The Kinetics of β -Granule Formation: A Morphometric Study

P.M. Dean*

Dept. of Pharmacology, Univ. of Cambridge, Medical School, Cambridge, England

Summary. The rate of formation of β -granules in mouse islet cells has been studied by morphometry using experimental conditions in which insulin secretion is known to be blocked. The number of β -granules in a cell is increased by 120%, 45 minutes after a continued glucose stimulus of 16.7 mM. The implications of the results are discussed with respect to the construction of kinetic models for the secretion of insulin by exocytosis.

Key words: B-cell morphometry, kinetics of β -granule formation, exocytosis models, D-glucose-induced granule formation.

Evidence from electronmicrographs of islet B-cells suggests that insulin is secreted from granular stores by exocytosis, although the biochemical evidence for exocytosis in B-cells is not yet as complete as for that in the chromaffin cell [22]; there may be some insulin-release by intracellular granulolysis [5]. By analogy with neurotransmitter release from nerve terminals, exocytosis of insulin from islet cells is likely to be a stochastic process, that is an increase in the rate of release of discrete quantities of insulin. Therefore any attempt to formulate an accurate stochastic model describing the kinetics of insulin secretion must be based on values for the numbers of granules available for release and the numerical rate of formation of granules. The number of granules in islet cells can be determined by ultra-structural morphometry from electron micrographs of cell sections. Thus, if an experimental procedure can be designed such that insulin secretion is stopped completely whilst insulin syn-

thesis remains unaffected, it should be possible to measure, at least initially, the rate of formation of β -granules to different biosynthetic stimuli. D-glucose induces both insulin secretion and insulin biosynthesis [3, 4, 10, 15], whereas adrenaline blocks secretion to a glucose stimulus without appreciably affecting glucose oxidation or insulin biosynthesis [1, 4, 16]. Electron micrographs taken from cells incubated in different glucose concentrations in the presence of adrenaline could be used for determining by morphometry changes in particle synthetic rate. The rationale assumes that adrenaline does not affect the incorporation of insulin precursors into the β -granule – an assumption which may not be wholly justified.

Material and Methods

The procedure for evaluating quantitatively the ultrastructure of islet cells has been described in detail previously [6]. Islets were isolated by microdissection from mouse pancreas and incubated for one hour in Krebs-Henseleit solution containing 2.8 mM D-glucose (ref. [6] for details). They were then superfused with Krebs solution containing 1 μ M adrenaline for 5 minutes before the solutions were changed for either Krebs solution containing 1 μ M adrenaline without D-glucose or Krebs solution containing 1 μ M adrenaline and 16.7 mM glucose. All perfusing solutions contained sodium pyruvate 4.9 mM, sodium fumarate 2.7 mM and sodium glutamate 4.9 mM as alternative energy sources in the presence or the absence of glucose. The islets were subsequently superfused for varying times up to one hour (10 minutes intervals) and fixed in 2% glutaraldehyde buffered with sodium phosphate 0.15 M at pH 7.4 for 18 hours; the buffer solution contained 1 μ M adrenaline. Tissues were then post fixed with osmic acid 1% for 2 hours, em-

* Beit Memorial Research Fellow

bedded in epon, sectioned and stained for electron microscopy. Six or more islets were used for sectioning at each time interval and glucose concentration. Electron micrographs were taken randomly from stained sections and the granule numbers counted in an area of 225 cm^2 on micrographs at a final magnification of 29040. Random sampling of the microscopic field was achieved by using a specified corner of the copper grid as a reference system for each micrograph [21]. The numerical density N_{Vi} of β -granules/ cm^3 was calculated from equation:

$$N_{Vi} = N_{Ai}/D \quad \text{Eq. 1}$$

where N_{Ai} is the number of profiles/ cm^2 of islet cell section and D is the diameter of the β -granule – 289 nm [6]. The results were then expressed as numbers of β -granules per B-cell volume of $1434 \text{ }\mu\text{m}^3$ [6]. The mean diameter of β -granules was obtained by a Wicksell transformation of measured profile diameters; cell volumes were previously obtained by point counting morphometry [6].

The criterion for deciding whether a profile represented a section through a β -granule was taken to be an approximately circular profile containing at least the remnants of an electron dense material, but not clear profiles. It is appreciated that not all section planes will pass through the central core and such profiles would be difficult to distinguish from the vacuolated areas of smooth endoplasmic reticulum. Therefore it is expected that the estimated granule numerical density will be less than the real value, although this would not affect the calculation of granule synthetic rate.

Results

The changes in particle numbers morphometrically estimated during incubation in the absence and in the presence of 16.7 mM D-glucose are shown in Fig. 1. Applying a two-tailed 't' test to the results from 10 minute incubation onwards showed significant differences ($P < 0.05$) between granule counts for the two incubation conditions. The smooth curves were fitted by a weighted least-squares polynomial approximation to the data points by Forsythe's [9] method using an orthogonal quadratic equation, where the weighting factor is taken to be the reciprocal $(\text{SEM})^2$. The equation is of the form:

$$N = a_0 + a_1t + a_2t^2 \quad \text{Eq. 2}$$

where N is the total granule number per cell and t is the time in minutes. The values of the coefficients are given in Table 1. Since there was appreciable particle synthesis on changing from the preincubation con-

centration of 2.8 mM glucose to zero glucose, it is quite likely that the curve for 16.7 mM glucose is a compounded curve and does not reflect exactly the effect of 16.7 mM glucose alone on inducing β -granule synthesis, which could be found by subtraction (broken line Fig. 1). Equation 2 applies only to curve fitting to experimental data points and is not designed to be extrapolated for further time intervals.

Discussion

The practical and theoretical problems encountered in the morphometry of islet cells have been discussed previously [6] and it has been pointed out that the real number of granules per cell is likely to be more than the values in Fig. 1 because of the difficulty of assessing whether circular profiles without an electron dense centre are β -granules. Fukuma [8] has shown that in cultured rat islets the β -granules swell by a factor of 1.1 in the presence of 28 mM D-glucose; no attempt has been made in this paper to use a different mean granule diameter, if there were a similar proportional swelling then the granule number estimates would need to be multiplied by a factor of 0.91. Fur-

Table 1. Coefficients for the quadratic equation used to fit the experimental data

Curves for	a_0	a_1	a_2
Zero D-glucose	0.131×10^5	207	-2.41
16.7 mM D-glucose	0.131×10^5	696	-7.73
Broken line	0.131×10^5	489	-5.32

Fig. 1. The number of β -granules contained in B-cells at different time intervals after incubation with $1 \text{ }\mu\text{M}$ adrenaline and either zero D-glucose or 16.7 mM D-glucose. SEMs are indicated by the vertical bars. The broken curve was obtained by subtraction of the two fitted curves

thermore, it has been assumed that cell volume was unchanged by the two experimental procedures since isotonicity was maintained throughout. It has been shown in many species that adrenaline $1 \mu\text{M}$ blocks the release of insulin in response to a 16.7 mM glucose stimulus: they include mouse [3], rat [16] and rabbit islets [4]. In mouse islets $1 \mu\text{M}$ adrenaline inhibits the electrically gated entry of Ca^{2+} generated by 16.7 mM glucose, an event which is concomitant with insulin secretion [7]; however, at the same time glucose metabolism is unchanged [1]. *De novo* insulin biosynthesis is not inhibited by adrenaline $1 \mu\text{M}$ [15]. The results described here with isolated mouse islets, where it is assumed that adrenaline $1 \mu\text{M}$ inhibits insulin secretion, show that glucose is able to stimulate the synthesis of β -granules, suggesting that insulin biosynthesis and associated package into the normal storage form proceeds independently of secretion by exocytosis. The rate of granule formation can be obtained by differentiation of equation 2; thus, when biosynthesis is initiated by switching the glucose concentration, the initial rate of granule formation is given by the coefficient a_1 (particles per minute). However in the absence of exocytosis the granule synthetic rate falls to zero after 45 minutes exposure to 16.7 mM glucose, indicating that, under these experimental conditions, granule accumulation in B-cells is probably a self-limiting process. The increase in cell granule concentration on changing from 2.8 mM glucose to zero glucose could be explained by: 1) a basal granule synthetic mechanism independent of the presence of glucose, 2) a lingering effect of 2.8 mM glucose remaining from the preincubation conditions, 3) an enhancement of insulin synthesis by adrenaline for which there is some evidence from rat islets [15]. The chief problem in the experimental strategy described in this paper is that granule formation rates were measured under the constraint of blocked secretion; ideally one would like to know the rates without such a constraint. With an unhindered secretory mechanism operating it would appear more than likely that the granule synthetic rate would not have the same limiting constraints, so that one would expect the initial synthetic rates to be maintained over a longer period. Nevertheless, it is probable that the initial rates are similar under both conditions and the rates of formation measured here under the influence of the secretory restraint would be close to, but perhaps slightly less than, the initial rates in a freely secreting cell.

Insulin appears to be stored within the granule as a rhombohedral crystal of hexameric units [2, 11]; thus, from the repeat distance, 5 nm , the number of molecules of insulin can be calculated [11]. If the ratio of core to granule diameters is 0.54 [8] it can be shown

that $15,800$ hexamers would be present in a granule ($95,000$ insulin monomers). Insulin is manufactured as proinsulin [19, 20] which associates into a hexamer in the cytoplasm [2]. Therefore, for an initial rate of synthesis of 11.6 granules per second, the mouse islet cell has a capability of making proinsulin molecules at a rate of about 1.1×10^6 per sec. It is not yet clear how the proinsulin is incorporated into the granule, whether it is by specific uptake into an already formed vesicle or whether the vesiculation process encapsulates proinsulin solution from the Golgi apparatus [12, 14, 18]. If the latter alternative were to be true then it can readily be shown that the concentration of proinsulin hexamers in the Golgi tubules would be about 2 mM since that would be the concentration of hexamers needed to crystallize out in the granule.

Curve fitting to experimental data, by its very nature, is a hazardous and somewhat arbitrary procedure and a single curve passing through the origin was chosen to fit the data; therefore, although such curves are of limited use in accurate predictions they can be used to clarify certain questions about differences in synthetic rates. For example, if the rate constant for the conversion of proinsulin to insulin is known one can calculate for the experimental conditions used here the synthetic rates for insulin and proinsulin at any time using the following system of linear differential equations:

$$\frac{dN}{dt} = a_1 + 2a_2t$$

$$\frac{dP}{dt} = \frac{dN}{dt} - kP$$

$$\frac{dI}{dt} = \frac{dN}{dt} - \frac{dP}{dt}$$

where k is the rate constant for conversion of proinsulin to insulin (0.01155 min^{-1} [14], N is the number of *de novo* synthesized granules per cell, P and I represent the granule numerical equivalents containing proinsulin or insulin. Stepwise solutions of the differential equations give:

$$N = a_1t + a_2t^2 \quad \text{eq. 3}$$

$$P = \frac{1}{k} [a_1 + 2a_2(t - \frac{1}{k}) + e^{-kt}(\frac{2a_2}{k} - a_1)] \quad \text{eq. 4}$$

$$I = N_{(t)} - P_{(t)} \quad \text{eq. 5}$$

and are applicable in the region dN/dt positive. Multiplying P or I by 9.5×10^4 (i.e. the number of insulin monomers) gives a rough estimate of the number of molecules of proinsulin and insulin synthesized during time t . If it is assumed that at time $t = 0$ all the granules previously synthesized contained only insulin

(in all species so far studied the content of proinsulin in the resting B-cell is less than 10% of total immunoreactive insulin [13]), then after 45 minutes of a maintained 16.7 mM glucose stimulus (and in the presence of a secretory restraint), the granule number would have increased by 120% whereas the insulin content would have increased by only 35%. During secretion triggered by exposure of islets to high glucose over an extended time period, one may expect that continuously varying ratios of insulin, *de novo* proinsulin and *de novo* insulin would be released if the surface properties of the granules were unaltered by maturation. On that account it may well be better to include measurements for the release of each of these three components if accurate models are to be formulated for a stochastic mechanism of insulin secretion.

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Dr. P.M. Dean
Dept. of Pharmacology
Univ. of Cambridge
Medical School
Hills Road
Cambridge CB2 2QD
England