The effect of ascorbic acid on glucose-induced insulin release from single pancreatic islets was measured using a new, ultra-sensitive enzyme-linked immunosorbent insulin assay. Within 20 s ascorbic acid inhibited insulin secretion; inhibition was dose dependent and completely reversible. There was a 50% inhibition of the secretory response with 200 μM ascorbic acid and 90% inhibition with 400 μM ascorbic acid. The decrease in insulin secretion was recorded as a reduction of the amplitudes of the fast insulin transients, which give rise to the oscillatory nature of insulin secretion. The inhibition of glucose-induced insulin release by ascorbic acid was associated with hyperpolarization of the pancreatic β-cell. Suppression of glucose-induced membrane depolarization was evident after 20 s, was dose dependent, and was completely reversible. The data here may provide the first explanation of why plasma ascorbate concentrations are tightly controlled.

Glucose is the main physiologic stimulator of insulin release in pancreatic β-cells (1). In human neutrophils glucose regulates the transport of ascorbic acid (2, 3). Glucose and ascorbic acid are structurally similar, and transport and accumulation of ascorbic acid in pancreatic β-cells could affect glucose-induced insulin release. The presence of the ascorbic acid-dependent enzyme peptidylglycine α-amidating monoxygenase in islets (4–7) also implies the existence and action of the vitamin in pancreatic islets. Furthermore, the oxidized form of ascorbic acid, dehydroascorbic acid, has a proposed role in the development of diabetes (8); its accumulation can be regulated by glucose (9).

For all of these reasons we investigated the content, accumulation, depletion, and repletion of ascorbic acid in mouse and rat pancreatic islets and its effects on glucose-induced insulin release and membrane depolarization. It became possible to perform these experiments using a newly developed enzyme-linked immunosorbent insulin assay with a sensitivity of 50 amol of insulin (10, 11).

**MATERIALS AND METHODS**

**Islet Isolation, Incubation, and Subcellular Fractionation.**—Pancreatic islets from 4-month-old male Sprague-Dawley rats and 10–11-month old ob/ob-mice (12) were either isolated by collagenase digestion or micro dissection. The micro dissected islets were used for studies of membrane potential. Freshly collagenase-isolated islets were used directly for studies of insulin release or cultured in modified RPMI 1640 medium containing 5.5 mM glucose and 10% fetal calf serum in the presence or absence of 100 μM ascorbic acid. After culture with or without ascorbic acid for the times indicated in the figures, islet samples were washed twice in media without serum and used either for ascorbic acid determinations or dry weight measurements.

Subcellular fractionation of isolated pancreatic islets was performed as described, with minimal disruption of subcellular components (13). Insulin secretion from single mouse islets was studied as described (10, 11) using a micro perifusion system. The perifusion medium contained 125 mM NaCl, 5.5 mM KCl, 1.2 mM MgCl₂, 1.25 mM CaCl₂, 25 mM HEPES, and 1 mg/ml bovine serum albumin titrated to pH 7.4 with NaOH. After isolation the islet was perfused in the presence of 3 mM glucose. After 60 min the glucose concentration was raised to 11 mM. Sampling was begun 15 min after the increase to 11 mM glucose (10). Ascorbic acid was introduced in concentrations and durations as indicated in the figures.

Membrane potential was measured as described (14–16). The mouse islet was perfused with Krebs buffered solution (140 mM NaCl, 25 mM NaHCO₃, 5 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂, and 11 mM glucose at pH 7.4). Ten min after the burst pattern was observed, the islet was perfused with 11 mM glucose and the indicated concentration of ascorbic acid. The membrane potential in all experiments was monitored for at least 30 min. Records shown are representative of experiments repeated in at least six different cells from different animals. Perfusion solutions were prewarmed to 37 °C and saturated with 95% O₂, 5% CO₂. Stock solutions of ascorbic acid were prepared in ice-cold Krebs immediately prior to experiments. Ascorbic acid addition to perfusion solutions had no effect on pH. Temperature in the chamber was kept constant at 37 °C.

**Assays—**Insulin was measured with a competitive enzyme-linked immunosorbent assay technique as recently described (10) using insulin-peroxidase, tetramethylbenzidine, and mouse insulin antibodies raised against guinea pigs at the University of Uppsala. The optical density was read at 370 nm with a spectrophotometric plate reader (Lems, Labsystems Oy, Helsinki, Finland). Amounts of insulin down to 50 amol were detected. Ascorbic acid (<500 μM) did not interfere with the insulin assay.

Ascorbic acid was determined by high performance liquid chromatography with coulometric electrochemical detection (17) in islets placed in an ice-cold solution of 60% methanol, 1 mM EDTA. Samples were immediately vortexed for 30 s and frozen in the dark at −70 °C until analysis. Experimental points represent the mean ± S.D. of at least three samples unless otherwise indicated. Error bars were omitted when the standard deviation was less than the size of the symbol.

Intracellular ascorbic acid concentration was determined by relating amounts of vitamin measured to freeze-dried islets weighed on a quartz fiber balance. Rat islets averaged 0.45 μg/islet, whereas ob/ob-mouse islets averaged 6.0 μg/islet.

Results were obtained from commercial sources except as indicated above and were the highest grade available.

**RESULTS**

Glucose and ascorbic acid, being derived from glucose, have similar chemical structures. Furthermore, glucose has been found to regulate ascorbic acid accumulation (2, 3, 9). Therefore, we proposed that ascorbic acid might participate in regulating glucose-induced insulin release in pancreatic β-cells. Since endogenous concentrations of ascorbic acid might affect
Upon addition of 100 mM Ascorbic acid, 200 mM ascorbic acid was added to media not containing ascorbic acid of some islets at 42 times for ascorbic acid determination. Islets were maintained in modified RPMI 1640 media containing 5.5 mM glucose and 10% fetal calf serum from 0 to 90 h in the absence (□) or presence (●) of 100 μM ascorbic acid. To maintain the external ascorbic acid concentration, ascorbic acid was added approximately every 12–14 h (19), and media samples were taken for ascorbic acid determination. Islets were taken at the indicated times for ascorbic acid determination. 100 μM ascorbic acid was added to media not containing ascorbic acid of some islets at 42 and 50 h (△), or at 90 and 98 h (○); samples were taken at the indicated times for ascorbic acid determination. B, reuptake of ascorbic acid into mouse pancreatic islets. Isolated mouse pancreatic islets were incubated in modified RPMI 1640 media without ascorbic acid for 46 h. Ascorbic acid 200 μM was then added to the media, and samples for ascorbic acid determination were taken from 0 to 240 min after ascorbic acid addition.

The data indicate that freshly isolated islets contained millimolar ascorbic acid concentrations, which depleted unless ascorbic acid was provided. Although ascorbic acid was maintained in cultured islets, in subsequent experiments we used freshly isolated islets to study insulin release, since culture might otherwise affect glucose-induced insulin secretion (18). Since islets incubated with ascorbic acid in culture did not have higher ascorbic acid concentrations than acutely isolated islets, it is unlikely the isolation process itself caused acute ascorbic acid depletion.

Insulin release is pulsatile in normal humans (19), and in diabetics this pattern disappears (20). Pulsatile insulin release has been proposed to prevent down-regulation of insulin receptors (21). With a newly developed ultrasensitive assay for insulin, it was possible to measure dynamically the 2–5 min pulsations from a single mouse islet: these are insulin oscillations (10). When the glucose-induced oscillations were further resolved at 3-s fractions, a new pattern of insulin secretory activity was demonstrated with periods <25 s, insulin transients (11).

The effect of 400 μM ascorbic acid on glucose-induced insulin secretion is shown in Fig. 2A–C. Ascorbic acid was present during the period indicated by the bar in Fig. 2A. The insulin secretory pattern at 11 mM glucose prior to ascorbate addition is seen in Fig. 2B, which is an expansion from Fig. 2A of one insulin oscillation resolved into its insulin transients. Insulin transients lasted 15–20 s, and the duration of the oscillation was 2 min. These are the predicted patterns of glucose-induced insulin release (11). When 400 μM ascorbic acid was added to the perfusion the insulin secretory rate was inhibited >90% within 20 s (Fig. 2, A and C). The inhibition is due to reduced amplitude of the insulin transients as shown in Fig. 2C, an expanded part of Fig. 2A. Although insulin values <1 pmoVg dry weight/s could not be reliably distinguished from 0 because of the detection limit of the assay, the data still show that the frequency of the oscillations persisted. After ascorbic acid was withdrawn from the perfusion medium, the insulin transients reappeared within 20 s with similar frequency and amplitude as before the introduction of the vitamin (Fig. 2A).

The effect of 200 μM ascorbic acid on insulin secretion in the presence of 11 mM glucose is shown in Fig. 3, A and B. Ascorbic pancreatic islets over shorter time periods is shown in Fig. 1B. Mouse pancreatic islets were incubated for 2 days without ascorbic acid and then incubated for 240 min with 200 μM ascorbic acid. These data indicate that ascorbic acid is accumulated against a concentration gradient within 30 min and that the accumulation probably occurs in β-cells. We investigated the intracellular distribution of ascorbic acid in mouse pancreatic islets by cell fractionation (Table I). Ascorbic acid was localized to the post-microsomal cytosol, with approximately 10% of ascorbic acid in secretory granules.
Ascorbic Acid and Insulin Secretion

Fig. 2. Effect of ascorbic acid (400 μM) on insulin release. A, insulin release from an individual mouse islet exposed to 11 mM glucose in the presence and absence of 400 μM ascorbic acid. The rate of insulin secretion was obtained with a sampling time of 3 s. The bar indicates the period when 400 μM ascorbic acid was added to the perfusion medium. The x-axis shows the time after raising the glucose from 3 to 11 mM (see “Materials and Methods”). Representative of seven experiments. B, expansion of one insulin oscillation with the resolved insulin transients recorded 19 min after raising the glucose concentration to 11 mM glucose. From A. C, expansion of one insulin oscillation with the resolved insulin transients recorded 12 min after adding 400 μM ascorbic acid to the perfusion medium. The inset is a 20-fold enlargement of the oscillation. Secretory rates below 1 pmol/g dry weight/s cannot be distinguished from 0 in view of non-linearity of the insulin standard curve (10, 11). From A.

acid was introduced during the time indicated by the bar in Fig. 3A. 200 μM ascorbic acid inhibited insulin secretion by approximately 50%, manifested by decreased amplitudes of the insulin transients without changing the frequency of the oscillations and transients (Fig. 3B). Inhibition occurred within 20 s of ascorbic acid exposure. When ascorbic acid was removed the insulin transients returned to initial amplitude within 20 s. Both before and after ascorbic acid perfusion, the pattern of insulin transients and oscillations was as expected (Fig. 3A, data not shown; also refer to Fig. 2).

When perfusion media contained 11 mM glucose and 100 μM ascorbic acid, no inhibition of insulin secretion occurred (Fig. 4).

We attempted to determine the mechanism of ascorbic acid action on insulin secretion. Ascorbic acid could regulate mechanisms for increasing intracellular Ca²⁺ concentration, or could regulate exocytotic events occurring after intracellular Ca²⁺ concentrations had increased. To distinguish between these possibilities, we measured the effects of extracellular ascorbic acid on membrane potential in β-cells. Pancreatic islets were perifused with 11 mM glucose to induce the characteristic P-cell bursting electrical activity. After 10 min of electrical activity, 50–400 μM ascorbic acid was added (Fig. 5, A–D). Although 50 μM ascorbic acid had no effect on β-cell membrane potential (Fig. 5A), 100 μM ascorbic acid transiently suppressed the electrical activity within 20 s (Fig. 5B). At 200 μM ascorbic acid, suppression occurred for approximately 2 min, followed by depolarization (Fig. 5C). Ascorbic acid at 400 μM hyperpolarized islet cells for >5 min (Fig. 5D). The effect of ascorbic acid was
Ascorbic Acid and Insulin Secretion

**FIG. 4. Effect of ascorbic acid (100 μM) on insulin release.** Insulin release from an individual mouse islet exposed to 11 mM glucose in the presence and absence of 100 μM ascorbic acid. The rate of insulin secretion was obtained with a sampling time of 3 s. The bar indicates the period when 100 μM ascorbic acid was added to the perfusion medium. The x axis shows the time after raising the glucose from 3 to 11 mM (see “Materials and Methods”). Representative of five experiments.

**FIG. 5. Ascorbic acid and pancreatic β-cell membrane potential.** A microdissected mouse islet was perfused with Krebs buffered solution. Membrane potential was measured as described (16–18). Records shown are representative of at least six experiments. Ten min after the burst pattern was obtained, the islet was perfused with 11 mM glucose and (as indicated by arrows): 50 μM ascorbic acid (A); 100 μM ascorbic acid (B); 200 μM ascorbic acid (C); 400 μM ascorbic acid (D); 200 μM ascorbic acid for 30 s followed by removal of ascorbic acid (E).

also completely reversible within 20 s after the vitamin was removed (Fig. 5E). These effects on membrane potential were specific for ascorbic acid and could not be reproduced by the reducing agents glutathione or homocysteine (data not shown). As expected, we found that ascorbic acid in the islet cell perifusate remained as ascorbic acid and had not oxidized to dehydroascorbic acid during the perfusion (data not shown). We predicted that intracellular Ca2+ concentrations would be changed by ascorbic acid action. In preliminary experiments using fura-2 (22), decreased cytoplasmic Ca2+ concentrations were recorded under conditions when there was an inhibition of insulin secretion by 200 μM ascorbate (data not shown).

**DISCUSSION**

It is reported here that ascorbic acid within 20 s produced dose-dependent, reversible inhibition of glucose-induced insulin secretion from single pancreatic islets. The inhibition was monitored by a newly developed assay for ultrasensitive measurements of insulin; inhibition was detected as reduced amplitudes of the fast insulin transients (11). The transients may produce the minute variations in the secretion of insulin normally occurring in humans (19).

Since ascorbic acid inhibited membrane depolarization and prevented an increase in intracellular Ca2+ concentration, the mechanism of the vitamin’s action is proximal to exocytosis itself. There are several possibilities to explain ascorbate’s action. The effect of ascorbic acid on glucose-induced insulin release might be by changing ion channel conductance directly or indirectly, by affecting glucose metabolism, or by directly interacting with the entry of glucose. Regarding the last possibility, recent experiments indicate that dehydroascorbic acid is transported via GLUT 1 (9). However, dehydroascorbic acid (200 μM) did not inhibit membrane depolarization of islet cells. Additional experiments to delineate any role for dehydroascorbic acid and the mechanism for ascorbic acid action are underway.

The findings in this paper provide the first explanation of why systemic ascorbic acid concentrations in humans are strictly controlled. Across a broad range of oral doses of ascorbic acid, plasma concentrations have not exceeded 150 μM, despite oral doses as much as 40 times the recommended dietary allowance. Since ascorbic acid is reported to have little toxicity in humans (3–36), it has not been understood why its plasma concentrations are so tightly controlled. The findings here provide a novel explanation for these observations. If systemic ascorbic acid concentrations exceed 200 μM in plasma, especially during food ingestion, insulin release would be inappropriately inhibited.

Although systemic concentrations of ascorbic acid above 200 μM could be detrimental, local transient concentrations of ascorbic acid above 200 μM might have a beneficial paracrine regulatory function on hormone release. In adrenal cortex and medulla, where millimolar ascorbic acid is also localized predominant to cell cytoplasm as found here for islets, the vitamin is secreted from cytosol in response to hormones and secretagogues (37–39). Cytosolically secreted ascorbic acid from endocrine and neuronal tissue has been proposed to have a local regulatory function (37–42). In analogy, the effects of extracellular ascorbic acid on inhibiting insulin release together with the findings of high intracellular concentrations of ascorbic acid in pancreatic β-cell cytosol suggests a possible endogenous regulatory role of ascorbic acid. Cytoplasmic ascorbic acid could be secreted from islets and locally regulate insulin secretion. Experiments to test this possibility await development of radiolabeled ascorbic acid analogues with much higher specific activity than those currently available. These compounds are currently being synthesized.

The findings of millimolar intracellular concentrations of ascorbic acid in pancreatic islet cytosol agree with findings in neonatal rat islets (43, 44). The high intracellular ascorbic acid concentration has been suggested to also play a role in the activity of a number of intracellular enzymes, such as glutathione reductase, glucose-6-phosphate dehydrogenase, and thioredoxin reductase (45–47). The high concentration may also be involved in the regulation of glucose metabolism in the islet cell (48–50). In summary, our findings suggest that ascorbic acid concentrations above 200 μM in plasma, especially during food ingestion, may contribute to the regulation of insulin secretion in humans.

2 M. Levine, R. Welch, Y. Wang, K. R. Dhariwal, C. Conry-Cantilena, L. Cantilena, and J. King, unpublished observations.
of peptidylglycine α-amidating monoxygenase, a vitamin C-dependent enzyme (4–7, 45, 46). In the endocrine pancreas proteins like thyrotropin-releasing hormone, pancreatic polypeptide, and amylin are terminally amidated; amidation confers hormone stability and may have other unrecognized consequences (45, 46).

The role of plasma ascorbic acid as well as its oxidized form dehydroascorbic acid is unclear in diabetes. Although controversial, some studies indicate lower levels of ascorbic acid both in diabetic patient plasma and experimental animal models (47–49). A lower plasma and presumably intracellular concentration of ascorbic acid might decrease its ability to act as an inhibitory factor and to modulate the secretory rate. It has been observed that an early sign in diabetes is the disappearance of the pulsatile nature of plasma insulin levels (20). The studies here suggest that ascorbic acid could be a candidate physiologic participant in the regulation of pulsatile insulin release.

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