

# Rebaudioside A Potently Stimulates Insulin Secretion From Isolated Mouse Islets: Studies on the Dose-, Glucose-, and Calcium-Dependency

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Extracts of leaves of the plant *Stevia rebaudiana* Bertoni (SrB), have been used for many years in traditional treatment of diabetes in South America. *Stevia* leaves contain diterpene glycosides, stevioside and rebaudioside A being the most abundant. Recently, it was demonstrated that stevioside stimulates the insulin secretion both *in vitro* and *in vivo*. Subsequently, we wanted to elucidate the influence of rebaudioside A on the insulin release from mouse islets using static incubations, as well as perfusion experiments. Rebaudioside A ( $10^{-16}$  to  $10^{-6}$  mol/L) dose-dependently stimulated the insulin secretion in the presence of 16.7 mmol/L glucose ( $P < .05$ ). The stimulation of insulin release occurs at a concentration of  $10^{-14}$  mol/L rebaudioside A, and maximal insulin response was obtained at  $10^{-10}$  mol/L ( $P < .01$ ). Rebaudioside A stimulates insulin secretion in a glucose-dependent manner (3.3 to 16.7 mmol/L) and only potentiated insulin secretion at glucose  $> 6.6$  mmol/L. The effect of rebaudioside A is critically dependent on the presence of extracellular  $Ca^{2+}$ , ie, rebaudioside A-induced insulin stimulation at high glucose disappears in the absence of extracellular  $Ca^{2+}$ . In conclusion, rebaudioside A possesses insulinotropic effects and may serve a potential role as treatment in type 2 diabetes mellitus.

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**T**YPE 2 DIABETES has 2 hallmark features, ie, insulin resistance and compromised function of  $\beta$  cells. Extracts of leaves from the plant *Stevia rebaudiana* Bertoni (SrB) have been used for many years in traditional medicine in South America in the treatment of diabetes.<sup>1</sup> *Stevia* leaves contain diterpene glycosides that are responsible for the typical sweet taste, Stevioside and rebaudioside A being the most abundant accounting for 5% and 2% of the dry matter, respectively.<sup>2</sup> A 35% reduction in blood glucose has been observed in diabetic subjects after oral intake of SrB extracts.<sup>3</sup> Previously, we have demonstrated that stevioside exerts a direct insulinotropic action in isolated mouse islets and in clonal  $\beta$ -cell lines (INS-1 and INS-1E)<sup>4</sup> and possesses antihyperglycemic effects.<sup>5,6</sup> The question arises if rebaudioside A does also possess an insulinotropic action. Rebaudioside A shares the main structure with stevioside, ie, consisting of steviol, the aglucone of stevioside, with 4 moles of D-glucose, while the structure of stevioside is steviol with 3 moles of D-glucose.<sup>2</sup> Our hypothesis was that rebaudioside A also possesses insulinotropic actions. The aim was to evaluate the effect on insulin secretion of rebaudioside A using in isolated mouse islets. To the best of our knowledge, this is the first investigation on the influence of rebaudioside A on the pancreatic function.

## MATERIALS AND METHODS

### Materials

Tissue culture medium RPMI 1640 was obtained from GIBCO BRL (Paisley, UK). A guinea pig antiporcine insulin antibody, mono-<sup>125</sup>I-(Tyr A14)-labeled human insulin and porcine insulin were from Novo Nordisk (Bagsvaerd, Denmark). Collagenase P was obtained from Boehringer Mannheim GmbH (Mannheim, Germany) and Hanks' balanced salt solution, bovine serum albumin (BSA), EGTA, and other chemicals were obtained from Sigma Chemical (St Louis, MO). Rebaudioside A was purchased from Wako Pure Chemical Industries (Tokyo, Japan).

### Isolation of Islets of Langerhans

Islets were isolated from adult female NMRI mice (Bomholtgård Breeding and Research Center, Ry, Denmark) weighing 22 to 25 g by collagenase digestion, as described previously<sup>4,7</sup> and maintained overnight in RPMI 1640 culture medium containing 11 mmol/L glucose and supplemented with 10% fetal calf serum, 2.06 mmol/L L-glutamine, 100 IU/mL penicillin (all GIBCO BRL).

### Measurements of Insulin Release From Incubated Pancreatic Islets

Islets were preincubated in a modified Krebs-Ringer bicarbonate buffer (KRB) containing 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, supplemented with 1 mg/mL BSA, with 3.3 mmol/L glucose for 30 minutes at 37°C. For experiments performed in  $Ca^{2+}$ -free medium,  $CaCl_2$  was replaced by 0.5 mmol/L EGTA, and the preincubation time was extended to 60 minutes.<sup>8</sup> After preincubation, a single islet was selected and incubated in 100  $\mu$ L KRB for 60 minutes at 37°C with appropriate test substances. An aliquot of 50  $\mu$ L was drawn for insulin assay after incubation.

### Measurements of Insulin Release From Perfused Pancreatic Islets

Twenty-five islets was placed into the perfusion chambers and perfused with KRB at a flow rate of 200  $\mu$ L/min at 37°C.<sup>4</sup> Fractions were collected every 2 minutes. The experiment was designed as follows: (1) 10-minute preperfusion at 3.3 mmol/L glucose, (2) 30-minute perfusion at 3.3 mmol/L glucose in the presence or absence of  $10^{-10}$  mol/L rebaudioside A, (3) 30-minute perfusion at 3.3 mmol/L glucose, (4) 30-minute perfusion at 16.7 mmol/L glucose in the pres-

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Submitted February 5, 2004; accepted April 13, 2004.

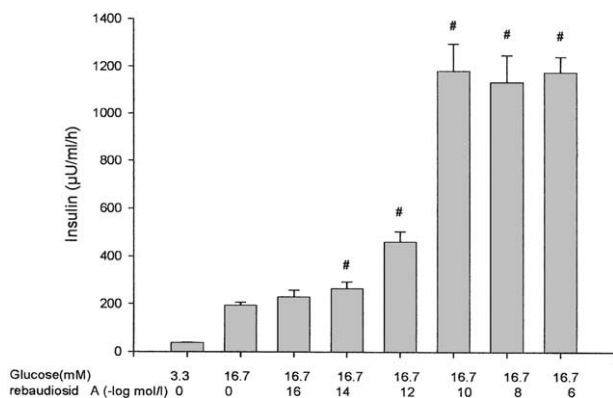
Supported by the Novo Nordisk Foundation, Institute of Experimental Clinical Research, Aarhus University, the Danish Research Agency, Danish Medical Research Council, and Danish Council for Development Research (RUF).

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0026-0495/04/5310-0046\$30.00/0

doi:10.1016/j.metabol.2004.04.014



**Fig 1.** Effect of rebaudioside A ( $10^{-16}$  mol/L to  $10^{-6}$  mol/L) on glucose (16.7 mmol/L)-stimulated insulin secretion from isolated mouse islets incubated for 60 minutes. # $P < .05$  v control (glucose 16.7 mmol/L). Each bar represents the mean  $\pm$  SEM from 16 incubations of a single islet.

ence or absence of  $10^{-10}$  mol/L rebaudioside A, (5) 30-minute perfusion at 3.3 mmol/L glucose, (6) 20-minute preperfusion at 16.7 mmol/L glucose.

#### Insulin Assay

Insulin was analyzed by radioimmunoassay using a guinea pig antiporcine insulin antibody and mono- $^{125}\text{I}$ -Tyr A14-labeled human insulin as tracer and porcine insulin as standard. The separation of bound and free radioactivity was performed using ethanol. Rebaudioside A at the concentration used did not interfere with the insulin assay.

#### Statistical Analysis

Data are expressed as means  $\pm$  SEM. Each treatment was compared with control, and statistical significance between 2 groups was evaluated using the Student's  $t$  test. A  $P$  value of less than .05 was considered statistically significant.

## RESULTS

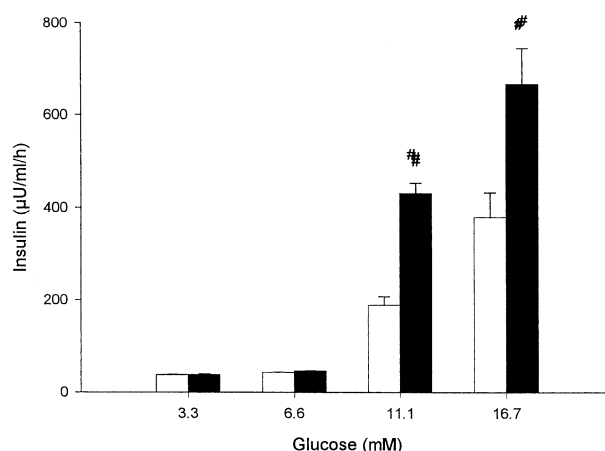
### The Dose- and Glucose-Dependent Effects of Rebaudioside A on Insulin Release

Figure 1 demonstrates the effect of rebaudioside A ( $10^{-16}$  to  $10^{-6}$  mol/L) on glucose-stimulated (16.7 mmol/L) insulin release from mouse islets. The addition of increasing concentrations of rebaudioside A elicited a dose-dependent increase in the insulin secretion in the presence of 16.7 mmol/L glucose. The maximal insulin response was obtained at the concentration of  $10^{-10}$  mol/L ( $P < .01$ ) and the lowest effective dose of rebaudioside was  $10^{-14}$  mol/L.

Figure 2 shows the effects of rebaudioside A ( $10^{-10}$  mol/L) on insulin secretion from mouse islets at the glucose concentrations of 3.3, 6.6, 11.1, and 16.7 mmol/L, respectively. Rebaudioside A potentiated the insulin secretion at glucose levels of 11.1 mmol/L or higher ( $P < .05$ ), whereas no stimulatory effect was detected at normal or low glucose concentrations.

### Effects of Rebaudioside A on the Dynamic of Insulin Release From Perfused Mouse Islets

In the presence of 3.3 mmol/L glucose, the addition of rebaudioside A did not change basal insulin secretion. As

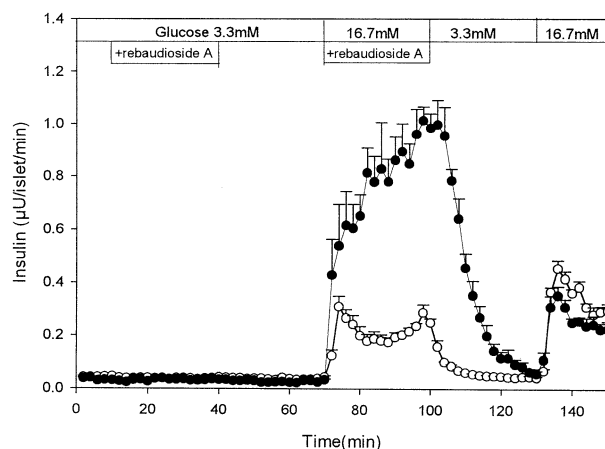


**Fig 2.** Effect of rebaudioside A ( $10^{-10}$  mmol/L, ■) on insulin secretion from isolated mouse islets in the presence of glucose concentrations of 3.3, 6.6, 11.1, and 16.7 mmol/L, respectively. # $P < .05$  v control (in the absence of rebaudioside A, □). Each bar represents the mean  $\pm$  SEM from 16 incubations of a single islet.

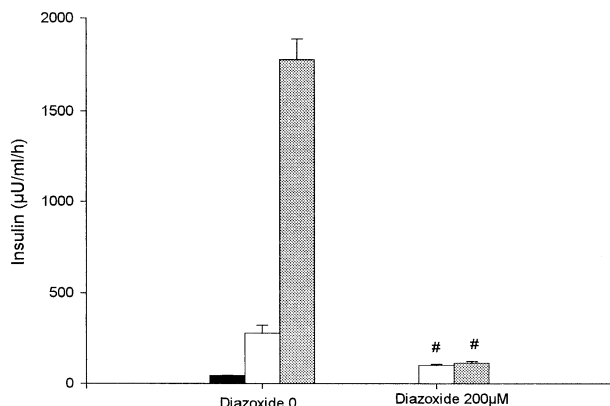
expected, the increase in the prevailing glucose level from 3.3 to 16.7 mmol/L caused a biphasic insulin response from perfused islets. In the presence of high glucose (16.7 mmol/L),  $10^{-10}$  mol/L rebaudioside A elicited a pronounced and sustained monophasic increase in insulin release (Fig 3).

### $K^+$ -Adenosine Triphosphate Channel Dependency of the Insulinotropic Effect of Rebaudioside A

As seen in Fig 4, the insulin response from islets incubated at 16.7 mmol/L glucose was diminished in the presence of 200  $\mu\text{mol/L}$  diazoxide. Addition of  $10^{-10}$  mol/L rebaudioside A was unable to counteract the inhibition of glucose-induced insulin release mediated by diazoxide.



**Fig 3.** Insulin secretion from perfused isolated mouse islets in the absence (control, ○) or presence of rebaudioside A ( $10^{-10}$  mol/L, ●) at low (3.3 mmol/L) and high (16.7 mmol/L) glucose. Each curve is the mean  $\pm$  SEM from 6 perfusion experiments with 25 islets in each.



**Fig 4. Effects of diazoxide (200 µmol/L) on insulin release in the absence (□) or presence of rebaudioside A (10<sup>-10</sup> mol/L, ▨) in the presence of high glucose (16.7 mmol/L). #P < .05 v control (absence of diazoxide). (■) Represents insulin secretion at 3.3 mmol/L glucose in the absence of diazoxide. Each bar represents the mean ± SEM from 16 incubations of a single islet.**

#### Ca<sup>2+</sup> Dependency of the Insulinotropic Effect of Rebaudioside A

The dependence on extracellular Ca<sup>2+</sup> was examined at 16.7 mmol/L glucose. As can be seen in Fig 5, 16.7 mmol/L glucose alone or 16.7 mmol/L glucose in the presence of 10<sup>-10</sup> mol/L rebaudioside A did not stimulate insulin secretion in the absence of extracellular Ca<sup>2+</sup>.

### DISCUSSION

We tested the hypothesis that an insulinotropic effect was harbored in the diterpene glycoside, rebaudioside A. The study is the first to demonstrate that rebaudioside A causes a dose- and glucose-dependent stimulation of insulin secretion from isolated mouse islets. Rebaudioside A elicits a typical monophasic insulin response. Interestingly, the stimulatory effect of rebaudioside A disappears in the presence of normal or low glucose. In the absence of extracellular calcium, the insulinotropic effect of rebaudioside A vanishes.

Type 2 diabetes is a chronic metabolic disorder that results from insufficient insulin secretion. The necessity to identify new molecules that safely stimulate endogenous insulin biosynthesis and secretion is considerable.<sup>9,10</sup> We have demonstrated that stevioside and steviol, glucosides present in SrB, both have the capability to potentiate insulin secretion from isolated mouse islets in a dose- and glucose-dependent manner.<sup>4</sup> Rebaudioside A seems to be more potent than stevioside and steviol, eliciting clear-cut insulin stimulation even at a concentration as low as 10<sup>-14</sup> mol/L. The reason for the higher potency of rebaudioside A is not known. The difference in number and positions of the glucose molecules in these glycoside molecules may play a role in this respect. While steviol is an aglucon, rebaudioside A contains 4 glucose molecules versus 3 in stevioside. The purity of rebaudioside A is from 95% to 98% according to the information provided from the producer. Although, in theory, minor impurities of the tested substances

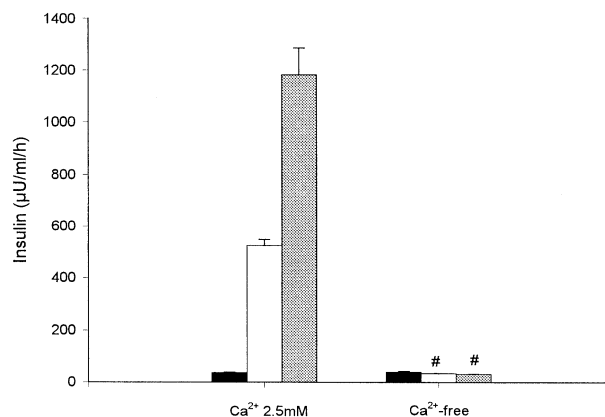
may influence the findings, it seems unlikely that they should play a major role.

The insulinotropic effects of stevioside and steviol were critically dependent on the prevailing glucose concentration, i.e., they potentiated insulin secretion only at or above 8.3 mmol/L glucose.<sup>4</sup> The requirement of a supranormal glucose level as a costimulus for rebaudioside A to be insulinotropic is important, because this may reflect that Rebaudioside A, as stevioside<sup>4</sup> seems to carry a lower risk of hypoglycemia than sulphonylureas. Thus the effects of rebaudioside A fade at glucose concentrations comparable with normoglycemia. In this context, it is noteworthy that we previously demonstrated that a bolus injection of stevioside in the fasting state did not cause hypoglycemia in the normal Wistar rat or the diabetic Goto-Kakizaki (GK) rat.<sup>5</sup>

In perfusion experiments, we demonstrated that rebaudioside A elicits a distinct monophasic insulin response, similarly to what we have previously found for stevioside.<sup>4</sup> Despite the apparent lack of a first-phase insulin response of stevioside *in vitro*,<sup>4</sup> long-term oral stevioside treatment improves first-phase insulin response in the diabetic GK rat.<sup>5</sup>

To understand the mechanisms of action of rebaudioside A on the β cells, we incubated pancreatic islets with diazoxide, a compound that inhibits the insulin release by increasing K<sup>+</sup> conductance of the β-cell membrane through opening of the adenosine triphosphate (ATP)-sensitive K<sup>+</sup> channels.<sup>11,12</sup> Rebaudioside A was unable to reverse the inhibitory effect of 200 µmol/L diazoxide on glucose-induced insulin release. This suggests that rebaudioside A may not affect ATP-sensitive K<sup>+</sup> channels in the β cells like we previously demonstrated for stevioside.<sup>4</sup> Further studies are, however, needed to clarify whether or not rebaudioside A does affect the ATP-sensitive K<sup>+</sup> channels directly.

In the present study, rebaudioside A (10<sup>-10</sup> mol/L) did not stimulate insulin release at 16.7 mmol/L glucose in the absence of extracellular Ca<sup>2+</sup>. This suggests that the insulinotropic effect of rebaudioside A is critically dependent on Ca<sup>2+</sup> influx



**Fig 5. Effect of rebaudioside (10<sup>-10</sup> mol/L) on insulin release in Ca<sup>2+</sup>-free medium. Isolated mouse islets were incubated for 60 minutes in the absence or presence (control) of extracellular calcium at glucose concentrations of 3.3 (■), 16.7 (□), and 16.7 mmol/L plus rebaudioside A (10<sup>-10</sup> mol/L, ▨). Each bar represents the mean ± SEM from 16 incubations of single islets. #P < .05 v control.**

from the extracellular space. At first glance this may occur puzzling considering our previous findings indicating that the insulinotropic effect of both stevioside and steviol is preserved in the absence of extracellular  $\text{Ca}^{2+}$ .<sup>4</sup> However, it is noteworthy that the diterpene concentration needed to stimulate the insulin secretion in a  $\text{Ca}^{2+}$ -free medium was extremely high ( $10^{-3}$  to  $10^{-2}$  mol/L). Stevioside, as well as steviol, in a concentration causing maximal effect ( $10^{-6}$  mol/L) were unable to enhance the insulin release in the absence of extracellular  $\text{Ca}^{2+}$ . The augmentation of the insulin release in  $\text{Ca}^{2+}$ -free medium in our previous studies may consequently be due to unspecific effects at extremely high levels of the glucosides. It seems unlikely that it is ascribed to an irreversible toxic effect on  $\beta$  cells, because the islets subsequently responded

with a normal insulin response to carbacholine in the presence of normal extracellular calcium during a perfusion study.<sup>4</sup>

In conclusion, rebaudioside A potently stimulates the insulin secretion from isolated mouse islets in a dose- and glucose-dependent manner. Interestingly, rebaudioside A does not cause a stimulation of insulin release at near-normal glucose levels, which is likely to reduce or eliminate the risk of hypoglycemia. Whether rebaudioside A may serve in the treatment or prevention of type 2 diabetes remains to be elucidated.

#### ACKNOWLEDGMENT

We thank Kirsten Eriksen, Dorte Rasmussen, Lene Trudsø, and Tove Skrumsager Hansen for skillful technical assistance.

#### REFERENCES

1. Soejarto DD, Kinghorn AD, Farnsworth NR: Potential sweetening agents of plant origin. III. Organoleptic evaluation of Stevia leaf herbarium samples for sweetness. *J Nat Prod* 45:590-599, 1982
2. Shibata H, Sawa Y, Oka T, et al: Steviol and steviol-glycoside: Glucosyltransferase activities in Stevia rebaudiana Bertoni—Purification and partial characterization. *Arch Biochem Biophys* 321:390-396, 1995
3. Oviedo CA, Franciani G, Moreno R, et al: Action hipoglucemiant de la stevia rebaudiana bertoni. Seventh Congress of the International Diabetes Federation, Buenos Aires, Argentina, 1970 (abstr 208)
4. Jeppesen PB, Gregersen S, Poulsen CR, et al: Stevioside acts directly on pancreatic beta cells to secrete insulin: Actions independent of cyclic adenosine monophosphate and adenosine triphosphate-sensitive  $\text{K}^{+}$ -channel activity. *Metabolism* 49:208-214, 2000
5. Jeppesen PB, Gregersen S, Rolfsen SE, et al: Antihyperglycemic and blood pressure-reducing effects of stevioside in the diabetic Goto-Kakizaki rat. *Metabolism* 52:372-378, 2003
6. Gregersen S, Thomsen JL, Brock B, et al: Endothelin-1 stimulates insulin secretion by direct action on the islets of Langerhans in mice. *Diabetologia* 39:1030-1035, 1996
7. Lacy PE, Kostianovsky M: Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* 16:35-39, 1967
8. Komatsu M, Schermerhorn T, Aizawa T, et al: Glucose stimulation of insulin release in the absence of extracellular  $\text{Ca}^{2+}$  and in the absence of any increase in intracellular  $\text{Ca}^{2+}$  in rat pancreatic islets. *Proc Natl Acad Sci USA* 92:10728-10732, 1995
9. Bailey CJ, Day C: Traditional plant medicines as treatments for diabetes. *Diabetes Care* 12:553-564, 1989
10. Ivorra MD, Paya M, Villar A: A review of natural products and plants as potential antidiabetic drugs. *J Ethnopharmacol* 27:243-275, 1989
11. Henquin JC, Meissner HP: Opposite effects of tolbutamide and diazoxide on  $^{86}\text{Rb}^{+}$  fluxes and membrane potential in pancreatic B cells. *Biochem Pharmacol* 31:1407-1415, 1982
12. Henquin JC, Charles S, Nenquin M, et al: Diazoxide and D600 inhibition of insulin release. Distinct mechanisms explain the specificity for different stimuli. *Diabetes* 31:776-783, 1982