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# Regulation of Glucagon Release: Effects of Insulin on the Pancreatic $A_2$-Cell of the Guinea Pig

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**Summary.** The effect of exogenous insulin on glucagon release by guinea pig $A_2$-cells of isolated islets from normal and streptozotocin treated animals has been studied to test the hypothesis that insulin directly affects glucagon secretion. Glucose utilization and ATP concentration were also measured. In addition, the effects of exogenous somatostatin on glucagon release and glucose utilization of these cells have been investigated. In the $A_2$-cell rich islets from streptozotocin treated guinea pigs glucagon release was 76.9 ± 7.8 pg/μg islet dry weight/h in 1.7 mmol/l glucose, not being significantly inhibited when the glucose concentration was increased up to 16.7 mmol/l. Glucagon release was, however, strongly suppressed by 30 mU/ml insulin independent of the glucose concentration, while release from the normal islets was unaffected by exogenous insulin. Glucose utilization increased four-fold in the $A_2$-cell rich islets when the glucose level was raised from 1.7 to 16.7 mmol/l, but was nevertheless at all times less than 70 per cent of that of the normal islets. Addition of exogenous insulin caused a further significant stimulation (40–100 per cent) in the $A_2$-cell rich islets, but not in the normal islets. The ATP concentration of the $A_2$-cell rich islets increased in parallel with the glucose concentration. Addition of insulin effected the highest ATP levels, about 15 mmol/kg islet dry weight, irrespective of the glucose concentration. Somatostatin (2.5 μg/ml) strongly inhibited glucagon release, but failed to affect glucose utilization. The present observations support the view that the $A_2$-cell is sensitive to insulin, and suggest that the suppressive effect of insulin on glucagon release is mediated via an increased intracellular ATP concentration generated by stimulated glucose metabolism.

**Key words:** Glucagon release, guinea pig, streptozotocin, $A_2$-cell rich islets, insulin, somatostatin, glucose utilization, ATP content.

It is now well established that in normal man the rate of glucagon secretion from the islet $A_2$-cell is depressed by a rise of the blood glucose concentration [1, 2]. However, in both juvenile and maturity onset diabetes mellitus there is a paradoxical hypersecretion of glucagon, which is not suppressed by a further elevation of the blood glucose [2–5]. Up to now the most active agents rectifying glucagon secretion in diabetes have been found to be two other islet cell hormones, namely insulin and somatostatin, each of which is able to suppress the hyperglucagonaemia seen in diabetic patients [6–9].

Since hyperglycaemia without a concomitant rise in insulin does not inhibit glucagon secretion [10, 11], it has been suggested that the $A_2$-cell is critically dependent on insulin with respect to its glucose metabolism and that the rate of intracellular glucose degradation is inversely proportional to the rate of glucagon release [11, 12]. A direct study of the glucose metabolism of the $A_2$-cell and its relationship to glucagon release is, however, hampered by the predominance of B-cells in the normal mammalian islets. The availability of $A_2$-cell rich islets in streptozotocin treated animals provides a means of attacking this problem [13, 14]. In the present study the effects of insulin on glucagon release, glucose utilization and ATP concentration of $A_2$-cell rich islets isolated from the guinea pig have been examined. Since somatostatin strongly inhibits glucagon secretion the effect of this polypeptide on the glucose utilization of the $A_2$-cell has also been evaluated.

## Material and Methods

*Preparation and Isolation of $A_2$-Cell Rich Islets.* 154 male guinea pigs weighing approximately 200 g were used; 138 received streptozotocin (kindly provided by Dr. W. E. Dulin, The Upjohn Co., Kalamazoo, Mich., USA) in a single IP injection of 375 mg/kg bodyweight. Immediately before injection the drug was dissolved in 0.01 mol/l citrate buffer, pH 4.5, at a concentration of 75 mg/ml. After the treatment the animals were monitored for body weight, the occurrence of glucose in the urine (Clinistix®, Ames Co., Slough Bucks., U.K.) and blood glucose concentration [15].

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1–3 weeks after streptozotocin administration animals were killed by decapitation and their pancreata removed. A small piece of each pancreas was fixed in Bouin's solution, while the remaining part was used for isolation of pancreatic islets using a collagenase (Worthington Biochemical Corp., Freehold, N.J., USA) technique [16]. The isolation protocol was identical in the experimental and control animals.

After isolation islets were either used immediately for studies of glucose utilization or maintained in tissue culture [17, 18] for 24–48 h for subsequent assay of glucagon release and concentration of adenine nucleotides. The cultured islets were isolated aseptically and transferred to plastic Petri dishes containing 4 ml TCM 199, 1 ml calf serum (Statens Bakteriologiska Laboratorium, Stockholm, Sweden), antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin; Glaxo Laboratories Ltd., Greenford, Middx., U.K.) and glucose (5.5 mmol/l).

*Morphological Studies.* The Bouin fixed pancreatic specimens were embedded in paraffin, cut in 7 μm thick sections and stained with aldehyde fuchsin trichrome [19]. At least five sections from each animal were studied by light microscopy.

*Glucagon Release.* Batches of 12 $A_2$-cell rich islets were incubated in a shaking water bath (100 strokes/min) at 37° C for 60 or 120 min in 250 μl of a bicarbonate buffer of the following composition: NaCl 111.2 mmol/l, $Na_2HPO_4$ 0.8 mmol/l, $NaHCO_3$ 27.0 mmol/l, KCl 5.1 mmol/l, $KH_2PO_4$ 0.2 mmol/l, $CaCl_2$ 1.4 mmol/l, $MgSO_4$ 0.3 mmol/l and $MgCl_2$ 1.0 mmol/l [20], containing bovine plasma albumin (2 mg/ml; Armour Pharmaceutical Co., Eastbourne, U.K.). Glucose, insulin (Beef insulin, 10 × crystallized, kindly donated by Novo Terapeutisk Laboratorium A/S, Bagsvaerd, Denmark) and somatostatin (AB Kabi, Stockholm, Sweden) were added at concentrations shown in Table 1. The gas phase consisted of 95% $O_2$:5% $CO_2$. After incubation, samples of the medium were immediately frozen at −20° C for subsequent assay of glucagon [21], with porcine glucagon as a standard. Control studies showed that no correction was needed for the small amounts of glucagon present in the insulin added to the medium, nor did exogenous somatostatin interfere with the glucagon radioimmunoassay. After incubation dry weights of the islets were determined [22], using a Cahn Electrobalance (model 4100) sensitive to 0.1 μg.

*Glucose Utilization.* The utilization of glucose by isolated $A_2$-cell rich islets, or normal guinea pig islets, was determined as the formation of $^3H_2O$ from 5-$^3$H-glucose [23]. Batches of 15 islets were incubated in glass vials [24], containing 20 μl bicarbonate buffer [20]. 5-$^3$H-Glucose (spec. activity 2.4 Ci/mmol; The Radiochemical Centre, Amersham, U.K.) was added with unlabelled glucose to give a final concentration of 1.7, 3.3 or 16.7 mmol/l glucose. The specific activities were 60, 30 and 6 mCi/mmol, respectively. Insulin and somatostatin were dissolved directly in the incubation medium at final concentrations of 30 mU/ml and 2.5 μg/ml respectively. The vials were placed inside glass scintillation flasks containing 0.5 ml redistilled water and sealed with rubber membranes. Groups of islets isolated from control animals were incubated similarly.

After 60 min incubation at 37° C under continous shaking (100 strokes/min) in a gas phase of 95% $O_2$:5% $CO_2$, islet cell metabolism was stopped by an injection through the rubber membrane of 10 μl of 0.2 mol/l HCl into the incubation vial. The scintillation flasks were then incubated at room temperature for another 18–24 h to allow the $^3H_2O$ formed to equilibrate with the water in the outer flask. Radioactivity in the water was then measured by liquid scintillation, using a mixture of 2.5 ml redistilled water and 3.5 ml Instagel® (Packard Instruments Co., Inc., Downers Grove, Ill., USA) added to the water, and a Packard (Tri-carb model 3255) liquid scintillation spectrometer. After incubation islets were collected and dry weight determined as described above. Controls without islets were always included in order to allow for a correction of the initial $^3H_2O$ in the 5-$^3$H-glucose. The recovery of $^3H_2O$ during the equilibration period as tested by measurements of the diffusion of known amounts of $^3H_2O$ was estimated to be approximately 70%.

*Concentration of Adenine Nucleotides.* The effects of glucose and insulin on the concentrations of ATP and the sum of ATP, ADP and AMP of $A_2$-cell rich islets were evaluated using a bioluminescence firefly luciferase technique [25]. Groups of 5–10 cultured islets were incubated for 60 min in a bicarbonate buffer [20] as described above. Within 15 seconds after the incubation the islets were frozen at −70° C and freeze-dried over-night. They were then carefully dissected free from adhering salt crystals and weighed on a quartz fibre balance [26] before being assayed for their concentration of ATP and the sum of adenine nucleotides.

*Expression of Results.* The rate of glucagon release was calculated as pg glucagon released/μg islet dry weight/h. Glucose utilization rates were calculated as pmol glucose utilized/μg islet dry weight/h, according to Ashcroft et al. [23]. The islet concentration of adenine nucleotides was expressed as mmol/kg islet dry weight. The results in each experimental group were expressed as the mean ± SEM of a series of experiments each one representing the mean value of 2–4 observations on islets obtained from the same animal. Statistical significances were evaluated with the aid of Student's t-test [27] and in one case (as indicated in Table 1) with Mann-Whitney's U-test [28].

## Results

*Characterization of the Experimental Animals.* All guinea pigs injected with streptozotocin had a strongly positive reaction for urinary glucose and a marked retardation of growth; mean body weight at death was 168 ± 5 g (n = 30) compared to 297 ± 11 g (n = 21) for controls ($P < 0.001$). The blood glucose concentration at this time was 7.1 ± 0.4 mmol/l (n = 15) in the streptozotocin treated animals and 8.9 ± 0.6 mmol/l (n = 5) in the controls ($P < 0.05$). Light microscopy of pancreatic sections showed that in every streptozotocin injected animal the islets were predominantly composed of $A_2$-cells with only a few remaining B-cells.

*Glucagon Release.* The effects of glucose and of insulin on the glucagon release of the $A_2$-cell rich islets in vitro are shown in Table 1. As expected the highest glucagon release was found at 1.7 mmol/l glucose. Raising the glucose concentration to 16.7 mmol/l caused no significant decrease of the glucagon release. Addition of insulin (30 mU/ml) to the medium in the presence of 1.7 mmol/l glucose inhibited glucagon release by more than 50%. There was no further major decrease of glucagon release when the glucose concentration was raised to either 3.3 mmol/l or 16.7 mmol/l in the presence of insulin. Insulin itself, in the absence of glucose, did not influence glucagon secretion. In control experiments with normal guinea pig islets the glucagon release

decreased from 28.8 ± 3.5 to 20.6 ± 2.1 pg/μg/h, when the glucose level of the incubation medium was raised from 1.7 to 16.7 mmol/l (n = 10; $P < 0.05$). Addition of insulin (30 mU/ml) had no effect at any glucose concentration.

*Glucose Utilization.* In the absence of exogenous insulin glucose utilization was significantly higher in the normal than in the $A_2$-cell rich islets (Table 2). In the normal islets addition of insulin (30 mU/ml) to the high glucose incubation medium (16.7 mmol/l) did not affect glucose utilization significantly. In contrast, when insulin was added at a similar concentration to $A_2$-cell rich islets, a significant stimulation of glucose utilization was noted at all glucose concentrations. The magnitude of this stimulation was most pronounced at the lowest glucose concentration, 1.7 mmol/l, when it was over 100 per cent of the value in the absence of insulin. At 3.3 mmol/l glucose the stimulatory action of insulin was 42% and at 16.7 mmol/l glucose was 50%.

*Adenine Nucleotides.* As shown in Table 3 the level of ATP of the $A_2$-cell rich islets increased significantly when the glucose concentration was raised from 1.7 or 3.3 mmol/l to 16.7 mmol/l. However, there was a further significant increase at all glucose concentrations when the media were supplemented with insulin (30 mU/ml). Under all conditions the sum of ATP, ADP and AMP remained within the narrow range of 26.7 to 33.4 mmol/kg islet dry weight (not shown in the table).

*Effects of Somatostatin.* Addition of somatostatin (2.5 μg/ml) reduced the glucagon release of the $A_2$-cell rich islets by more than 50% at both 1.7 mmol/l and 3.3 mmol/l glucose (Table 1). In contrast, somatostatin failed to affect islet glucose utilization at either of these glucose concentrations (Table 2).

**Table 1.** Glucagon release from $A_2$-cell rich islets of the guinea pig

| Glucose concentration (mmol/l) | Addition to the medium | Number of experiments | Glucagon release (pg/μg islet dry weight/h) |
|---|---|---|---|
| 0 | – | 6 | 51.1 ± 3.3 |
| 0 | Insulin (30 mU/ml) | 7 | 48.0 ± 5.2 |
| 1.7 | – | 13 | 76.9 ± 7.8 |
| 1.7 | Insulin (30 mU/ml) | 13 | 35.1 ± 3.8[a] |
| 1.7 | Somatostatin (2.5 μg/ml) | 8 | 37.4 ± 10.6[b] |
| 3.3 | – | 16 | 61.4 ± 4.6 |
| 3.3 | Insulin (30 mU/ml) | 13 | 26.6 ± 2.9[c] |
| 3.3 | Somatostatin (2.5 μg/ml) | 8 | 29.1 ± 3.0[c] |
| 16.7 | – | 11 | 58.4 ± 6.9 |
| 16.7 | Insulin (30 mU/ml) | 10 | 36.2 ± 3.5[d] |

Islets were incubated for 60 min in a bicarbonate buffer with the additions as indicated. Glucagon release is expressed as the mean value ± SEM (pg/μg islet dry weight/h). [a] $P < 0.001$ vs 1.7 mmol/l glucose; [b] $P < 0.02$ vs 1.7 mmol/l glucose (Mann-Whitney's U-test); [c] $P < 0.001$ vs 3.3 mmol/l glucose; [d] $P < 0.01$ vs 16.7 mmol/l glucose

**Table 2.** Glucose utilization by normal and $A_2$-cell rich islets of the guinea pig

| Glucose concentration (mmol/l) | Addition to the medium | Glucose utilization (pmol/μg islet dry weight/h) | | |
|---|---|---|---|---|
| | | Normal islets | $A_2$-cell rich islets | |
| 1.7 | – | 24.3±2.9 [7] | 16.6±2.0[d] | [11] |
| 1.7 | Insulin (30 mU/ml) | – | 33.9±2.8[a] | [7] |
| 1.7 | Somatostatin (2.5 μg/ml) | – | 17.9±2.9 | [11] |
| 3.3 | – | 35.9±3.4 [5] | 23.7±2.8[d] | [13] |
| 3.3 | Insulin (30 mU/ml) | – | 33.6±1.4[b] | [9] |
| 3.3 | Somatostatin (2.5 μg/ml) | – | 25.1±2.5 | [13] |
| 16.7 | – | 94.4±2.3 [5] | 64.6±5.8[e] | [12] |
| 16.7 | Insulin (30 mU/ml) | 110.5±8.8 [5] | 97.2±8.1[c, f] | [11] |

Glucose utilization was measured as the formation of $^3H_2O$ from 5-$^3$H-glucose. Islets were incubated for 60 min in a bicarbonate buffer with the additions listed in the Table. Number of experiments are shown in parentheses. Results are given as mean values ± SEM (pmol/μg islet dry weight/h) [a] $P < 0.001$ vs 1.7 mmol/l glucose; [b] $P < 0.05$ vs 3.3 mmol/l glucose; [c] $P < 0.01$ vs 16.7 mmol/l glucose; [d] $P < 0.05$ vs corresponding group of normal islets; [e] $P < 0.01$ vs corresponding group of normal islets; [f] $P > 0.05$ vs corresponding group of normal islets

## Discussion

*Methodological Considerations.* An inherent requirement of the present experimental models is the use of streptozotocin to dispose of the B-cells and obtain islets rich in $A_2$-cells. Apart from possible direct effects on the $A_2$-cell, streptozotocin causes a severe hyperglycaemia in most laboratory animals [29, 30], which may in itself affect the regulation of glucagon release from this cell [31]. However, the present study shows, in conformity with previous investigations, that guinea pigs remain normoglycaemic, or may even become slightly hypoglycaemic, after streptozotocin treatment [13]. Presumably, this paradoxical reaction reflects a severe tubular necrosis with heavy loss of glucose into the urine [13]. There is, furthermore, no evidence of a direct toxic effect of streptozotocin on the $A_2$-cell, which remains normal both morphologically and functionally after administration of this agent [13, 14].

Another potential hazard is the collagenase isolation procedure, which has previously been reported to impair the normal regulation of hormonal discharge from both B- and $A_2$-cells [32]. Tissue culture of islets after exposure to collagenase has, however,

**Table 3.** Concentrations of ATP in $A_2$-cell rich islets of the guinea pig

| Glucose concentration (mmol/l) | Additions to the medium | Number of experiments | ATP (mmol/kg) |
|---|---|---|---|
| 1.7 | – | 6 | 9.2 ± 0.8 |
| 1.7 | Insulin (30 mU/ml) | 6 | 15.1 ± 2.1[a, d] |
| 3.3 | – | 11 | 9.0 ± 0.3 |
| 3.3 | Insulin (30 mU/ml) | 5 | 13.9 ± 0.6[b, d] |
| 16.7 | – | 16 | 11.5 ± 0.5[a, b] |
| 16.7 | Insulin (30 mU/ml) | 11 | 14.4 ± 0.4[c] |

Islets incubated for 60 min in a bicarbonate buffer with the given additions. Each experiment represents the mean of 2–4 determinations on islets from one animal. Results are expressed as mean values ± SEM (mmol/kg islet dry weight). [a] $P < 0.05$ vs 1.7 mmol/l glucose; [b] $P < 0.001$ vs 3.3 mmol/l glucose; [c] $P < 0.001$ vs 1.7, 3.3 or 16.7 mmol/l glucose; [d] $P < 0.05$ vs 16.7 mmol/l glucose

been shown to restore the islet cell function [33], and the present culture of islets for 24–48 hours appears to have been sufficient for recovery. It should be noted in this context that the glucose utilization of the $A_2$-cell rich islets was quite sensitive to insulin even without a preceding culture period.

In any studies of functional variables other than the specific hormone production of the islets the question must be raised to what extent the results are representative for a particular islet cell type. Previous data indicate that injection of streptozotocin in the guinea pig changes the proportion of $A_2$-cells from the normal value (15–20 per cent) to 60–70 per cent [13, 14]. At the same time the islet glucagon content is doubled, whereas insulin falls to about 10 per cent of the normal value [14]. These observations strongly support the notion that the present data on glucose utilization and ATP concentrations of the $A_2$-cell rich islets mainly reflect the status of this cell type. It is worthy of note, however, that islets devoid of B-cells remain much smaller than those containing a normal complement of these cells. To make possible relevant comparisons between the two sets of islets all results were therefore expressed on the basis of islet dry weight rather than the number of islets.

*Effects of Insulin on the Glucagon Release.* The present data indicate that in the presence of glucose insulin exerted a powerful depressant action on the glucagon release of the $A_2$-cell rich islets, whereas no significant effect of glucose alone was observed. In normal islets glucagon release was, however, not affected by exogenous insulin and only to a small extent by glucose. The lack of insulin effects on the latter islets presumably was due to accumulation in these preparations of endogenous insulin sufficient to saturate putative receptors on the $A_2$-cells. It has actually been calculated from studies on the perfused pancreas that during maximal glucose stimulation the local insulin concentration around the islet cells may exceed 100 mU/ml [34]. In contrast the $A_2$-cells used in the present study had been deprived of most of their surrounding B-cells for at least a week before the experiment and also maintained at normoglycaemia, both in vivo and in tissue culture. This probably resulted in grossly suboptimal intra-islet insulin concentrations, which may well have made the $A_2$-cells more sensitive to acute exposure to exogenous insulin. The precise molecular mode of interaction between the $A_2$-cell and insulin, however, remains to be elucidated. There is, for example, nothing known on the intra-islet circulation and the rate of exchange of extracellular fluid around the $A_2$-cell.

The observations discussed above indicate that the $A_2$-cell is, indeed, insulin sensitive and agree in this regard with a number of previous reports dealing with insulin effects on glucagon release [6, 34–40]. Of particular interest in this context are experimental studies indicating that the release of gastric glucagon from the perfused canine stomach is sensitive to insulin, even in the physiological range of concentration [41]. Cells manufacturing gastric glucagon, which is physicochemically and immunologically indistinguishable from pancreatic glucagon, are devoid of adjacent B-cells and may therefore serve as an interesting model for studies of the regulation of glucagon release in the total absence of insulin. In addition, Samols and Harrison [37], using the perfused pancreas from streptozotocin treated dogs, demonstrated a significant depression of glucagon release when 25 mU/ml of exogenous insulin was added to the perfusate, whereas lower concentrations were ineffective.

*Effects of Insulin on the Glucose Metabolism of the $A_2$-Cell.* The demonstration that insulin inhibits glucagon release raises the question whether this effect is mediated via an altered glucose catabolism of the $A_2$-cells. Indeed, the present observation that insulin selectively stimulates the glucose utilization rate of islets rich in $A_2$-cells strongly suggests that this is the case. The results also show that the glucose usage of the $A_2$-cell is below that of the B-cell, except in the presence of adequate amounts of insulin, such as could be expected to occur in the extracellular space of the normal islet. Glucose utilization was, however, significantly enhanced by insulin already at glucose concentrations far below the plasma levels. This suggests that glucose uptake by the $A_2$-cell is rate limiting and may be regulated by insulin in a way comparable to that observed with other insulin sensitive cells. However, the insulin concentration required to achieve this effect is apparently far above that required to affect the glucose uptake of, for instance, muscle [42] or fat cells [43]. This, of course, could be expected in view of the high insulin environ-

ment of the normal $A_2$-cell, which may lead to a sustained down-regulation of the insulin receptor concentration of the plasma membrane of this cell. The insulin sensitivity of the $A_2$-cell, therefore, may show characteristics quite different from those of other cell types.

Previous studies have provided indirect evidence for an essential role of ATP in the regulation of glucagon release from the $A_2$-cell [44–46]. The present results lend more direct support to this hypothesis in that the most pronounced suppression of glucagon release was always observed when the ATP concentration of the $A_2$-cell rich islets reached an apparent maximum. Moreover, these high ATP concentrations occurred only in the presence of exogenous insulin and irrespective of whether the glucose concentration was low or high. Glucose utilization was, however, faster at 16.7 mmol/l alone than at 1.7 mmol/l glucose plus insulin, suggesting that the rate of glucagon release is more closely correlated with the level of ATP than the flow of glucose metabolites in the $A_2$-cells. A variable production of lactate could explain why intracellular ATP values are not directly proportional to the glucose utilization rate of the $A_2$-cell. An effect other than the promotion of glucose uptake of a high insulin concentration on this cell is also a challenging possibility.

*Stimulus-Secretion Coupling of the Glucagon Release.* The observations discussed above conform to the idea that regulation of glucagon release, at least as controlled by glucose, is mediated through energy-yielding reactions of the $A_2$-cell. In further support of this, inhibitors of ATP generation, such as malonate, iodoacetate and 2,4-dinitrophenol, stimulate glucagon secretion [44, 47]. Thus, it appears that suppression, rather than stimulation, of glucagon release is an energy-requiring process, and that a lowered phosphate potential of the $A_2$-cell may be a signal for hormone discharge. Although there is no definite explanation of this seemingly paradoxical relationship, it is noteworthy that recent investigations indicate a reduced calcium uptake and a net outflow of calcium from $A_2$-cells exposed to high glucose [48]. Calcium deprivation has been shown to impair glucagon release and redistribution of this ion in response to an altered phosphate potential might provide a basis for regulation of the hormone discharge. However, the role of calcium in the regulation of glucagon release at present appears very complex [49–52] and the intimate relationship between the energy metabolism and calcium fluxes of the $A_2$-cell must await further study.

The present observation that somatostatin failed to affect glucose utilization of the $A_2$-cell rich islets, while at the same time markedly inhibiting glucagon discharge, suggests the existence of alternative mechanisms for the regulation of secretion of the $A_2$-cell.

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