (data on the concentration of ATP are not provided). Kennedy et al. (39), using MIN6 insulinoma cells that express luciferase in the cytoplasm, observed an ~16% increase in luminescence upon increasing glucose from 3 to 30 mmol/l; based on their accompanying data, this translates into cytosolic ATP increasing from 1.0 to 1.3 mmol/l. In similarly treated mixed rat islet cells, the increase in luminescence amounted to only ~10% (39). In MIN6 cells, in 3 mmol/l glucose, the concentration of ATP was similar in the cytoplasm, beneath the plasma membrane, and inside the mitochondria (1.0, 0.9, and 1.2 mmol/l, respectively) (39). Furthermore, the maximal glucose-dependent changes in the luminescence originating from luciferase inside mitochondria, anchored to the plasma membrane, or free in the cytosol were similar, though the half-times to plateau ranged from 20 to 55 s (39).

Detimary et al. (42) suggested that ATP contained in secretory granules contributes appreciably (~30%) to the total islet ATP content, and that this ATP leads one to underestimate relative changes in cytoplasmic ATP content. However, our βHC9 insulin-secreting cells contained only ~0.6 pg insulin/cell, whereas the whole mouse islets used by Detimary et al. contained ~63 pg insulin/cell. Whether βHC9 cells also contain fewer secretory granules (and thus less vesicular ATP) than normal islet B-cells is not known. It is therefore conceivable that fuel-induced changes in the concentration of cytosolic ATP of βHC9 cells are in fact larger than those reported here.

The concentration of phosphatidylinositol bisphosphate in the plasma membrane affects the sensitivity of K_{ATP} channels for ATP (43,44). Whether glucose and amino acids affect the concentration of phosphatidylinositol bisphosphate remains to be investigated. We expect such effects to be small, because glucose- and amino acid–induced insulin release via the K_{ATP}-independent pathway is appreciable.

A two-component model may apply not only to amino acid–induced insulin release but also to glucose-induced insulin release. If so, glucokinase plays a dual role: one as the pacemaker of glycolysis and one as a signaling molecule by

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itself. K_{ATP} channels attenuate this signal when cellular energy stores are low. The effect of glucose on the concentrations of ATP and ADP is most pronounced at concentrations of glucose well below half-maximal glucose saturation of glucokinase. Indeed, as is evident from Fig. 6, the concentration of free ADP falls exponentially as the concentration of glucose increases. Because glucokinase relaxes only slowly from a glucose-induced conformational change (13), it is a suitable candidate as an intracellular signal. Compatible with such a role, glucose stimulates insulin release even when K_{ATP} channels are held open with diazoxide and the plasma membrane is partially depolarized with extracellular KCl (12). Despite the very significant activity of glucokinase below 5 mmol/l glucose, insulin release is negligible below 5 mmol/l glucose; this may well be due to the activation of K_{ATP} channels. Our data suggest that K_{ATP} channels in B-cells work as guardians, attenuating glucose- and amino acid–induced insulin release in the hypoglycemic range. In agreement with this notion, βHC9 cells show a pronounced increase in glucose oxidation as glucose is raised from 0 to 5 mmol/l glucose, but there is no accompanying increase in insulin release (11). Interestingly, a large number of signaling pathways has been excluded from involvement in the K_{ATP}-independent pathway of glucose-induced insulin release (45), but the involvement of glucokinase has not yet been tested rigorously. Finally, it is worth noting that conformational changes in hexokinases also appear to be involved in glucose sensing by yeast and plants (46–48).

Evidence continues to accumulate that non-B islet cells also contain K_{ATP} channels (21–26). Now that we know that K_{ATP} channels are not unique to B-cells among islet cells, it is time to consider a more generally applicable role for K_{ATP} channels in stimulus-secretion coupling. K_{ATP}-independent pathways have now been demonstrated for insulin release induced by amino acids, glucose, fatty acids, and α-ketoisocaproate (this work; 12,49–51). A role of K_{ATP} channels in attenuating hormone release when cells have a low phosphorylation potential could fit all four types of islet cells and still allow for differences in fuel sensitivity between cell types.

ACKNOWLEDGMENTS

Supported in part by National Institutes of Health Grand DK-51016 to P.R.

The hormone assays were carried out by the RIA Core Facility of the University of Pennsylvania Diabetes Research Center in Philadelphia, PA.

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