The Stimulus-Secretion Coupling of Glucose-Induced Insulin Release

INSULIN RELEASE DUE TO GLYCOGENOLYSIS IN GLUCOSE-DEPRIVED ISLETS

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1. When pancreatic islets are preincubated for 20 h in the presence of glucose (83.3 mM) and thereafter transferred to a glucose-free medium, theophylline (1.4 mM) provokes a dramatic stimulation of insulin release. This phenomenon does not occur when the islets are preincubated for either 20 h at low glucose concentration (5.6 mM) or only 30 min at the high glucose concentration (83.3 mM). 2. The insulinotropic action of theophylline cannot be attributed to contamination of the islets with exogenous glucose and is not suppressed by mannoheptulose. 3. The secretory response to theophylline is an immediate phenomenon, but disappears after 60 min of exposure to the drug. 4. The release of insulin evoked by theophylline is abolished in calcium-depleted media containing EGTA. Theophylline enhances the net uptake of ^{45}Ca by the islets. 5. Glycogen accumulates in the islets during the preincubation period, as judged by both ultrastructural and biochemical criteria. Theophylline significantly increases the rate of glycogenolysis during the final incubation in the glucose-free medium. 6. The theophylline-induced increase in glycogenolysis coincides with a higher rate of both lactate output and oxidation of endogenous ^{14}C -labelled substrates. 7. These data suggest that stimulation of glycolysis from endogenous stores of glycogen is sufficient to provoke insulin release even in glucose-deprived islets, as if the binding of extracellular glucose to hypothetical plasma-membrane glucoreceptors is not an essential feature of the stimulus-secretion coupling process.

The relative insulinotropic potency of different sugars closely parallels their ability to undergo glycolysis in pancreatic islets (for review, see Malaisse, 1977). However, the possible relevance of glycolysis to the process of glucose recognition by the B-cell is obscured by the fact that at the same time the sugar is present in the extracellular milieu and metabolized in the islet cells. Thus, under such conditions, it is difficult to distinguish between the respective roles of glucose binding to hypothetical membrane receptors, glucose transport across the plasma membrane and glucose metabolism as possible determinants of the secretory response. The aim of the present study, reported in abstract form elsewhere (Malaisse *et al.*, 1976a), is to find out whether stimulation of glycolysis from endogenous stores of glycogen is sufficient to provoke insulin release, even in the absence of exogenous glucose.

Experimental

Preincubation

In most of the present experiments, isolated islets removed from fed rats (Lacy & Kostianovsky, 1967)

were preincubated for 20 h at 37°C in a tissue-culture medium (Medium 199 with Hanks salts; Gibco Bio-cult, Glasgow, Scotland, U.K.) mixed with calf serum (10%, v/v; Gibco Bio-cult), equilibrated in an atmosphere of CO_2/O_2 (1:19), and containing streptomycin (0.1 mg/ml), penicillin (100 i.u./ml) and glucose in various concentrations.

At the end of the preincubation period, the islets were washed, at low temperature (5–15°C), once or more in a glucose-free bicarbonate-buffered medium (Malaisse-Lagae & Malaisse, 1971). The validity of this washing procedure was usually assessed by measuring the concentration of glucose in the final incubation medium (see the Results section). Since a critical point in the present study was the removal of extracellular glucose, an additional control was performed by preincubating groups of 25 islets for 20 h in the presence of L-[1- ^{14}C]glucose (1.7 $\mu\text{Ci}/\text{ml}$), which has been used as an extracellular tracer in pancreatic islets (Hellman *et al.*, 1971). After preincubation, the islets were washed twice, incubated for 10 min, and eventually resuspended in 1.0 ml of washing medium. The radioactivity of the final solution was examined in two samples (0.4 ml each), the first sample free of islets and the second sample

obtained after sonication (Malaisse *et al.*, 1976b) of the islets in the remaining medium. Under these experimental conditions, no significant contamination of the islets themselves was detectable (apparent contamination by the preincubation medium: 0.12 ± 0.22 nl/islet; $n = 10$). Further, the radioactivity derived from L-[1- 14 C]glucose and recovered in the incubation medium (1.0 ml) indicated that only 0.18 ± 0.03 μ l ($n = 10$) of the initial culture medium (0.5 ml) had been transferred to the incubation flasks.

None of these findings rules out the presence or generation of free glucose inside the B-cells during the final incubation period.

Incubation

After preincubation, the washed islets were usually incubated for 60 min in a glucose-free medium for measurement of insulin release, or used for determination of different metabolic parameters. The methods used for the incubation or perfusion of the islets (Brisson *et al.*, 1972), for the measurement of insulin content and release (Malaisse *et al.*, 1967a), net 45 Ca uptake (Malaisse-Lagae & Malaisse, 1971), lactate output (Sener & Malaisse, 1976) and glucose oxidation (Malaisse *et al.*, 1974) in the islets, and for their ultrastructural examination (Malaisse *et al.*, 1972), were all previously described.

In all experiments, the release of insulin found in the presence of theophylline (1.4 mM) was compared with that recorded in its absence (basal value). Occasionally, the basal release of insulin was abnormally elevated, the highest value recorded in any individual experiment averaging 173.3 ± 16.3 μ i.u./60 min per islet ($n = 20$). Such a high basal release does not appear to correspond to an active process of secretion (F. Malaisse-Lagae, M. Ravazzola & W. J. Malaisse, unpublished work).

Glycogen determination

To measure glycogen, groups of 50 islets each were placed in 0.25 ml of 0.1 M-NaOH and sonicated (Malaisse *et al.*, 1976b). The homogenate was then heated at 80°C for 20 min to destroy contaminating glucose (Lust *et al.*, 1975), and stored at -20°C. From each homogenate, three portions of 60 μ l each were mixed with 5 μ l of acetic acid (1.5 M) and 300 μ l of sodium acetate buffer (10 mM; pH 4.6–5.0). Two of the three samples were then exposed for 60 min, at room temperature (20°C), to amylo- α -1,4- α -1,6-glucosidase (0.07 unit; EC 3.2.1.3; Boehringer, Mannheim, Germany), the concentration of the latter enzyme being selected (i) to avoid significant interference by the glucose contaminating the enzyme preparation, and (ii) to achieve full conversion of glycogen into glucose (Nahorski & Rogers, 1972). The third sample of the homogenate served as a

control. The amount of glucose present in each sample was assayed fluorimetrically. For this purpose, the homogenate was transferred to the assay cuvette together with 1.6 ml of Tris/HCl buffer (100 mM; pH 7.8) containing MgCl₂ (4 mM). The assay cuvette contained, in a final volume of 2.0 ml, ATP (82.5 μ M), NADP⁺ (63.5 μ M) and yeast glucose 6-phosphate dehydrogenase (0.18 unit/ml; EC 1.1.1.49; Boehringer). The reaction was initiated by addition of yeast hexokinase (final concentration 0.35 unit/ml; EC 2.7.1.1; Boehringer). After the reaction had reached completion, standard amounts of glucose (0.5–2.0 nmol) were added to the cuvette. The mean value derived from each duplicate measurement was expressed as pmol of glucose residue/islet, after subtraction of the blank value obtained for the third sample of the homogenate, which was treated in exactly the same manner except for the omission of amyloglucosidase. Control experiments indicated that, under these experimental conditions, no significant amount of contaminating glucose could be detected, the amount of glucose used in these control experiments being close to that known to contaminate the islet preparation at the end of the preincubation period (i.e. 1.5–1.7 nmol of glucose per cuvette). The recovery of glycogen, also used in amounts comparable with that found in the islet homogenate (i.e. 0.8–3.1 nmol of glucose residue per cuvette), averaged 96.8 ± 5.1 % ($n = 5$).

Presentation of results

All results are expressed as the means $\pm$ s.e.m. together with the numbers of individual determinations (n).

Results

Insulin content

The islets had a much lower insulin content after (0.32 ± 0.09 m-i.u./islet; $n = 6$) than before (2.10 ± 0.13 m-i.u./islet; $n = 4$) the 20 h preincubation in the presence of 83.3 mM-glucose. The number of secretory granules also appeared to be markedly decreased in most B-cells (see Plate 1).

Insulin release

When groups of eight islets each were preincubated for 20 h in the presence of glucose (83.3 mM) and then transferred to a glucose-free medium (1.0 ml), theophylline provoked a dramatic stimulation of insulin release (Table 1, lines 1 and 2). Such behaviour differs from that seen in islets not preincubated. In the latter islets, theophylline fails to stimulate insulin release provided that the glucose concentration of the medium does not exceed 2.8 mM (Brisson *et al.*, 1972).

Table 1. *Insulin output by islets after preincubation*

Mean values ($\pm$ S.E.M.) for insulin output are shown together with the experimental conditions prevailing during preincubation, the number of washes performed after preincubation, the composition of the final incubation medium, the number of individual determinations (in parentheses) and the statistical significance (*P*) of differences between appropriate control (lines 1, 4, 6, 9 and 13) and experimental values (NS, not significant, i.e. $P>0.3$).

Preincubation				Incubation				<i>P</i>
Line no.	Glucose (mM)	Duration (min)	No. of washes	Glucose (mM)	Theophylline (mM)	Mannoheptulose (mM)	Insulin output (μ -i.u./60 min per islet)	
1	83.3	1200	1-2	—	—	—	53.4 $\pm$ 13.3 (23)	
2	83.3	1200	1-2	—	1.4	—	218.8 $\pm$ 23.8 (23)	<0.001
3	83.3	1200	1-2	—	1.4	10.0	247.7 $\pm$ 22.2 (20)	<0.001
4	83.3	1200	4	—	—	—	30.1 $\pm$ 13.8 (14)	
5	83.3	1200	4	—	1.4	—	207.0 $\pm$ 10.7 (14)	<0.001
6	83.3	30	1	—	—	—	45.1 $\pm$ 19.0 (19)	
7	83.3	30	1	—	1.4	—	71.4 $\pm$ 17.0 (24)	NS
8	83.3	30	1	16.7	—	—	236.4 $\pm$ 14.0 (24)	<0.001
9	5.6	1200	1-2	—	—	—	42.4 $\pm$ 9.6 (40)	
10	5.6	1200	1-2	16.7	—	—	39.3 $\pm$ 10.2 (27)	NS
11	5.6	1200	1-2	—	1.4	—	40.8 $\pm$ 9.8 (34)	NS
12	5.6	1200	1-2	16.7	1.4	—	184.8 $\pm$ 8.9 (20)	<0.001
13	16.7	1200	1	—	—	—	51.8 $\pm$ 15.9 (6)	
14	16.7	1200	1	—	1.4	—	202.3 $\pm$ 26.1 (6)	<0.001

Several findings suggest that the effect of theophylline in the islets preincubated with glucose (83.3 mM) cannot be attributed to contamination of the final incubation medium with free glucose. First, when assayed, the glucose concentration of the medium never exceeded 0.24 ± 0.03 mM ($n = 4$), the latter value having been recorded when as many as 60 islets were incubated in a small volume of 0.1 ml. Secondly, mannoheptulose failed to decrease the secretory response evoked by theophylline (Table 1, lines 2 and 3). In islets not preincubated, mannoheptulose inhibits both the insulinotropic action of glucose and its potentiation by theophylline (Malaisse *et al.*, 1967b). Thirdly, when the preincubated islets were submitted to four repeated washes at room temperature (total duration of the washing period: approx. 60 min), theophylline still caused a marked increase in insulin output (Table 1, lines 4 and 5).

Preincubation of the islets for a sufficient length of time in the presence of sufficient glucose appeared to be prerequisite for theophylline to stimulate release from the islets eventually incubated in the glucose-deprived medium. Thus, when the islets were preincubated at high glucose concentration (83.3 mM) but only for 30 min, the theophylline-induced increment in secretion rate did not achieve statistical significance, whereas glucose alone displayed its usual insulinotropic capacity (Table 1, lines 6-8). Further, when the islets were preincubated for 20 h at a low glucose concentration (5.6 mM), theophylline again did not stimulate insulin release (Table 1, lines 9 and 11). The latter islets were also not stimulated

by glucose alone, whereas the combination of glucose and theophylline provoked a marked secretory response (Table 1, lines 9-12). Such behaviour is reminiscent of that found in islets removed from starved animals (Levy *et al.*, 1976a). Indeed, starvation suppresses the insulinotropic action of glucose, which, however, tends to be restored in the concomitant presence of theophylline.

In view of these findings, further experiments were invariably performed with islets preincubated for 20 h at high glucose concentration (83.3 mM). It should be noted, however, that a lower concentration of glucose may be sufficient to allow the present phenomenon, since theophylline also markedly stimulated release when the islets had been first preincubated for 20 h with 16.7 mM-glucose (Table 1, lines 13 and 14).

The dynamics of theophylline-induced insulin release in perfused islets were characterized by a rapid onset followed by a progressive decline towards control values found in the absence of the methylxanthine (Fig. 1). Within 2 min of exposure to theophylline, the rate of secretion was $17.3 \pm 4.6\%$ ($P < 0.05$) higher than that found just before the introduction of the methylxanthine to the perfusate. When corrected for control values found in the absence of theophylline, the increment in secretion rate attributable to the latter drug was fairly stable between 4 and 20 min after the addition of the methylxanthine to the perfusate. However, during the later period of perfusion, the effect of theophylline progressively decreased, so that at 60 min the theo-

Fig. 1. Dynamics of theophylline-induced insulin release

Islets preincubated for 20 h in the presence of glucose (83.3 mM) were immediately transferred from the preincubation medium to the perfusion chamber, and thereafter exposed to glucose-free media. The individual rates of insulin release obtained at each time-interval were expressed as percentage of the mean value found within the same experiment between 14 and 20 min after placing the islets in the perfusion chamber. Such a reference value averaged $6.4 \pm 1.0 \mu\text{-i.u./min}$ per islet. In the inset, the pattern of insulin release (expressed as mentioned above) in control islets (no theophylline present) is illustrated as a function of the time after introducing the islets into the perfusion chamber. In the main Figure, the increment in secretion rate attributable to theophylline was calculated, at each time-interval, as the difference between the normalized rates of secretion found in the control and theophylline-treated islets respectively. Theophylline was introduced at 20 min of perfusion (vertical broken line). Mean values ($\pm$ S.E.M.) refer to four individual experiments in each group.

phylline-induced increment in secretion rate did not achieve statistical significance. Over the 60 min exposure to theophylline, the integrated increment in insulin output attributable to the drug amounted to $145.3 \pm 23.8 \mu\text{-i.u./islet}$, a value close to that found in the static experiments.

Participation of calcium

The release of insulin evoked by theophylline during the final incubation was considerably decreased when the incubation medium was deprived of extracellular Ca^{2+} and enriched with a calcium-chelating agent. Thus in the Ca^{2+} -depleted medium the theophylline-induced increment in insulin output ($+11.2 \pm 27.9 \mu\text{-i.u./60 min}$ per islet; degrees of freedom

Fig. 2. Time course for ^{45}Ca accumulation

The islets were preincubated for 20 h in the presence of glucose (83.3 mM) and thereafter incubated in a glucose-free medium with (○) or without (●) theophylline (1.4 mM). Mean values ($\pm$ S.E.M.) are expressed relative to the mean uptake of ^{45}Ca found, in the absence of theophylline, at 20 min of incubation.

= 38) was much lower ($P < 0.02$) than that found, in the same experiments, at normal Ca^{2+} concentration ($+107.0 \pm 26.9 \mu\text{-i.u./60 min}$ per islet; degrees of freedom = 38), and actually did not achieve statistical significance.

The net uptake of ^{45}Ca by the islets preincubated for 20 h in the presence of glucose (83.3 mM) was measured at 20 and 60 min of incubation in the glucose-free medium. These times were selected because, as shown in Fig. 1, they correspond to periods at which the insulinotropic action of theophylline is either close to its optimal value (20 min) or decreasing (60 min). In the control islets (no theophylline present), the time course for net ^{45}Ca uptake was similar to that previously found in islets not preincubated but also exposed to a glucose-free medium, the net uptake of ^{45}Ca increasing from $33.8 \pm 3.4 \text{ pg/islet}$ at 20 min to $45.4 \pm 4.7 \text{ pg/islet}$ at 60 min (Fig. 2). After 20 min of incubation, the accumulation of ^{45}Ca in the islets exposed to theophylline (1.4 mM) was significantly higher than in the control islets (Fig. 2 and Table 2). Mannoheptulose (10.0 mM) failed to abolish the stimulant action of theophylline (Table 2). After 60 min of incubation, the accumulation of ^{45}Ca in the islets was almost identical under all three experimental conditions

EXPLANATION OF PLATE I

Glycogen deposition in the B-cell

Presence of glycogen particles (arrows) in a B-cell of an islet preincubated for 20 h in the presence of glucose (83.3 mM). The cell is poorly granulated (sg: secretory granule). Calibration bar represents 1.0 μm.

Table 2. *Calcium uptake by islets after preincubation*

The uptake of ^{45}Ca by islets preincubated for 20 h in the presence of glucose (83.3 mM) was measured after 20 or 60 min incubation in a glucose-free medium (C: control). Each measurement refers to a group of 10 islets. Theophylline (T; 1.4 mM) and mannoheptulose (MH; 10.0 mM) were added to the medium, as required. For reasons given elsewhere (Malaisse-Lagae & Malaisse, 1971), the values for ^{45}Ca uptake are expressed as a percentage of that found in a paired group of islets. Mean values ($\pm$ S.E.M.) for such ratios are shown together with the number of paired comparisons (in parentheses) and the statistical significance ($*P < 0.01$; $\dagger P < 0.005$) of the changes evoked by theophylline alone (T) or the combination of theophylline and mannoheptulose (T+MH). Absolute values for ^{45}Ca uptake are quoted in the text.

Time (min)	T/C	(T+MH)/C	(T+MH)/T
20	149.4 $\pm$ 14.2 (15) $\dagger$	126.3 $\pm$ 8.1 (15)*	92.4 $\pm$ 8.7 (15)
60	103.2 $\pm$ 9.0 (14)	101.9 $\pm$ 11.3 (14)	107.4 $\pm$ 13.5 (13)

Fig. 3. *Glycogen accumulation and glycogenolysis* Accumulation of glycogen in islets preincubated for 20 h in the presence of glucose (83.3 mM) and thereafter incubated for 30 min in a glucose-free medium in the absence (----) or presence (hatched column, and —) of theophylline (1.4 mM). No significant amount of glycogen could be detected in the islets before the preincubation. Results are means $\pm$ S.E.M. of 11–12 measurements.

(Table 2). These findings indicate that, like its insulinotropic action, the stimulant effect of theophylline on net ^{45}Ca uptake was a transient phenomenon. Such a stimulant action of theophylline, which is reminiscent of that evoked by glucose (Malaisse-Lagae & Malaisse, 1971), sharply contrasts with the situation found in islets not preincubated and exposed to theophylline in a glucose-free medium. Indeed, under the latter experimental conditions, theophylline provokes a modest but significant inhibition of net ^{45}Ca uptake (Brisson *et al.*, 1972).

Glycogen metabolism

After 20 h preincubation in the presence of glucose (83.3 mM), glycogen particles were found scattered in a number of B-cells (Plate 1). Such particles are

not observed in B-cells of islets not preincubated.

Glycogen accumulation in the islets averaged 75.8 ± 11.5 pmol of glucose residue/islet ($n = 12$) at the end of the preincubation period in the presence of 83.3 mM-glucose (Fig. 3). Incubation of these islets for 30 min in the absence of glucose but in the presence of theophylline (1.4 mM) provoked a marked decrease ($P < 0.005$) in glycogen content, to 32.9 ± 3.0 pmol of glucose residue/islet ($n = 11$). The latter value was significantly lower ($P < 0.02$) than that found in islets incubated for 30 min in the absence of theophylline, i.e. 53.1 ± 7.1 pmol of glucose residue/islet ($n = 11$). As illustrated in Fig. 3, the residual amount of glycogen recovered in the islets exposed to theophylline would not have been sufficient to maintain for a long period the same mean rate of glycogenolysis as that recorded during the first 30 min of incubation in the presence of the methylxanthine (i.e. 42.9 ± 12.4 pmol of glucose residue/islet per 30 min; degrees of freedom = 21).

Glycolysis

The output of lactate during the final incubation was more than doubled ($P < 0.001$) in the presence of theophylline, relative to the control value found in its absence (Table 3). When compared with previously reported data (Sener & Malaisse, 1976), the output of lactate in the absence and presence of theophylline was comparable with that found in islets not preincubated and exposed to extracellular glucose at concentrations close to 2.0 and 6.0–7.0 mM respectively.

When $[\text{U-}^{14}\text{C}]\text{glucose}$ (54–78 $\mu\text{Ci/ml}$) was present in the preincubation medium, theophylline significantly increased ($P < 0.02$) the output of $^{14}\text{CO}_2$ during the final incubation (Table 3). This stimulant action of theophylline is unlikely to be due to contamination with exogenous $[\text{U-}^{14}\text{C}]\text{glucose}$. Indeed, in islets not preincubated, theophylline fails to stimulate $[\text{U-}^{14}\text{C}]\text{glucose}$ oxidation whether at low (2.0 mM; W. J. Malaisse, unpublished work) or high (16.7 mM; see Sener *et al.*, 1976) glucose concentration.

Table 3. *Metabolic data in islets after preincubation*

The islets were preincubated for 20h in the presence of glucose (83.3mM) and thereafter incubated in a glucose-free medium with or without theophylline (1.4mM). Mean values ($\pm$ s.e.m.) for metabolic parameters are expressed as pmol of glucose residue/30min per islet, and are shown together with the number of individual determinations (in parentheses). The rate of glycogenolysis was calculated from the mean glycogen content of the islets before and after the final incubation (see Fig. 3). The output of $^{14}\text{CO}_2$ was converted into pmol of glucose residue by using the same specific radioactivity as that of the exogenous [$\text{U-}^{14}\text{C}$]glucose present in the preincubation medium.

Metabolic rate	Control	Theophylline
Glycogenolysis	22.7	42.9
Lactate output	9.0 $\pm$ 1.6 (9)	19.4 $\pm$ 0.7 (9)
$^{14}\text{CO}_2$ output	7.4 $\pm$ 0.8 (10)	18.0 $\pm$ 4.1 (9)

The high radioactivity (78 $\mu\text{Ci/ml}$) used during the preincubation did not exert any obvious deleterious effect on the secretory function of the islets. Indeed, during the final incubation in the absence of glucose, the theophylline-induced increment in insulin output above control values averaged $199.3 \pm 26.9 \mu\text{-i.u./60 min}$ per islet (degrees of freedom = 12; $P < 0.001$), a value of the same order of magnitude as that found in islets not exposed to labelled glucose (see Table 1).

Taken as a whole, these findings are compatible with the view that theophylline stimulated glycogenolysis, and hence increased glycolytic flux, in the glycogen-enriched islets.

Discussion

Exogenous glycogen was used by Maier *et al.* (1975) to stimulate insulin release and the data were interpreted in support of the glucoreceptor theory for the insulinotropic action of glucose. The present experiments were undertaken with exactly the opposite view since they were designed to find out whether stimulation of glycolysis from endogenous stores of glycogen may be sufficient to cause insulin release, even in the absence of exogenous glucose.

The model used here for such a purpose was adapted from that outlined in a previous study, in which theophylline was found to provoke insulin release in the absence of glucose, provided that the pancreatic tissue was taken from hyperglycaemic rats (Malaisse *et al.*, 1967b). Both models make use of the following facts. First, glycogen accumulates in B-cells exposed for a sufficient time to high concentrations of glucose (Lazarow, 1963). Secondly, theophylline is known to increase the concentration of cyclic AMP in pancreatic endocrine cells (Charles *et al.*, 1973; Montague & Cook, 1971; Turtle & Kipnis, 1967)

and, in turn, cyclic AMP is reported to stimulate glycogenolysis in islet tissue (Hellman & Idahl, 1970).

The results outlined in Table 1 indicate that theophylline was indeed able to stimulate insulin release in the islets first preincubated for 20h at high glucose concentration (83.3mM). Such behaviour contrasts with that found in islets not preincubated (Brisson *et al.*, 1972), and could not be ascribed to contamination of the final incubation medium with free glucose.

In interpreting these findings, one could argue that the release of insulin evoked by theophylline occurred through a mechanism unrelated to the stimulation of glycolysis. For instance, there are converging indications (Brisson *et al.*, 1972; Brisson & Malaisse, 1973; Howell & Montague, 1975) that theophylline provokes in the B-cell a glucose-independent translocation of calcium from an organelle-bound pool into the cytosol, and by doing so enhances insulin release. However, it is equally well documented that such an intracellular translocation of calcium is unable to provoke sustained release of insulin, if the B-cell is not concomitantly exposed to another insulinotropic agent, such as glucose, mannose, glyceraldehyde, leucine or sulphonylurea (Brisson *et al.*, 1972). Therefore it is reasonable to assume that, under the present experimental conditions, the insulinotropic action of theophylline resulted from the combination of two concomitant events: a stimulation of glycolysis (mimicking the effect of exogenous glucose) and a redistribution of intracellular calcium. The participation of these two events is evidenced by the following considerations.

Several lines of evidence indicate that, in the present experiments, the insulinotropic action of theophylline was dependent on the stimulation of glycogenolysis, and hence on an increased glycolytic flux.

First, the occurrence of a secretory response to theophylline was dependent on the prior exposure of the islets for a sufficient length of time to a sufficiently high glucose concentration (Table 1). It is reasonable to assume therefore that sufficient glycogen had to accumulate in the islets to allow theophylline to exert its insulinotropic action.

Secondly, glycogen was indeed found in the islets preincubated for 20h with glucose (83.3mM). After such a preincubation, the glycogen content of the islets averaged 76 pmol of glucose residue/islet, corresponding to approx. 63 mmol/kg dry wt. This value is about 25 times lower than that found in human liver (Hultman & Nilsson, 1975). The number of glycogen particles in the B-cells was also much lower than that usually found in the hepatocyte. The present value for glycogen content is 2–6 times that measured in islets removed from genetically obese-hyperglycaemic (*ob/ob*) mice (Hellman & Idahl, 1970). The fall in glycogen content found during incubation in the glucose-free medium containing no theophylline

confirms previous experiments in which more than 90% of the glycogen found in the islets of *ob/ob* mice (i.e. approx. 10 mmol/kg dry wt.) was mobilized over 45 min incubation in the absence of glucose (Hellman & Idahl, 1970).

Thirdly, theophylline significantly increased the rate of glycogenolysis, lactate output and oxidation of endogenous ^{14}C -labelled substrates. In both the absence and the presence of theophylline, the fall in glycogen content was closely matched by the amount of carbon appearing as either lactate or $^{14}\text{CO}_2$ (Table 3).

Finally, the stimulant action of theophylline on both insulin release (Fig. 1) and net ^{45}Ca uptake (Fig. 2) disappeared after 60 min incubation, at a time when the endogenous stores of glycogen were likely to be exhausted in the theophylline-treated islets (Fig. 3).

In addition to the enhanced utilization of endogenous nutrients, intracellular translocation of calcium probably also participated in the insulinotropic action of theophylline. In the presence of theophylline, the rate of glycolysis, as judged from the output of lactate, was comparable with that otherwise found in islets not preincubated and exposed to extracellular 6.0–7.0 mM-glucose (see under 'Glycolysis' in the Results section). At such a glucose concentration, the release of insulin from islets not preincubated occurs at a rate close to 30–60 $\mu\text{i.u.}/60\text{ min}$ per islet (Brisson *et al.*, 1972; Levy *et al.*, 1976b). In the present experiments, despite the degranulation of the B-cell in the islets exposed for 20 h to the high glucose concentration (83.3 mM), the theophylline-induced increment in insulin output averaged $152.8 \pm 14.1 \mu\text{i.u.}/60\text{ min}$ per islet (degrees of freedom = 120). It is thus clear that the rate of insulin release evoked by theophylline in the glycogen-enriched islets was much higher than that normally expected from the actual rate of glycolysis. The same dissociation between the rate of glycolysis and that of insulin release is found in islets not preincubated but concomitantly exposed to glucose and theophylline (Levy *et al.*, 1976a). Because the latter situation is currently ascribed to the effect of theophylline on the intracellular distribution of calcium (Brisson *et al.*, 1972; Brisson & Malaisse, 1973), it is tempting to believe that such an effect was also operative under the present experimental conditions. However, it should be stressed once again that the theophylline-induced intracellular translocation of calcium is insufficient in itself to cause sustained stimulation of insulin release in the present as well as other (Somers *et al.*, 1976) systems *in vitro*.

In conclusion, the present data suggest that, at least in the presence of theophylline, stimulation of glycolysis from endogenous stores of glycogen mimics the insulinotropic action of exogenous glucose, even when the outer face of the B-cell mem-

brane is not exposed to high concentrations of glucose. In other words, the inward transport of glucose into the B-cell and, if it ever occurs, the binding of extracellular glucose to a hypothetical glucoreceptor located at the plasma membrane (Kawazu *et al.*, 1976) apparently do not themselves represent essential features in the stimulus–secretion coupling for glucose-induced insulin release.

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