

A. KLIBER, T. SZKUDELSKI, J. CHICHŁOWSKA

ALLOXAN STIMULATION AND SUBSEQUENT INHIBITION OF INSULIN RELEASE FROM *IN SITU* PERFUSED RAT PANCREAS

Department of Animal Physiology and Biochemistry, Academy of Agriculture, Poznań, Poland

In the performed experiment the effect of alloxan on insulin secretion by *in situ* perfused rat pancreas was determined. It was found that alloxan added to the perfusion medium results in a sudden, short-term release of insulin. This effect is independent on the presence of glucose (6.66 mmol/l) in the perfusion medium. Furthermore, it was observed that glucose at 16.66 mmol/l concentration after alloxan perfusion does not stimulate insulin secretion.

Key words: *alloxan, insulin secretion, rat pancreas*

INTRODUCTION

Alloxan shows a strongly diabetogenic activity and that is why it has been used for a long time to induce experimental diabetes in animals (1). Numerous experiments have already been carried out with the aim to identify its properties and to learn more about its mode of action since such information is essential for better understanding of changes caused by this compound in experimental animals. It is known that alloxan easily penetrates into insulin-secreting pancreatic cells (2, 3) undergoing changes resulting in the formation of hydrogen peroxide as well as superoxide and hydroxyl radicals (4—6). The avalanche formation of these active oxygen species leads to impairment of B cells (5, 7, 8). It has also been shown that certain compounds — including glucose — exhibit protective action against alloxan cytotoxicity (8, 9). However, changes in the level of many biochemical parameters which occur *in vivo* shortly after the administration of this diabetogenic agent to animals (among others variations of glycemia) seem to suggest that the effects of its action are more complex and may concern not only pancreatic cells of the

pancreas. Furthermore, hyperglycemia may be accompanied by an elevated level of insulin in blood (10, 11).

The aim of this study was to ascertain the direct effect of alloxan on insulin secretion by the *in situ* perfused rat pancreas in the presence or absence of glucose in the perfusion medium. Moreover, the effect of high concentration of glucose on insulin secretion by the pancreas previously perfused by alloxan was also determined.

MATERIAL AND METHODS

In the experiment male Wistar rats of 250 ± 5 g body weight were used. Animals were kept in standard conditions with continual access to food and water. Prior to the experiment, animals were fasted for 16 hours. Experiments were carried out according to the method of *in situ* pancreas perfusion described by Penhos et al. (12) with Kliber's modification (13). Applied modifications concerned only the composition of the basic perfusion medium and the way of rats' anaesthesia.

In the experiment, basic perfusion medium (B) was Krebs-Ringer-bicarbonate buffer containing 0.2 bovine serum albumin (fraction V) and 4% dextran T-70000 instead of 4% bovine serum albumin alone proposed by Penhos et al. (12). Besides, sodium amytal used to anaesthesia of rats was replaced by ether, but general procedure of the perfusion was unchanged.

After surgical preparation of rats (15—25 minutes) performed under general anaesthesia, pancreases were stabilized for the period of thirty minutes using the basic perfusion medium of constant temperature 37.5 ± 0.5 and $\text{pH} = 7.4\text{—}7.5$ obtained by blowing the medium through with carbogen (95% O_2 and 5% CO_2). After this period, the proper experiment began.

Depending on the experimental design, the perfusion medium contained D-glucose (6.66 or 16.66 mmol/l) alloxan or D-glucose (6.66 mmol/l) and alloxan.

The surgically prepared rats were placed in a chamber in which the temperature was maintained at $37.5 \pm 0.5^\circ\text{C}$ and relative humidity at 100%.

Alloxan was diluted in cold distilled water directly before use and was added by means of infusion pump to the perfusion medium (just before aorta) which was supplied to pancreas *via* cannula placed in the aorta. This prevented its rapid decomposition in the perfusion medium. The alloxan concentration in the medium perfusing the pancreas was 4.1 mmol/l which, at the flow rate of 3.1 ml/min. and 10 min. duration of perfusion by this compound, corresponded to 75 mg of alloxan per 1 kg rat body weight.

Two experimental designs were applied. In the first of them ($n = 6$) the pancreas was perfused for 15 min. with the basic medium containing 6.66 mmol glucose/l which was followed by further 10 min. (15—25 min.) perfusion during which alloxan was added to the medium. Next, the pancreas was perfused for 20 min. with medium containing 16.66 mmol glucose/l.

In the second experimental design ($n = 6$), after 15 min. perfusion with the medium containing 6.66 mmol glucose/l, the pancreas was perfused for 10 min. with medium without glucose and then, for further 10 minutes, alloxan was added to the medium. Next, from the 35th to 45th minute of the duration of experiment, the medium contained glucose at the concentration of 16.66 mmol/l. Control perfusions ($n = 6$) were run according to the same design with the exception that the perfusion medium was supplemented with water in the amount identical to that used to dilute alloxan.

In order to determine insulin, the perfusion medium flowing out of the pancreas by a cannula inserted in the portal vein was collected into separate test-tubes from every two minutes of perfusion, while during the perfusions with the medium containing alloxan (or water) the perfusion

medium flowing out of pancreas was collected every minute. Insulin was determined using a radioimmunological RIA-INS test (Świerk, Poland).

Results of individual experimental groups were presented on figures as mean percentage increments of insulin level in individual times in relation to "0" time and total amounts of secreted insulin in individual time intervals together with their statistical assessment with t-Student test in comparison with the control treatment of the appropriate experimental variant.

RESULTS

The effect of alloxan on insulin secretion stimulated by 6.66 mmol/l glucose concentration is presented on *Fig. 1B*, while *Fig. 2B* shows the influence of alloxan on insulin secretion in the absence of glucose. *Fig. 1A* and *2A* represents control.

Alloxan added to the perfusion medium containing glucose (*Fig. 1B*) caused an immediate, rapid insulin secretion reaching its maximum already in the first minute after its addition. During the following minutes of alloxan perfusion, the hormone secretion decreased markedly but its total quantity discharged from the pancreas between the 15th and 25th minute was over twice higher in comparison with the respective perfusion time with glucose alone. After the withdrawal of alloxan from the perfusion medium, the stimulation of the pancreas with 16.66 mmol/l concentration of glucose did not cause insulin secretion; to the contrary, further drop in its secretion was observed (*Fig. 1B*). The total amount of insulin released after perfusion with alloxan (25—45 min.) was five times lower in comparison with control perfusions.

Alloxan added to the perfusion medium without glucose (*Fig. 2B*) caused a rapid, persisting for four minutes release of insulin. Later the hormone secretion dropped markedly but its amount released during the perfusion with alloxan without glucose (25 to 35 min.) was over four times higher than in the corresponding time of perfusion with the basic medium (*Fig. 2A*). When alloxan was removed from the perfusion medium, the stimulation of the pancreas with glucose (16.66 mmol/l) did not result in a conspicuous response, and the quantity of the secreted hormone between 35th and 45th minute was nearly four times lower in comparison with control perfusions.

DISCUSSION

In the performed experiment it was found that alloxan added to the perfusion medium at the concentration of 4.1 mmol/l results in a rapid discharge of insulin from the rat pancreas. The effect is of short duration and occurs directly after the addition of alloxan to the medium perfusing pancreas. The release of insulin affected by alloxan is independent of the presence of glucose at 6.66 mmol/l concentration and can also occur when the sugar is

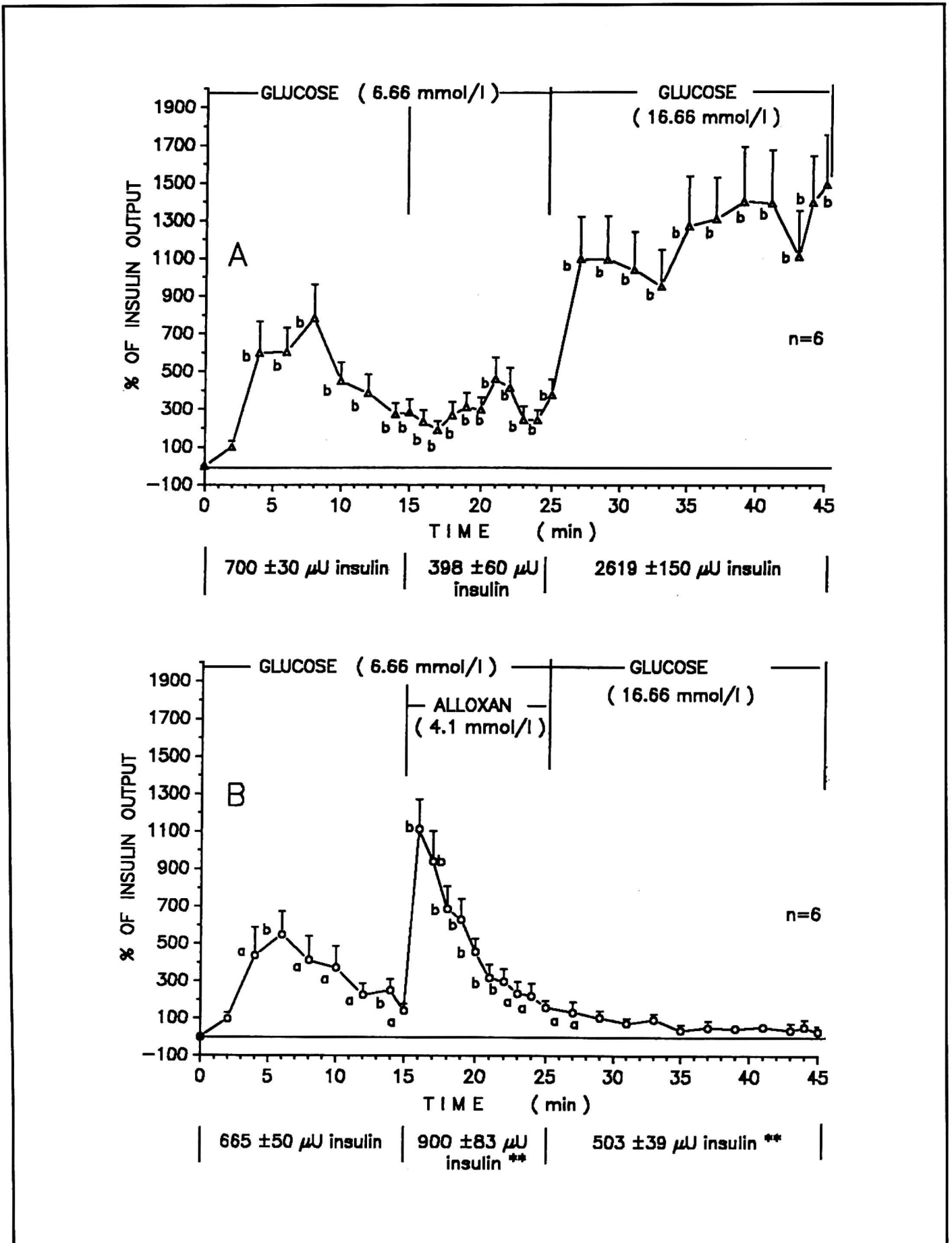


Fig. 1. Effect of perfusion of rat pancreas with alone glucose (A) or with alloxan in presence of glucose (B) on insulin secretion (mean percentage increment) of insulin concentration in perfusion medium \pm SEM). Statistically significant differences in comparison to time "0" are expressed: a — $P \leq 0.05$, b — $P \leq 0.01$. ** $P \leq 0.01$ — statistically significant differences of total quantity of insulin released in marked time intervals in comparison to adequate intervals in control perfusions (A).

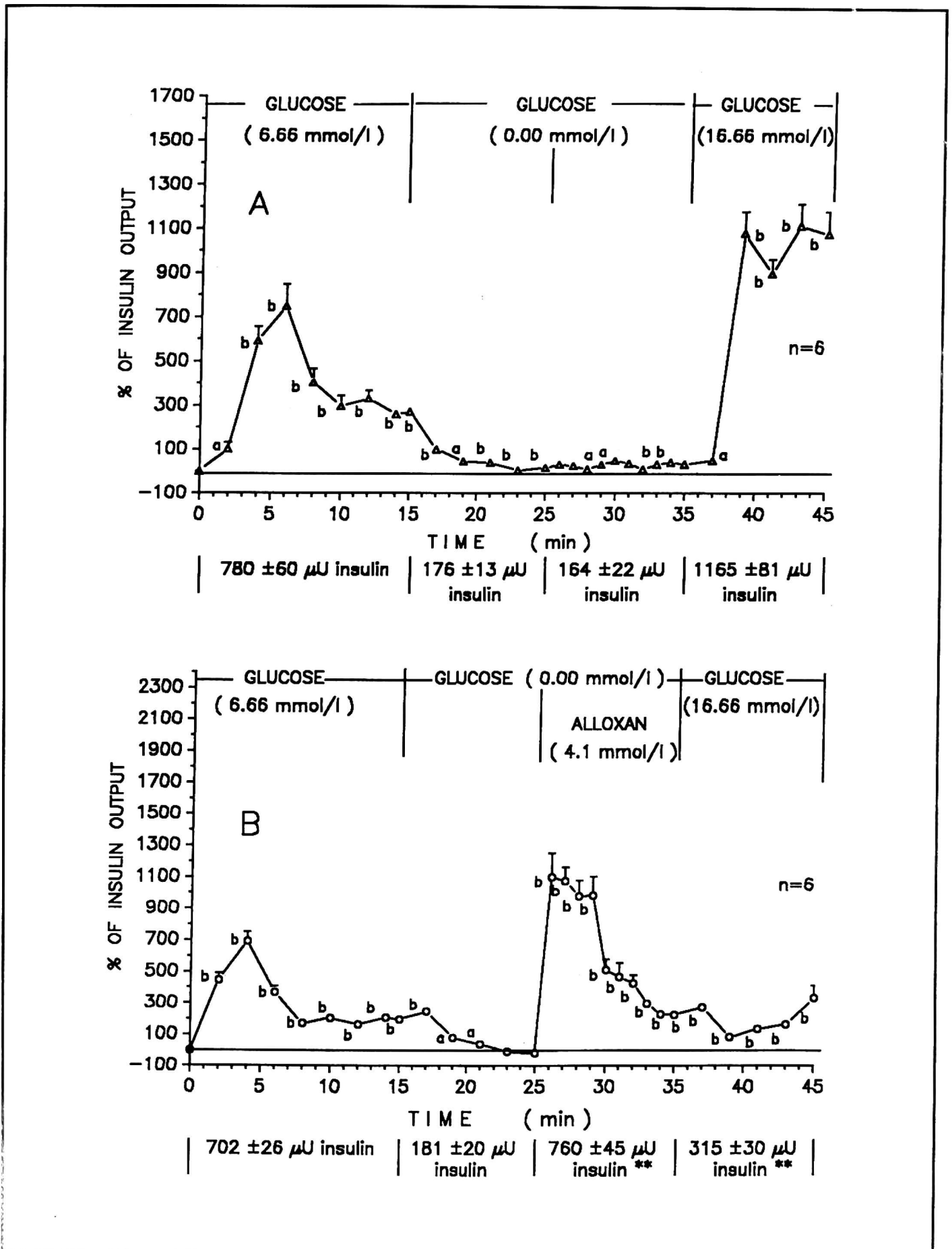


Fig. 2. Effect of perfusion of rat pancreas with alloxan without glucose in medium (B) on insulin secretion (mean percentage increment of insulin concentration in perfusion medium \pm SEM). Statistically significant differences in comparison to time "0" are expressed: a — $P \leq 0.05$, b — $P \leq 0.01$. ** $P \leq 0.01$ — statistically significant differences of total quantity of insulin released in marked time intervals in comparison to adequate intervals in control perfusions (A).

absent in the perfusion medium. This implies that alloxan alone is capable of a short-term stimulation of insulin secretion, probably by increasing calcium ion concentration in the cytosol of B cells. Dean and Matthews (14) observed that the cell membrane of pancreatic B cells undergoes depolarization in the presence of alloxan. This results in the opening of voltage-dependent calcium channels (15). High gradient of these ions maintained between the cytosol of B cells and extracellular fluid causes that even small changes in membrane permeability for Ca^{2+} may result in a considerable increase of their concentration in cytosol leading to insulin secretion (16). The influx of calcium ions into the insulin secreting cells stimulated by alloxan seems to constitute an important link in the mode of action of this drug in B cells. It was observed that in *in vivo* conditions, calcium channel antagonists (i. e. diltiazem) prevent diabetes produced in rats by alloxan even when high dose of this diabetogenic agent was administered (17).

In the present experiment, after the removal of alloxan from the perfusion medium, high level of glucose (16.66 mmol/l) did not stimulate pancreas to secrete insulin. This was probably the effect of the impairment of B cells by alloxan which can easily penetrate into their interior. In experiments with [2- ^{14}C] alloxan, it was demonstrated that B cells uptake of this compound is more considerable in comparison with the remaining pancreatic cells (2, 3). Alloxan-induced superoxide and hydroxyl radicals formation is responsible for the cytotoxic activity of this compound (5). Numerous experiments showed that glucose exhibits protective properties against alloxan cytotoxicity by preventing glucokinase inactivation (8) and leading to an increase in the concentration of reducing equivalents (9) employed to reduce the oxidized form of glutathione. This allows glutathione to take part in radical neutralization (4, 18). However, this protective action of glucose depends on its own concentration of alloxan and the time of cell exposure to action of this drug (8, 19). In the described experiment, alloxan concentration at which physiological level of glucose (6.66 mmol/l) might constitute intracellular protection against the toxicity of this compound was exceeded. Furthermore, glucose does not act as an agent limiting alloxan penetration into the pancreatic B cells (2). This explains why the response of pancreas to alloxan and later stimulation with a high level of glucose was similar in both experimental treatments (during perfusion with alloxan with and without glucose in the perfusion medium).

The rapid, short-term release of insulin caused by alloxan and the subsequent absence of stimulation response of insulin secretion by the high glucose level suggest that alloxan caused inhibition of insulin biosynthesis and the hormone released by pancreas was the insulin earlier stored in B cells. This finding is in keeping with observations made by Takasu et al. (7) showing that hydrogen peroxide formed in the result of alloxan metabolism is responsible for

the destruction of DNA structure in cells. On the other hand, activation of the DNA repair system leads to the inhibition of the proinsulin biosynthesis (20).

It can be concluded, that the effect of alloxan on insulin secretion is achieved in two stages. Initially, it is a stimulating effect — a rapid, albeit short-term secretion of the hormone is observed, presumably due to the influx of calcium ions into the cytosol of B cells. Shortly afterwards, insulin release is completely stopped and is not resumed until the end of perfusion, even during pancreas stimulation by glucose at 16.66 mmol/l concentration. This is caused by B cell destruction by alloxan.

REFERENCES

1. Dunn JS, Sheehan HL, McLethie NGB. Necrosis of islets of Langerhans produced experimentally. *Lancet* 1943; 1: 484—487.
2. Gorus FK, Malaisse WJ, Pipeleers DG. Selective uptake of alloxan by pancreatic B-cells. *Biochem J* 1982; 208: 513—515.
3. Weaver DC, McDaniel M, Lacy PE. Alloxan uptake by isolated rat islets of Langerhans. *Endocrinology* 1978; 102 (6): 1847—1855.
4. Sakurai K, Ogiso T. Inhibitory effect of glutathione on the generation of hydroxyl radicals in the reaction system of glutathione-alloxan. *Chem Pharmacol Bull* 1991; 39 (3): 737—742.
5. Zhang H, Zdolsek JM, Brunk UT. Alloxan cytotoxicity involves lysosomal damage. *APMIS* 1992; 100: 309—316.
6. Munday R. Dialuric acid autooxidation. Effect of transition metals on the reaction rate and on the generation of "active oxygen" species. *Biochem Pharmacol* 1988; 37 (3): 409—413.
7. Takasu N, Asawa T, Komiya I, Nagasawa Y, Yamada T. Alloxan-induced DNA strand breaks in pancreatic islets. Evidence for H_2O_2 as an intermediate. *J Biol Chem* 1991; 266 (4): 2112—2114.
8. Lenzen S, Mirzaie-Petri M. Inhibition of glucokinase and hexokinase from pancreatic B-cells and liver by alloxan, alloxantin, dialuric acid and t-butylhydroperoxide. *Biomed Res* 1991; 12 (5): 297—307.
9. Malaisse-Large F, Sener A, Malaisse WJ. Contrasting modes of action of D-glucose and 3-O-methyl-D-glucose as protectors of the rat pancreatic B-cells against alloxan. *Biochim Biophys Acta* 1983; 762: 36—43.
10. Korec R. Alloxan diabetes. In: Experimental diabetes mellitus in the rat. Publishing House of the Slovak Academy of Sciences, Bratislava 1967; pp. 108—135.
11. Kliber A, Szkudelski T. Changes in some metabolic parameters and insulin after alloxan treatment in rats. (in Polish) *Zeszyty Poznańskiego Towarzystwa Przyjaciół Nauk* 1994; LXXVII: 125—132.
12. Penhos JC, Wu ChH, Basabe JC, Lopez N, Wolff PW. A rat pancreas-small gut preparation for the study of intestinal factors and insulin release. *Diabetes* 1969; 18: 733—738.
13. Kliber A. The stimulatory effect of L-tryptophane on insulin secretion studied with the method in situ perfusion of rat pancreas. (in Polish) *Endrokryol Pol* 1987; 38: 309—316.
14. Dean PM, Matthews EK. The bioelectrical properties of pancreatic islet cells: Effect of diabetogenic agents. *Diabetologia* 1972; 8: 173—178.

15. Komatsu M, Yokokawa N, Takeda T, Nagasawa Y, Aizawa T, Yamada T. Pharmacological characterization of the voltage-dependent calcium channel of pancreatic B-cell. *Endocrinology* 1989; 125: 2008—2014.
16. Boyd AE, Royan AS, Gaines KL. Regulation of insulin release by calcium. In: Molecular and cellular biology of diabetes mellitus. Insulin secretion. vol. 1. New York 1989 pp. 93—105.
17. Katsumata K, Katsumata K Jr, Katsumata Y. Protective effect of diltiazem hydrochloride on the occurrence of alloxan- or streptozotocin-induced diabetes in rats. *Horm Metab Res* 1992; 24: 508—510.
18. Zhang H, Gao G, Brunk UT. Extracellular reduction of alloxan results in oxygen radical-mediated attack on plasma and lysosomal membranes. *AMPIS* 1992; 100: 317—325.
19. Tomita T, Lacy PF, Matschinsky FM, McDaniel ML. Effect of alloxan on insulin secretion in isolated rat islets perfused *in vitro*. *Diabetes* 1974; 23: 517—524.
20. Okamoto H, Yamamoto H. DNA strand breaks and poly (ADP-ribose) synthetase activation in pancreatic islets — a new aspects to development of insulin-dependent diabetes and pancreatic B-cell tumors. *Japan Sci Soc Press Tikyo/VNU Science Press, Utrecht*, 1993; 297—308.

Received: September 19, 1995

Accepted: February 15, 1996

Author's address: A. Kliber, Department of Animal Physiology and Biochemistry, Academy of Agriculture, 35 Wołyńska Str., 60-637 Poznań, Poland