Hexamminecobalt(III) Chloride Inhibits Glucose-induced Insulin Secretion at the Exocytotic Process*

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Yoshiharu Tsubamoto‡, Kazuhiro Eto‡, Mitsuhiko Noda‡, Samira Daniel§, Sechiko Suga¶, Shigeo Yamashita‡, Haruo Kasai, Makoto Waku, Geoffrey W. G. Sharp§, Satoshi Kimura‡, and Takashi Kadowaki‡‡

From the Departments of Metabolic Diseases and Physiology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan, the Department of Molecular Medicine, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853-6401, and the Department of Physiology, Tokyo University School of Medicine, 5 Zaifu-cho, Hiroaki 036-8562, Japan

Hexamminecobalt(III) (HAC) chloride was found to have a potent inhibitory effect on glucose-induced insulin secretion from pancreatic islets. HAC at 2 mM inhibited the secretion in response to 22.2 mM glucose by 90% in mouse islets. Perfusion experiments revealed that the first phase of insulin secretion was severely suppressed and that the second phase of secretion was completely abrogated. Removal of HAC from the perfusate immediately restored insulin secretion with a transient overshooting above the normal level. However, HAC failed to affect glucose-induced changes in intracellular calcium concentration, or calcium influx into mitochondria. Furthermore, HAC inhibited 50 mM potassium-stimulated insulin secretion by 77% and 10 μM mastoparan-stimulated insulin secretion in the absence of extracellular calcium by 80%. The results of a co-immunoprecipitation study of lysates from insulin-secreting βHC9 cells using anti-synaptotagmin and anti-vesicle-associated membrane protein antibodies for immunoprecipitation or Western blotting suggested that HAC inhibited disruption of the SNARE complex, which is normally observed upon glucose challenge. These results suggest that the inhibitory effect of HAC on glucose-induced insulin secretion is exerted at a site(s) distal to the elevation of cytosolic [Ca2+]c, possibly in the exocytotic machinery per se; thus, HAC may serve as a useful tool for dissecting the molecular mechanism of insulin exocytotic processes.

Glucose-induced insulin secretion from pancreatic β cells is regulated by generation of ATP through glucose metabolism and an increase in the cytosolic calcium concentration ([Ca2+]c)1–4. The following cascade has been generally accepted as the glucose-induced insulin secretory pathway. When glucose is metabolized in the cytosol and mitochondria, ATP is generated to promote closure of ATP-sensitive potassium (KATP) channels, and this depolarizes the plasma membrane potential. The depolarization of the plasma membrane leads to activation of voltage-dependent Ca2+ channels, with subsequent Ca2+ entry into the cytosol. The rise in [Ca2+]c is thought to finally trigger exocytosis of insulin from secretory vesicles (1–3). Thus, inhibitors of glucose metabolism (5) and Ca2+ channel-blocking agents, such as verapamil (6), nifedipine (7), and divalent cations (8–14), suppress glucose-induced insulin secretion. Moreover, recent studies showed that the intramitochondrial Ca2+ concentration ([Ca2+]m) increases in accordance with the rise in [Ca2+]c, upon glucose stimulation via a calcium transporter on the inner mitochondrial membrane (calcium uniporter) (15, 16) and that the increase in [Ca2+]m is closely associated with glucose-induced insulin secretion (17, 18).

To examine the involvement of [Ca2+]m in glucose-induced insulin secretion from isolated islets, we tested the effects of ruthenium red and hexamminecobalt(III) (HAC), both of which have been reported to be inhibitors of the mitochondrial calcium uniporter (15, 18–20), on the secretion. Ruthenium red was also reported to inhibit insulin secretion from permeabilized β cells through inhibition of the calcium uniporter (18). We also observed that ruthenium red at 100 μM severely suppressed glucose-induced insulin secretion in mouse islets. However, under these conditions, Ca2+ influx through the plasma membrane was almost completely inhibited. These results indicated that ruthenium red was not a suitable agent to study the relationship of [Ca2+]m to glucose-induced insulin secretion in intact cells, different from the case of permeabilized cells. In contrast, HAC at 2 mM suppressed glucose-induced insulin secretion to the same extent as 100 μM ruthenium red, without affecting the increase in [Ca2+]c in response to glucose. However, different from our initial expectations, HAC failed to affect glucose-stimulated increases in [Ca2+]m under these conditions. In this study, we tried to determine which step of the glucose-induced insulin secretory pathway was inhibited by HAC. HAC failed to suppress oxidation of glucose, glucose-stimulated generation of ATP, or Ca2+ current through the plasma membrane, indicating that the inhibitory effect of HAC on insulin secretion was exerted distal to [Ca2+]c, elevation. Our results suggested that the dissociation of the SNARE complex formed in the sequence of exocytotic events was prevented in the presence of HAC. Thus, HAC may serve as a useful probe to monitor steps distal to elevation of [Ca2+]c in the glucose-stimulated insulin secretory pathway.

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** To whom correspondence should be addressed. Tel.: 81-3-5800-8818; Fax: 81-3-5689-7209; E-mail: kadowaki-3im@h.u-tokyo.ac.jp.

† The abbreviations used are: [Ca2+]c, cytosolic calcium concentration; [Ca2+]m, intramitochondrial calcium concentration; HAC, hexamminecobalt(III); SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; VAMP, vesicle-associated membrane protein; KRB, Krebs-Ringer bicarbonate; IBMX, 3-isobutyl-1-methylxanthine.
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EXPERIMENTAL PROCEDURES

Materials—HAC, chlortetracycline, rhodamine 123, and the ATP bioluminescent assay kit were purchased from Sigma. Fura-2/ace- toxymethyl ester was from Molecular Probes, Inc. (Eugene, OR). n-6-14C)(Glucose was from American Radiolabeled Chemicals (St. Louis, MO). Solvable was from Pickard Instrument Co. The insulin radioimmunoassay kit, CAMP enzyme-linked immunoassay kit, and ECL protein detection kit were from Amersham Pharmacia Biotech (Uppsala, Sweden). Anti-syntaxin and anti-vesicle-associated membrane protein (VAMP) antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). Nitrocellulose membranes were from Schleicher & Schuell (Dassel, Germany).

Preparation of Islets—Islets were isolated by collagenase digestion and manual picking from the pancreases of 10–16-week-old male C57BL/6N mice. These islets were used for the experiments immediately after isolation.

Cell Culture—HC9 cells were maintained as described elsewhere (22). They were cultured in Dulbecco’s modified Eagle’s medium containing 25 mmol/liter glucose, 1 mmol/liter pyruvate, 15% horse serum, 2.5% fetal bovine serum, 100 µg/ml streptomycin, and 100 units/ml penicillin at 37 °C in a 95% air and 5% CO2 atmosphere. In the course of experiments, cells were maintained with one passage/week.

Insulin Secretion from Islets—Insulin release from pancreatic islets was measured in static incubation or perfusion incubation with Krebs-Ringer bicarbonate (KRH) buffer composed of 129 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 2.5 mM CaCl2, 5 mM NaHCO3, 0.2% bovine serum albumin, and 10 mM HEPES (pH 7.4) (4). HAC was present, if used, throughout the experiment. In static incubation experiments, islets were freshly isolated and were preincubated with the test agents at 37 °C for 20 min in KRH buffer containing 2.8 mM glucose. The preincubation solutions were replaced with KRH buffer containing test agents, and batches of islets were incubated at 37 °C for 60 min. Insulin released in these supernatants was measured by radioimmunoassay. In perfusion incubation, 30 freshly isolated islets were suspended in 500 µl of Bio-Gel G-10 beads in each perfusion chamber and perfused with KRH buffer containing 0.1 mM glucose at a rate of 6 µl/min. Islets were perfused for 30 min in the presence of 2.8 mM glucose prior to stimulation with 16.7 mM glucose. Perifusate fractions were collected and insulin in these samples was measured by radioimmunoassay.

Insulin Secretion from HC9 Cells—Insulin release from HC9 cells was measured in static incubation experiments (23). HC9 cells grown in 60-mm diameter wells were preincubated at 37 °C for 20 min in KRH buffer containing 0.1 mM glucose. The medium used for preincubation of 22.2 mM glucose, HAC also failed to significantly change insulin secretion from isolated islets (Fig. 1B). In the presence of 2.8 mM glucose, islets released 0.5 ng insulin/islet (n = 4), and HAC at 2 mM failed to exert significant inhibition of insulin secretion under these conditions (0.4 ± 0.1 ng/islet, n = 4). In the presence of 22.2 mM glucose, insulin secretion from isolated islets increased to 3.2 ± 0.1 ng/islet (n = 4), and HAC inhibited this secretion in a dose-dependent manner. Thus, the inhibition by 0.1, 0.5, 1, and 2 mM HAC amounted to 0, 50, 71, and 90% of the glucose-stimulated increase in insulin secretion, respectively.

Perifusion experiments revealed the effect of HAC on the kinetics of glucose-induced insulin secretion from isolated islets (Fig. 1B). An increase in glucose concentration from 2.8 to 16.7 mM induced a biphasic insulin secretion in control islets (Fig. 1B, ). In the presence of 2 mM HAC, the first phase of insulin secretion (0–10 min) over the basal values was inhibited by 80%, and the second phase was completely abrogated (Fig. 1B, ). Removal of HAC from the perifusate at 20 min caused a prompt and full restoration of insulin secretion with a transient overshooting above the second phase level of the control (Fig. 1B, A).

Effect of HAC on Mitochondrial Metabolism and ATP Generation—To examine the effect of HAC on mitochondrial metabolism eventually leading to ATP generation, we measured glucose-induced changes in n-[6-14C]glucose oxidation, the level of NADPH, mitochondrial inner membrane potential, and ATP content in isolated HC9 cells incubated in n-[6-14C]glucose (24), which well reflects the tricarboxylic acid cycle activity at the level of isocitrate dehydrogenase and α-ketoglutarate dehydrogenase (24), was increased by 13-fold when extracellular glucose was raised from 2.8 to 22.2 mM in control islets (0.6 ± 0.1 and 8.5 ± 1.5 pmol/h/islet, respectively; n = 4) (Table 1). HAC at 2 mM did not affect n-[6-14C]glucose oxidation in the presence of 2.8 mM glucose (0.6 ± 0.2 pmol/h/islet, n = 4). In the presence of 22.2 mM glucose, HAC also failed to significantly change
Next, we measured the ATP content of islets to directly monitor the efficiency of mitochondrial ATP synthesis. After a 1-h static incubation with 22.2 mM glucose at 37 °C, the ATP content was increased by 40% (12.0 ± 0.8 pmol/islet, n = 4) compared with that with 2.8 mM glucose in control islets (8.5 ± 0.4 pmol/islet, n = 4) (Table I). When 2 mM HAC was added, the ATP content of islets incubated with 2.8 mM glucose remained unchanged (9.1 ± 0.5 pmol/islet, n = 4), whereas a slight, but not significant increase in ATP content over the control islets was noted in the presence of 22.2 mM glucose (14.5 ± 1.0 pmol/islet, n = 4). These results indicated that HAC has no inhibitory effects on ATP generation through mitochondrial glucose metabolism.

Effect of HAC on the Glucose-induced Rise in Cytosolic and Mitochondrial Ca2+ Concentrations—To investigate the effect of HAC on the increase in [Ca2+]c, in response to glucose stimulation, we monitored the fluorescence of fura-2/acetoxymethyl ester excited at 340 and 380 nm in perfused islets (Fig. 2C). When the glucose concentration was increased from 2.8 to 22.2 mM, [Ca2+]c, which was expressed as the 340/380 nm fluorescence ratio, was increased in control islets by 0.143 ± 0.023 (n = 5). In the presence of 2 mM HAC, [Ca2+]c, also rose in response to glucose stimulation (0.123 ± 0.018, n = 6; p = 0.51 compared with control islets), although the time taken to increase to a plateau was delayed by 1 min compared with the controls. Moreover, the duration (2.6 ± 0.1 min, n = 6) and magnitude (0.024 ± 0.002, n = 6) of "phase 0" [Ca2+]c, change below a base-line level in islets treated with HAC were not different from those of controls (duration, 2.8 ± 0.1 min, n = 5; magnitude, 0.022 ± 0.003, n = 5). We also directly measured the plasma membrane potential and Ca2+ currents of single β cells with patch clamp techniques (30, 31) and found that HAC at 2 mM did not interfere with 22.2 mM glucose-responsive changes in this potential or currents (data not shown).

It was previously reported that HAC inhibits the mitochondrial calcium uniporner, which mediates Ca2+ entry into the mitochondrial matrix in response to changes in the mitochondrial membrane potential (15, 16). It was also reported that [Ca2+]m, which rises in accordance with [Ca2+]c, upon glucose stimulation, is associated with glucose-induced insulin secretion (17). We thus measured [Ca2+]m with chlortetracycline. Chlortetracycline is a fluorescent Ca2+ probe that allows continuous monitoring of an intramitochondrial Ca2+ pool bound to the inner mitochondrial membrane, possessing a substantial fluorescence intensity suitable for quantitative measurement (Fig. 2D) (25). In control islets, chlortetracycline fluorescence was increased above the basal level by 233 ± 17 arbitrary units (n = 8) in response to 22.2 mM glucose. In the presence of 2 mM HAC, an increase in chlortetracycline fluorescence in response to glucose (234 ± 17 arbitrary units, n = 7) was also observed and was not significantly different in magnitude from that of control islets. However, the onset of the increase was delayed by 1 min compared with controls.

Effect of HAC on KCl- or Mastoparan-stimulated Insulin Secretion—A high concentration of extracellular KCl decreases K+ conductance and leads to depolarization of the plasma membrane, with subsequent activation of voltage-dependent Ca2+ channels (32). This effect of KCl causes elevation of [Ca2+]c, and results in insulin secretion by β cells. We examined whether HAC also inhibits this depolarization-induced insulin secretion, which does not require mitochondrial glucose metabolism or ATP synthesis. HAC did not affect insulin secretion at the basal concentration of KCl (4.8 mM). When 50 mM KCl was added to the medium, insulin secretion was increased to 3.6 ± 0.6 ng/h/islet (n = 4). However, 2 mM HAC decreased KCl-
stimulated insulin secretion to $1.3 \pm 0.1 \text{ng/h/islet}$ ($n = 4$) (Fig. 3A).

Mastoparan, a tetradecapeptide purified from wasp venom, activates heterotrimeric G-proteins and stimulates insulin secretion from $\beta$ cells even in the absence of extracellular Ca$^{2+}$ (33, 34). We examined the effect of HAC on mastoparan-stimulated insulin secretion in the absence of extracellular Ca$^{2+}$ (Fig. 3B). Mastoparan at 10 $\mu$M increased insulin secretion to $3.5 \pm 0.4 \text{ng/h/islet}$ ($n = 4$) in the absence of external Ca$^{2+}$, whereas 22.2 mM glucose elicited no significant increase ($0.6 \pm 0.1 \text{ng/h/islet}$, $n = 4$) under these Ca$^{2+}$-free conditions. HAC at 2 mM inhibited mastoparan-stimulated insulin secretion by 80%.

**Effect of HAC on cAMP Generation**—An increase in cAMP, which is synthesized from ATP by adenylyl cyclases and eliminated by phosphodiesterases, potentiates glucose-stimulated insulin secretion.

### TABLE I

| Glucose oxidation | ATP content |
|-------------------|-------------|
| **2.8 mM glucose** | **22.2 mM glucose** |
| Control           | 0.6 ± 0.1   | 8.5 ± 1.5   |
| HAC               | 0.6 ± 0.2   | 11.5 ± 1.8 |

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- **Effect of HAC on glucose oxidation and ATP generation in islets.** Glucose oxidation was measured by generation of $^{14}$CO$_2$ from $\text{D-[6-}^{14}$C$\text{]glucose}$. Batches of 10 mouse islets were incubated at 37 °C for 90 min in KRB buffer containing the indicated concentrations of glucose with or without 2 mM HAC. The $^{14}$CO$_2$ produced was made volatile by adding HCl, captured by Solvable, and measured by liquid scintillation counting. The ATP content of islets was measured after incubation at 37 °C for 60 min in KRB buffer containing the indicated concentrations of glucose with or without 2 mM HAC. Batches of 10 islets were used for each condition. The incubation was stopped by addition of ice-cold HClO$_4$, and extracts from islet homogenates were neutralized by addition of NaOH. ATP contents in these lysates were measured by a luminometric method. The results are expressed as means ± S.E. ($n = 4$).

- **Effect of HAC on $\text{KCl}$- or mastoparan-stimulated insulin secretion from islets.** Batches of 10 freshly isolated islets were incubated in KRB buffer containing the indicated concentrations of glucose, KCl, and HAC at 37 °C for 60 min after preincubation for 20 min in the presence of 2.8 mM glucose and 4.8 mM KCl. HAC was present, if used, throughout the experiments. The results are expressed as means ± S.E. ($n = 4$).

- **Effect of HAC on $\text{Ca}^{2+}$**—The fluorescence of which was excited in a dual-wavelength ratiometric mode at 340 and 380 nm. The emission wavelength was filtered at 500 nm in the presence of 4.8 mM glucose. HAC was present, if used, throughout the experiments. The results are expressed as means ± S.E. ($n = 4$).
insulin secretion through the activation of cAMP-dependent protein kinases (35). In control islets, the cAMP contents were 45.7 ± 4.5 fmol/islet (n = 4) at 2.8 mM glucose and 49.3 ± 3.0 fmol/islet (n = 4) at 22.2 mM glucose. The nonselective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) at 1 mM elevated cAMP levels to 611.7 ± 18.7 fmol/islet (n = 4) at 2.8 mM glucose and to 837.8 ± 58.2 fmol/islet (n = 4) at 22.2 mM glucose (Fig. 4A). However, IBMX potentiated insulin secretion (21.5 ± 1.3 ng/h/islet, n = 4) only in the presence of 22.2 mM glucose (Fig. 4B). In the presence of HAC at 2 mM, IBMX also elevated cAMP levels at both 2.8 and 22.2 mM glucose (755.5 ± 54.1 and 727.1 ± 27.3 fmol/islet, respectively; n = 4) (Fig. 4A). However, IBMX-mediated potentiation of insulin secretion in response to 22.2 mM glucose was virtually negated to 4.0 ± 0.5 ng/h/islet (n = 4) in the presence 2 mM HAC (Fig. 4B).

**Effect of HAC on the Exocytosis Machinery**—To examine whether HAC affects the formation or dissociation of the exocytosis machinery, we performed a co-immunoprecipitation experiment using anti-syntaxin and anti-VAMP antibodies (23). Be-cause of difficulties in isolating the large amounts of pancreatic b cells needed to detect the complex of SNARE proteins (data not shown), we used mouse insulin-secreting bHC9 cells, which preserve the characteristics of progenitor mouse islets (22). Insulin secretion from bHC9 cells was increased 5-fold in response to 22.2 mM glucose compared with the basal secretion level in the presence of 0.1 mM glucose, and this glucose-induced increase in insulin secretion was inhibited by 50% in the presence of 2 mM HAC (Fig. 5A). In the absence of HAC, VAMP co-immunoprecipitated with syntaxin (Fig. 5B, first lane) and syntaxin co-immunoprecipitated with VAMP (Fig. 5C, first lane) were clearly detectable under the basal glucose conditions, and both were significantly decreased to 54 and 28% of the original levels, respectively, by 22.2 mM glucose stimulation. In the presence of HAC at 2 mM, the amounts of co-immunoprecipitated VAMP (Fig. 5B, third lane) and syntaxin (Fig. 5C, third lane) were not affected at the basal glucose concentration compared with those in the absence of HAC. These results indicated that the SNARE complex in the docked state was retained even in the presence of HAC under basal glucose conditions. However, under stimulated conditions with 22.2 mM glucose, HAC suppressed the decrease in co-immunoprecipitated VAMP (Fig. 5B, fourth lane) and syntaxin (Fig. 5C, fourth lane), so these two proteins remained to form the complex, as if they became stabilized by HAC. In each lane, the amounts of VAMP protein detected by Western blotting without immunoprecipitation were constant (data not shown), indicating that HAC did not induce a decrease in or degradation of proteins.

**DISCUSSION**

HAC ([Co(NH₃)₆]³⁺) is a trivalent complex cation of Co(III) and amine that has been known and used as an inhibitor of the mitochondrial calcium uniporter (15, 20) and a radiation-sensitizing agent (36). The importance of [Ca²⁺]ᵢ in regulation of glucose-induced insulin secretion from pancreatic b cells (18) prompted us to study the effect of HAC on the secretory pathway. We found that HAC strongly inhibited glucose-induced insulin secretion and designed the present experiments to further evaluate the mechanism of HAC inhibition of insulin secretion using different types of insulin secretagogues. HAC inhibited glucose-induced insulin secretion from pancreatic islets without affecting Ca²⁺ uptake into mitochondria. This inhibitory effect of HAC on the secretion was acutely and completely reversed with a transient overshooting by removing HAC from the perfusate. This indicated that the insulin secretory mechanisms had not been irreversibly altered by HAC. It is noteworthy that HAC showed practically no inhibition of basal insulin secretion, indicating that the inhibitory effects of HAC are exerted at sites specific to stimulated secretion. Features similar to the inhibitory and withdrawal effects of HAC on glucose-induced insulin secretion have been observed in the case of inhibition by several divalent cations, including Co²⁺, Zn²⁺, Ni²⁺, Mn²⁺, and Mg²⁺ (8–13). The inhibitory effects of these divalent cations are exerted mainly on stimulated insulin secretion (8, 9, 11–13). In particular, the inhibitory effect of Co²⁺, Mg²⁺, and Mn²⁺ on glucose-induced insulin secretion is reversed with a transient overshooting by removal of these cations from the perfusate (8, 12, 13). Moreover, the inhibitory effects of Co²⁺, Zn²⁺, and Ni²⁺ on insulin secretion show no parallel effects on the oxidation of glucose (8, 9, 11), resembling the effect of HAC. However, each of these cations is known to be a blocker of Ca²⁺ channels and has been reported to inhibit glucose-stimulated calcium uptake into β cells (8, 12, 13). Furthermore, Co²⁺ and Zn²⁺ have been reported to inhibit the electrical activities of the plasma membrane induced by glucose (10). It is generally accepted that a rise in the cytosolic free Ca²⁺ concentration is essential for insulin secretion from β cells (1). Thus, with most of these divalent cations, interference with Ca²⁺ influx from the extracellular space is the main cause of suppression of insulin secretion (8, 10, 12, 13). In contrast to these divalent cations, HAC failed to suppress glucose-stimulated Ca²⁺ influx and the plasma membrane electrical activities. These results suggest that HAC inhibits insulin secretion at a site(s) distal to the elevation of [Ca²⁺]. The inhibitory effect of HAC on depolarization-induced insulin secretion also validates this localization. We also examined whether HAC
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FIG. 5. Effect of HAC on the SNARE complex in whole cells. A, glucose-induced insulin secretion from βHC9 cells. βHC9 cells were incubated in KRB buffer with the indicated concentrations of glucose and HAC at 37 °C for 60 min after a 20-min preincubation in the presence of 0.1 mM glucose. Insulin was present, if used, throughout the experiments. Insulin secreted in the media was measured by radioimmunoassay. The results are expressed as means ± S.E. (n = 4). B and C, detection of the SNARE complex by the co-immunoprecipitation method. βHC9 cells were incubated for 5 min with the indicated concentrations of glucose and HAC subsequent to a 30-min preincubation with 0.1 mM glucose at 37 °C. HAC (2 mM) was present, if used, during the preincubation period to the end of the incubation. The lysates obtained from these cells were used for immunoprecipitation (IP) with anti-syntaxin (B) or anti-VAMP (C) antibody. The precipitated samples were boiled in Laemmli sample buffer; separated on 12% SDS-polyacrylamide gels; transferred to polyvinylidene difluoride membranes; and probed with anti-VAMP (B) or anti-syntaxin (C) antibody, followed by detection with ECL. The relative intensities of immunoprecipitated proteins on the Western blot (WB) membranes, compared with controls at 0.1 mM glucose without HAC, are shown beneath the representative blots (B, means ± range, n = 2; C, means ± S.E., n = 3).

affects the generation and action of cAMP, which is one of the major second messengers and has been reported to potentiate insulin secretion via its action on the K$_{ATP}$ channel-independent pathway (37). HAC clearly suppressed the potentiation of glucose-induced insulin secretion by cAMP without inhibiting its generation.

The suppressive effect of HAC on mastoparan-stimulated insulin secretion in the absence of extracellular Ca$^{2+}$ provides a clue to the mechanism of HAC inhibition of insulin secretion. Mastoparan is thought to directly stimulate insulin secretion from β cells by activation of exocytosis-linked heterotrimeric guanosine triphosphate-binding proteins (G$_{q}$) without employing influx of Ca$^{2+}$ through the plasma membrane (33, 38). As in neurotransmitter release, the molecular machinery of exocytosis for regulated insulin secretion involves SNAREs, which have been highly conserved from yeast to mammals (39). SNAREs have been identified as VAMP (soluble NSF attachment proteins to mediate a membrane fusion process) and syntaxin on the vesicular membrane and as SNAP-25 (synaptosome-associated protein of 25 kDa) and syntaxin on the plasma membrane (40–45). SNAREs form a very stable core complex called the SNARE complex in the presence of Ca$^{2+}$ (46), and this complex is dissociated by the action of NSF (N$\_2$-ethylmaleimide-sensitive fusion protein) and soluble NSF attachment proteins to mediate a membrane fusion process (47–49). In pancreatic β cells, it has been shown that the SNARE complexes are disrupted through the insulin secretion process (23) and that the specific cleavage of SNAREs by botulinum toxins inhibits Ca$^{2+}$-dependent insulin release (40, 42, 43), in which Ca$^{2+}$ is thought to mediate exocytosis through synaptotagmin, which, depending on the concentration of free cytosolic Ca$^{2+}$, interacts with phospholipids and syntaxin (50). On the other hand, G$_{q}$ is thought to be directly coupled with exocytosis in a number of cell types and to stimulate exocytosis independently of any elevation of [Ca$^{2+}$]$_{cytosol}$ (33, 38). Moreover, an interaction between heterotrimeric G-proteins and syntaxin was demonstrated in a recent study (51). HAC was demonstrated to inhibit both Ca$^{2+}$-dependent and Ca$^{2+}$-independent insulin secretion. These results are compatible with a concept that the inhibitory effect of HAC is exerted on the exocytotic process per se, or at sites closely proximal to this, within the final processes of stimulus-secretion coupling.

The results of a co-immunoprecipitation study of the SNARE complex demonstrated that the complex was clearly detectable under basal glucose conditions even in the presence of HAC, but that the disruption of the complex triggered by glucose stimulation was prevented by HAC (Fig. 5). A recent study reported that the co-immunoprecipitated SNARE complex reflects a physiologically releasable pool of docked insulin-containing granules and is decreased during the early phase of glucose-stimulated insulin release (23). Thus, the results of our co-immunoprecipitation study indicated that HAC essentially failed to inhibit the docking of granules with the plasma membrane and that HAC exerted its inhibitory effect after the docking step in the exocytotic machinery. This finding narrows the possible sites of HAC action directly to the disruption of the SNARE complex per se or to the cascade that triggers the disruption. In this regard, the molecules interacting with HAC might be key components of the exocytotic machinery, and identification of these molecules should be a focus of future investigations. In addition to the SNARE complex molecules, the secretory vesicle-acidifying machinery might be among these candidate molecules since glutamate, generated through α-ketoglutarate from glucose, was reported to be a metabolic signal that augments fuel-stimulated insulin secretion via its action on secretory vesicle acidification (52), although the relationship between the fusing and acidifying machineries of β cells has not been elucidated.

In conclusion, we have found that HAC inhibits glucose-induced insulin secretion from isolated pancreatic islets without inhibiting glucose metabolism and Ca$^{2+}$ influx into the cytosol. Furthermore, HAC also extends its inhibitory effect to mastoparan-stimulated insulin secretion in the absence of extracellular Ca$^{2+}$. Although the sites on which HAC exerts its
inhibitory effect have not been completely elucidated, this compound is likely to affect exocytosis per se and/or triggers of exocytosis in a reversible manner. Botulinum toxins, which cleave SNAREs irreversibly, have been the only agents reported to inhibit the exocytotic machinery to date, except for antibodies against proteins in the SNARE complex. Therefore, HAC may potentially serve as a powerful tool to study the exocytotic processes in β cells. It is also expected that further experiments on the mechanism of the inhibitory action of HAC will clarify new aspects of the exocytotic machinery in numerous other tissues such as neuronal synapses.

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