The cytoplasmic calcium concentration (Ca\textsuperscript{2+}) was measured in suspensions of fura-2 loaded mouse pancreatic \(\beta\)-cells by continuously recording the 340/380 nm fluorescence excitation ratio. When the glucose concentration was raised from 3 to 20 mM, there was an initial lowering of Ca\textsuperscript{2+}, followed by a sustained increase. Whereas the reduction in Ca\textsuperscript{2+} was related to the extracellular calcium concentration in a hyperbolic manner, the increasing component exhibited a sigmoidal dose-response relationship. Both effects became maximal at 15–20 mM of the sugar. Qualitatively similar bimodal Ca\textsuperscript{2+} responses were obtained with 30 mM mannose, 2 mM \(\alpha\)-ketoisocaproic acid, 10 mM leucine, and 10 mM metabolism-stimulating leucine analogue \(\beta\)-2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid. Fructose (30 mM) had virtually no effect on Ca\textsuperscript{2+} in the presence of extracellular Ca\textsuperscript{2+}, and 10 mM arginine induced only a rise. The results indicate that nutrient secretagogues stimulate both the entry of Ca\textsuperscript{2+} into the \(\beta\)-cells and its elimination from the cytoplasm by processes like organelle sequestration and outward transport. Consequently, the Ca\textsuperscript{2+} level determining insulin secretion results from the balance between two opposing actions.

By using \(\beta\)-cell-rich suspensions from the pancreatic islets of \(\text{ob/ob}\) mice it was recently established that glucose-induced stimulation of insulin secretion is associated with an initial lowering of the cytoplasmic Ca\textsuperscript{2+} concentration (Ca\textsuperscript{2+}; Ref. 1). Moreover, the subsequent increase of Ca\textsuperscript{2+}, could not be attributed to a proposed intracellular mobilization of the cation (2–4) but was found to depend solely on an influx of external Ca\textsuperscript{2+}. Studies of individual cells confirmed that the pattern of glucose-induced changes of Ca\textsuperscript{2+}, were representative for the insulin-secreting \(\beta\)-cell and not accounted for by the minor contamination of glucose-producing \(\alpha\)-cells (5).

Glucose-induced lowering of Ca\textsuperscript{2+} can be attributed to intracellular sequestration and stimulated outward transport (1). Indeed, the Ca\textsuperscript{2+} incorporated in response to the sugar can be mobilized by inositol 1,4,5-triphosphate (6–8). It is therefore evident that the glucose-induced Ca\textsuperscript{2+} sequestration is relevant also for the action of other types of secretagogues. If there is negative feedback inhibition of high Ca\textsuperscript{2+} concentrations on the entry of Ca\textsuperscript{2+} (1), elimination of Ca\textsuperscript{2+} from the submembrane space may be a prerequisite for maintaining a sustained glucose-stimulation of insulin release.

In the present study the action of glucose on Ca\textsuperscript{2+}, was further investigated and the effects of other insulin secretagogues analyzed. It will be shown that the glucose-induced increase of Ca\textsuperscript{2+}, mimics insulin release (9, 10) in being sigmoidally related to glucose concentration, whereas the lowering effect exhibits a hyperbolic dependence like the metabolism of glucose (9–11). Moreover, other secretagogues stimulating \(\beta\)-cell metabolism were found to have glucose-like bimodal actions, whereas the non-nutrient secretagogue arginine only increased Ca\textsuperscript{2+}.

**Experimental Procedures**

**Materials**—Reagents of analytical grade and deionized water were used. Collagenase and HEPES\textsuperscript{1} were obtained from Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany (F.R.G.), and leucine, arginine, glutamine, \(\alpha\)-ketoisocaproic acid, bovine serum albumin (fraction V), EGTA, Tris, and Triton X-100 were from Sigma. Mannose and diethylstilbestrol testosterone acetate were from Merck (Darmstadt, F.R.G.), glucose from BDH Chemicals (Poole, England), TPEN and BCH from Behring Diagnostics. Molecular Probes Inc. (Eugene, OR) supplied the tetratopotiassium salt of fur-2 and its acetoxymethylene and Bio-Rad supplied Chexol 100.

**Preparation of \(\beta\)-Cells**—Adult obese hyperglycemic mice (gene symbol \(\text{ob/ob}\)) from a local colony (12) were starved overnight. The animals were killed by decapitation and pancreatic islets isolated by a collagenase technique. Previous studies indicated that these islets contain more than 90% \(\beta\)-cells (12), which respond normally to glucose and other regulators of insulin release (13). The islets were dispersed into single cells by shaking in a Ca\textsuperscript{2+}-deficient medium, and \(\beta\)-cells were separated from debris by centrifugation through a medium containing Ca\textsuperscript{2+} and supplemented with 40 mg/ml bovine serum albumin (14). A suspension of the cells in RPMI 1640 culture medium supplemented with 10% fetal calf serum was then gently shaken for 1–3 h in an incubator at 37 °C with an atmosphere of 5% CO\textsubscript{2} in humidified air.

**Measurements of Cytoplasmic Ca\textsuperscript{2+}**—For each experiment 2–6 × 10\textsuperscript{6} cells, representing the yield from one mouse, were washed in a HEPES-buffered (pH 7.4) medium physiologically balanced in cations with Cl\textsuperscript{−} as the sole anion (15). This medium was supplemented with 1 mg/ml bovine serum albumin and contained 3 mM glucose and 1.28 mM Ca\textsuperscript{2+}. The cells were then suspended in 5 ml of the washing medium and 5 μl of 1 mM fur-2 acetoxyethyl ester in dimethyl sulfoxide was added. After incubation for 40 min, the cells were centrifuged and washed in an identical medium lacking the indicator and containing zero or 3 mM glucose and zero or 1.28 mM Ca\textsuperscript{2+} as stated in the legends to the figures. The cells were then suspended in 1 ml of the same medium and transferred to a 1-cm quartz cuvette placed in the thermostatically controlled (37 °C) cuvette holder of a time-sharing multichannel spectrophotofluorometer (16) set for continuous recording of the 340/380 nm fluorescence excitation ratio. The dynamic response of this equipment exceeded by at least 2 orders of magnitude the most rapid effects observed here. Further information

\textsuperscript{1}The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, \([\text{ethyleneglycolbis(oxyethylenenitrilo)}]tetraacetic acid; TPEN \text{N,N',N',N'-tetraakis(2-pyridylmethyl)ethylenediamine; BCH} \text{\(\beta\)-2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid.}
tion about the system and its properties have been previously described in detail (1).

There was some leakage of the fura-2 indicator from the cells which was enhanced by stimulators of secretion. This increase was difficult to estimate in the individual experiments (1). The observation periods after addition of insulin secretagogues were therefore limited to 5-6 min, assuming the same rate of leakage as before stimulation of secretion. The contribution of extracellular fura-2 to the fluorescence ratio signal was estimated by quenching the extracellular fura-2 fluorescence by addition of 100 μM Ce³⁺ early in the experiments followed by chelation of the cation by 150 μM diethylenetriaminepentaacetic acid (1).

At the end of each experiment the cells were disrupted by the addition of 0.05% Triton X-100. The 340/380 nm fluorescence excitation ratio and the 380 nm excitation fluorescence were then obtained both at saturating Ca²⁺ concentrations and at <1 nM Ca²⁺. The latter was accomplished by the addition of excess EGTA as well as Tris to raise pH above 8.3. Ca²⁺ could now be calculated as previously described (17), assuming a K/D₅₀ for the Ca²⁺-fura-2 complex of 231 nM (18).

During the course of the studies it was discovered that α-ketoisocaproic acid interfered with the fluorescence of fura-2. This nutrient has previously been found to quench also the fluorescence from the Ca²⁺ indicator quin-2 (19). The present interference could not be attributed to contamination with heavy metals, since it was not affected by treatment with the resin Chelex 100 or addition of the cell-permeant heavy metal chelator TPEN to the suspension. To minimize the interference the concentration of α-ketoisocaproic acid was limited to 2 mM.

Statistical Analyses—Results are presented as means ± S.E. Statistical analyses were by Student’s t test for paired data.

RESULTS

The basal Ca²⁺ of β-cells incubated in 3 mM glucose and 1.28 mM Ca²⁺ was 90-100 nM (Figs. 1-4). Increase of the glucose concentration to 20 mM resulted in lowering of Ca²⁺, with a maximal decrease of 13.1 ± 3.2 nM (p < 0.02) at 45 s. Ca²⁺ then rose to more than 180 nM within 3 min (Fig. 1). If instead of increasing of the glucose concentration, 50 mM mannose was added, the initial lowering of Ca²⁺ was delayed and diminished (5.8 ± 1.2 nM at 90 s, p < 0.01). The subsequent increase was also more sluggish and less pronounced, about 140 nM being reached after 6 min. Fructose (30 mM) only in some experiments tended to have bimodal effects on Ca²⁺, although the magnitudes were very small (Fig. 2). The presence of 3 mM glucose had virtually no effect on the response to fructose.

The amino acid leucine (10 mM) or its deamination product α-ketoisocaproic acid (2 mM) induced glucose-like effects in similarly designed experiments (Fig. 3). The leucine-induced lowering of Ca²⁺ was at least as pronounced as that induced by glucose, but was maximal already at 30 s when it corresponded to 17.5 ± 3.5 nM (p < 0.01). However, the subsequent increase was slightly less marked, 165 nM being reached within 3 min. The lowering of Ca²⁺, caused by 2 mM α-ketoisocaproic acid was more like that obtained with 20 mM glucose with a maximal change of 14.9 ± 1.3 nM (p < 0.001) at 45 s. This concentration of α-ketoisocaproic acid subsequently raised Ca²⁺, to about 150 nM.

The nonmetabolizable leucine transport analogue BCH (10 mM) had leucine-like effects with an initial lowering of Ca²⁺, of 14.0 ± 1.5 nM (p < 0.001) at 30 s and 165 nM being reached within 3 min (Fig. 4). Addition of 10 mM glutamine 1 min prior to the introduction of BCH caused a slight increase of Ca²⁺, (6.8 ± 0.7 nM, p < 0.001) and the BCH-induced lowering of Ca²⁺ at 30 s was now diminished (5.9 ± 0.3 nM, p < 0.001), although the subsequent increase was virtually identical.

In contrast to the other insulin secretagogues, 10 mM arginine did not lower Ca²⁺, but induced a rapid and relatively modest increase, to a final concentration of about 125 nM (Fig. 5).

Fig. 1. Effects of increasing the glucose concentration from 3 to 20 mM or adding 30 mM mannose on the cytoplasmic Ca²⁺ in β-cells. β-Cells in suspension were loaded with fura-2 by a 40-min incubation with 1 μM fura-2 acetoxymethylester in a medium containing 3 mM glucose and 1.28 mM Ca²⁺. Subsequent rinsing and incubation were performed in a similar medium lacking the indicator and containing 0 (●) or 3 mM (●) glucose. Fluorescence is indicated by the horizontal bar. Means ± S.E. for five experiments.

Fig. 2. Effects of 30 mM fructose on the cytoplasmic Ca²⁺ in β-cells incubated in 0 or 3 mM glucose. β-Cells in suspension were loaded with fura-2 by a 40-min incubation with 1 μM fura-2 acetoxymethylester in a medium containing 3 mM glucose and 1.28 mM Ca²⁺. Subsequent rinsing and incubation were performed in a similar medium lacking the indicator and containing 0 (●) or 3 mM (●) glucose. Fluorescence is indicated by the horizontal bar. Means ± S.E. for four (●) or five (●) experiments.

Fig. 3. Effects of 10 mM leucine or 2 mM α-ketoisocaproic acid on the cytoplasmic Ca²⁺ in β-cells incubated in 3 mM glucose, β-Cells in suspension were loaded with fura-2 by a 40-min incubation with 1 μM fura-2 acetoxymethylester in a medium containing 3 mM glucose and 1.28 mM Ca²⁺. Subsequent rinsing and incubation were performed in a similar medium lacking the indicator. The presence of leucine (●) or α-ketoisocaproic acid (●) is indicated by the horizontal bar. Means ± S.E. for five experiments.
FIG. 4. Effects of 10 mM BCH on the cytoplasmic Ca²⁺ in β-cells incubated in 3 mM glucose in the absence or presence of 10 mM glutamine. β-Cells in suspension were loaded with fura-2 by a 40-min incubation with 1 μM fura-2 acetoxymethylester in a medium containing 3 mM glucose and 1.28 mM Ca²⁺. Subsequent rinsing and incubation were performed in a similar medium lacking the indicator. In one series of experiments BCH (open bar) was added alone (○) and in another glutamine (filled bar) was included 1 min before the addition of BCH (●). Means ± S.E. for five experiments.

FIG. 5. Effects of 10 mM arginine on the cytoplasmic Ca²⁺ in β-cells. β-Cells in suspension were loaded with fura-2 by a 40-min incubation with 1 μM fura-2 acetoxymethylester in a medium containing 3 mM glucose and 1.28 mM Ca²⁺. Subsequent rinsing and incubation were performed in medium lacking the indicator. This medium contained either the same glucose and Ca²⁺ concentrations ([ ]) or was depleted of glucose and Ca²⁺ and supplemented with 2 mM EGTA ([ ]). Arginine was present as indicated by the horizontal bar. Means ± S.E. for five (□) or four (●) experiments.

FIG. 6. Effect of glucose concentration on the relative increase of the cytoplasmic Ca²⁺ in β-cells. β-Cells in suspension were loaded with fura-2 by a 40-min incubation with 1 μM fura-2 acetoxymethylester in a medium containing 3 mM glucose and 1.28 mM Ca²⁺. Subsequent rinsing and incubation were performed in a similar medium lacking the indicator and glucose. The glucose concentration was increased from zero to 4, 6, 8, or 12 mM followed by a further increase to 20 mM after a stable increase of Ca²⁺ had been attained. The relative increase of Ca²⁺ is expressed in percent of the effect at 20 mM glucose. Means ± S.E. for 9, 9, 10, and 5 experiments representing 4, 6, 8, and 12 mM glucose, respectively.

FIG. 7. Effects of different glucose, mannose, or fructose concentrations on the lowering of the cytoplasmic Ca²⁺ in β-cells incubated in Ca²⁺-deficient medium. β-Cells in suspension were loaded with fura-2 by a 40-min incubation with 1 μM fura-2 acetoxymethylester in a medium containing 3 mM glucose and 1.28 mM Ca²⁺. Subsequent rinsing and incubation were performed in a similar medium lacking the indicator glucose and Ca²⁺. The incubation was started by the addition of 2 mM EGTA followed by gradually increasing concentrations of glucose (○), mannose (●), or fructose (△). Means ± S.E. for five (○, △) or 10 (●) experiments.

FIG. 8. Effects of different leucine or α-ketoisocaproic acid concentrations on the lowering of the cytoplasmic Ca²⁺ in β-cells incubated in Ca²⁺-deficient medium. β-Cells in suspension were loaded with fura-2 by a 40-min incubation with 1 μM fura-2 acetoxymethylester in a medium containing 3 mM glucose and 1.28 mM Ca²⁺. Subsequent rinsing and incubation were performed in a similar medium lacking the indicator glucose and Ca²⁺. The incubation was started by the addition of 2 mM EGTA followed by gradually increasing concentrations of leucine (■) or α-ketoisocaproic acid (△). Means ± S.E. for five experiments.

Fig. 5 shows that there is a sigmoidal dependence of the increase of Ca²⁺, on glucose concentration, with a lowest detectable increase at about 4 mM of the sugar. The corresponding thresholds for increase of Ca²⁺, in a glucose-free medium by mannose, α-ketoisocaproic acid, and BCH were about 0.1, 0.1, and 0.1 mM, respectively, whereas leucine slightly increased Ca²⁺ even at 0.01 mM.

Characterizations of the dose-response relationships for reduction of Ca²⁺, were facilitated by the fact that only lowering is obtained in a Ca²⁺-deficient medium. The basal Ca²⁺, in glucose-free medium lacking Ca²⁺ and supplemented with 2 mM EGTA was 60–70 nM (data from Figs. 7–9). The maximal lowering from this level was about 20 nM, and all substances with effects had hyperbolic dose-response relationships. The approximately half-maximal lowerings (10 nM) were obtained at 6, 13, and 22 mM for glucose, mannose, and fructose, respectively (Fig. 7). Lowering of Ca²⁺ was considerably more sensitive to leucine, α-ketoisocaproic acid (Fig. 8), and BCH (Fig. 9), with half-maximal effects at 0.3, 0.5, and 0.5 mM, respectively. Addition of 10 mM glutamine induced a small lowering of Ca²⁺, and this amine decreased also...
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Fig. 9. Effects of different BCH concentrations on the lowering of the cytoplasmic Ca^{2+} in ß-cells incubated in Ca^{2+}-deficient medium in the absence and presence of 10 mM glucose. ß-Cells in suspension were loaded with fura-2 by a 40-min incubation with 1 μM fura-2 acetoxymethylester in a medium containing 3 mM glucose and 1.28 mM Ca^{2+}. Subsequent rinsing and incubation were performed in a similar medium lacking indicator glucose and Ca^{2+}. The incubation was started by the addition of 2 mM EGTA followed by gradually increasing concentrations of BCH alone (○), or 10 mM glucose was added prior to the gradual increase of the BCH concentration (●). In the latter case the effect of glucose alone was used as the starting point of the curve. Means ± S.E. for five experiments.

the Ca^{2+}, lowering effect of BCH (Fig. 9). Arginine had no effect on Ca^{2+}; in a Ca^{2+}-deficient medium (Fig. 5).

DISCUSSION

Although ^45Ca fluxes have been studied extensively in pancreatic islets for more than 15 years, unequivocal interpretation of the glucose effects was not possible until information about corresponding changes of Ca^{2+}, became available. It is therefore not surprising that even recently there has been considerable disagreement as to the mechanisms involved in the regulation of Ca^{2+}. The initial effect of glucose on Ca^{2+}, represented such a controversy. There have thus been arguments presented both for an early increase of Ca^{2+}; by inhibited outward transport (2, 20) and/or intracellular mobilization of the cation (2-4), and lowering of Ca^{2+}, by enhanced organelle sequestration and stimulated outward transport (10, 21).

With the present technique it was possible for the first time to show that, under physiological conditions, the glucose-induced changes in Ca^{2+}, are similar to ^45Ca efflux (10, 22, 23) with an initial lowering followed by increase due to influx of Ca^{2+} (1). The current study provides further evidence for such a similarity with ^45Ca efflux (9, 10, 23) in demonstrating that the lowering exhibits a hyperbolic dependence on the glucose concentration, whereas the increase is sigmoidally related. The lowering action also parallels the hyperbolic dependence of glucose metabolism with half-maximal effects at 5-8 mM of the sugar (9, 10, 23). Moreover, the threshold concentration of glucose for increase of Ca^{2+}, as well as the concentrations for half-maximal and maximal effects are similar to the characteristics of glucose-induced insulin release (9, 10).

Glucose-induced sequestration of Ca^{2+}, is important for providing the Ca^{2+} mobilized by some other secretagogues (6-8) and may be a prerequisite for the maintenance of a sustained glucose stimulation of insulin release (1). In inducing lowering of Ca^{2+}, glucose may, under certain conditions, suppress insulin release (24, 25), and initial inhibition of secretion is even of significance in human diabetes (26-28). The recent observation that glucose-induced lowering of Ca^{2+}, accounts also for the physiological inhibition of glucagon secretion (29) indicates that it may be of more general relevance. To clarify the underlying mechanism it was important to evaluate whether nutrient secretagogues other than glucose have similar bimodal actions on Ca^{2+}, of the pancreatic ß-cells. In performing such studies it was considered important to prevent depletion of ATP before the addition of the test substance (4, 30). Most studies of the bimodal actions were therefore performed in the presence of 3 mM glucose, which is sufficient to secure adequate ß-cell concentrations of ATP (31).

The data obtained indicate that nutrient secretagogues other than glucose have bimodal actions on Ca^{2+}, of the ß-cells. Also in the cases of mannose and fructose the concentration dependencies for lowering Ca^{2+}, corresponded to those of the metabolism of the sugars (32, 33). The threshold for increase of Ca^{2+}, by mannose was close to that for stimulation of insulin release (32), whereas the fructose-induced increase only compensated for its lowering effect. Fructose is poorly metabolized by the ß-cells and is a weak insulin secretagogue (32, 33). It has been reported that its small secretory response is bimodal with initial inhibition and later stimulation (34), a phenomenon mimicking the actions of nutrient secretagogues on Ca^{2+}. Although low concentrations of glucose facilitate fructose-stimulated insulin release (32), the presence of 3 mM glucose did not significantly affect the fructose-induced changes of Ca^{2+}.

Leucine and its deamination product α-ketoisocaproic acid are potent insulin secretagogues with reported threshold concentrations for stimulation of about 3 mM (35-37). This concentration is more than an order of magnitude higher than the minimum required for increasing Ca^{2+}. The reason for this discrepancy remains unclear until detailed laborious dose-response studies have been performed like that presented here for glucose. There is no distinct concentration threshold for stimulation of secretion with the nonmetabolizable leucine transport analogue BCH (38), which is more consistent with the characteristics of its increasing effect on Ca^{2+}.

When comparing leucine and α-ketoisocaproic acid lowering of Ca^{2+}, with their metabolism, there were also discrepancies. Although the dose-response relationships were hyperbolic also for the degradation of these compounds, approximately 10-fold higher concentrations were required for half-maximal effects (36, 37, 39). The contradiction became even more pronounced with BCH, which is not metabolized at all (38). This paradox can be explained by stimulation of metabolism unrelated to the degradation of the compounds per se. Leucine is known to activate allosterically ß-cell glutamate dehydrogenase (40), which is probably of significance for the action of this amino acid on insulin release (35). Moreover, whereas α-ketoisocaproic acid does not directly influence the activity of glutamate dehydrogenase (41), it may do so after conversion to leucine. Since the first demonstration of BCH activation of glutamate dehydrogenase, which might explain its stimulation of insulin secretion (41), the metabolism activating properties of this model amino acid have received considerable attention (35, 42-45). It is now established that the action of BCH is also consistent with the fuel hypothesis for stimulation of insulin release (43-45).

BCH has been found to stimulate the degradation of glutamine in rat ß-cells (43). Although this amine alone does not increase insulin release, it markedly amplifies the secretory response to BCH (35). Consistent with the lack of action of glutamine on secretion, Ca^{2+}, was only marginally affected. However, unexpectedly, glutamine did not make the ß-cells more responsive with respect to BCH-induced changes in Ca^{2+}. Whether this implies that glutamate dehydrogenase already has sufficient substrate concentrations in the absence
of added glutamine in the ob/ob-mouse β-cells remains to be elucidated.

Considering the metabolism-stimulating actions of leucine and BCH, as well as the good correlation between the metabolism of glucose, mannose, and fructose and lowering of Ca\textsuperscript{2+}, it seems likely that the latter phenomenon simply reflects enhanced energy production. Consistent with such a view, arginine, which is essentially nonmetabolizable by pancreatic β-cells (39), had no Ca\textsuperscript{2+}-lowering effect. This positively charged amino acid depolarizes by entering the β-cells, and the subsequent influx of Ca\textsuperscript{2+} explains the stimulation of insulin secretion (46).

The present data are consistent with the idea that the increase of Ca\textsuperscript{2+}, follows depolarization due to inhibition of specific K\textsuperscript{+} channels by the ATP formed during nutrient degradation (47, 48). However, the results indicate that stimulation of metabolism also increases Ca\textsuperscript{2+} removal from the cytoplasm. The concept that the Ca\textsuperscript{2+}, level determines insulin secretion results from the balance between Ca\textsuperscript{2+} influx due to depolarization and removal of the cation by organelle sequestration and stimulated outward transport (1, 10) is consequently not only valid for glucose but can now be extended to nutrient secretagogues in general.

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