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DIURNAL VARIATIONS IN INSULIN SECRETION AND K^+ PERMEABILITY IN ISOLATED RAT ISLETS

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SUMMARY

1. The effects of glucose on insulin secretion and ^{86}Rb efflux from isolated rat islets were studied at six different times during a 24-h period (00.00, 04.00, 08.00, 12.00, 16.00 and 20.00 h).

2. In the absence of glucose and in the presence of sub-stimulatory concentrations (2.8 mmol/L) of the sugar, insulin secretion did not vary with the time of day. At a glucose concentration of 5.6 mmol/L the stimulated insulin secretion was greater than basal levels only at 20.00 h.

3. At a higher sugar concentration (8.3 mmol/L) the increase in insulin secretion and the reduction in ^{86}Rb efflux rate were more marked during the dark period. No effect of the time of day on insulin secretion was observed at glucose concentrations above 8.3 mmol/L (except in 27.7 mmol/L).

4. The time of day appears to affect insulin secretion mainly at glucose concentrations close to physiological values (5.6–8.3 mmol/L).

5. This result agrees with the ability of physiological amounts of glucose to alter the ^{86}Rb-permeability of pancreatic B cells at the same time intervals.

Key words: circadian rhythm, diurnal variations, glucose, insulin release, insulin rhythm, K^+ permeability, rat.

INTRODUCTION

In pancreatic B cells, glucose metabolism increases the cytosolic adenosine-triphosphate/adenosine diphosphate (ATP/ADP) ratio,[1] which in turn leads to inhibition of a class of K^+ channels in the islet cell membrane.[2] As a consequence, there is a relative intracellular accumulation of K^+ which results in membrane depolarization, the opening of voltage-dependent Ca^{2+} channels and finally Ca^{2+} influx. The resulting increase in cytosolic Ca^{2+} activates the secretory machinery (i.e. microtubules, microfilaments and plasma membrane) and triggers the exocytosis of insulin-containing granules.[3,4]

The existence of circadian variation in glycaemia and insulinaemia has been demonstrated in rats[5–7] and in humans[8,9] after a glucose load. In humans, vespertine hyperglycaemia is the first sign of glucose intolerance[10,11] and has been associated with a concomitant reduction in insulin secretion.[8,12] A loss of the diurnal variation in insulin secretion characterizes the onset of diabetes mellitus.[13]

The rhythm of insulinaemia appears to be endogenously determined[6,14] and can be detected at any time of the year, regardless of the type of light (natural or artificial) present.[15] However, care should be exercised in the interpretation of insulinaemia rhythms because plasma insulin levels reflect not only variations in the secretion rate but also the effects of dilution, distribution and degradation.[16] In addition, changes in plasma insulin levels are not merely a consequence of corresponding alterations in the glucose concentration.[17] In this regard, the existence of a circadian variation in B cell sensitivity to insulinotropic stimuli has been suggested.[18] There is also evidence that the B cell response to the ingestion of a mixed meal may be influenced by the time of day that it is provided.[19] In contrast, the rhythm of plasma insulin levels is independent of the carbohydrate intake,[20] a finding confirmed by the observation that fasting does not appear to influence the diurnal variation of insulin secretion.[7,18] A strong exogenous effect of meal times upon this rhythm, however, has been registered.[21]

The mechanism for circadian variation in insulin secretion is unknown.[22] In rats, diurnal insulin secretion may be controlled directly by a circadian oscillator that influences pancreatic secretion and glucose metabolism through a neural signal modulated by the suprachiasmatic nucleus.[23] Data on circadian variation in insulin secretion using the isolated pancreas are scarce, with the first attempt at such a study being performed 25 years ago with pieces of rat pancreas.[24] Very recently, circadian rhythm of insulin secretion from isolated rat islets, perifused for a prolonged period of time, was also observed.[25]

In the present study, we re-evaluated the existence of a diurnal rhythm in insulin secretion in isolated rat islets, obtained at six different hours of the day. In addition, we examined whether there is any correlation between insulin secretion and the ability of glucose to reduce K^+-permeability in islets at the same time of day.

METHODS

Male Wistar albino rats, 3–7 months old, were housed individually under a 12 h light:dark cycle (lights on at 06.00 h) with free access to water and food. The room temperature was maintained at 23±2°C and illumination was provided exclusively by fluorescent lights controlled by an automatic device. The cleaning of cages was performed after 15.00 h. The rats were maintained under these housing conditions for at least 10 days before the experiments.

For each experiment two rats were killed by a blow to the head. Collagenase-digested islets were isolated by the same individual using the same batch of enzyme. Static incubation studies were carried out using groups

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of five islets per well. The islets were first incubated in 0.75 mL Krebs' bicarbonate solution (composition: (in mmol/L) NaCl 115; $NaHCO_3$ 24; KCl 5; $CaCl_2$ 2.56; $MgCl_2$ 1; 1 mg bovine serum albumin/mL; physiological pH was achieved by equilibration with a mixture of 95 : 5 O_2 : CO_2) containing 5.6 mmol/L glucose for 30 min at 37°C. At the end of this period, the solution was replaced with fresh buffer and the islets further incubated for 60 min under various experimental conditions.[26] The insulin content of each sample was measured by a conventional radioimmunoassay[27] using rat insulin as the standard.

The method used to measure ^{86}Rb efflux (a substitute for ^{42}K) has been described in detail elsewhere.[28] Briefly, groups of 100 islets were preincubated for 75 min in 0.3 mL Krebs' bicarbonate solution (pH 7.4, 37°C) containing $^{86}RbCl$ (approximately 400 kBq), 5.6 mmol/L glucose and 1 mg bovine serum albumin/mL. The islets were washed three times with non-radioactive medium and transferred to the perifusion system. The effluent was collected over successive 2-min periods and the ^{86}Rb efflux expressed as the fractional outflow rate (% instantaneous islet content/min).

The rats were killed at exactly 00.00, 04.00, 08.00, 12.00, 16.00 and 20.00 h and static insulin secretion and ^{86}Rb efflux experiments were initiated approximately $2^1/_2$ h later.

The data from the ^{86}Rb efflux were analysed using four different mathematical approaches: (i) the area under the curve G 5.6 mmol/L represents the B cell membrane permeability to K^+; (ii) slope of temporal decay for ^{86}Rb (22–40 min) represents the deceleration of the ^{86}Rb fractional efflux rate; (iii) percentage change in ^{86}Rb efflux after G 8.3 mmol/L; and (iv) area over the curve (%) G 8.3 mmol/L (42–60 min) represents the inhibition on the ^{86}Rb efflux induced by the increase in glucose concentration from 5.6 to 8.3 mmol/L.

The data are presented as the mean ± SEM and were statistically analysed by one-way parametric analysis of variance (ANOVA) followed by a Student–Newman–Keuls' test, as appropriate. Student's *t*-test was used for comparison between light versus dark groups depicted in the inset of Fig. 1. When parametric test assumptions were violated, the data were analysed using a Kruskal–Wallis ANOVA followed by Dunn's multiple comparison test (comparison of all points). Dunnett's test was also used for multiple comparisons with a single control point. When required, linear correlation analysis was also employed. The confidence limit for significance was 5%.

RESULTS

Insulin secretion

In the absence of glucose there was no significant variation in the levels of insulin secretion during the hours of the day examined. Minimal and maximal secretion values of 3.6±0.50 ($n = 15$) and 4.4±0.45 ng/islet per 60 min ($n = 26$) were observed at 16.00 and 08.00 h, respectively ($P > 0.05$).

In order to minimize the variability in the basal levels of secretion the values obtained at different glucose concentrations were

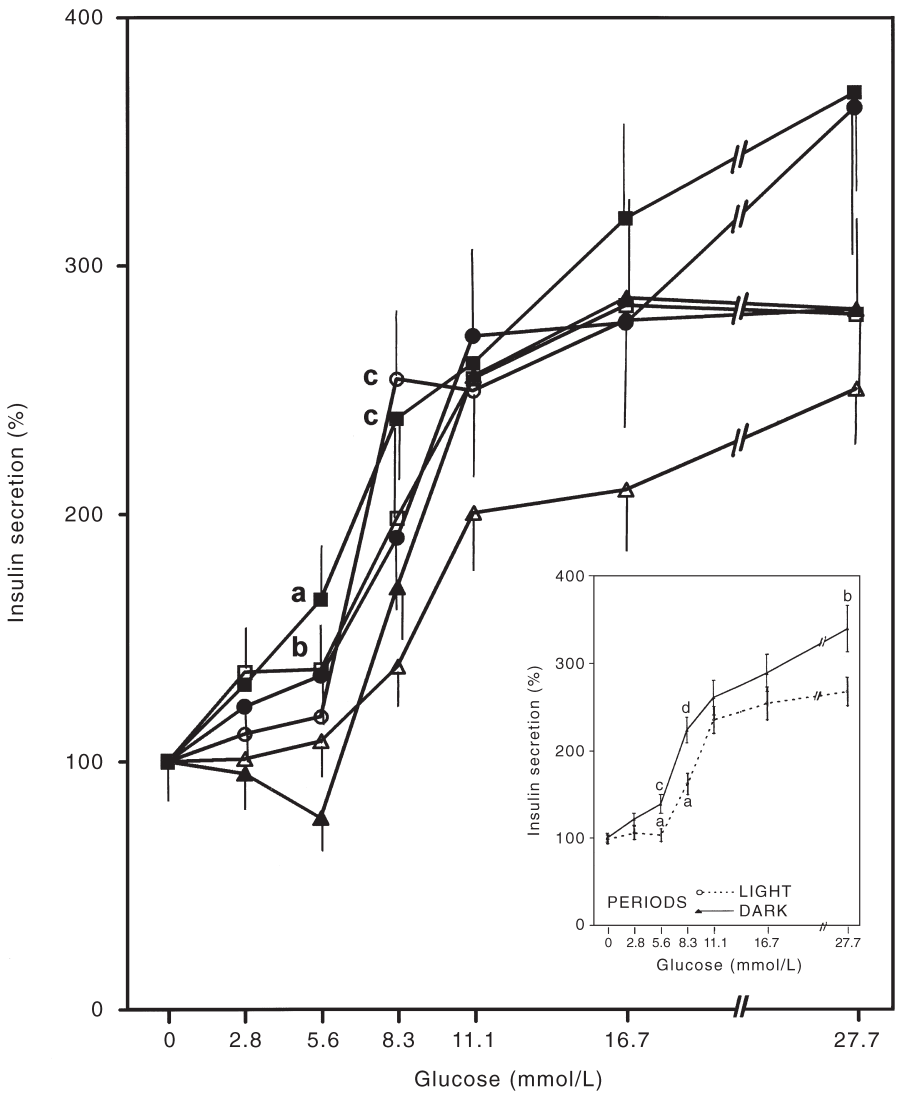

Fig. 1 Influence of the time of day on glucose-induced insulin secretion. Groups of five islets each were preincubated for 30 min at 37°C after which the preincubation medium was replaced with Krebs' solution containing increasing concentrations of glucose (0–27.7 mmol/L). The points represent cumulative (60 min) insulin secretion, at the indicated concentration of glucose, expressed as a percentage of the insulin secreted in the absence of glucose (100%) in the same experiment. Each point represents the mean ± SEM of the insulin secretion in 11–30 experiments. Basal insulin secretion, expressed as ng/islet per 60 min in the absence of glucose (G0), was: 4.0±0.35, 3.9±0.50, 4.4±0.45, 4.05±0.45, 3.6±0.5 and 4.2±0.50 for 00.00, 04.00, 08.00, 12.00, 16.00 and 20.00 h, respectively. (a) $P < 0.05$ versus G0 and $P < 0.002$ versus G 5.6 mmol/L at 12.00 h; (b) $P < 0.05$ versus 12.00 h; (c) $P < 0.01$ versus 08.00 hours. Inset: Values of insulin secretion obtained at 08.00 (△), 12.00 (▲) and 16.00 h (□) were pooled together representing the light period, while those of 00.00 (○), 04.00 (●) and 20.00 h (■) indicate the dark period. (a) $P < 0.05$ versus G0; (b) $P < 0.025$ versus light period; (c) $P < 0.005$ versus light period; (d) $P < 0.0025$ versus light period.

expressed as a percentage of the basal insulin secretion obtained in the absence of glucose (Fig. 1). The minimal and maximal insulin secretion observed at 2.8 mmol/L glucose was 95±12% ($n = 25$) and 136±15% ($n = 16$) of the basal values at 12.00 and 16.00 h, respectively ($P > 0.05$ compared with basal levels). When the dose–response curves, obtained during light (08.00, 12.00 and 16.00 h) and dark (00.00, 04.00 and 20.00 h) periods, were pooled together (inset Fig. 1) the threshold values for glucose-induced insulin secretion were 5.6 and 8.3 mmol/L for light and dark periods, respectively. Secretion values for dark period at 5.6, 8.3 and 27.7 mmol/L glucose were significantly higher than for light periods for the same glucose concentrations ($P < 0.005$; $P < 0.0025$; $P < 0.025$, respectively). At 5.6 mmol/L glucose, insulin secretion increased significantly above basal values only at 20.00 h (165±18%, $n = 19$, $P < 0.05$). At this same concentration of glucose, the insulin secretion at 20.00 and 16.00 h (137±15%, $n = 16$, above basal for the latter) was significantly higher ($P < 0.002$

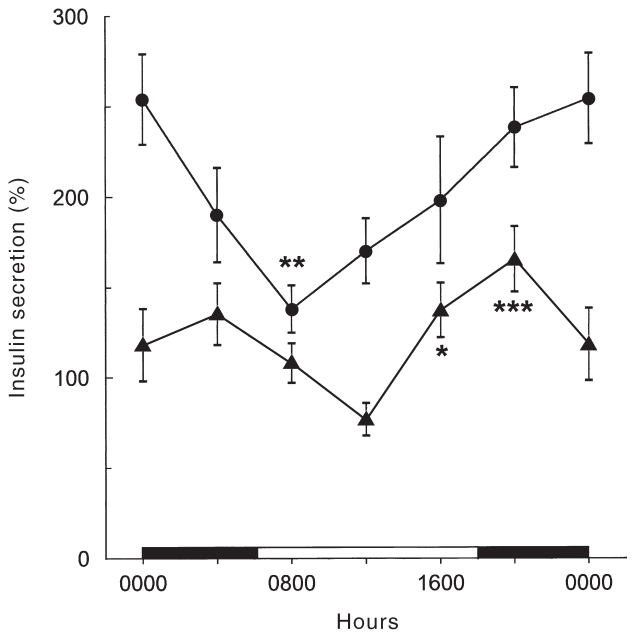

Fig. 2 Diurnal variation in the insulin secretion by isolated islets. Insulin secretion from islets incubated for 60 min at 37°C in the presence of 5.6 (▲) or 8.3 mmol/L (●) glucose was measured at the indicated hours. The data points, derived from Fig. 1, are expressed as a percentage of the basal insulin secretion (no glucose) and represent the mean±SEM of the secretion in 11–26 experiments. The black and white bars in the lower part of the figure represent the dark and light phases of the photoperiod, respectively. *$P < 0.05$ versus 12.00 h; **$P < 0.01$ versus 00.00 h; ***$P < 0.002$ versus 12.00 h.

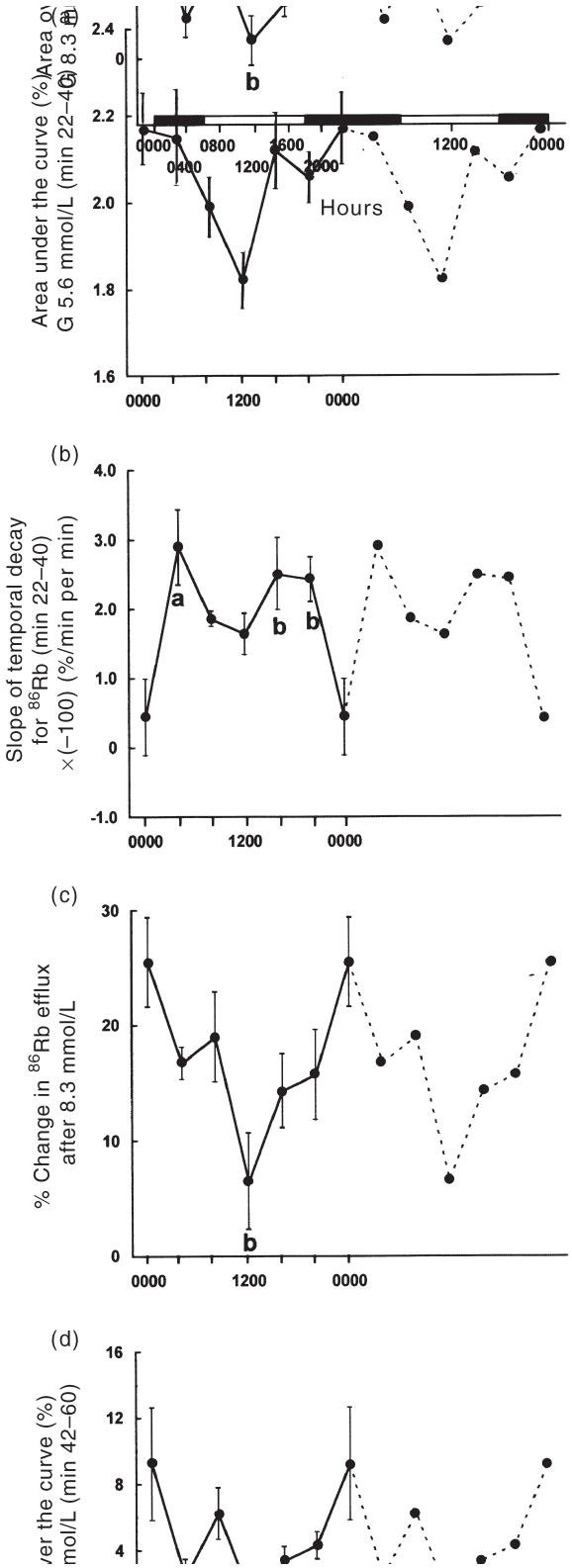

Fig. 3 Diurnal variation in selected parameters of ^{86}Rb fractional outflow rate. (a) Integrated area under the curves between min 22 and 40 of perifusion (G 5.6 mmol/L; $F_{5,17} = 2.06$; $P > 0.05$). (b) Slope of the regression line calculated for the interval between 22 and 40 min of perifusion ($F_{5,17} = 4.52$; $P < 0.01$). (c) Decrease (%) of ^{86}Rb efflux between mean values calculated before (36–40 min) and after the addition of 8.3 mmol/L glucose (56–60 min; $H = 15.01$; $P < 0.025$). (d) Integrated area over the curves between 42 and 60 min of perifusion (G 8.3 mmol/L; $F_{5,17} = 2.66$; $P = 0.06$). The data were taken from Fig. 3. (a) $P < 0.01$ versus 00.00 h; (b) $P < 0.05$ versus 00.00 h. Solid black bars indicate the dark phase of the photoperiod.

and $P < 0.05$, respectively) than that observed at 12.00 h (77±9% of basal, $n = 22$; Figs 1,2). Maximal secretion at 8.3 mmol/L glucose was obtained at 00.00 h (254±25% of basal, $n = 18$) and 20.00 h (238±22% of basal, $n = 19$) and was significantly different ($P < 0.01$ in both cases) from that recorded at 08.00 h (138±13% of basal, $n = 20$; Figs 1,2). Interestingly, the insulin secretion induced by 8.3 mmol/L glucose at 20.00 and 00.00 h was similar to that observed in the presence of 27.7 mmol/L glucose at 08.00 h. At glucose concentrations higher than 8.3 mmol/L, there was no significant variation in the levels of insulin secretion at the different times studied (Fig. 1), except in 27.7 mmol/L (inset Fig. 1).

^{86}Rb efflux

Statistical analysis of some parameters of the results obtained at various hours of the day, concerning the ^{86}Rb efflux (Fig. 3), revealed that: (i) there were no differences in the area under the curve during exposure to 5.6 mmol/L glucose (22–40 min of perifusion) at any of the intervals tested (Fig. 3a); (ii) the slope of the regression line for this perifusion period was minimal at 00.00 h and was significantly different from the corresponding slope calculated for experiments done at 04.00 h ($P < 0.01$), 16.00 and 20.00 h ($P < 0.05$ for the latter two; Fig. 3b); (iii) the maximal effect of 8.3 mmol/L glucose in reducing the ^{86}Rb efflux rate was observed at 00.00 h (25±3.9%) while the minimal effect was registered at 12.00 h (6.5±4.2%; $P < 0.05$; Fig. 3c); and (iv) the area over the curve during perifusion with 8.3 mmol/L glucose (42–60 min) was maximal at 00.00 h (9.2±3.4%) and minimal at 12.00 h (1.1±1.49%; Fig. 3d). These last two parameters show a very good correlation throughout the hours of the day (Fig. 3c,d) with a correlation coefficient of 0.94 ($P < 0.001$). Figure 4 shows the correlation between the effect of 8.3 mmol/L glucose on insulin secretion and the reduction in fractional ^{86}Rb outflow rate (area over the curve during 42–60 min). As can be seen in Fig. 4, the maximal and minimal insulin secretion correlates well with the maximal and minimal reduction in ^{86}Rb efflux. This ultimately represents the ability of glucose to provoke membrane cell depolarization by the regulating entry of Ca^{2+} into B cells.

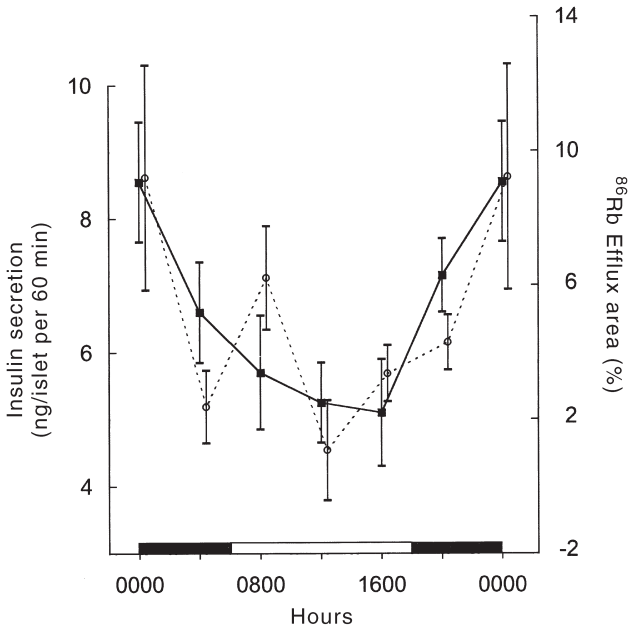

Fig. 4 Relationship between insulin secretion and the areas over the curves of ^{86}Rb efflux with 8.3 mmol/L glucose. Insulin secretion from islets incubated for 60 min (■) in the presence of 8.3 mmol/L glucose at different times of the day are plotted together with the values for the area over the curves for ^{86}Rb efflux at the same glucose concentration (42–60 min; ○). Each point represents the mean±SEM of 11–22 experiments for insulin secretion and four for ^{86}Rb efflux.

DISCUSSION

In the present study, we examined the diurnal variations in insulin secretion and alterations in K^+-permeability induced by increasing concentrations of glucose in collagenase-isolated rat islets. The data confirm and extend previous observations that insulin secretion undergoes some daily variation both *in vivo*[12] as well as *in vitro*.[24] Our data also indicate that at physiological glucose concentrations (5.6–8.3 mmol/L) the dose–response curves obtained during the dark phase are shifted to the left relative to those obtained during the light phase. We observed no effect of the time of day on insulin secretion at glucose concentrations lower than 5.6 mmol/L. This finding contrasts with earlier observations in the presence of 3.3 mmol/L glucose.[24] A possible explanation for this discrepancy may be that the incubation medium used by the latter study also included 5 mmol/L of other secretagogues (e.g. glutamic, fumaric and pyruvic acids).

In agreement with earlier observations, 5.6 mmol/L glucose increased the insulin secretion above basal levels only at 20.00 h. This could indicate a higher sensitivity of B cells to sugar at this hour of the day.[24] As food intake by rats occurs predominantly at night,[29] and because the sensitivity to glucose is higher in rats during meal periods (despite alterations in the time of day that the animals had access to food),[6] the greater sensitivity to glucose apparently coincides with the dark period. In humans, a reduced tolerance to glucose during the evening and at night has been associated with a diminished sensitivity and/or responsiveness of B cells to glucose.[30–32]

It was suggested that nourishment acts as a *zeitgeber* on the rhythms of insulinaemia and glycaemia.[14,33] However, circadian rhythms of insulin secretion were observed in fasted[7] or fed rats,[34] as well as in rats[7] and humans,[9] submitted to hyperglycaemic clamp. In addition, an endogenous control of the circadian rhythm of insulinaemia may be present,[34,35] as demonstrated in different animal species submitted to starvation including humans,[18] rats,[6] and rabbits.[36] Interestingly, an effect of the time of day was present mainly at physiological concentrations of glucose. In general, increasing the glucose concentration from 5.6 to 8.3 mmol/L had a greater effect on insulin secretion during the dark phase. Incidentally, the acrophase of insulin secretion in fed rats occurs at the beginning of the dark period.[14] In addition, rats submitted to a high carbohydrate or standard diet and maintained under conditions similar to those used in this work, showed maximal insulin secretion at 21.00 and 00.00 h and minimal secretion at 12.00 and 18.00 h.[37] Similar results were obtained in mice.[38] If one considers that the clearance of insulin is not affected by the time of day,[39] we can assume that at least some of the variations in plasma insulin levels reflects alterations in insulin secretion. The observation that in the presence of 8.3 mmol/L glucose, insulin secretion at 00.00 and 20.00 h was higher than that measured during the light period is consistent with the fact that human and rat plasma insulin levels are greater at

the end of the period of rest or in the first part of the period of activity.[5–9,12,14,18,19,40] However, our data do not agree with the observation that the insulin levels of blood collected from the portal vein were greater at 08.00 and lower at 20.00 h.[41] Again, one must remember that fluctuations in plasma insulin levels reflect not only variations in secretion rate but also the effect of dilution, distribution and degradation.[16]

Insulin secretion was affected by the time of day mainly at physiological concentrations of glucose. However, although not significantly different, the insulin secretion induced by 16.7 mmol/L glucose at 20.00 h was 52% greater than that at 08.00 h as observed earlier.[24] Our results obtained at 00.00 h contrast with those of others who observed that 16.7 mmol/L glucose induced minimal insulin secretion at 00.00 h.[24] Again, the discrepancy between these data could be explained by the different species used in the two studies (rat *vs* mouse), the different incubation times (60 min in our experiments *vs* 20 min in Gagliardino and Hernández, the later interval would reflect mainly the first phase of insulin secretion) and the different types of preparations (isolated islets *vs* pieces of pancreas).[24]

Diurnal variations in B cell physiology are accompanied by multiple changes of different parameters in those cells such as nuclear size,[42] surface density of rough endoplasmic reticulum, number of secretory granules, volume densities of lysosomes and crinophagosomes;[41] peptide synthesis and insulin content of islets.[43]

Figure 4 shows the correlation between insulin secretion and ^{86}Rb efflux data at different hours of the day in the presence of 8.3 mmol/L glucose. As can be seen, the maximal secretion coincides with the maximal reduction in ^{86}Rb efflux induced by glucose at 20.00 and 00.00 h. It is suggestive that the most predominant effect of the hour of the day on insulin secretion was obtained at similar glucose concentrations (8.3 mmol/L) used recently to demonstrate circadian rhythm of insulin secretion in isolated islets.[25] The closure of K^+_{ATP} channels is a pivotal step in the mechanism of glucose-induced insulin secretion,[2] which is a consequence of an increase in the ATP/ADP ratio generated by glucose metabolism.[1] Although not yet fully elucidated, the existence of a circadian rhythm in insulin secretion could be linked to oscillations in the ATP generation in B cells. Ultradian oscillations (periods of approximately 15 min) in glycolysis in pancreatic B cells have been demonstrated and bear a marked resemblance to the patterns of lactate and insulin oscillations in islets during perifusion with 16.7 mmol/L glucose.[44] Oscillations in the ATP/ADP ratio (periods of approximately 10 min) have also been detected in insulinoma induced by radiation m5F cells.[45] These oscillations could provoke oscillations in the activity of ATP-sensitive K^+ channels, the membrane potential and intracellular-free Ca^{2+} that lead to insulin secretion.[46] Interestingly, the presence of ionic channels regulated by a circadian clock has recently been described in neurons from the retina of molluscs[47] and in cultured chicken pineal cells.[48] In summary, pancreatic rat B cells stimulated by physiological concentrations of glucose release more insulin during the first half of the activity period. This increased secretion is tightly coupled to the ability of glucose to reduce K^+ permeability in these cells.

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