A New Approach to Kinetic Analysis of Glucose-Induced Insulin Release by Hill's Equation

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Synopsis

Kinetic analyses of glucose-induced insulin release from the perfused rat pancreas and the perfused rat islets were performed by Lineweaver-Burk's and Hill's equations. All dose-response curves tested here were sigmoid with K_m values of 4-5 mM and 8-9 mM glucose, respectively in a K^+ -depleted and the normal media in the perfusion system, and with a K_m value of 8-9 mM glucose under either a square-wave or a slow-rise stimulation in the perfusion system. Each Lineweaver-Burk plot was represented as a parabolic profile and therefore each insulin secretory response seem to be n reaction orders ($n > 1$) for perfusion and the perfusion with the normal medium, except for the perfusion with the K^+ -depleted medium. All Hill plots for the rate of insulin release from the perfused pancreas and perfused islets under all experimental conditions were demonstrated as straight lines with various slopes.

Each kinetic constant, *e.g.*, K_m (Michaelis constant), K (equilibrium constant) and n (Hill's constant or reaction order) was calculated from Hill's equation applied to the insulin secretory response, and particularly each K_m value was very similar to that described above. It was concluded that Hill's method seemed to be available for the kinetic analysis of the rate of glucose-induced insulin release.

Although D-glucose has been well known to be an important nutritional substance and a major physiological stimulus for insulin release, it still remains unsolved whether glucose-induced insulin release from the pancreatic B cell is fundamentally due to metabolism of glucose molecule (Coore and Randle, 1964) or glucoreceptor mechanism (Niki *et al.*, 1974; Grodsky *et al.*, 1974).

Recent studies have shown that α anomer of D-glucose predominantly stimulated insulin release as compared with β anomer of D-glucose, although the former was incorporated to B cell and metabolized less than the latter (Matschinsky *et al.*, 1975),

and it was also demonstrated that the protection of the α anomer against the diabetogenic action of alloxan was more effective than the β anomer (Rossini *et al.*, 1974). Such specific effects of the α anomer as described above seem to confirm that the pancreatic B cell contains glucoreceptors directly controlling insulin release. However, the anomeric specificity of the α anomer of D-glucose may not yet fully elucidate the character of glucoreceptor, since some differences in binding manner or affinity between α and β anomers to glucoreceptor have been suggested (Kobayashi *et al.*, 1976).

In order to investigate, further, the glucoreceptor mechanism involved in insulin release and, moreover, to make the charac-

teristics of insulin release stimulated with glucose clear, the kinetic analysis on the dynamics of insulin release induced with glucose under various stimulations containing square-wave and slow-rise systems will be needed.

Currently, although the classical Lineweaver-Burk analysis was applied to the interrelationship between the rate of insulin release and D-glucose or L-arginine (Gerich *et al.*, 1974), it does not seem to give sufficient information about the glucoreceptor mechanism, because the Lineweaver-Burk plot for the rate of insulin release was not a linear but a parabolic profile under the most of stimulation system.

Now, we applied Hill's equation (Gutfreund, 1972) to kinetic analysis of the insulin secretory response from the rat perfused pancreas and perfused islets, which seemed to make possible calculation of the K_m value and the affinity for various stimuli such as D-glucose, its anomers and L-arginine. Although the transmission mechanisms of the insulin releasing signal in B cell have not been clarified, this analysis is based on a simple relationship between the amounts of insulin as a biological response from the B cells and the glucose concentrations, that the rate of insulin release will be proportional to the concentration of "receptor-stimulating substance complex".

We would like to report here that Hill's equation was applicable to the kinetic analysis of the rate of glucose-induced insulin release under either a square-wave or a slow-rise stimulation.

Materials and Methods

Reagents: D-glucose, theophylline and dextran were products of Wako Pure Chemical Industries Ltd., Japan. Bovine serum albumin (fraction V) was purchased from The Armour Laboratories, USA and collagenase from Sigma Chemical Co., USA. Standard pork, 125 I-pork insulin, anti-insulin guinea

pig serum and anti- γ -G-sheep serum were purchased from Dainabot Radioisotope Laboratories Ltd., Japan.

Perfusion system: Male Wistar rats made to fast overnight, weighing 120–200 g, served as a donor of pancreas. In brief, removal of pancreas was performed by the method of Grodsky (Grodsky *et al.*, 1963). The ionic compositions of "normal medium" are as follows (mM): Na^+ 142.6, K^+ 6.2, Mg^{++} 1.2, Cl^- 131.6, HCO_3^- 18.6, H_2PO_4^- 1.2 and SO_4^{--} 1.2, K^+ -free media were prepared by substituting KCl and KH_2PO_4 by equivalent amounts of NaCl and NaH_2PO_4 . Dextran (4%) was supplemented in the perfusate to provide an osmotic pressure similar to the serum, and the medium was equilibrated with a mixed gas containing O_2 and CO_2 (95:5, v/v) to maintain pH at 7.35 and the average flow rate of the perfusate was 1.0 ml/min. Two series of experiments were performed under following conditions; (1) in the absence of K^+ : during the 10-min control period the perfusions were carried out with a K^+ - and Ca^{++} -free medium containing glucose at various levels and then the perfusions were performed with a medium during the 10-min period in which Ca^{++} was elevated to 1.3 mM.

(2) in the presence of K^+ 6.2 mM: during the 10-min control period the preperfusions were carried out with a Ca^{++} -depleted medium containing glucose at various levels and then the perfusions were performed with a medium during 10-min period in which Ca^{++} was elevated to 1.3 mM. The perfusates were collected by a fraction collector at 1-min intervals.

Perifusion system: Pancreatic islets of Langerhans were isolated from male Wistar rats made to fast overnight, weighing 120–200 g by the method of Lacy and Kostianovsky (Lacy and Kostianovsky, 1967). The medium used in all experiments was Krebs Henseleit bicarbonate buffer (pH 7.35), supplemented with 0.5% bovine serum albumin and 1 mM theophylline, equilibrated with mixed gas described above. One hundred and twenty islets collected from three rats were perfused in a plastic flow cell maintained at constant temperature (37°C) and the average flow rate of the perfusate was 0.5 ml/min. During the 30-min control period each perifusion system was equilibrated with a medium containing 2.8 mM glucose and then the perfusions were carried out under a square-wave stimulation with glucose at various levels or a slow-rise stimulation by glucose during the period of 150-min. The perfusate was collected by a fraction collector at 2-min intervals.

Measurement of insulin and glucose: Insulin (immunoreactive insulin, IRI) and glucose contents from the same effluent fraction were measured by a double-antibody radioimmunoassay and Glucose Analyser (ERA 2001, Beckman Instrument, INC., USA) respectively.

Calculations: All calculations were performed using a digital computer (Canola F-20, Canon INC., Japan).

Lineweaver-Burk's and Hill's equations for the rates of insulin release with glucose: Assuming that insulin is released through the glucoreceptor mechanisms and then the rate of insulin release is directly proportional to the concentration of "receptor-stimulating substance complex", formulas as shown below are derived.

$$K = \frac{(S^nR)}{(S)^n(R)} \quad (2)$$

$$v = k(S^nR) \quad (3)$$

$$V_m = k[(S^nR) + (R)] \quad (4)$$

K: equilibrium constant

v: rate of insulin release

V_m: maximal rate of insulin release

(S): concentration of stimulating substance

(R): concentration of receptor

(SⁿR): concentration of "receptor-stimulating substance-complex"

k: kinetic rate constant

n: reaction order (Hill's constant)

In the case that the insulin secretory reaction is that first order, Michaelis-Menten type equation (5) is introduced from the equations (2), (3) and (4).

$$\frac{1}{v} = \frac{1}{V_m} \left[1 + \frac{1}{K(S)} \right] \quad (5)$$

If K_m equals the stimulating substance concentration at which the velocity of the first reaction is half maximal (i.e., (R)=(SⁿR)), we can obtain the following equation (Lineweaver-Burk's equation) (6) from the equation (5).

$$\frac{1}{v} = \frac{1}{V_m} \left[1 + \frac{K_m}{(S)} \right] \quad (6)$$

In the case that the insulin secretory reaction is n order, the equation (5) is represented as the equation (7).

$$\frac{1}{v} = \frac{1}{V_m} \left[1 + \frac{1}{K(S)^n} \right] \quad (7)$$

Since the equation (7) is not a linear but a parabolic line, it cannot give valuable information on kinetic analysis of insulin release. Therefore, we can obtain Hill's equation (8) simply by taking the logarithms of both sides of the equation (7).

$$\log \frac{v}{V_m - v} = n \log (S) + \log K \quad (8)$$

If K_m equals the stimulating substance concentration at which the velocity is half maximal, K_m is obtained from the intercept on the abscissa of Hill plots or the calculation by the equation (9).

$$K_m = 10^{-\frac{\log K}{n}} \quad (9)$$

Results

Kinetics of insulin release from the perfused rat pancreas under a square-wave stimulation by glucose

The dose-response curve from the rat pancreas performed with the normal medium as shown in Fig. 1 was sigmoid with a K_m of 8–9 mM glucose, and showed that the threshold of glucose was around 2.7 mM and insulin release reached a plateau (98 ± 10 μU/ml/min, N=4) at about 16.7 mM glucose. On the other hand, the amounts of insulin released in a K⁺-depleted medium from the perfused rat pancreas were much

Fig. 1. Dose-response curves for the secretory rates of insulin released from the perfused rat pancreas. The rates of insulin release were obtained from the perfused rat pancreas under square-wave glucose stimulations in K⁺-depleted (●—●, N=2–11 pancreases) and 6.2 mM K⁺ (○—○, N=3–6 pancreases) media. The results were indicated as the mean ± S.E.M. of N experiments.

larger at each glucose concentration (256 ± 9 $\mu\text{U/ml/min}$, $N=3$ at 16.7 mM glucose) than those in the normal medium.

As illustrated in Fig. 1 the dose-response curve was sigmoid with a K_m of 4–5 mM glucose, which was a glucose level lower than that in the normal medium described above.

Each Lineweaver-Burk plot was represented as a parabolic and a straight line (correlation coefficient, $r=0.98$), respectively for the perfusion with the normal and the K^+ -depleted media, and each maximal rate of insulin release calculated from the intercept on the abscissa was 118 $\mu\text{U/ml/min}$ in the normal medium and 333 $\mu\text{U/ml/min}$ in a K^+ -depleted medium, respectively (Fig. 2).

However, Hill plots for the insulin release to glucose were illustrated as straight lines ($r=0.97$) with different slopes in both media and the slope represented as a Hill's constant was a smaller value ($n=1.2$) in a K^+ -depleted medium as compared to that in the normal medium (Fig. 3).

Kinetics of insulin release from the perfused rat islets under a square-wave and slow-rise stimulation by glucose

The mean rates of the insulin release per 2 min during the 150-min period under a square-wave stimulation with glucose at 2.8, 5.6, 11.1, 16.7 and 27.8 mM were determined.

The dose response relationship was indicated as a sigmoid curve reaching the maximum at about 11.1 mM glucose and a K_m was 7–8 mM glucose as shown in Fig. 4 and Table 1. On the other hand, the dynamics of insulin release were also determined under the slow-rise stimulation by glucose linearly increased at a changing rate with 0.1 mM/min from 2.8 mM glucose. As illustrated in Fig. 4, the dose-response relationship obtained under a slow-rise glucose stimulation was also a sigmoid curve with a K_m of 7–8 mM glucose and

Fig. 2. Lineweaver-Burk plots for the secretory rates of insulin released from the perfused rat pancreas. Each point was calculated from the dose-response curves as shown in Fig. 1 and indicated as the mean $\pm$ S.E.M. of N experiments.

Fig. 3. Hill plots for the secretory rates of insulin release from the perfused rat pancreas. Each point was calculated from the dose-response curves as shown in Fig. 1 and indicated as the mean $\pm$ S.E.M. of N experiments.

with a tendency to plateau around 11.1 mM of glucose.

Lineweaver-Burk plots for the rate of insulin release from the perfused islets under such stimulation as a square-wave and a slow-rise manner were represented in either case as parabolic functions as illustrated in Fig. 5 and each maximum rate of insulin release by the extrapolating method was the same value as 0.8 $\mu\text{U/ml/islet/min}$. Each Hill plot for the rate of insulin release was represented as a straight line ($\gamma=0.97$) as shown in Fig. 6 and each slope represented as Hill's constant(n) was 3.1 and 6.2, respectively for the perfusion under square-wave and slow-rise manners. Each kinetic constant calculated by Hill's equation was demonstrated in Table 1.

Table 1. Several constants from analysis of Hill's equation

stimulation system	n	log K (mM ⁻ⁿ)	Km (mM)		N
			calcd.	observed	
perfusion					
square-wave	3.1	-2.8	7.9	8-9	(3)
slow-rise	6.2±0.6	-5.4±0.6	7.3±0.5	8-9	6
perfusion					
K ⁺ 6.2 mM	2.4	-2.3	8.9	8-9	(3-6)
K ⁺ 0 mM	1.2	-0.9	5.5	4-5	(2-11)

Each constant represents the values calculated by Hill's equation using the mean insulin secretory rates of N experiments shown in the parentheses (Fig. 1 and Fig. 4) except a slow-rise perfusion, in which the constants are shown as the mean±S.E.M. of 6 experiments.

Fig. 4. Dose-response curves for the secretory rates of insulin released from the perfused rat islets. The rates of insulin release were obtained from the perfused rat islets under square-wave (○.....○, N=3) and slow-rise (●.....●, N=6) glucose stimulations. Each point represents the mean value of N experiments.

Fig. 5. Lineweaver-Burk plots for the secretory rates of insulin released from the perfused rat islets. Each point was calculated from the dose-response curves as shown in Fig. 4 and represents the mean value of N experiments (○.....○, square-wave; ●.....●, slow-rise).

Fig. 6. Hill plots for the secretory rates of insulin released from the perfused rat islets. Each point was calculated from the dose-response curves as shown in Fig. 4 and represents as the mean values of N experiments ($\circ$ $\circ$, square-wave; $\bullet$ — $\bullet$, slow-rise).

Discussion

Since the Lineweaver-Burk plot for the rate of insulin release was represented as a straight line and Hill's constant was 1.2 in the perfused system under the square-wave stimulation by glucose in the K^+ -depleted medium, this insulin secretory response seemed to be about the first order reaction.

On the other hand, the Lineweaver-Burk plot in the square-wave or slow-rise perfused system was represented as a parabolic profile in either case and each Hill's constant was calculated as 3.1 and 6.2 respectively. Therefore, it was suggested that those insulin secretory response to glucose may be n order secretory reaction greater than the first order one.

Because Hill plots for the rate of insulin release in all experiments were represented as straight lines with high correlation coefficients ($r > 0.97$) and particularly, each K_m value calculated from Hill's equation agreed very closely with that observed from those dose-response curves, Hill's equation seemed to be applicable to

the kinetic analysis of the rates of glucose-induced insulin release from pancreatic B cells.

When each kinetic constant from Hill's equation was compared with two kinds of stimulation of perfused systems containing square-wave and slow-rise manners each value of n (6.2) and $\log K$ (-5.4 mM^{-n}) in the slow-rise system was respectively larger and smaller than that ($n=2.4-3.1$, $\log K = -2.3$ — -2.8 mM^{-n}) in the square-wave system. These results showed that the values of n decreased, while the $\log K$ increased in contrary under such a rapid elevation of glucose level as a square-wave stimulation as compared with those under a slow-rise stimulation.

It has been known that a rapid change of the glucose level caused high depolarization on the B cell membranes (Dean and Matthews, 1970), and also it has been suggested to depolarize more weakly under the slowrise stimulation system than under the square-wave stimulation system since the first phase of insulin release could not be observed at the dynamics of insulin release under a slow-rise stimulation by glucose (Curry, 1971). Furthermore, it has been reported that B cell membranes were depolarized by changing from 6.2 mM to a higher or lower K^+ concentration (Dean and Matthews, 1971).

The mechanism of insulin release from the pancreatic B cells due to depolarization might be elucidated by the following concepts; depolarization on pancreatic B cell membranes evokes a rapid influx of extracellular Na^+ , which leads to accumulation of intracellular Na^+ and Na^+/Ca^{++} pump is activated simultaneously, and then accumulation of intracellular Ca^{++} stimulates insulin release by promotion of microtubulus-microfilamentous system (Henquin and Lambert, 1974). The present results which showed the maximal rate of insulin release shown as a value of $256 \pm 9 \mu\text{U/ml/min}$ in the K^+ -depleted medium and as a value of $98 \pm 10 \mu\text{U/ml/min}$ in the normal medium seemed

to support the mechanism of insulin release described above.

Consequently, it was suggested that the variation of n value correlates with the intensity of depolarization on the B cell membranes. The value of $\log K$ indicating the affinity of glucoreceptor to a stimulus became larger in a condition which decreased the n value, and the n value has been known to represent the reaction order together with the intensity of allosteric interactions of the glucoreceptor (Gutfreund, 1974). Therefore, it seemed that the intensity of interaction decreased according to the increase in depolarization on B cell membranes, and at this time the insulin release was more enhanced by depolarization and increase in affinity.

Additionally, since the kinetic analysis was performed by insulin and glucose contents measured with the same effluent fraction, and moreover an absolute change rate of the glucose level raised from 0.10 to 0.22 mM/min did not influence the sigmoidal dose response curve of insulin release and the kinetic constants, the slow-rise glucose seems to stimulate correspondingly insulin release without any delay (Kobayashi *et al.*, 1977).

In conclusion, the present study characterizes a new approach to kinetic analysis of the rate of insulin release induced with glucose from the perfused rat pancreas and the perfused rat islets under various stimulation systems and is in progress in order to clarify an intrinsic significance of K_m , n and $\log K$, and an interrelationship between them.

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