EFFECT OF DILTIAZEM ON INSULIN SECRETION
I. EXPERIMENTS IN VITRO

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Accepted June 8, 1977

Abstract—Effect of diltiazem on glucose-induced insulin secretion was investigated in
the rat islets of Langerhans isolated by a collagenase digestion technique. It was
found that B-cells, main constituents of isolated islet preparations, had a well-preserved
ultrastructural appearance immediately following isolation or after incubation with
glucose or glucose and diltiazem. The islets released a large amount of insulin upon
stimulation with glucose and CaCl₂. Diltiazem (10⁻⁸-10⁻⁴ M) produced a dose-
related inhibition of glucose-induced insulin secretion and this effect was antagonized
by the increase in extracellular concentration of CaCl₂. The inhibitory effect of dil-
tiazem on the insulin secretion was also counteracted by dibutryryl-3',5'-cyclic AMP or
by theophylline. Among calcium-antagonists tested, nifedipine produced the most
powerful inhibitory action on the insulin secretion, while the effect of verapamil was
similar to or somewhat stronger than that of diltiazem. It was suggested that diltiazem
may reduce the intracellular concentration of free calcium ion, thus causing an in-
hibitory effect on the glucose-induced insulin secretion by the isolated islets of
Langerhans.

Calcium ions play an essential role in secretion of several hormones by endocrine glands
(1). It has been reported that secretion of insulin from the pancreatic B-cell by stimulation
with glucose depends upon the extracellular concentration of calcium ions (2–5). An
increase in the net uptake of calcium ions by the B-cell has also been demonstrated, when
insulin secretion was stimulated by glucose (6). It is assumed that intracellular accumulation
of calcium ions is responsible for glucose-induced insulin secretion (6, 7).

Diltiazem, a new 1,5-benzothiazepine derivative with a potent coronary vasodilating
activity (8), has been shown to have calcium-antagonistic properties in the myocardium
(9, 10) and smooth muscles (11–15). Therefore, the compound may also act on the humoral
secretion which depends on calcium ions. From this point of view, the effect of diltiazem
on glucose-induced insulin secretion was investigated using isolated islets of Langerhans
from the rat pancreas. Ultrastructures of the same materials as those used in the present
experiments were also examined.

MATERIALS AND METHODS

Isolation of islets

Islets of Langerhans were prepared from rat pancreas by the method of Lacy et al. (16,
17). Sprague-Dawley rats (330–350 g) were exsanguinated and the abdominal cavity
opened. The common bile duct was cannulated and modified Hank's solution (8 to 10 ml) was injected into the pancreas. Body and tail of the pancreas were dissected, chopped into small pieces after removing the attached adipose tissue and then washed with Hank's solution. The chopped preparations were poured into a small plastic tube and Hank's solution containing 15 mg of collagenase (Sigma Chemical Company, No. C-0130) was added to a total volume of approx. 5 ml. The tube was incubated for 15-16 min at 37°C. During incubation, the tube was shaken by hand vigorously three times for 1 min at 2 min intervals and then slowly for 30 sec at 2 min intervals. After incubation, the collagenase-treated samples were washed at least eight times with 15-20 ml of Hank's solution, suspended in 25 ml of the same solution and finally transferred into a Petri dish, shaking vigorously. Islets of Langerhans were picked up with a micropipette under a dissecting microscope. Usually, 70 to 100 islets were isolated from each pancreas.

In experiments, 5 islets were put into a plastic tube containing 2 ml of ice-cold Krebs-bicarbonate solution, which was aerated with a gas mixture of 95% O₂ and 5% CO₂. The tube was incubated for a given period at 37°C and 0.5 ml of aliquots for insulin analysis were kept overnight in an ice-box. The released insulin was measured by a radioimmunoassay technique (INSI K-3, Midori Juji) (18). The composition of modified Hank's solution included (in mM): NaCl 137.0, KCl 5.37, CaCl₂ 1.26, MgCl₂ 1.05, Na₂HPO₄ 0.42, KH₂PO₄ 0.40, MgSO₄ 0.83, NaHCO₃ 4.29, glucose 1.67. Krebs-bicarbonate solution was composed of (in mM): NaCl 119.0, KCl 4.7, CaCl₂ 1.25, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.0, Na-pyruvate 5.0, Na-glutamate 5.0, glucose 1.67. Bovine serum albumin (Fraction V, Nakarai Chemicals) was added to Krebs-bicarbonate solution (final concentration 0.2%). The concentration of CaCl₂ or glucose in Krebs-bicarbonate solution varied according to the experimental conditions.

Electronmicroscopy

Islets of Langerhans isolated after a collagenase digestion technique were prepared by double fixation in 2.5% glutaraldehyde and 1% osmium tetroxide in Millonig's buffer solution (pH 7.2), dehydrated in graded alcohol and propylene oxide, and embedded in Epon 812. Sectioning was done using an ultramicrotome, JEOL's JUM-7. Ultra-thin sections were double-stained with uranyl acetate and lead citrate. Observations were carried out using an electron microscope, JEOL's JM-100C.

Drugs

The compounds used in the present experiments were as follows; theophylline (Tokyo Kasei), N-2-O-dibutyryl-adenosine-3',5'-cyclic-monophosphate monosodium (Boehringer Manheim), diltiazem-HCl, verapamil-HCl and nifedipine. The last three compounds were synthesized in our Organic Chemistry Research Department for the present work. All compounds except nifedipine were dissolved in Krebs-bicarbonate buffer solution. Nifedipine was finely suspended in the buffer solution with a droplet of Tween-80 (Wako Pure Chemicals). It was ascertained that this amount of Tween-80 did not influence the glucose-induced insulin secretion.
Statistical analysis

The statistical analysis was based on a group comparison between control and experimental data, using Student's t test. Standard error of mean value relative to the control was estimated by applying Fieller's equation (19, 20) (in Figs. 5 and 7, and in experiments with various Ca-antagonists).

RESULTS

Electron microscopic findings on the preparation of isolated islets

In islets fixed immediately after isolation by collagenase digestion, B-cells were the most common constituents, and A-cells were rarely observed. The ultrastructure of the B-cells appeared normal without evidence of swelling of mitochondria, dilation of the ergastoplasm or disruption of the plasma membrane. Essentially the same findings were obtained in islets incubated with diltiazem (Fig. 1).

Insulin secretion from islets

Effect of incubation time: Fig. 2 shows the time course for insulin secretion from isolated islets by stimulation with either 1.7 or 16.7 mM glucose. Islets were incubated for a period of 45, 90 and 120 min in the presence of 1.25 mM CaCl₂. As shown in Fig. 2, the insulin secretion stimulated by 1.7 mM glucose was only 28.4 ± 3.2 μU/islet after 120 min incubation. In this case, the basal insulin secretion was 0.9 ± 0.62 μU/islet. When the islets were incubated with 16.7 mM glucose, a significant increase in insulin secretion was observed in proportion to the incubation time. That is, the secretion of insulin, which was only 0.98 ± 0.44 μU/islet at the beginning of experiments, increased to 78.8 ± 16.9, 156.6 ± 10.0 and 193.1 ± 10.4 μU/islet after incubation for 45, 90 and 120 min, respectively.

Effect of glucose: Fig. 3-A represents the effect of glucose on insulin secretion, measured after 90 min incubation. The concentration of CaCl₂ was kept at 1.25 mM. Glucose at
FIG. 2. The time course of insulin secretion. Concentrations of glucose were 1.7 mM (---) and 16.7 mM (•---•). Each point is the mean ± SE from 10–12 experiments. IRI: immunoreactive insulin.

FIG. 3. Effects of glucose and CaCl₂ on the insulin secretion from isolated islets. Incubation period is 90 min. In experiments with glucose (Fig. 3-A), the concentration of CaCl₂ was kept at 1.25 mM. When the effect of CaCl₂ was examined (Fig. 3-B), insulin secretion was stimulated by 16.7 mM glucose. Each point is the mean ± SE from 10–15 experiments.

A concentration of 5.6 mM or below produced a slight increase in insulin secretion (20 ± 4.2 and 31 ± 2.9 μU/islet for 1.7 and 5.6 mM glucose, respectively), while the insulin secretion considerably increased when the concentration of glucose was elevated to 11.1 mM (128.0 ± 9.5 μU/islet). In the presence of 16.7 and 27.8 mM glucose, insulin secretion was 156.0 ± 10.0 and 174.0 ± 15.6 μU/islet, respectively. The result indicates that the islets of Langerhans from rat are highly responsive to glucose at a concentration over 5.6 mM and that a significant amount of insulin is thus released.

Effect of CaCl₂: The effect of CaCl₂ on insulin secretion was examined by stimulation with 16.7 mM glucose. Extracellular concentration of CaCl₂ was changed from 0.125 to 3.75 mM and the release of insulin was estimated after 90 min incubation. As illustrated in Fig. 3-B, the glucose-induced insulin secretion was increased by increasing extracellular concentration of CaCl₂; the secretion of insulin was 59.9 ± 4.0, 122.2 ± 11.4, 168.1 ± 15.4 and...
179.6±15.4 μU/islet in the presence of 0.125, 0.41, 1.25 and 3.75 mM CaCl₂, respectively.

The present results demonstrated that the islets of Langerhans isolated from rat pancreas by means of the collagenase digestion technique are so active that a large amount of insulin can be released when the cells are stimulated with glucose and CaCl₂. It was also evident that the glucose-induced insulin secretion in the presence of CaCl₂ increased in proportion to the incubation time. In the following experiments, the effects of diltiazem and other calcium-antagonists on the insulin secretion were investigated by stimulation with 16.7 mM glucose in the presence of 1.25 mM CaCl₂. The islets were incubated for 90 min.

Effect of diltiazem on the glucose-induced insulin secretion

Diltiazem at a concentration of 10⁻⁶ M or higher caused a dose-related decrease in insulin secretion of isolated islets. The results are shown in Fig. 4. A lower concentration of diltiazem (10⁻⁷ M) produced no significant influence on secretion of insulin. At the concentration of 10⁻⁶ M, diltiazem reduced the insulin secretion by 19.4% of the control (P<0.05). When the concentration of diltiazem was increased to 10⁻⁵ M or 10⁻⁴ M, the insulin secretion was suppressed by 51.9 or 83.1%, respectively.

To determine whether or not the inhibitory action of diltiazem on the glucose-induced secretion depends upon the extracellular concentrations of calcium ions, experiments were carried out with various concentrations of CaCl₂ in the presence of 16.7 mM glucose. As illustrated in Fig. 5, inhibition of insulin secretion caused by 10⁻⁶ M diltiazem was 34.4% of the control when the extracellular concentration of CaCl₂ was 0.41 mM, whereas it was diminished by 20.5% in the presence of 1.25 mM CaCl₂. When the concentration of CaCl₂ was increased to 3.75 mM, 10⁻⁶ M diltiazem exhibited no significant inhibitory effect (inhibition: 6.7% of the control, P>0.10). In the presence of 10⁻⁵ M diltiazem, the insulin secretion was reduced by 60, 52 and 33% of the control in the presence of 0.41, 1.25 and 3.75 mM CaCl₂, respectively. Thus, the inhibitory effect of diltiazem on the glucose-induced insulin secretion was diminished as the extracellular concentration of calcium ion was increased.
Effect of diltiazem on glucose-induced insulin secretion in the presence of dibutyryl cyclic AMP (db-cAMP) or theophylline: Since cyclic AMP and theophylline potentiate the insulin secretion induced by glucose (21-23), the effect of diltiazem on the glucose-induced insulin secretion was investigated in the presence of db-cyclic AMP or theophylline. As illustrated in Fig. 6, an incubation of islets with 0.4 mM db-cyclic AMP for 90 min caused a 1.5 fold increase in insulin secretion in response to 16.7 mM glucose (CaCl$_2$ 1.25 mM). Diltiazem at the concentration of 10$^{-5}$ M reduced the glucose-induced insulin secretion by approx. 30% of the control in the presence of db-cyclic AMP, whereas the inhibition was more marked without db-cyclic AMP, being approx. 55% of the control. Butyric acid itself (0.8 mM) produced no significant influence either on the glucose-induced insulin secretion or on the inhibitory action of diltiazem.

Theophylline also increased the insulin secretion evoked by glucose; the secretion was accelerated by 1.7 times after incubation with 1.4 mM theophylline. As was the case with db-cyclic AMP, the inhibitory effect of diltiazem on the glucose-induced insulin secretion was considerably lowered when theophylline was present. Fig. 7 shows that 10$^{-6}$ M diltiazem caused no significant influence on the insulin secretion in the presence of theophylline (1.4 mM), while diltiazem at the same concentration reduced the secretion by approx. 20% of the control when the islets were incubated without theophylline. Diltiazem at the concentration of 10$^{-5}$ M decreased the insulin secretion by approx. 10 and 50% of the control with and without theophylline, respectively. Even at a higher concentration (10$^{-4}$ M), diltiazem elicited only a 30% reduction of secretion in the presence of theophylline. On the contrary, 10$^{-4}$ M diltiazem without theophylline decreased insulin secretion by approx. 80%.

Comparison of effects between diltiazem and other calcium-antagonists: The effect of 10$^{-6}$ M diltiazem on the glucose-induced insulin secretion was compared with that of calcium-antagonists such as verapamil (11, 24-26) or nifedipine (11, 27, 28) at the same concentration. Islets were incubated with the test compound for 90 min in the presence of 1.25 mM CaCl$_2$.  

![Fig. 6. Effect of diltiazem on glucose-induced insulin secretion in the presence of dibutyryl cyclic AMP (db-cAMP, 0.4 mM). Each column is the mean±SE from 10 experiments. Glu: glucose, Dil: diltiazem.](image)

![Fig. 7. Inhibitory effects of diltiazem on glucose-induced insulin secretion with and without theophylline (1.4 mM). Insulin secretion was stimulated by 16.7 mM glucose. Each point is the mean ± SE from 10-12 experiments.](image)
Diltiazem reduced the release of insulin to 78±7% of the control, while verapamil and nifedipine decreased it to 69±6 and 31±5% of the control, respectively (N=10). Thus, nifedipine produced the most potent inhibitory effect on insulin secretion, whereas the effect of verapamil was similar to, or somewhat stronger than that of diltiazem.

**DISCUSSION**

Insulin secretion by pancreatic B-cells *in vitro* is accelerated by increasing the concentration of glucose (29, 30) and glucose-induced insulin secretion depends upon the extracellular concentration of calcium ion (2–7). In the present experiments, it was demonstrated that the islets isolated by the collagenase digestion method released a large amount of insulin in response to glucose and calcium ion. In addition, the electron microscopic study revealed that B-cells were the main constituents of the islets and such exhibited a well-preserved fine structural appearance.

The role of calcium ion in the glucose-induced insulin release has not been fully elucidated. Malaisse (31) postulated that glucose elevates intracellular concentration of free calcium ion by accelerating the net uptake of calcium ion and thus the microtubular-microfilamentous system is activated. As a result, insulin secretion of islets may be evoked.

As demonstrated in the present experiments, diltiazem, which is known to have calcium-antagonistic properties in the myocardium (9, 10) and smooth muscles (11–15), caused a dose-related inhibition of the glucose-induced insulin secretion from isolated islets. A similar effect of diltiazem has also been reported by Ishibashi et al. (32) who applied the perifusion technique to estimate the insulin release of isolated rat islets of Langerhans. They found that the inhibition of insulin secretion caused by diltiazem was not affected by extracellular concentration of calcium ion. The present results, however, clearly demonstrated that the inhibitory effect of diltiazem on the glucose-induced insulin secretion was manifested at a lower concentration of calcium ion and that the effect was reduced as the concentration of calcium ion was increased. Such findings indicate that diltiazem antagonizes calcium ion in the glucose-induced insulin secretion from isolated rat islets. Since the glucose-induced insulin secretion is assumed to be due to an increase in the intracellular concentration of free calcium ion (31), it is inferred that diltiazem may reduce the free calcium ion concentration in the B-cell, thus causing an inhibition of the release of insulin. It has been suggested that in islets of Langerhans, verapamil inhibits the glucose-induced insulin secretion by interfering with the transmembrane influx of calcium ion (26, 33). As shown in the present experiments, among the calcium-antagonists tested, nifedipine (11, 27, 28) produced the most potent inhibitory effect on insulin secretion, while the effect of verapamil was similar to, or somewhat more potent than that of diltiazem.

It has been reported that db-cyclic AMP or theophylline, which in itself has practically no effect on insulin secretion, potentiates the glucose-induced insulin secretion by increasing the intracellular level of cyclic AMP (21–23). As to the mechanism, Malaisse et al. suggested that the elevation of intracellular cyclic AMP level provokes an intracellular translocation of calcium from an organelle-bound pool into the cytosol, and such a shift of
calcium ion potentiates insulin secretion stimulated by glucose (23, 31). The present experiments show that the inhibitory effect of diltiazem on the glucose-induced insulin secretion was attenuated in the presence of db-cyclic AMP or theophylline. The results may be explained by assuming that the calcium-antagonistic action of diltiazem may be counteracted by increasing the concentration of intracellular calcium ion caused by db-cyclic AMP or theophylline.

Acknowledgements: We wish to thank Prof. Emeritus H. Kumagai, University of Tokyo and Dr. K. Abe, Director of Biological and Chemical Research Laboratories, Tanabe Seiyaku Co., Ltd., for pertinent advice and encouragement. Thanks are also due to Dr. A. Okaniwa and Mr. A. Yasojima for electron microscopic examinations.

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