Stevioside Acts Directly on Pancreatic β Cells to Secrete Insulin: Actions Independent of Cyclic Adenosine Monophosphate and Adenosine Triphosphate–Sensitive K^+-Channel Activity

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The natural sweetener stevioside, which is found in the plant *Stevia rebaudiana Bertoni,* has been used for many years in the treatment of diabetes among Indians in Paraguay and Brazil. However, the mechanism for the blood glucose–lowering effect remains unknown. To elucidate the impact of stevioside and its aglucon steviol on insulin release from normal mouse islets and the β-cell line INS-1 were used. Both stevioside and steviol (1 nmol/L to 1 mmol/L) dose-dependently enhanced insulin secretion from incubated mouse islets in the presence of 16.7 mmol/L glucose ($P < .05$). The insulinotropic effects of stevioside and steviol were critically dependent on the prevailing glucose concentration, ie, stevioside (1 mmol/L) and steviol (1 μmol/L) only potentiated insulin secretion at or above 8.3 mmol/L glucose ($P < .05$). Interestingly, the insulinotropic effects of both stevioside and steviol were preserved in the absence of extracellular Ca^{2+}. During perifusion of islets, stevioside (1 mmol/L) and steviol (1 μmol/L) had a long-lasting and apparently reversible insulinotropic effect in the presence of 16.7 mmol/L glucose ($P < .05$). To determine if stevioside and steviol act directly on β cells, the effects on INS-1 cells were also investigated. Stevioside and steviol both potentiated insulin secretion from INS-1 cells ($P < .05$). Neither stevioside (1 to 100 μmol/L) nor steviol (10 nmol/L to 10 μmol/L) influenced the plasma membrane K^+ adenosine triphosphate (K^+_{ATP})-sensitive channel activity, nor did they alter cyclic adenosine monophosphate (cAMP) levels in islets. In conclusion, stevioside and steviol stimulate insulin secretion via a direct action on β cells. The results indicate that the compounds may have a potential role as antihyperglycemic agents in the treatment of type 2 diabetes mellitus.

PLANTS PROVIDE a vast resource of compounds with the potential to become new antidiabetic agents. The diterpene glycoside stevioside and its aglucon, steviol, are contained in the leaves of *Stevia rebaudiana Bertoni,* a herbaceous member of the *Compositae* family.[1-4] Extracts of the plant have been used for many years in the treatment of diabetes among Indians in Paraguay and Brazil.[5-8] However, there is only scanty information on the action of these postulated antidiabetic substances.[7,8] An antihyperglycemic effect has been found in the rat when supplementing the diet with dried *S rebaudiana* leaves.[6] Curi et al[7] found a slight suppression of plasma glucose when extracts of *S rebaudiana* leaves were taken orally during a 3-day period. Furthermore, Oviedo et al[6] reported that tea prepared from the leaves caused a 35% reduction in blood glucose in man. This indicates that stevioside and steviol possess a blood glucose–lowering effect and may have potential in the treatment of diabetes mellitus. However, the mechanisms of action underlying the antihyperglycemic action are unknown.

The question arises as to whether stevioside and steviol have a direct stimulatory effect on pancreatic β cells. The aim of this study was to determine if stevioside and steviol stimulate insulin release from normal mouse islets and the β-cell line INS-1. Furthermore, we wished to elucidate if stevioside and steviol act via interference with K^+ adenosine triphosphate (K^+_{ATP})-channel activity and/or cyclic adenosine monophosphate (cAMP) levels in β cells.

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MATERIALS AND METHODS

Animals and Isolation of Islets

Adult female NMRI mice (Bomholtgård Breeding and Research Center, Ry, Denmark) weighing 22 to 25 g were used. The animals were kept on a standard pellet diet and tap water ad libitum before the experiments and a light/dark cycle of 12 hours. Islets were isolated by the collagenase digestion technique.[9,10] In brief, the animals were anesthetized with pentobarbital (50 mg/kg intraperitoneally) and a midline laparotomy was performed. The pancreas was retrogradely filled with 3 mL ice-cold Hanks balanced salt solution ([HBSS] Sigma Chemical, St Louis, MO) supplemented with 0.3 mg/mL collagenase P (Boehringer Mannheim GmbH, Mannheim, Germany). The pancreas was subsequently removed and incubated for 19 minutes at 37°C. After rinsing in HBSS, the islets were handpicked under a stereomicroscope and incubated overnight at 37°C and 95% normal atmosphere/5% CO_2 in 10 mL RPMI 1640 containing 11.1 mmol/L glucose supplemented with 10% fetal calf serum, 2.06 mmol/L L-glutamine, 100 IU/mL penicillin G, and 100 μg/mL streptomycin (all GIBCO BRL, Paisley, UK). Islets for incubation and perifusion studies were obtained from 6 to 10 mice to compensate for individual differences.

Incubation and Perifusion of Islets

After overnight culture, the islets were rinsed twice with a modified Krebs-Ringer buffer (KRB) supplemented with 3.3 mmol/L glucose and 0.1% human serum albumin (Sigma). The KRB contained 125 mmol/L NaCl, 5.9 mmol/L KCl, 1.2 mmol/L $MgCl_2$, 1.28 mmol/L $CaCl_2$, and 25 mmol/L HEPES (pH 7.4; all Sigma). For experiments performed in Ca^{2+}-free medium, $CaCl_2$ was replaced by 0.5 mmol/L EGTA (Sigma). After preincubation for 60 minutes in normal atmosphere, single islets were incubated in 100 μL KRB containing glucose and stevioside or steviol according to the protocols. After incubation, 50 μL of the medium was frozen for analysis of insulin.

In the perifusion experiments, 25 islets were transferred to each of 4 perifusion chambers.[10] The experiments were designed as follows: (1)

10-minute preperifusion at 3.3 mmol/L glucose, (2) 20-minute perifusion at 16.7 mmol/L glucose, (3) 20-minute perifusion at 16.7 mmol/L glucose and stevioside (1 mmol/L) or steviol (1 μmol/L), (4) 10-minute perifusion at 16.7 mmol/L glucose, and (5) 10-minute perifusion at 16.7 mmol/L glucose and 0.1 mmol/L carbacholine (Sigma). The flow rate was 100 μL/min. Samples were collected every 2 minutes.

Stevioside (19-*O*-β-glucopyranosyl-13-*O*[β-glucopyranosyl(1-2)]-β-g lucopyranosylsteviol, (95% purity) was obtained from Sigma, and steviol (13-hydroxy kaur-16-en-19oic acid, 90% purity) was kindly supplied by Dr Maitree Suttajit, Department of Biochemistry, Chiang Mai University, Chiang Mai, Thailand.

Incubation of INS-1 Cells

INS-1 cells were cultured in modified RPMI 1640, except that HEPES (10 mmol/L), sodium pyruvate (1 mmol/L), and β-mercaptoethanol (5 μmol/L) were supplemented.[11] Cultures were incubated at 37°C in a humidified 95% normal atmosphere/5% CO_2. Floating cell clusters were harvested by trypsinization of early culture passages. The cells were centrifuged at 1,000 × *g* at 4°C for 5 minutes and washed once in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (GIBCO). The pellets were suspended in 0.025% trypsin-EDTA solution (1 mL per 5 to 15 × 10^6 cells) and incubated at 37°C for 4 minutes. A 10-mL vol of ice-cold modified RPMI 1640 was then added, and the clusters were dispersed by 3 to 4 passages through a 10-mL glass pipette and washed in modified RPMI 1640. The cells used in the experiments were from passage 48 to 50. For secretion studies, modified KRB was used for preincubation (60 minutes at 3.3 mmol/L glucose). After preincubation, the cells were incubated for 60 minutes in modified KRB containing glucose and stevioside or steviol according to the protocols. After incubation, 300 μL incubation medium was removed and centrifuged (1,000 × *g* for 1 minute), and 100 μL was frozen for subsequent analysis of insulin.

Insulin Assay

Insulin was analyzed by radioimmunoassay using a guinea pig anti–porcine insulin antibody (Novo Nordisk, Bagsvaerd, Denmark) and mono-^{125}I-(Tyr A14)–labeled human insulin (Novo Nordisk) as tracer and porcine insulin as standard (Novo Nordisk). The separation of bound and free radioactivity was performed using ethanol. Stevioside and steviol at the concentrations studied did not interfere with the insulin assay.

Electrophysiology

Islets were dispersed into single cells by shaking in a low calcium concentration as described elsewhere.[12] The conventional whole-cell configuration of the patch-clamp technique was used to record whole-cell K^+ currents.[13] Membrane currents were recorded using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA), digitized, and stored in a computer using the Digidata AD-converter (Axon Instruments) and software pClamp (version 6.0; Axon Instruments). The K^+_{ATP}-channels were activated by dialysis of the cell interior with a pipette solution containing 0.3 mmol/L ATP and 0.3 mmol/L ADP, and whole-cell K^+ currents were measured in response to voltage clamp steps moving from a holding potential of −70 mV to −60 or −80 mV, respectively. The currents were digitized at 2 kHz and filtered at 1 kHz (−3 dB, 4-pole Bessel filter).[12,13] The extracellular solution consisted of the following (in mmol/L): 138 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES, and 5.6 glucose, with pH set at 7.40 using NaOH. The pipette solution was composed of 125 KCl, 30 KOH, 10 EGTA, 1 $MgCl_2$, 5 HEPES, 0.3 Mg-ATP, and 0.3 K-ADP, with a pH of 7.15. The cytoplasm is replaced by the pipette solution, which means that the cytosolic components required for glucose metabolism are washed out of the cell interior. As a consequence, the extracellular glucose concentration will not affect the ATP/ADP ratio in the cell and thus may not modulate K^+_{ATP}-channel activity.[14]

cAMP Measurement

Intracellular cAMP levels were measured in groups of 30 islets. The islets were first incubated in KRB for 30 minutes in the presence of 1 mmol/L 3-isobutyl-1-methylxantin (IBMX) and subsequently transferred to Eppendorf tubes and stimulated with 100 μmol/L stevioside or 1 μmol/L steviol for 12 minutes in the continued presence of IBMX (Sigma). Forskolin (2 μmol/L) was added after 2 minutes, and was present for the last 10 minutes of the incubation. The reaction was terminated by addition of HCl to the incubation medium (final concentration, 50 mmol/L). The samples were neutralized with NaOH, and total cAMP content was measured using a cAMP [^{125}I] scintillation proximity assay following the acetylation protocol (RPA 542; Amersham, UK).

Statistical Analysis

Statistical analysis was performed with Student's unpaired *t* test and 1-way ANOVA. Differences were considered significant at a level P less than .05. Data are expressed as the mean ± SEM.

RESULTS

Effects of Stevioside and Steviol on Glucose-Stimulated Insulin Secretion

Stevioside (1 nmol/L to 1 mmol/L) potentiated insulin secretion evoked by 16.7 mmol/L glucose, with an apparent maximal effect obtained in the presence of about 1 mmol/L stevioside (Fig 1A). Also, steviol (1 nmol/L to 1 mmol/L) increased insulin secretion at 16.7 mmol/L glucose, with a maximal effect at 1 μmol/L (Fig 1B). However, a stimulatory effect was found only at a concentration of at least 0.1 nmol/L for both substances (data not shown). The stimulatory effect of the 2 diterpenes diminished at concentrations higher than 1 mmol/L (data not shown).

To examine if the actions of stevioside and steviol are glucose-dependent, the influence of the maximal stimulatory concentration of stevioside (1 mmol/L) and steviol (1 μmol/L) was studied over a range of glucose concentrations (0 to 16.7 mmol/L glucose; Fig 2A and B). Stevioside and steviol both potentiated insulin secretion at glucose levels of 8.3 mmol/L or higher ($P < .05$), whereas no effect was detected at glucose concentrations of 3.3 and 0 mmol/L.

Ca^{2+} Dependence

The dependence on the presence of extracellular Ca^{2+} was studied at 16.7 mmol/L glucose (Fig 3A and B). Stevioside (≥1 mmol/L, $P < .05$) and steviol (≥0.01 mmol/L, $P < .05$) both increased insulin output even in the absence of extracellular Ca^{2+}. At lower concentrations, neither stevioside nor steviol possessed any insulin-releasing capacity.

Perifusion Studies

Perifusion experiments were performed to study the dynamic insulin response to stevioside and steviol. As expected, a biphasic insulin release was found when glucose was increased

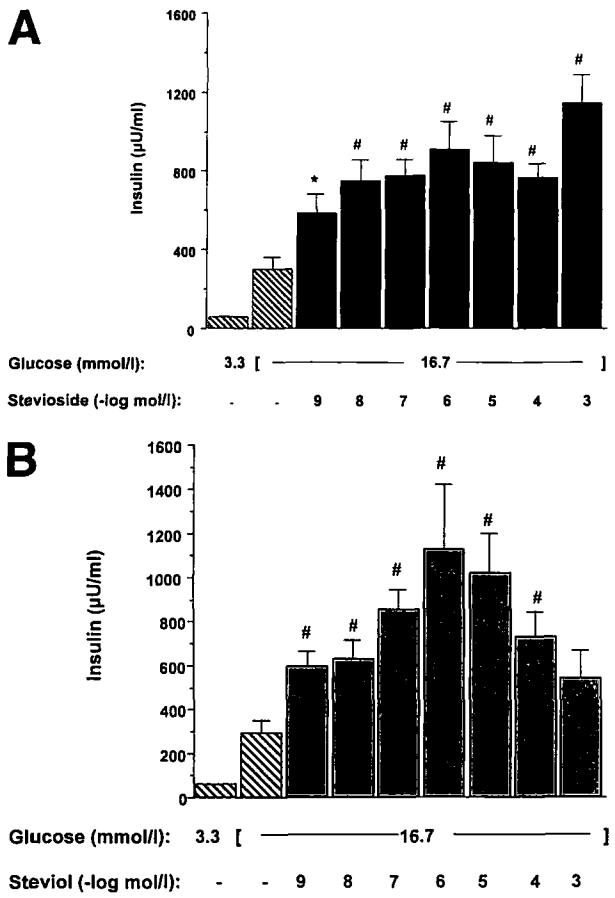

Fig 1. Effects of (A) stevioside and (B) steviol (1 nmol/L-1 mmol/L) on glucose (16.7 mmol/L)-stimulated insulin secretion from isolated mouse islets incubated for 60 minutes. Each bar represents the mean ± SEM from 24 incubations of single islets. #$P < .01$, *$P < .05$: probability level of random differences for incubations in the presence v absence of stevioside or steviol.

from 3.3 to 16.7 mmol/L. Both stevioside (1 mmol/L) and steviol (1 µmol/L) potentiated insulin secretion in the presence of 16.7 mmol/L glucose ($P < .01$; Fig 4A and B). The insulin response to the diterpenes was monophasic and apparently reversible, since insulin secretion declined toward the prestimulatory level in the washout period. β-Cell responsiveness was confirmed by the prompt secretory response to carbacholine (0.1 mmol/L) at the end of the experiment.

Effects of Stevioside and Steviol on INS-1 Cells

At 16.7 mmol/L glucose, both stevioside and steviol (1 nmol/L to 1 mmol/L) significantly ($P < .05$) increased insulin secretion from INS-1 (Fig 5A and B). At lower diterpene concentrations, no modulatory effect was found.

Effects of Stevioside and Steviol on ATP-Sensitive K^+-Channel Activity

The effects of stevioside and steviol on β-cell plasma membrane K^+_{ATP}-channels were tested in the whole-cell configuration. Low concentrations of stevioside (1 or 10 µmol/L) added to the bathing solution failed to affect K^+_{ATP}-channel activity induced by a pipette solution containing a mixture of 0.3 mmol/L ATP and 0.3 mmol/L ADP. The currents were 107% ± 10% (n = 5) and 94% ± 2% (n = 3) of the control in 1 and 10 µmol/L stevioside, respectively. However, 100 µmol/L stevioside caused a transient 35% ± 9% block of K^+_{ATP}-channel activity ($P < .025$; n = 5; Table 1). This block induced by stevioside reached a peak value about 1 minute after application of the substance and returned to 89% ± 9% of the control current within 3 minutes of continued application (Fig 6). Tolbutamide 100 µmol/L caused a rapid block to 6.5% ± 1.1% of the control current ($P < .001$; n = 10). Steviol failed to produce any significant effects on K^+_{ATP}-channel activity in the concentration range applied (0.01 to 10 µmol/L; n = 5).

Intracellular cAMP

The effects of stevioside and steviol on intracellular cAMP levels in normal mouse islets were studied next. cAMP levels were not affected by stevioside (100 µmol/L) or steviol (1 µmol/L) (Fig 7). As expected, a 4-fold increase in intracellular cAMP was found when forskolin (2 µmol/L) was added.

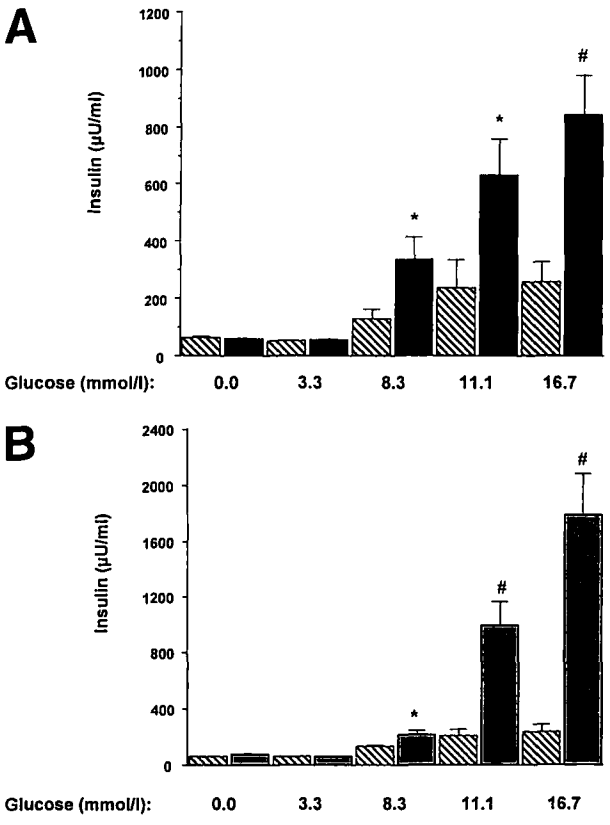

Fig 2. Effects of (A) stevioside (1 mmol/L) and (B) steviol (1 µmol/L) on insulin secretion from isolated mouse islets in the presence of glucose concentrations between 0 and 16.7 mmol/L. Each column represents the mean ± SEM from 16 incubations of single islets. #$P < .01$, *$P < .05$: probability level of random differences for incubations in the presence (■, ▩) and absence (▧) of stevioside or steviol, respectively.

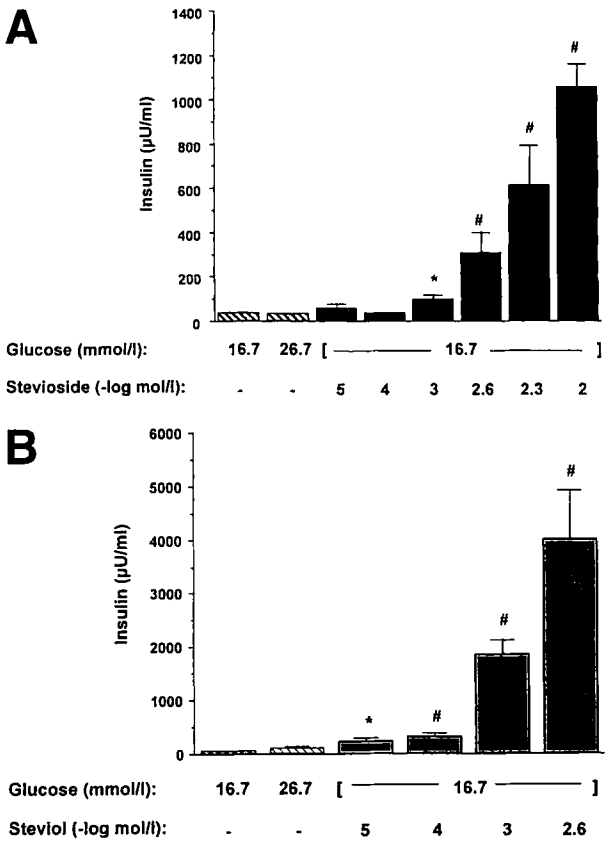

Fig 3. Effects of (A) stevioside (10 µmol/L-10 mmol/L) and (B) steviol (10 µmol/L-2.5 mmol/L) on glucose (16.7 mmol/L)-stimulated insulin secretion from isolated mouse islets incubated in the absence of extracellular Ca^{2+}. Each bar represents the mean ± SEM from 16 incubations of single islets. #$P < .01$, *$P < .05$: probability level of random differences for incubations in the presence *v* absence of stevioside or steviol.

DISCUSSION

There is increasing scientific validation for the use of certain traditional antidiabetic plants, and this has encouraged the search for new antidiabetic agents. However, due to the vast number of plants and complicated purification procedures, progress has been slow.[15,16] Although many plants are claimed to offer special benefits in the treatment of diabetes, few have undergone careful scientific investigation. Interestingly, this study shows that the purified substances from the leaves of *S rebaudiana Bertoni*, stevioside and the aglucon steviol, both have the capability to potentiate insulin secretion from isolated mouse islets in a dose- and glucose-dependent way. To our knowledge, this is the first demonstration of a direct effect of these compounds on pancreatic insulin secretion. Consequently, the previously described antihyperglycemic effect may be ascribed, at least in part, to a direct insulinotropic effect of the 2 diterpenes.[6,7]

The insulinotropic effects of stevioside and steviol are present in a broad concentration range, with insulin secretion being approximately tripled by both substances at the maximal effective concentration applied. Steviol is the aglucon of stevioside, the latter of which contains 3 D-glucose molecules. It is unlikely that the release of D-glucose from stevioside to the medium plays any role in the insulinotropic action, since glucose was not detected in the glucose-free medium after deposition of stevioside (data not shown). We are aware that the purity of the tested substances is 90% to 95%. Although, in theory, minor impurities of the tested substances may influence the findings, it is unlikely that they play a major role. We found different potencies for the 2 diterpenes, with steviol being the most potent substance. This is corroborated by our incubation studies in a Ca^{2+}-free medium demonstrating that steviol is more potent than stevioside. The reason for this is not clear.

We found that both substances stimulated insulin release in a dose-dependent manner. Both diterpenes caused a clear-cut insulin release at glucose concentrations of 8.3, 11.1, and 16.7

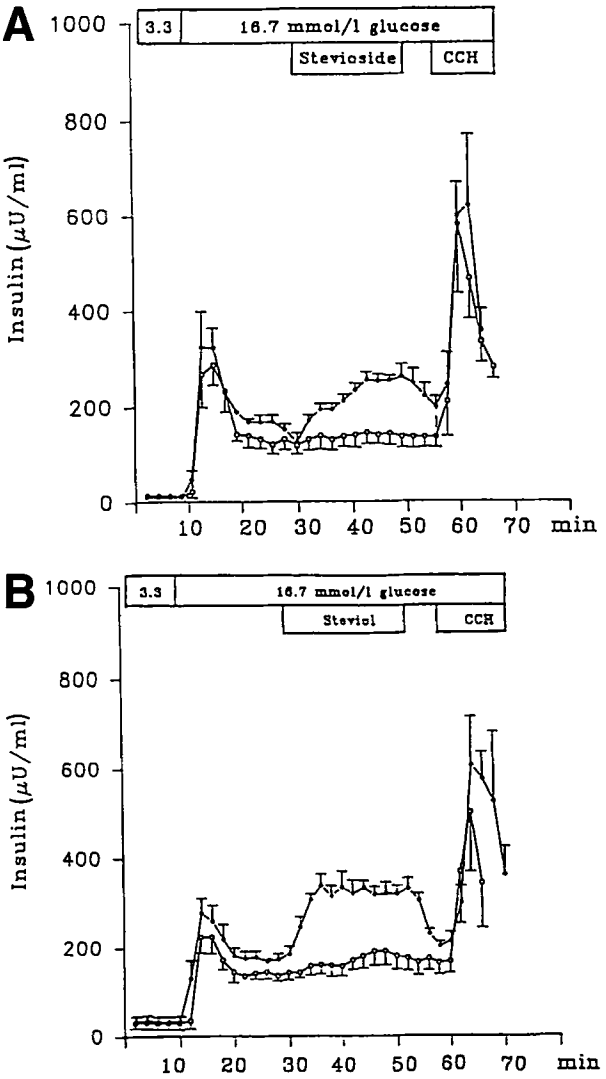

Fig 4. Effects of (A) 1 mmol/L stevioside and (B) 1 µmol/L steviol on glucose (16.7 mmol/L)-stimulated insulin secretion from perifused mouse islets. Each curve is the mean ± SEM from 4 perifusion columns with 25 islets each. CCH, carbacholine 0.1 mmol/L; ●, stevioside or steviol; ○, control.

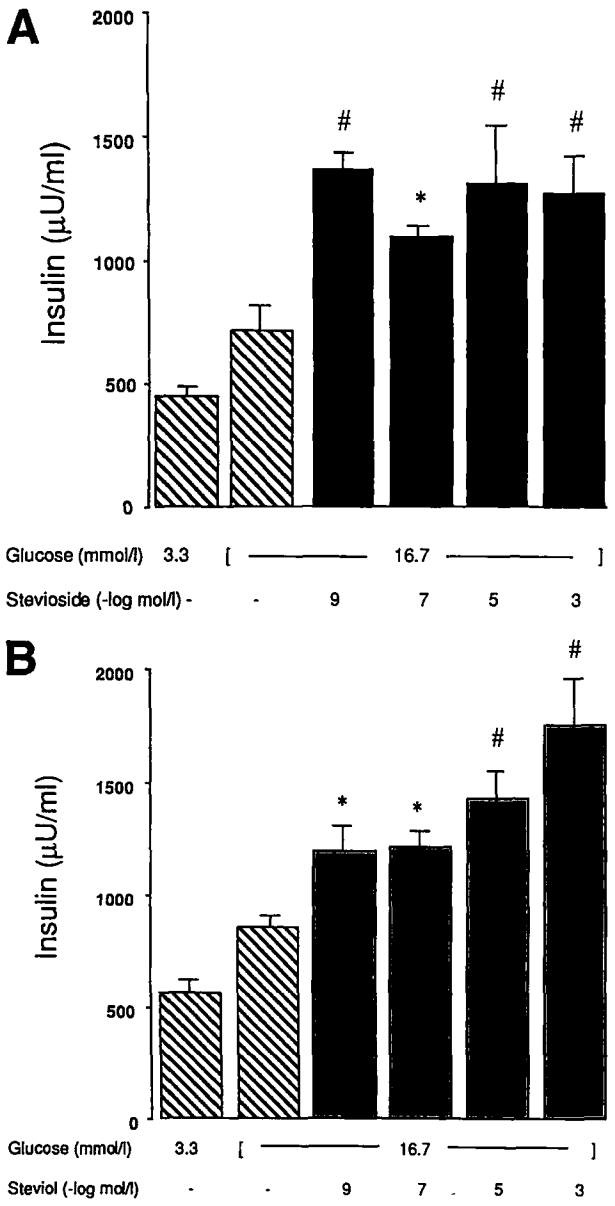

Fig 5. Effects of (A) stevioside and (B) steviol (1 nmol/L-1 mmol/L) on glucose (16.7 mmol/L)-stimulated insulin secretion from INS-1 cells incubated for 60 minutes. Each bar represents the mean ± SEM from 12 incubations of $0.2 \cdot 10^6$ cells each. #$P < .01$, *$P < .05$: probability level of random differences for incubations in the presence *v* absence of stevioside or steviol.

mmol/L (Fig 2). Even at 6.6 mmol/L glucose, a small increment in insulin could be detected (data not shown). However, at low glucose of 3.3 mmol/L or less, no insulinotropic action was found. This corroborates the results of Usami et al,[17] who found no effect of a similar stevioside concentration at 2.8 mmol/L glucose using the isolated perfused rat pancreas. Our findings seem to indicate that stevioside and steviol may possess potential in the treatment of type 2 diabetes, since the insulinotropic action seems to fade when blood glucose decreases toward normal levels. The interesting possibility exists that this property may prevent the development of hypoglycemia.

To explore if the diterpenes act directly on β cells, we studied the effects using the β-cell line INS-1. In agreement with the results obtained in whole islets, we found a stimulation of insulin release from INS-1 cells, pointing to direct effects on the β cell. The question arises as to whether the insulinotropic effects of the 2 diterpenes are mediated via the same mechanisms as the classic sulfonylureas, which bind to receptor proteins of β cells to block the K^+_{ATP}-channels and depolarize the β-cell plasma membrane and ultimately induce insulin release. The relevance of this question is substantiated by the fact that sulfonylureas have previously been reported to have adverse cardiovascular effects caused by the closure of K^+_{ATP}-channels in ventricular myocytes, thus causing arrhythmias.[18,19] Consequently, we were especially interested in examining the action of stevioside and steviol on K^+_{ATP}-channels in β cells. In this context, it is interesting to observe that neither stevioside nor steviol resulted in any lasting block of the plasma membrane K^+_{ATP}-channel on the β cell. As a consequence, the insulinotropic effects of stevioside or steviol are not mediated via membrane depolarization caused by closure of K^+_{ATP}-channels in the β-cell membrane.

We also explored the influence of the 2 diterpenes on cAMP levels in mouse islets, since cAMP is known to be an important second messenger in β cells.[20] In the presence of IBMX, a cAMP phosphodiesterase inhibitor, stevioside and steviol caused no change in intracellular cAMP levels in mouse islets. This indicates that the cAMP system does not play a major role in the insulinotropic effect of stevioside and steviol.

Interestingly, both substances caused insulin release even in the absence of extracellular Ca^{2+}. However, the impact of the substances was reduced when the medium was devoid of Ca^{2+}. The diterpene concentrations needed to enhance insulin secretion in the absence of Ca^{2+} were very high. Whether the diterpenes in the absence of extracellular Ca^{2+} induce the release of intracellularly stored Ca^{2+}, which then initiates insulin secretion, remains to be studied.

The lack of influence of the diterpenes on the islet cAMP level and β-cell K^+_{ATP}-channels now encourages the search for the impact on other effector pathways in β cells potentially involved in the acute stimulation of insulin release, eg, phospho-

Table 1. K_{ATP}^+-Channel Activity of Mouse β-Cells

Agent	-log (mol/L)	K_{ATP}^+-Channel Activity (% of control)	*P*	No.
Stevioside	6 (3 min)	107 ± 10	NS	5
	5 (3 min)	94 ± 2	NS	3
	4 (1 min)	65 ± 9	<.025	5
	4 (3 min)	89 ± 9	NS	5
Steviol	8 (3 min)	105 ± 10	NS	3
	7 (3 min)	106 ± 3	NS	3
	6 (3 min)	95 ± 3	NS	3
	5 (3 min)	95 ± 4	NS	3

NOTE. Data are whole-cell K^+ currents from β cells exposed to 1-100 µmol/L stevioside and 0.01-10 µmol/L steviol during the periods indicated. Only stevioside at a concentration of 100 µmol/L had a short-term effect on the K_{ATP}^+-channel (see Fig 6).

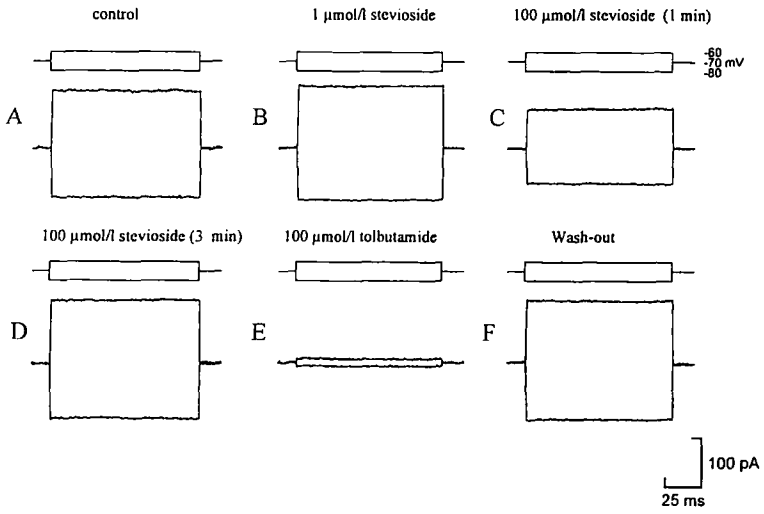

Fig 6. Effects of stevioside on K^+_{ATP}-channel currents. (A) Whole-cell K^+ currents were visualized by applying voltage steps moving from a holding potential of −70 to −60 and 80 mV. The pipette solution contained 0.3 mmol/L ATP and 0.3 mmol/L ADP. (B) Current responses in the presence of 1 μmol/L stevioside. (C) K^+_{ATP}-channel activity in the presence of 100 μmol/L stevioside 1 minute after application of stevioside. (D) "Run-down" of the inhibitory effect of stevioside 3 minutes after application of the compound. (E) Block of whole-cell K^+ current induced by 100 μmol/L tolbutamide. (F) Washout of tolbutamide.

lipase C and D. It is also important to study whether diterpenes interact with a membrane-bound receptor to induce the observed acute effects on insulin secretion.

Most in vivo studies have demonstrated an antihyperglycemic effect of the diterpenes found in the leaves or extracts of leaves from *S rebaudiana*.[6,7] However, Suanarunsawat and Chaiyabutr[21] observed that intravenous administration of stevioside at normal glucose levels caused a "paradoxical" elevation of blood glucose in rats, an observation that seems difficult to explain.

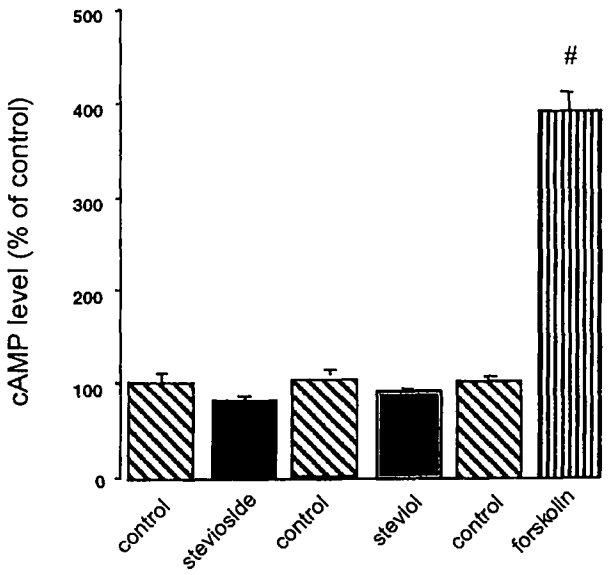

Fig 7. Effects of stevioside (0.1 mmol/L) and steviol (1 μmol/L) on intracellular cAMP levels in normal mouse islets (batches of 30 islets were used in each experiment). Forskolin (2 μmol/L) acts as a control. Each bar represents the mean ± SEM from 3-8 experiments. #$P < .01$.

Mechanisms other than the insulinotropic effect may play a role in the postulated antihyperglycemic action of stevioside and steviol. Thus, both substances exert an inhibitory action on ATP phosphorylation and on NADH-oxidase activity in rat liver mitochondria, thereby causing an increase in the rate of glycolysis and suppression of gluconeogenesis.[22,23] Interestingly, it has also been shown that extracts of *S rebaudiana* inhibit gluconeogenesis in isolated rat renal tubules.[24]

In conclusion, stevioside and steviol stimulate insulin secretion from mouse islets and INS-1 cells. Stevioside and steviol seem to possess antihyperglycemic effects that may be important in the treatment of type 2 diabetes. Stevioside and steviol seem to have an inherent advantage over the classic sulfonylureas, since the action of the diterpenes is not mediated via K^+_{ATP}-sensitive channels. Furthermore, the lack of insulin-stimulatory effects at subnormal glucose levels may reduce or eliminate the risk of hypoglycemia.

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