Regulation of Insulin Secretion in Relation to Nitric Oxide, Carbon Monoxide and Acid alpha-Glucoside Hydrolase Activities

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Insulin release transduction mechanism through acid glucan 1,4-α-glucosidase activation is Ca^{2+} regulated

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Salehi, Albert, Henrik Mosén, and Ingmar Lundquist. Insulin release transduction mechanism through acid glucan 1,4-α-glucosidase activation is Ca^{2+} regulated. *Am. J. Physiol.* 274 (*Endocrinol. Metab.* 37): E459–E468, 1998.—An important signal involved in glucose-stimulated insulin secretion is transduced through the action of a lysosomal acid, glucan 1,4-α-glucosidase. We investigated the Ca^{2+} dependency of this enzyme activity in relation to insulin release. In isolated islets, increased levels of extracellular Ca^{2+} induced a large increase in acid glucan 1,4-α-glucosidase activity accompanied by a similar increase in insulin release at both substimulatory and stimulatory concentrations of glucose. At low glucose the Ca^{2+} "inflow" blocker nifedipine unexpectedly stimulated enzyme activity without affecting insulin release. However, nifedipine suppressed $^{45}Ca^{2+}$ outflow from perifused islets at low glucose and at Ca^{2+} deficiency when intracellular Ca^{2+} was mobilized by carbachol. This nifedepine-induced retention of Ca^{2+} was reflected in increased acid glucan 1,4-α-glucosidase activity. Adding different physiological Ca^{2+} concentrations or nifedipine to islet homogenates did not increase enzyme activity. Neither selective glucan 1,4-α-glucosidase inhibition nor the ensuing suppression of glucose-induced insulin release was overcome by a maximal Ca^{2+} concentration. Hence, Ca^{2+}-induced changes in acid glucan 1,4-α-glucosidase activity were intimately coupled to similar changes in Ca^{2+}-glucose-induced insulin release. Ca^{2+} did not affect the enzyme itself but presumably activated either glucan 1,4-α-glucosidase-containing organelles or closely interconnected messengers.

pancreatic islets; lysosomal enzymes; nifedipine; emiglitate; carbachol; calcium ion

IT IS A WELL-KNOWN FACT (8, 25, 34, 35) that Ca^{2+} plays an essential role in the stimulus-secretion coupling for insulin release. The cytosolic accumulation and intracellular distribution of Ca^{2+} are important for a growing number of different events in the secretory process, which is affected not only by the major secretagogue glucose but also by a variety of cholinergic, adrenergic, and peptidergic influences (2, 3, 8–10, 12, 22, 25, 33–35). Moreover, glucose-induced insulin release itself is a complex cascade of events, the details of which are far from elucidated (2, 25, 35). We have previously proposed (14–20, 28–32) that one of these glucose-induced multiple signals is transduced via the vacuolar system, involving the activation of a lysosome-acid glucan 1,4-α-glucosidase system. The acid glucan 1,4-α-glucosidase (EC 3.2.1.3) and the acid α-glucosidase (EC 3.2.1.20) are members of the α-glucosidehydrolase family. The acid glucan 1,4-α-glucosidase is known to preferentially attack α-1,4-linked polymers such as glycogen (24) and thereby to have the ability to produce high local concentrations of nonphosphorylated glucose within the vacuolar system. This glucose production, in turn, could act as a transducer (e.g., metabolic, osmotic, or cybernetic) in the multifactorial process of insulin release. Glycogen is a normal constituent of islet tissue (11, 21), and it is known to display a surprisingly constant concentration at a wide range of blood glucose levels (21). Hence an important part of islet glycogen is probably not integrated in the metabolic pool of glucose phosphorylation processes in the cytoplasm but rather is restricted to a compartmentalized vacuolar pool of signal glycogen available to the acid glucan 1,4-α-glucosidase. It is worth noting that the phosphorolytic breakdown of glycogen in vitro from mouse islets is reportedly very slow (21).

In a recent report (28) we showed that the defective glucose-induced insulin release from isolated mouse islets in a Ca^{2+}-deficient medium was accompanied by markedly reduced activities of islet lysosomal α-glucosidehydrolases. In contrast, the activity of another lysosomal glycosidase, *N*-acetyl-β-D-glucosaminidase, was completely unaffected by Ca^{2+} deficiency. Likewise, islet activities of the lysosomal enzyme acid phosphatase and the neutral α-glucosidase (endoplasmic reticulum) were not influenced in a Ca^{2+}-deficient medium. A similar pattern of a greatly suppressed glucose-induced insulin release in parallel with a reduced acid α-glucosidehydrolase activity was accomplished by different selective α-glucosidehydrolase inhibitors such as miglitol, emiglitate, and acarbose (19, 20 28–32).

Hence, because the activity of the lysosomal acid α-glucosidehydrolases (but not the neutral α-glucosidase) seemed to be one of several important intracellular key factors in glucose-induced insulin release, we found it mandatory to study the Ca^{2+} dependency of these enzyme activities in more detail. In the present investigation we tested *1*) high concentrations of extracellular Ca^{2+}, *2*) the Ca^{2+} channel blocker nifedipine and the intracellular Ca^{2+} mobilizer carbachol, as well as *3*) the selective α-glucosidehydrolase inhibitor emiglitate (26) to further elucidate the role of Ca^{2+} in regulating islet acid α-glucosidehydrolase activities and insulin release.

MATERIALS AND METHODS

Animals

Adult female mice of the NMRI strain (B & K Universal, Sollentuna, Sweden), 3–4 mo old and weighing 25–30 g, fed ad libitum, and with free access to water, were used throughout the study. The experiments were approved by the Ethical Committee for Animal Research at the University of Lund.

Drugs and Chemicals

Collagenase (CLS 4) was obtained from Worthington Biochemicals (Freehold, NJ). Nifedipine, ethylene glycol-bis(β-

aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and carbachol as well as methylumbelliferyl-coupled substrates were purchased from Sigma Chemical (St. Louis, MO). Emiglitate, *N*-[β-(4-ethoxycarbonylphenoxy) ethyl]-1-deoxynojirimycin (Bay 0 1248), was kindly supplied by Bayer (Leverkusen, Germany). Bovine serum albumin was from ICN Biomedicals (High Wycombe, UK). $^{45}CaCl_2$ was from Radiochemical Centre (Amersham). All other chemicals were from British Drug Houses (Poole, UK) or Merck (Darmstadt, Germany). The radioimmunoassay kits for insulin determination were obtained from Novo Nordisk (Bagsvaerd, Denmark).

Experimental Procedure

Isolation of pancreatic islets from freely fed mice was accomplished by retrograde injection of a collagenase solution via the bile-pancreatic duct (5). The animals were killed from 8 to 9 AM by elongation of the neck and were immediately injected with collagenase.

Batch incubation of isolated islets. The freshly isolated islets were preincubated for 30 min at 37°C in Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4, supplemented with 10 mmol/l *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 0.1% bovine serum albumin, and 1 mmol/l glucose. Each incubation vial contained 30 islets in 1.5 ml buffer solution and was gassed with 95% O_2-5% CO_2 to obtain constant pH and oxygenation. After preincubation, the buffer was changed to a medium containing 1 or 16.7 mmol/l glucose ± test substances, and the islets were incubated for 120 min. Emiglitate, when used, was included also during the preincubation period (20). All incubations were performed at 37°C in an incubation box (30 cycles/min). It should be noted that, in the experiments with high concentrations of Ca^{2+}, phosphate and sulfate in the KRB-HEPES buffer were replaced with equimolar amounts of chloride (9). The change in osmotic pressure with high Ca^{2+} was compensated for by reduction of NaCl, as described by Hellman (9). Immediately after incubation, an aliquot of the medium was removed for assay of insulin. Insulin was determined radioimmunologically (7).

Lysosomal enzyme activities. If not otherwise stated, the islets were then thoroughly washed in glucose-free KRB buffer and collected and stored in 200 µl acetate-EDTA buffer (1.1 mmol/l EDTA and 5 mmol/l acetate, pH 5.0) at −20°C (14). Ancillary experiments showed that the collected islets could be kept frozen for several months without loss of enzyme activity. After sonication, islet homogenates were analyzed for lysosomal enzyme activities. In experiments in which we studied the direct influence of different Ca^{2+} concentrations added to islet homogenates on the lysosomal enzyme activities, the islets were washed in a glucose- and Ca^{2+}-free KRB buffer and collected and stored in 5 mmol/l acetate in the absence of EDTA. The procedures for determination of acid phosphatase (pH 4.5), acid α-glucosidase (pH 4.0 and 5.0), *N*-acetyl-β-D-glucosaminidase (pH 5.0), and neutral α-glucosidase (pH 6.5) with methylumbelliferyl-coupled substrates have previously been described (16). Islet glucan 1,4-α-glucosidase activity with glycogen as substrate was determined at pH 4.0, as described in detail elsewhere (14, 19). The acid α-glucosidase activity was assayed at both pH 4.0 and pH 5.0, because previous studies (23) have shown that inhibition of α-glucosidase by the lysosomotropic drug suramin was dependent on the prevailing pH value. Furthermore, it should be recalled that lysosomal enzyme activities are subjected to circadian and seasonal variations (see Ref. 6). Therefore, all experiments were always performed with both control groups and experimental groups at each occasion. Protein was analyzed according to the method of Lowry et al. (13).

Islet perifusion experiments. In the perifusion experiments, islets (150–200) were first incubated for 90 min in 800 µl of KRB medium containing 20 mmol/l glucose and 50 µl $^{45}CaCl_2$ (50–100 µCi), which was added from a stock solution with a specific activity of 10–40 mCi/mg Ca^{2+}. The islets were then washed three times with nonradioactive medium, divided into two or three groups with 75–100 islets per group, and transferred to perifusion columns. The islets were thereby sandwiched between two layers of gel (Bio-gel P-4, 200–400 mesh; Bio-Rad Laboratory, Richmond, CA) and perifused at a rate of 0.1 ml/min with the KRB buffer supplemented with 1 mmol/l glucose. Test substances were introduced according to the protocols. A Ca^{2+}-deficient medium was obtained by omitting calcium chloride and adding 0.5 mmol/l EGTA. The radioactivity lost by the islets was measured in effluent fractions collected every 2 min (50 µl of the sample were added to 5 ml of scintillation fluid) and counted in a scintillation counter (Packard Instrument, Downers Grove, IL). The fractional efflux rate was calculated for each period (radioactivity lost by islets during the time interval/radioactivity present in the islets during the same time interval), and the mean value calculated for *minutes 40* and *42* was then normalized to 100%. Insulin was determined with a radioimmunoassay (7).

Statistics

Probability levels of random differences were determined by Student's unpaired *t*-test or analysis of variance followed by Tukey-Kramer's multiple comparisons test where applicable.

RESULTS

Effect of a Maximal Concentration of Extracellular Ca^{2+} on Basal Insulin Release and Islet Lysosomal Enzyme Activities

Taking advantage of a previous dose-response study by Hellman (9) showing that increasing extracellular Ca^{2+} concentrations up to 30 mmol/l in the presence of a substimulatory glucose level could increase insulin release from isolated *ob/ob* islets, we performed a series of experiments at 1 mmol/l glucose with either a normal Ca^{2+} (2.5 mmol/l) or a high maximal (9) concentration of Ca^{2+} (30 mmol/l) in the extracellular medium. Figure 1 shows that increasing the Ca^{2+} concentration from 2.5 to 30 mmol/l induced an almost threefold increase in basal insulin release. This enhanced insulin secretion was accompanied by a marked increase in islet lysosomal α-glucosidehydrolase activities, i.e., acid glucan 1,4-α-glucosidase (3-fold increase), acid α-glucosidase pH 4.0 (+40%), and pH 5.0 (+95%), whereas other lysosomal enzyme activities such as acid phosphatase and *N*-acetyl-β-D-glucosaminidase were totally unaffected. In contrast, the activity of the neutral α-glucosidase, an enzyme attributed to the endoplasmic reticulum, was modestly reduced (−30%) by 30 mmol/l Ca^{2+} in the incubation medium.

Effects of Direct Addition of Different Concentrations of Ca^{2+} on Lysosomal Enzyme Activities in Islet Homogenates

Because a high concentration of extracellular Ca^{2+} greatly increased the acid α-glucosidehydrolase activi-

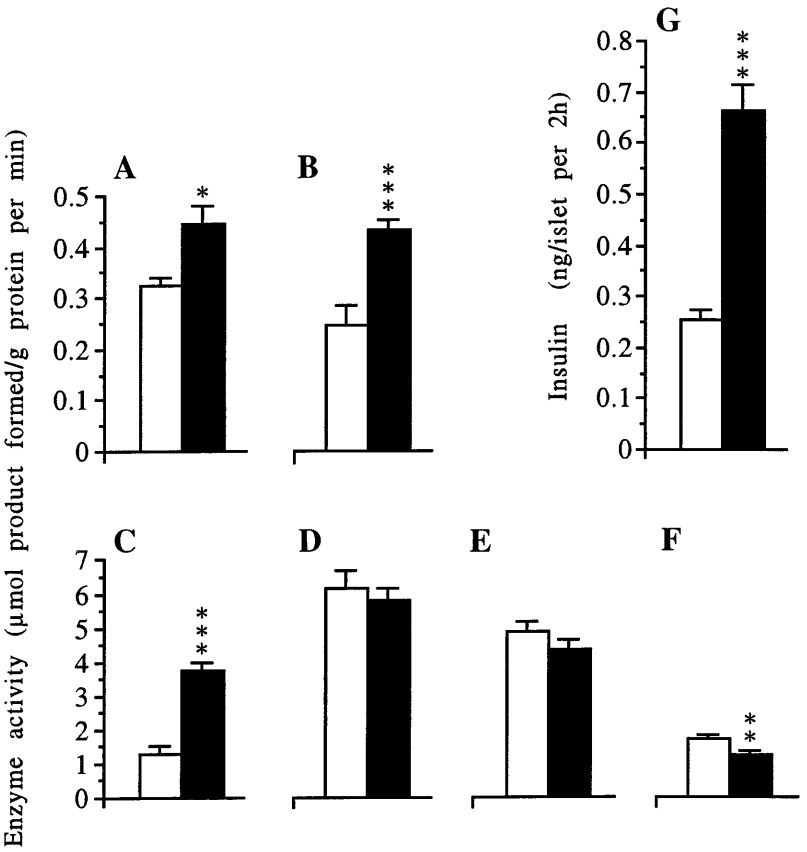


Fig. 1. Effect of Ca^{2+} on islet activities of different lysosomal enzymes and neutral α-glucosidase, as well as insulin secretion, at a low substimulatory concentration of glucose (1 mmol/l): acid α-glucosidase, pH 4.0 (*A*); acid α-glucosidase, pH 5.0 (*B*); acid glucan 1,4-α-glucosidase (*C*); acid phosphatase (*D*); *N*-acetyl-β-D-glucosaminidase (*E*); neutral α-glucosidase (*F*); insulin release (*G*). Islets were incubated for 2 h at 2.5 mmol/l Ca^{2+} (open bars) or at 30 mmol/l Ca^{2+} (solid bars). Enzyme activities are expressed as μmol glucose (acid glucan 1,4-α-glucosidase) or 4-methylumbelliferone liberated per g protein per min. Insulin secretion is expressed as ng insulin released per islet per 2 h. Values are means ± SE for 9–10 batches of islets in each group obtained from 3 independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

ties in intact islets (Fig. 1), we studied whether addition of Ca^{2+} to islet homogenates could influence these enzyme activities directly. The effects of direct addition to islet homogenates of a wide range of Ca^{2+} concentrations (0.05 μmol/l-30 mmol/l) on the different enzyme activities are illustrated in the absence (Fig. 2, *A* and *B*) and presence (Fig. 2, *C* and *D*) of calmodulin. No appreciable influence of Ca^{2+} was seen within known intracellular fluctuations of the cation. However, there was a large decrease in acid α-glucosidase activities (about −80%) and a marked increase in acid glucan 1,4-α-glucosidase activity (about +50%) at very high "unphysiological" intracellular concentrations of Ca^{2+} (2, 10, and 30 mmol/l).

Influence of the Ca^{2+} Channel Blocker Nifedipine on Islet Lysosomal Enzyme Activities and Insulin Release at Low and High Glucose Concentrations

To further investigate, in intact islets, the effect of Ca^{2+} perturbations on insulin release in relation to the activities of the acid α-glucosidehydrolases, we studied the influence of nifedipine on basal and glucose-induced insulin secretion and islet lysosomal enzyme activities. Figure 3*A* shows the effect of nifedipine on insulin secretion from incubated islets at low or high glucose. As expected, glucose-induced insulin secretion was greatly suppressed in the presence of nifedipine. Moreover, we found, unexpectedly, that the islet lysosomal acid α-glucosidehydrolase activities were significantly increased by nifedipine at basal glucose (Fig. 4), i.e., acid glucan 1,4-α-glucosidase (+35%) and acid α-glucosidase pH 4.0 (+45%) and pH 5.0 (+40%). However, nifedipine had no effects on these enzymes in the presence of high glucose (16.7 mmol/l), a glucose concentration which by itself markedly enhanced the acid α-glucosidehydrolase activities (Fig. 4).

Effects of Direct Addition of Nifedipine on Lysosomal Enzyme Activities in Islet Homogenates

To elucidate whether nifedipine could directly activate the islet α-glucosidehydrolases in islet homogenates, a dose-response study was performed. Table 1 shows the effect of nifedipine on the activities of the different lysosomal enzymes and the neutral α-glucosidase in islet homogenates. Nifedipine at 1 μmol/l induced a marked increase in *N*-acetyl-β-D-glucosaminidase activity. Furthermore, nifedipine at 30 μmol/l induced a modest suppression of the activities of the acid α-glucosidehydrolases (about −10 to −15%) and a pronounced decrease of *N*-acetyl-β-D-glucosaminidase activity (about −55%). Acid phosphatase activity was not influenced, whereas the activity of the neutral α-glucosidase was moderately reduced at 10 and 30 μmol/l nifedipine.

Effect of Nifedipine on $^{45}Ca^{2+}$ Efflux from Isolated Islets Either at Low (1 mmol/l) Glucose and Normal Ca^{2+} or in a Ca^{2+}-Deficient Medium in Which the Islets Were Stimulated by the Intracellular Ca^{2+} Mobilizer Carbachol at Low Glucose

Because high extracellular Ca^{2+} (Fig. 1) as well as nifedipine (Fig. 4) could increase the acid α-glucosidehy-

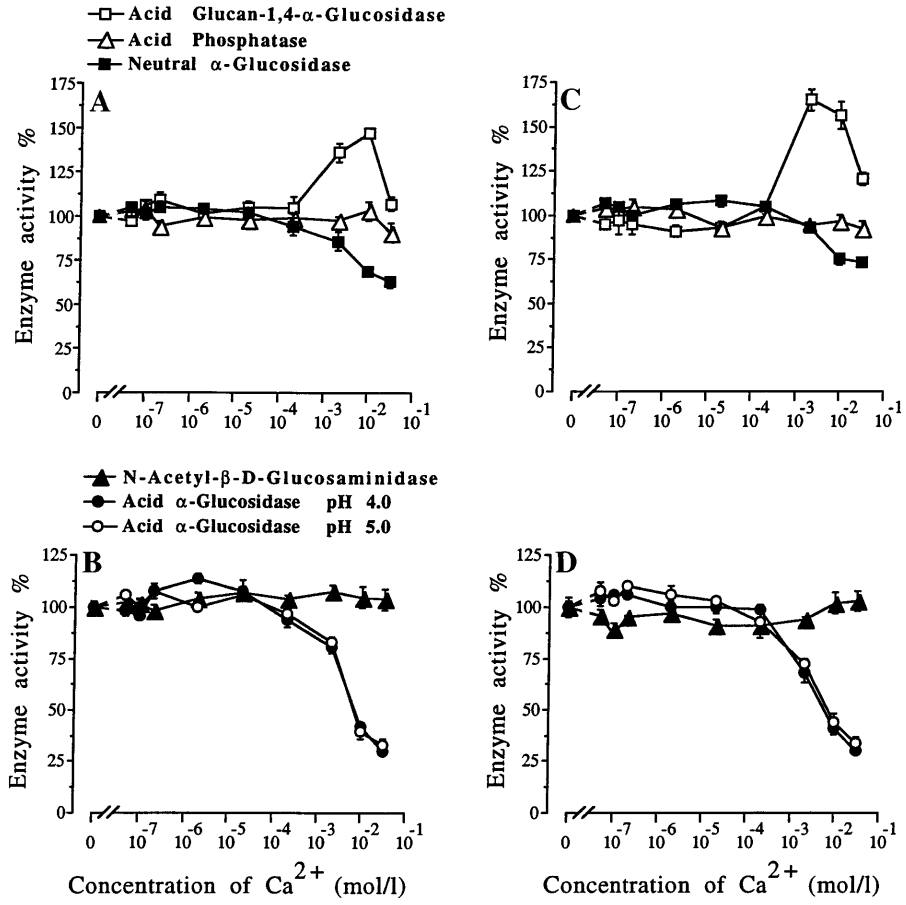
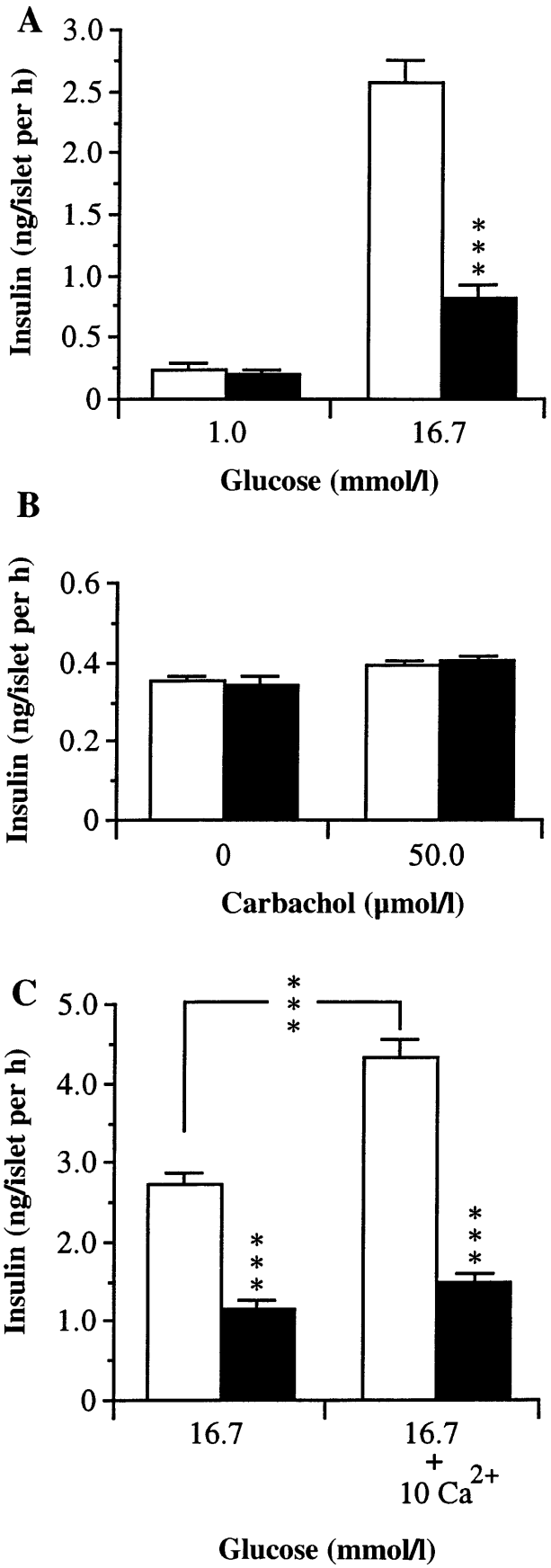


Fig. 2. Influence of different concentrations of Ca^{2+} added directly to enzyme assay mixtures containing aliquots of Ca^{2+}-free islet homogenates in the absence (*A* and *B*) or presence (*C* and *D*) of 25 U/ml calmodulin. Enzyme activities are expressed as percentage of control incubations. Values are means ± SE for 5–9 observations in each group obtained from 3 independent experiments.

drolase activities in intact islets, we searched for an effect of nifedipine on intracellular Ca^{2+} that was independent of its Ca^{2+} channel-blocking effects. Therefore, to study whether nifedipine could influence the efflux of Ca^{2+} from the β-cell, we performed a series of perifusion experiments with $^{45}Ca^{2+}$-loaded islets. The first experiment was conducted at substimulatory (1 mmol/l) glucose and normal Ca^{2+} (Fig. 5). It is seen that nifedipine induced a marked decrease of $^{45}Ca^{2+}$ efflux at this low glucose concentration, which indeed does not open the nifedipine-sensitive Ca^{2+} channels. The basal insulin release was not influenced by nifedipine (see also Fig. 3*A*). Because the cholinergic muscarinic receptor agonist carbachol is known to mobilize intracellularly stored Ca^{2+} (12), we performed another experiment at 1 mmol/l glucose with carbachol as a stimulus in a Ca^{2+}-deficient perifusion medium supplemented with 0.5 mmol/l EGTA, to preclude any influx of Ca^{2+} into the β-cells. Figure 6 shows the effect of nifedipine on $^{45}Ca^{2+}$ efflux and insulin release during stimulation with carbachol (50 μmol/l). In the absence of nifedipine, carbachol induced a clear biphasic increase of $^{45}Ca^{2+}$ efflux. Addition of nifedipine converted the initial carbachol-induced increase of $^{45}Ca^{2+}$ into a marked but transient decrease followed by a modest increase, which, however, was strongly suppressed compared with the carbachol controls. Thus nifedipine induced a powerful inhibition of carbachol-stimulated $^{45}Ca^{2+}$ efflux in a Ca^{2+}-deficient medium. As expected, carbachol did not notably influence insulin release from either controls or nifedipine-treated islets in the absence of extracellular Ca^{2+} (Fig. 6, *bottom*).

Effect of Nifedipine on Islet Lysosomal Enzyme Activities and Insulin Release in a Ca^{2+}-Deficient Medium in the Presence and Absence of the Intracellular Ca^{2+} Mobilizer Carbachol

The next experiment was performed to test whether the carbachol-mobilized Ca^{2+}, which was retained within the β-cell by the action of nifedipine (see Fig. 6), might be redistributed to affect the acid α-glucosidehydrolase activity. To avoid any influence of extracellular Ca^{2+}, the experiment was performed in a Ca^{2+}-deficient medium. We therefore incubated isolated islets for 60 min in the absence and presence of nifedipine and carbachol after a preincubation period of 40 min, i.e., largely mimicking the perifusion experiments. Figure 7 shows that nifedipine induced a large increase (almost 2-fold) in islet lysosomal acid α-glucosidehydrolase activities at basal glucose (1 mmol/l) and Ca^{2+} deficiency, i.e., a much higher increase than in the presence of a normal concentration of extracellular Ca^{2+} (see Fig. 4). The activities of other lysosomal enzymes and the neutral α-glucosidase were not influenced by nifedipine (Fig. 7). Carbachol had no notable influence on the different enzyme activities in the basal state and did not induce a greater increase in enzyme activities



together with nifedipine than that brought about by nifedipine itself, except for inducing a modest but significant increase in the acid glucan 1,4-α-glucosidase activity (+22%) in the presence of the Ca^{2+} channel blocker (Fig. 7). Hence, nifedipine stimulated acid glucan 1,4-α-glucosidase activity in both the absence and the presence of carbachol. As expected, no effect of either carbachol or nifedipine on insulin release in the Ca^{2+}-deficient medium was observed (Fig. 3*B*).

Effect of a Maximal Concentration of Extracellular Ca^{2+} on Glucose-Stimulated Insulin Release and Islet Lysosomal Enzyme Activities in the Absence and Presence of the Selective α-Glucosidehydrolase Inhibitor Emiglitate

In a further attempt to elucidate whether the Ca^{2+} dependency of islet acid α-glucosidehydrolases and insulin release in intact islets was intimately coupled in the secretory process, we performed a series of experiments with the selective α-glucosidehydrolase inhibitor emiglitate (20, 26, 29, 31) in the presence of high glucose and using either a normal (2.5 mmol/l) or a maximal concentration of Ca^{2+} (10 mmol/l). At this high glucose concentration 10 mmol/l Ca^{2+} is maximal, because higher Ca^{2+} concentrations are even inhibitory to insulin release (8). Figures 3*C* and 8 show that raising the extracellular Ca^{2+} from 2.5 to 10 mmol/l at 16.7 mmol/l glucose (open bars) did increase both insulin release (+55%; Fig. 3*C*) and acid glucan 1,4-α-glucosidase activity (+50%) as well as acid α-glucosidase pH 4.0 (+55%) and pH 5.0 (+40%; Fig. 8). The activities of acid phosphatase and *N*-acetyl-β-D-glucosaminidase, however, were significantly suppressed by high Ca^{2+} (−25%; Fig. 8). Moreover, addition of emiglitate almost totally suppressed both glucose-induced and Ca^{2+}-induced increase of acid α-glucosidehydrolase activities (Fig. 8) as well as the release of insulin (Fig. 3*C*).

DISCUSSION

The present results lend further support to our previous hypothesis (14–20, 28–32) that one of the multiple signals involved in glucose-induced insulin release is transduced through the activation of a glycogen-hydrolyzing acid, glucan 1,4-α-glucosidase.

Fig. 3. *A*: effect of 30 μmol/l nifedipine (solid bars) on basal (1 mmol/l glucose) and glucose-stimulated (16.7 mmol/l glucose) insulin secretion from isolated pancreatic islets at normal extracellular Ca^{2+} (2.5 mmol/l). Controls are shown by open bars. Values are means ± SE for 6–9 batches of islets in each group obtained from 4 independent experiments. ***$P < 0.001$ vs. controls. *B*: insulin release in a Ca^{2+}-deficient medium at 1 mmol/l glucose in the presence and absence of 50 μmol/l carbachol and 30 μmol/l nifedipine (solid bars). Controls are shown by open bars. Values are means ± SE for 8–10 batches of islets in each group obtained from 4 independent experiments. *C*: effect of Ca^{2+} (10 mmol/l) on insulin release at a high stimulatory concentration of glucose (16.7 mmol/l) in the absence (open bars) or presence (solid bars) of the selective α-glucosidehydrolase inhibitor emiglitate (1 mmol/l). Values are means ± SE for 10–12 batches of islets in each group obtained from 4 independent experiments. ***$P < 0.001$ vs. controls.

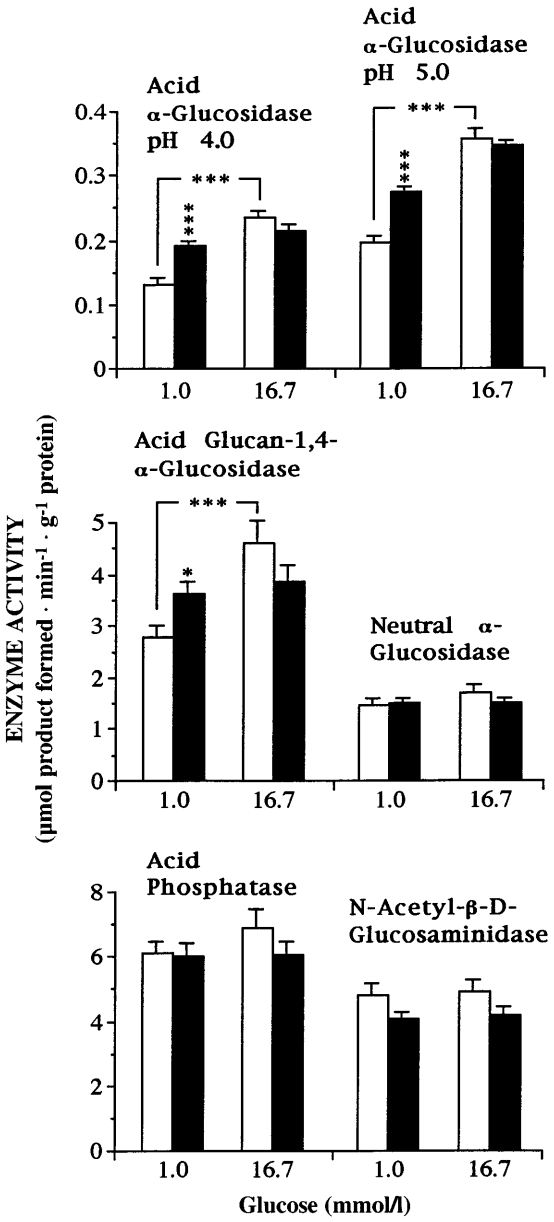


Fig. 4. Effect of 30 μmol/l nifedipine (solid bars) on activities of different lysosomal enzymes and neutral α-glucosidase in islets exposed to 1 or 16.7 mmol/l glucose. Controls are shown by open bars. Enzyme activities are expressed as micromoles glucose (acid glucan 1,4-α-glucosidase) or 4-methylumbelliferone liberated per g protein per minute. Values are means ± SE for 6–9 batches of islets in each group obtained from 4 independent experiments. $*P < 0.05$, $***P < 0.001$ vs. controls.

Effects of Ca^{2+} Deficiency and High Extracellular Ca^{2+}

In a recent study we reported (28) that the activities of the acid α-glucosidehydrolases were highly depressed in islets incubated in a Ca^{2+}-deficient medium. In contrast, classical lysosomal enzyme activities, such as acid phosphatase and *N*-acetyl-β-D-glucosaminidase, as well as the neutral α-glucosidase (endoplasmic reticulum), were unaffected by the absence of extracellular Ca^{2+}. In addition, we observed (28) that the presence of a high concentration of glucose in the incubate induced a significant increase of islet acid α-glucosidehydrolase activities also in a Ca^{2+}-deficient medium. The measured increase, however, never achieved the level of enzyme activity recorded in the presence of a normal physiological Ca^{2+} concentration (28). These observations suggested to us that both glucose and Ca^{2+} were needed for the full expression of islet acid α-glucosidehydrolase activity. We show here that a high concentration of Ca^{2+} itself, in the presence of a substimulatory concentration of glucose (1 mmol/l), greatly enhanced the islet acid α-glucosidehydrolase activities in isolated islets in parallel with an increased insulin release. These effects are most likely a result of increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), because recent data suggest that high extracellular Ca^{2+} can induce a rise in $[Ca^{2+}]_i$, which primarily is accounted for by Ca^{2+} influx through dihydropyridine- and voltage-insensitive, nonselective cation channels (33). Other lysosomal enzyme activities, such as acid phosphatase and *N*-acetyl-β-D-glucosaminidase, were unaffected by the high extracellular Ca^{2+} level. Moreover, a maximal (8) extracellular Ca^{2+} concentration at high glucose did further increase both insulin release and the acid α-glucosidehydrolase activities, supporting the idea that Ca^{2+} is a key regulatory factor for islet acid α-glucosidehydrolase activities.

Effects of Blockade of Ca^{2+} Influx by Nifedipine and of Intracellular Ca^{2+} Mobilization by Carbachol

Because high extracellular Ca^{2+} enhanced the acid α-glucosidehydrolase activity at low substimulatory glucose (Fig. 1, present study) and because high glucose could partially enhance the enzyme activity in the absence of extracellular Ca^{2+} (28), the question arose whether activation of the enzyme(s) was dependent on both the influx of extracellular Ca^{2+} and the redistribution/sequestration of intracellular Ca^{2+}. It is well established that glucose-induced insulin release is accompanied by closure of ATP-sensitive K^+ channels, followed by membrane depolarization (in normal mouse islets at glucose concentrations >7 mmol/l) (4), opening of the voltage-dependent Ca^{2+} channels, and influx of Ca^{2+}. The subsequent rise in intracellular Ca^{2+} elicits multiple signals that induce the recruitment and extrusion of secretory granules (2, 25, 35). The present data show that nifedipine, a well-known blocker of voltage-dependent Ca^{2+} channels, unexpectedly did induce a marked increase in acid α-glucosidehydrolase activity in the presence of a very low nondepolarizing concentration of glucose (1 mmol/l). Hence, nifedipine might have changed the intracellular distribution of Ca^{2+} and/or inhibited Ca^{2+} outflow, because its classical Ca^{2+} channel-inhibiting effects could not be operating at 1 mmol/l glucose. Our $^{45}Ca^{2+}$ efflux experiments showed that such an assumption was justified. Nifedipine induced a marked suppression of the basal efflux of $^{45}Ca^{2+}$ in the presence of 1 mmol/l glucose and a normal extracellular Ca^{2+} concentration. This effect was in all probability not solely, if at all, a consequence of an inhibitory effect of nifedipine on the influx of Ca^{2+} through nonvoltage-dependent Ca^{2+} channels, because $^{45}Ca^{2+}$ efflux in a Ca^{2+}-deficient medium with very low glucose (1 mmol/l)

Table 1. *Influence of nifedipine on different lysosomal enzyme activities and neutral α-glucosidase in islet homogenates*

	Control	Nifedipine, μmol/l			
		0.1	1	10	30
Acid phosphatase	100 ± 1.0	99 ± 1.1	100 ± 1.1	93 ± 1.0	90 ± 3.5
N-acetyl-β-D-glucosaminidase	100 ± 2.4	99 ± 2.0	224 ± 7.5‡	114 ± 2.5*	44 ± 8.6‡
Acid glucan 1,4-α-glucosidase	100 ± 2.1	105 ± 3.1	99 ± 2.2	95 ± 1.5	84 ± 6.2*
Acid α-glucosidase					
pH 4.0	100 ± 1.6	101 ± 2.8	105 ± 1.5	94 ± 1.5	84 ± 1.9‡
pH 5.0	100 ± 1.4	101 ± 1.8	102 ± 1.6	98 ± 4.0	90 ± 1.3†
Neutral α-glucosidase	100 ± 2.7	94 ± 1.0	97 ± 2.3	76 ± 4.1‡	84 ± 6.0†

Aliquots of pancreatic islet homogenates were assayed with nifedipine in a concentration range from 0.1 μmol/l to 30 μmol/l. Enzyme activities are expressed as percentage of control incubations. Values are means ± SE for 4–9 observations. *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.

was strongly inhibited by nifedipine also during stimulation by the intracellular Ca^{2+} mobilizer carbachol. Carbachol has previously been shown to be a very efficient mobilizer of intracellular Ca^{2+} in isolated islets (12). Hence, in addition to its blocking effect on voltage-dependent Ca^{2+} channels, nifedipine, at least under our experimental conditions, also inhibited the outflow of Ca^{2+} across the plasma membrane and/or caused a redistribution of intracellular Ca^{2+}, leading to accumulation of Ca^{2+} in acid α-glucosidehydrolase-containing organelles in the vacuolar system. Such an assumption is in accordance with a very recent finding (1), showing that a high concentration (30 μmol/l) of the nifedipine analog nicardipine did enhance the cytoplasmic Ca^{2+} concentration in mouse thymocytes in the absence of extracellular Ca^{2+}. Indeed, a Ca^{2+} redistribution effect of nifedipine (or a nifedipine-induced messenger) rather than, or in addition to, an inhibition of $^{45}Ca^{2+}$ efflux across the plasma membrane, is suggested by the observation that the initial acute increase in $^{45}Ca^{2+}$ efflux in the biphasic response to carbachol stimulation was converted into a marked initial decrease (negative peak; see Fig. 6) by nifedipine followed by a modest and highly suppressed second phase. Such a pattern is in

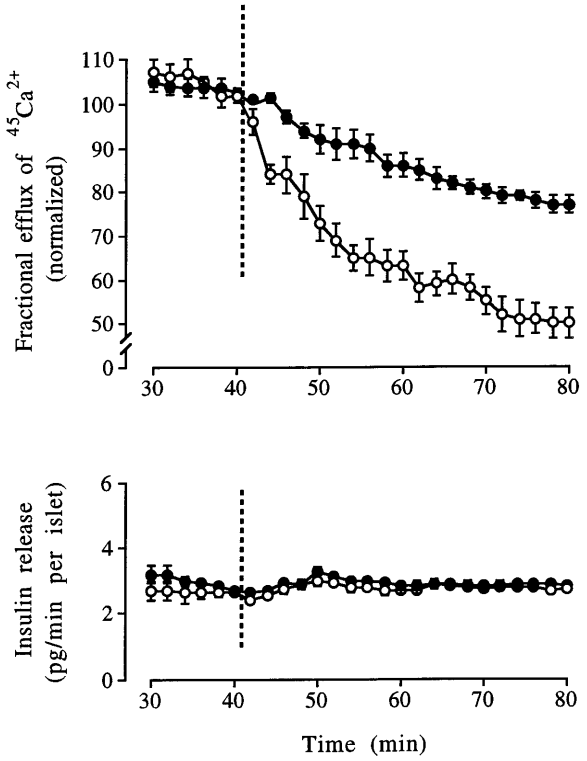


Fig. 5. Effect of nifedipine on $^{45}Ca^{2+}$ efflux (*top*) and insulin release (*bottom*) from perifused islets at a low substimulatory concentration of glucose (1 mmol/l) and normal Ca^{2+} (2.5 mmol/l). Nifedipine (○, 30 μmol/l) or solvent (controls, ●) was introduced at *minute 42*, as shown by dotted vertical line. Fractional efflux rate was normalized, as described in MATERIALS AND METHODS. Values are means ± SE for 6–7 perifusions in each group obtained from 3 independent experiments.

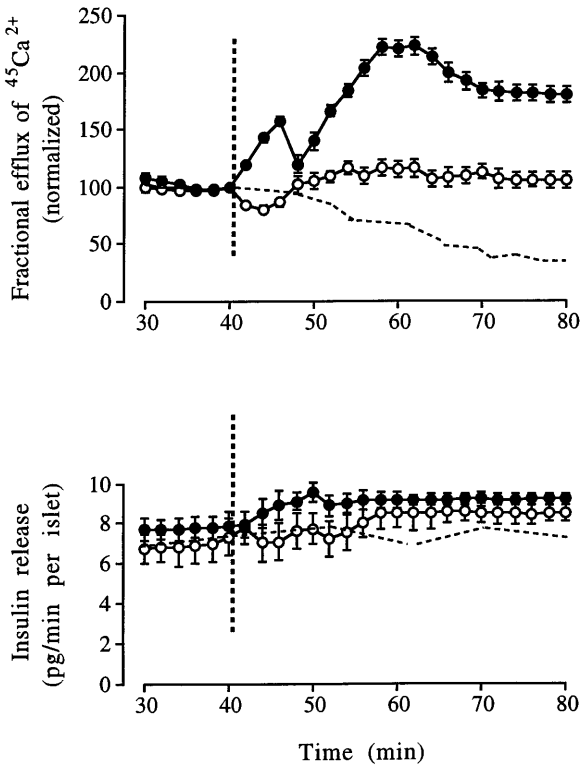


Fig. 6. Effect of carbachol on $^{45}Ca^{2+}$ efflux (*top*) and insulin release (*bottom*) in the presence (○) and absence (●) of nifedipine (30 μmol/l) at a low substimulatory concentration of glucose (1 mmol/l) in a Ca^{2+}-deficient medium supplemented with 0.5 mmol/l EGTA. Carbachol (50 μmol/l) was introduced at *minute 42*, as indicated by dotted vertical line. Basal controls in the absence of nifedipine and carbachol are shown by dashed line. Fractional efflux rate was normalized, as described in MATERIALS AND METHODS. Values are means ± SE for 5 perifusions in each group obtained from 4 independent experiments.

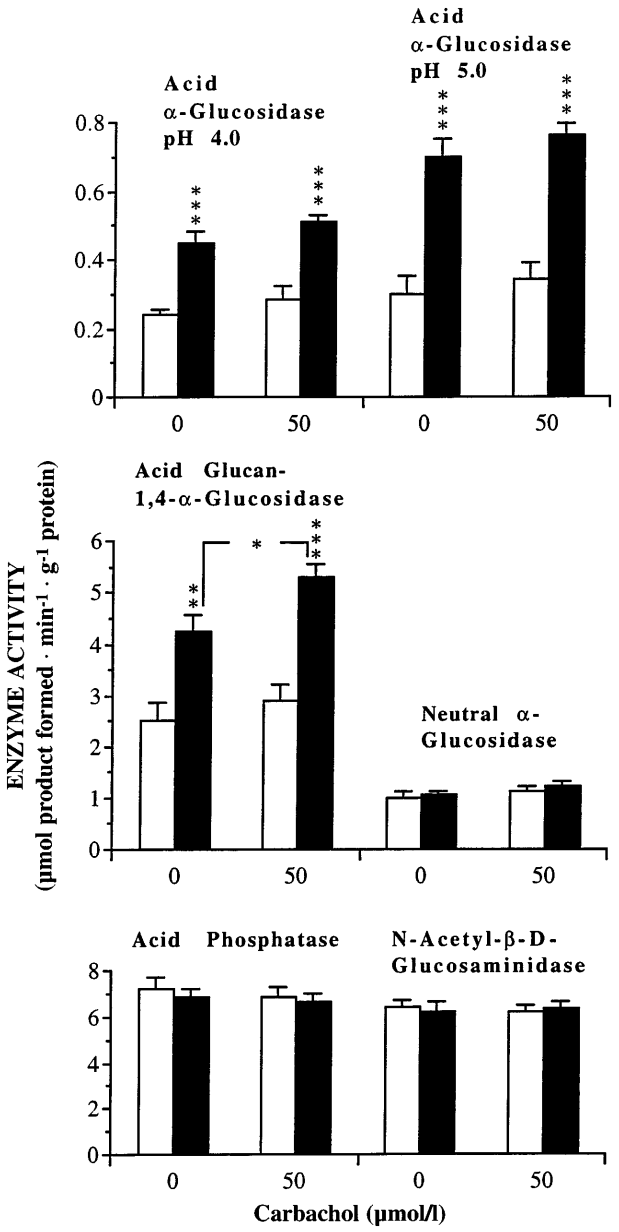


Fig. 7. Effect of 30 μmol/l nifedipine (solid bars) on activities of different lysosomal enzymes and neutral α-glucosidase in islets incubated in the absence and presence of 50 μmol/l carbachol at 1 mM glucose in a Ca^{2+}-deficient medium. Controls are shown by open bars. Enzyme activities are expressed as micromoles glucose (acid glucan 1,4-α-glucosidase) or 4-methylumbelliferone liberated per g protein per minute. Values are means ± SE for 8–10 batches of islets in each group obtained from 4 independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

fact much like the $^{45}Ca^{2+}$ efflux curve obtained by glucose stimulation (3) in the presence of extracellular Ca^{2+} (and in the absence of nifedipine).

The putative Ca^{2+} redistribution leading to activation of the acid α-glucosidehydrolases seemed in no way obligatory to the inflow of extracellular Ca^{2+} through the voltage-dependent Ca^{2+} channels, because enzyme activity could be induced by nifedipine in a Ca^{2+}-deficient medium. From these experiments it is also obvious that the nifedipine-stimulated Ca^{2+} redistribution and the subsequent increase in acid glucan 1,4-α-glucosidase activity are not sufficient by themselves to induce an insulin secretory response, because nifedipine at the same time blocks the voltage-dependent Ca^{2+} channels. Furthermore, it seems very unlikely that Ca^{2+} itself is directly modulating the enzyme(s), because physiological $[Ca^{2+}]_i$ (10, 25, 27, 33) in both the absence and presence of calmodulin did not display any notable effect on the enzyme activities after addition to islet homogenates. It should be noted, however, that very high concentrations of Ca^{2+} (2 mmol/l-30 mmol/l) did activate considerably the acid glucan 1,4-α-glucosi-

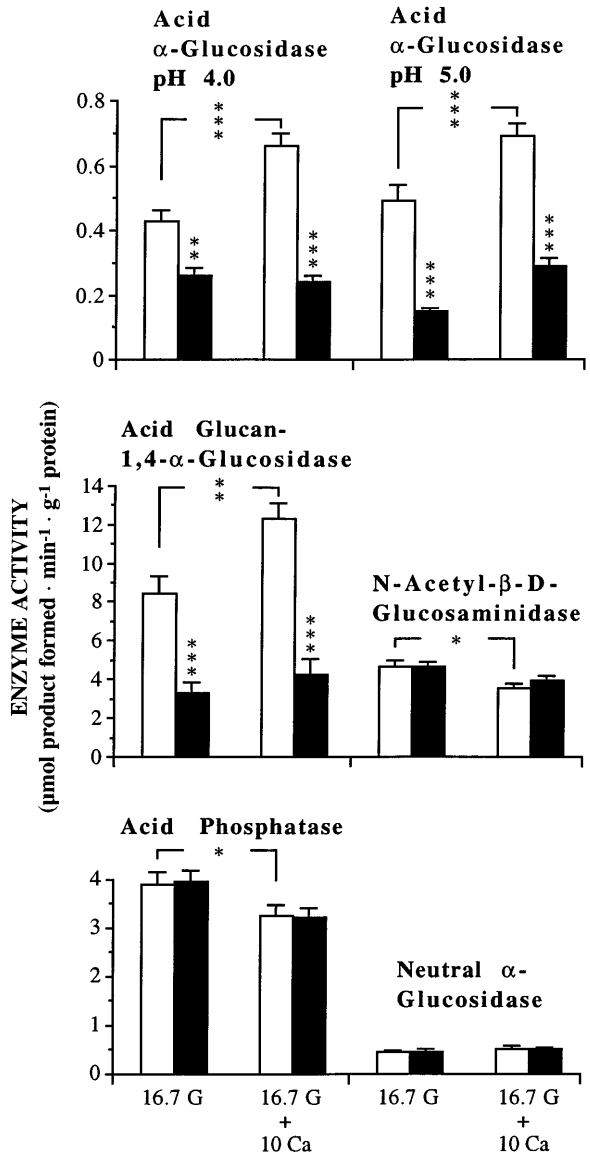


Fig. 8. Effect of Ca^{2+} on islet activities of different lysosomal enzymes and neutral α-glucosidase at a high stimulatory concentration of glucose (G; 16.7 mmol/l) in the absence (open bars) or presence (solid bars) of the selective α-glucosidehydrolase inhibitor emiglitate (1 mmol/l). Islets were incubated for 2 h at either 2.5 or 10 mmol/l Ca^{2+}. Enzyme activities are expressed as μmol glucose (acid glucan 1,4-α-glucosidase) or 4-methylumbelliferone liberated per g protein per min. Values are means ± SE for 10–12 batches of islets in each group obtained from 4 independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

dase but inhibited the acid α-glucosidases (Fig. 2). Such high Ca^{2+} concentrations are not likely to occur intracellularly, although theoretically they cannot be completely ruled out in certain Ca^{2+}-rich subcellular compartments and organelles. Furthermore, this highly differential action of 2 and 10 mmol/l Ca^{2+} on the acid glucan 1,4-α-glucosidase, compared with the acid α-glucosidases in islet homogenates, was indeed not reflected in the various experiments with isolated intact islets, where these activities were always increased by the supraphysiological extracellular Ca^{2+} concentrations used in our studies (see Figs. 1 and 8).

With regard to glucose-stimulated insulin release, it is conceivable that the initial glucose-induced decrease in $^{45}Ca^{2+}$ efflux (3), which was recently shown to be the result of the ability of glucose to induce sequestration of cytoplasmic Ca^{2+} in a slowly exchangeable "organelle pool" (10), may be a key event in this context. Interestingly, the glucose-induced redistribution and sequestration of intracellular Ca^{2+} are manifested earlier and at lower glucose concentrations than those required to open the voltage-dependent Ca^{2+} channels (10). It is not inconceivable that one of these sequestration targets is the acid glucan 1,4-α-glucosidase-containing organelles. Such an assumption is in accordance with previous data showing, in a Ca^{2+}-deficient medium, that an increase in glucose concentration from 1 to 4 mmol/l also increased the acid α-glucosidehydrolase activities (28). Hence, in addition to other factors, glucose-stimulated insulin release is apparently dependent on both a redistribution and a sequestration of intracellular Ca^{2+}, which in turn activate the lysosomal-acid glucan 1,4-α-glucosidase system as well as the inflow of extracellular Ca^{2+} through voltage-dependent Ca^{2+} channels at depolarizing glucose concentrations. This redistribution/sequestration hypothesis also conforms with recent observations (29) showing that the acid α-glucosidehydrolase activities were profoundly suppressed in islets incubated in a Ca^{2+}-deficient medium in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), which is known to induce perturbations of intracellular organelle-bound Ca^{2+} in the β-cell (2).

Effects of the Selective α-Glucosidehydrolase Inhibitor Emiglitate

Finally, our experiments with emiglitate strongly suggest that the activating effect of Ca^{2+} on the lysosome-acid glucan 1,4-α-glucosidase system and glucose-induced insulin release are closely interconnected. Emiglitate almost totally suppressed insulin release stimulated both by high glucose alone and by high extracellular Ca^{2+} in the presence of high glucose. This powerful inhibition of insulin release was accompanied by greatly suppressed activities of the islet acid α-glucosidehydrolases. Hence, it appears that a most important Ca^{2+} effect in the stimulus-secretion coupling of glucose-stimulated insulin release is exerted closely proximal to the action of the acid glucan 1,4-α-glucosidase, the inhibition of which greatly impairs the signal transduction. This inhibition of enzyme activity and subsequent insulin release apparently cannot be overcome by greatly increasing the Ca^{2+} concentration (see Fig. 8). Thus it is not inconceivable that this particular effect of Ca^{2+} is exerted on certain membrane components of acidic organelles and/or key factor(s) assisting the acid α-glucosidehydrolases in their in vivo catalytic function. It should be noted that emiglitate is reportedly (26) a selective α-glucosidehydrolase inhibitor. Hence, our results suggest a direct cause-effect relationship between islet acid glucan 1,4-α-glucosidase activity on the one hand and glucose-Ca^{2+}-induced insulin release on the other. The present data are thus in accordance with previous observations in our laboratory showing that nutrient-induced insulin release is greatly suppressed by different selective α-glucosidehydrolase inhibitors, such as the pseudotetrasaccharide acarbose or the deoxynojirimycin derivatives miglitol and emiglitate (20, 28–32), whereas Ca^{2+}-independent insulin secretion induced by IBMX is not (29). Moreover, receptor-activated insulin release induced by carbachol is unaffected by selective α-glucosidehydrolase inhibition (30, 32). These data also conform with the present results showing that carbachol itself had no influence on islet acid glucan 1,4-α-glucosidase activity in a Ca^{2+}-deficient medium (see Fig. 7). In contrast, glucose has previously been shown to greatly enhance the enzyme activity during Ca^{2+} deficiency (28).

In summary, in intact islets, high supraphysiological concentrations of extracellular Ca^{2+} brought about a marked enhancement of the islet acid α-glucosidehydrolase activities, accompanied by a large insulin release. The Ca^{2+} channel blocker nifedipine unexpectedly brought about an increase in acid α-glucosidehydrolase activity at low glucose. This increase was explained by showing that nifedipine suppressed $^{45}Ca^{2+}$ outflow from perifused islets at substimulatory glucose and normal Ca^{2+}, as well as after intracellular mobilization of $^{45}Ca^{2+}$ by carbachol in a Ca^{2+}-deficient medium. The inhibition of $^{45}Ca^{2+}$ efflux was probably accomplished through increased intracellular sequestration and impaired outflow of Ca^{2+} across the plasma membrane. The Ca^{2+}-induced effects were shown not to be exerted by a direct action of either nifedipine or Ca^{2+} on the acid α-glucosidehydrolases. Instead we suggest that this signal function of Ca^{2+} is exerted on a step closely proximal to enzyme activation, e.g., on certain membrane constituents of acidic organelles and/or key factor(s) modulating the acid α-glucosidehydrolases in their in vivo catalytic function. This was further emphasized by the finding that selective inhibition of the acid α-glucosidehydrolases by emiglitate almost abolished glucose-induced insulin release, an effect which could not be overcome by increased Ca^{2+}. Taken together with data on islet acid α-glucosidehydrolase activities obtained from previous experiments in Ca^{2+}-deficient media (28), a redistribution of Ca^{2+} induced by glucose (or by pharmacological agents such as nifedipine) that is directed to acid α-glucosidehydrolase-containing organelles appears an attractive mechanism in this context. The intimate details of Ca^{2+} redistribution, seques-

tration, and induction of acid glucan 1,4-α-glucosidase activity in nutrient-induced insulin release will await further investigations.

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