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EFFECTS OF METABOLIC INHIBITORS ON THE REGULATION OF PANCREATIC GLUCAGON RELEASE

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Summary

The effects of metabolic inhibition on the kinetics of glucagon release by pancreatic islets isolated from normal guinea pigs have been studied in a perifusion system. All three metabolic inhibitors, malonate, iodoacetate and 2,4-dinitrophenol, induced a marked stimulation of glucagon secretion, each displaying its own characteristic pattern of response. This was accompanied by a rapid and marked decrease in the ATP concentration of the A_2-cell as evidenced by measurements performed in A_2-cell rich islets, isolated from guinea pigs treated with streptozotocin. The ATP levels were reduced by about 90 percent following iodoacetate and 2,4-dinitrophenol exposure and by about 60 percent after exposure to malonate. The data support the hypothesis that the inhibition of glucagon release from the A_2-cell is regulated via an intracellular, energy-dependent mechanism.

Introduction

There is at present strong evidence to support the idea that insulin is necessary for the normal regulation of glucagon release by glucose [1—6]. It has thus been shown that the hyperglucagonaemia in diabetes mellitus can be corrected by the administration of high doses of insulin [2,3,7—9]. Furthermore, we have demonstrated that exposure of A_2-cell rich islets of streptozotocin-treated guinea pigs to high concentrations of exogenous insulin may lead to a depressed glucagon release, an enhanced glucose utilization and increased concentrations of ATP [10]. On the other hand, previous studies indicate that exposure of normal isolated guinea pig islets to various metabolic inhibitors (malonate, iodoacetate, 2.4-dinitrophenol and cyanide) causes an increased glucagon release [11].

These findings have led to the suggestion that an enhanced glucose metabolism and a raised ATP level in the A_2-cell are somehow related to the inhibition of glucagon release by glucose. Thus, the A_2-cell might depend on a raised intracellular energy level to be able to restrain its hormonal output, and conversely, energy deprivation may result in glucagon secretion. It was the aim of the present study to investigate in more detail the kinetics of glucagon release from guinea pig islets exposed to various metabolic inhibitors in a perifusion system. Moreover, the data have been related to changes in the ATP concentration of the A_2-cell, as shown by measurements of ATP in A_2-cell rich pancreatic islets isolated from streptozotocin treated guinea pigs.

Materials and Methods

Chemicals. Streptozotocin was kindly donated by Dr. W.E. Dulin, The Upjohn Co., Kalamazoo, MI, U.S.A. Collagenase was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Tissue culture medium (TCM 199) supplemented with 5.5 mM glucose, Hanks' solution and calf serum were supplied by Statens Bakteriologiska Laboratorium, Stockholm, Sweden. Penicillin and streptomycin were from Glaxo Laboratories Ltd., Greenford, Middx., U.K. Bovine plasma albumin (fraction V) was obtained from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Disodium malonate and sodium iodoacetate were provided by Sigma Chemical Co., St. Louis, MO, U.S.A. and 2,4-dinitrophenol was from Hopkin and Williams, Ltds., Chadwell Heath, Essex, U.K. ^{125}I-Labelled porcine glucagon, pork glucagon and anti-pork glucagon rabbit serum K 4023 were purchased from Novo A/S, Copenhagen, Denmark. Tris(hydroxymethyl)aminomethane, firefly luciferase and apyrase (EC 3.6.1.5) were supplied by Sigma Chemical Co., St. Louis, MO, U.S.A. ATP, ADP, AMP, phospho*enol*pyruvate, adenylate kinase (EC 2.7.4.3) and pyruvate kinase (EC 2.7.1.40) were obtained from Boehringer und Soehne, Mannheim, F.R.G. Ethylene diamine tetraacetate (EDTA) was from E. Merck AG, Darmstadt, F.R.G. All other chemicals were of purest grade commercially available.

Animals and preparation of islets. Islets intended for perifusion experiments were isolated aseptically from pancreatic glands of normal male guinea pigs (250—350 g), using a collagenase digestion method [12]. In order to reduce the potentially harmful effects of the collagenase digestion, the islets intended for perifusion were subsequently kept in tissue culture [13] for two days in culture medium 199 (TCM 199) supplemented with 5.5 mM D-glucose, 10% calf serum and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). Islets prepared for the determination of adenine nucleotide concentrations were obtained as above either from untreated guinea pigs or from guinea pigs injected i.p. with 375 mg/kg body weight streptozotocin 1—2 weeks prior to islet isolation [1,14,15]. These islets were cultured as described above [13,16] for one week prior to any further experimentation.

From each streptozotocin treated animal a small piece of the pancreatic gland was fixed in Bouin's solution for subsequent histological processing. Several sections from each gland were stained with aldehyde fuchsin trichrome [17] to evaluate, by light microscopy, the effect of streptozotocin on the

B-cells. Only animals with a predominance of A_2-cells in their islets (A_2-cell rich islets) were included in the further experiments.

Perifusion technique. The perifusion equipment was modified from similar, previously described systems [18,19]. In principle, the perifusion media were kept at +37° C in two reservoir flasks and gassed continuously with a gas mixture of O_2/CO_2 (95 : 5, v/v). The media circulated by a peristaltic pump at a constant flow rate of 0.5 ml per min, and passed through a Millipore chamber surrounded by water at +37°C. Each chamber contained 100—165 islets isolated from non-treated guinea pigs. Fractions of the effluent media were immediately frozen and stored for subsequent assay of glucagon immunoreactivity.

All perifusions were performed using a bicarbonate buffer composed of 111.2 mM NaCl, 0.8 mM Na_2HPO_4, 27.0 mM $NaHCO_3$, 5.1 mM KCl, 0.2 mM KH_2PO_4, 1.4 mM $CaCl_2$, 0.3 mM $MgSO_4$, 1.0 mM $MgCl_2$ [20], and supplemented with 0.5 mg/ml bovine plasma albumin and 6.1 mM D-glucose. As shown in Figs. 1—3, islets were exposed to each of the metabolic inhibitors (malonate, 7 mM; iodoacetate, 0.4 mM; 2,4-dinitrophenol, 0.1 mM) for a test period of 30 min. Each experiment lasted for 125 min and fractions were collected as follows: (a) a 35-min equilibration period to establish a baseline glucagon secretion rate. Fractions were collected every minute during the five minutes immediately preceding the test period; (b) a 30-min test period with fractions collected every minute for 15 min, then every fifth minute; (c) a 1-h post-test period comprising six 5-min fractions, followed by three 10-min fractions. Samples (100 μl) of the perifusate fractions were assayed for glucagon by radioimmunoassay [21].

Determination of ATP and total adenine nucleotides. To investigate the effects of metabolic inhibitors on the concentrations of adenine nucleotides in the A_2-cell, isolated islets from 16 non-treated and 17 streptozotocin-injected guinea pigs were used. Following tissue culture for one week (see above), groups of 5—10 A_2-cell rich islets were transferred to small glass incubation vials [22] containing 300 μl of a bicarbonate buffered medium [20] supplemented with 2 mg/ml bovine plasma albumin and 6.1 mM D-glucose. The medium contained either malonate (7 mM), iodoacetate (0.4 mM) or 2,4-dinitrophenol (0.1 mM). Incubations were performed in a shaking water bath (100 strokes/min) at +37°C in a gas phase of O_2/CO_2 (95 : 5, v/v) for 1, 5 or 30 min, after which time the islets were immediately frozen at —70°C and subsequently freeze-dried overnight. Groups of cultured, normal guinea pig islets were similarly incubated for 30 min in the three different media. Groups of both normal and A_2-cell rich islets, either harvested directly from the culture dish (0 min), or after a 30-min incubation in medium containing no inhibitor, served as controls. The freeze-dried islets were detached from adhering salt crystals and weighed on a quartz fiber balance before being assayed for their concentration of ATP and, in some cases, their sum of adenine nucleotides. Adenine nucleotides were measured in these islets by a photokinetic bioluminescence luciferase technique [23], in which the concentration of ATP was measured directly and the total pool of adenine nucleotides was determined after enzymatic conversion of ADP and AMP to ATP.

Expression of results. The rate of glucagon release was expressed as pg

glucagon per islet per min of perifusion. The islet concentration of adenine nucleotides was calculated as mmol/kg islet dry weight, and the results were expressed as the mean value ± S.E.M. of a series of experiments, each one representing the mean value of 1—3 determinations on islets from the same animal. Statistical significances were evaluated with the aid of Student's *t*-test for independent variables.

Results

Effects of metabolic inhibitors on glucagon release

The results of the perifusion experiments are shown in Figs. 1—3. Malonate, at a concentration of 7 mM (Fig. 1), effected a short-lived (approx. 5 min) monophasic stimulation of the glucagon release, which was followed by a gradual return to base-line levels. The mean secretory rate during the first five minutes of stimulation (as estimated from calculations of the total sum of secreted glucagon during this particular period) amounted to 2.37 ± 0.28 pg per islet per min, while base-line secretion during the ten preceding minutes was 1.50 ± 0.08 pg per islet per min ($n = 5$; $P < 0.01$). Addition of 0.4 mM iodoacetate (Fig. 2) caused a biphasic release pattern with a decrease in the secretion rate to base-line levels immediately after omission of the inhibitor. In this case, the basal release, 1.25 ± 0.17 pg per islet per min, was increased to 2.44 ± 0.26 pg per islet per min ($n = 5$; $P < 0.001$) during the first ten minutes of exposure to iodoacetate, and 2.37 ± 0.30 pg per islet per min ($P < 0.01$) during the sub-

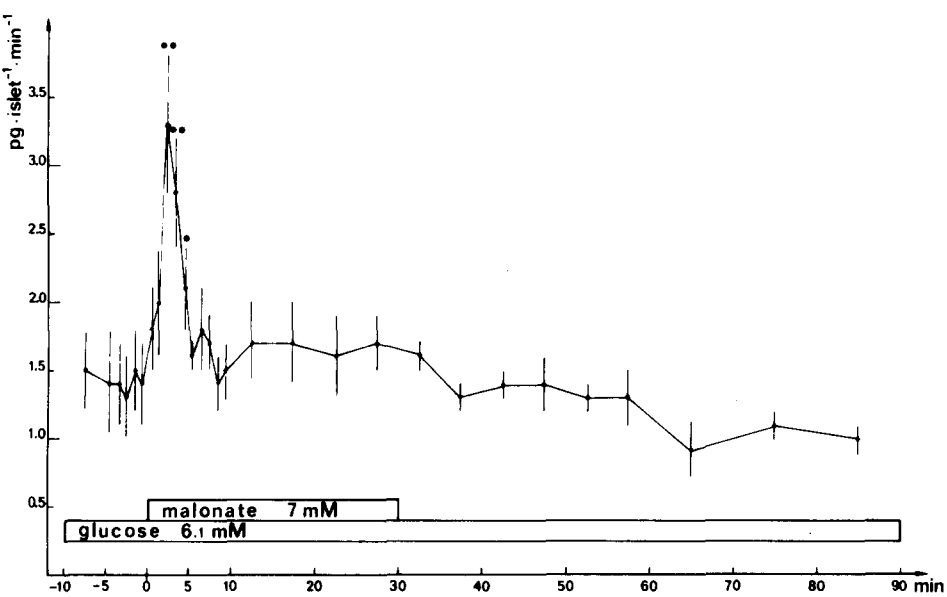

Fig. 1. Effect of malonate on glucagon release from isolated guinea pig islets in a perifusion system. In each of five experiments 100—165 islets were perifused with a bicarbonate buffered medium supplemented with 6.1 mM glucose. Malonate (7 mM) was added during the test period indicated in the figure. The flow rate was 0.5 ml per minute. The glucagon release (mean values ± S.E.M.) is expressed as pg per islet per min. Statistical significances between the base-line release (mean value for —10 to 0 min) and stimulated release are indicated as * $P < 0.05$, ** $P < 0.01$.

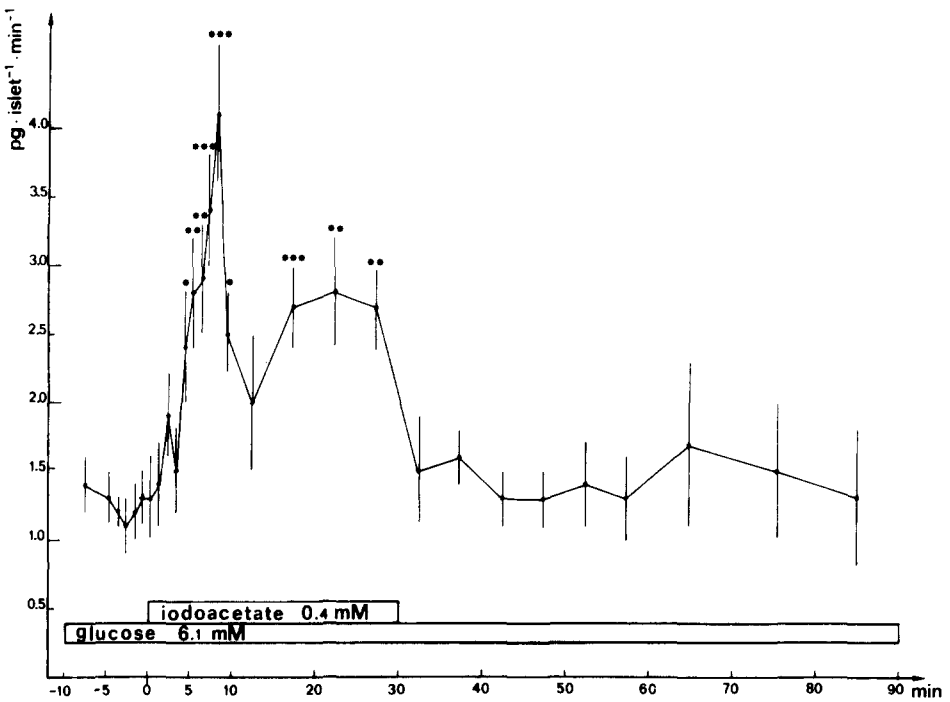

Fig. 2. Effect of iodoacetate on glucagon release from isolated guinea pig islets in a perifusion system. Conditions were similar to those described in the legend to Fig. 1, except that iodoacetate (0.4 mM) was added during the test period indicated in the figure. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

TABLE I

EFFECTS OF METABOLIC INHIBITORS ON ATP CONCENTRATION IN ISOLATED NORMAL AND A_2-CELL RICH GUINEA PIG ISLETS

Isolated pancreatic islets from either normal or streptozotocin-treated guinea pigs were cultured for one week in TCM 199 supplemented with 5.5 mM glucose. Batches of 5—10 islets from each animal were incubated for 1, 5 or 30 min in a buffered solution containing 6.1 mM glucose and one of the metabolic inhibitors, as given in the table. Groups of islets were also incubated without addition of inhibitor to the solution (controls), and some of them were assayed directly after the harvest from the culture dishes (0 min). ATP was determined in lyophilized islet specimens with the aid of a photokinetic bioluminescence method. The results are expressed as mmol ATP per kg islet dry weight. The number of animals in each group is given in parentheses. Statistical significances are related to non-incubated controls and indicated as follows: [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.

Type of islets	Duration of incubation (min)	Control (no addition)	Malonate (7 mM)	Iodoacetate (0.4 mM)	2,4-Dinitrophenol (0.1 mM)
A_2-cell rich islets	0	9.0 ± 0.9 (7)	—	—	—
	1	—	6.3 ± 0.9 (7) [a]	6.5 ± 0.8 (7) [a]	6.6 ± 0.7 (9) [a]
	5	—	4.7 ± 0.6 (8) [c]	6.2 ± 0.7 (12) [a]	4.9 ± 0.9 (7) [b]
	30	12.9 ± 1.0 (5)	2.9 ± 0.3 (11) [c]	0.3 ± 0.1 (5) [c]	0.7 ± 0.2 (6) [c]
Normal islets	0	11.2 ± 0.7 (11)	—	—	—
	30	14.1 ± 0.9 (5)	3.9 ± 0.5 (5) [c]	1.1 ± 0.3 (11) [c]	0.9 ± 0.3 (5) [c]

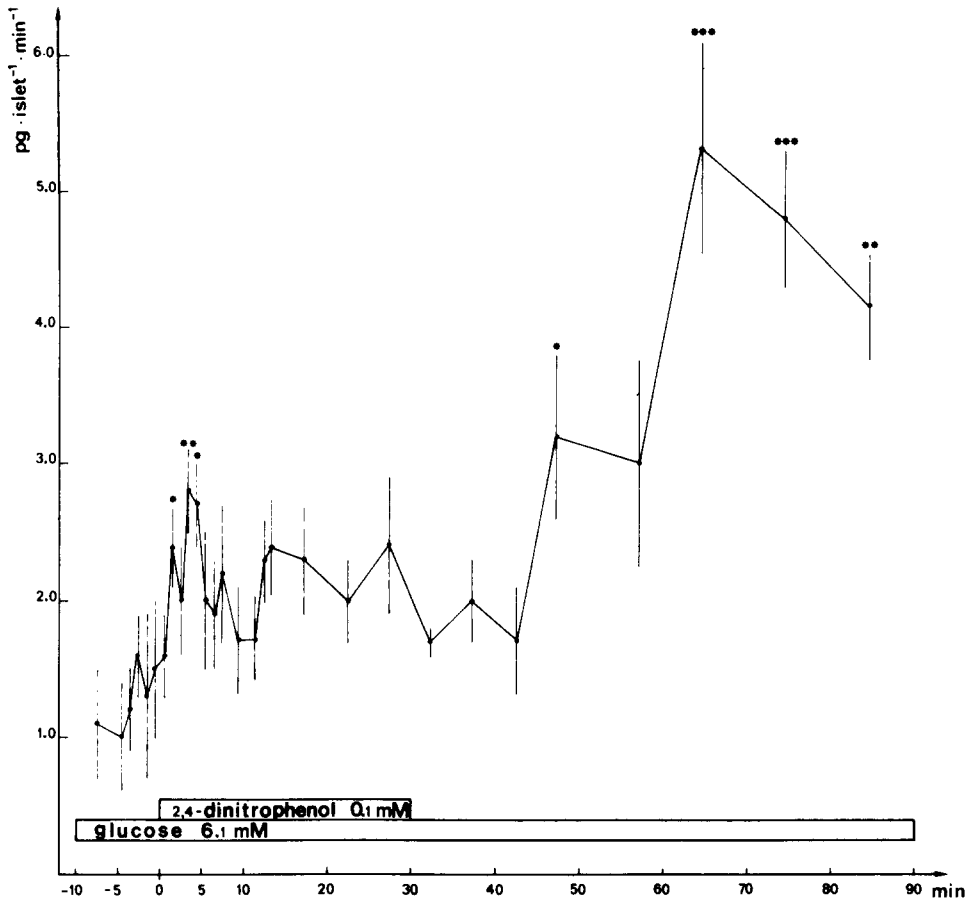

Fig. 3. Effect of 2,4-dinitrophenol on glucagon release from isolated guinea pig islets in a perifusion system. Conditions were similar to those described in the legend to Fig. 1, except that 2,4-dinitrophenol (0.1 mM) was added during the test period indicated in the figure. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

sequent 20 min. As seen in Fig. 3, 2,4-dinitrophenol significantly enhanced the glucagon release only during the first five minutes. Following omission of the inhibitor the secretory rate did not return to base-line levels but continued to rise in an apparently uncontrolled manner.

Effects of metabolic inhibitors on islet ATP concentration

Table I shows the results of the ATP determinations in A_2-cell rich and normal islets. The concentration of ATP in A_2-cell rich islets was approximately 80% of that in normal islets, when measured directly after one week of culture at 5.5 mM glucose. Exposure of A_2-cell rich islets to any of the tested inhibitors for only one minute, caused a significant decrease of the ATP level as compared to non-exposed islets ($P < 0.05$). Prolongation of the incubation period to 30 min induced very low ATP concentrations when either iodoacetate or 2,4-dinitrophenol was present ($P < 0.001$). The decrease in ATP was, however,

less pronounced after exposure to malonate. Incubation of normal islets for 30 min in the presence of inhibitors induced ATP concentrations similar to those of the corresponding A_2-cell rich islets, though significantly higher values were observed in the normal group exposed to iodoacetate ($P < 0.05$).

The sum of adenine nucleotides in the A_2-cell rich islets remained unchanged in all incubations lasting for 30 min, the mean values ranging from 24.5 to 26.3 mol/kg islet dry weight.

Discussion

The present data confirm that substances known to interfere with the metabolism of numerous different cell types also cause a marked decrease in the ATP concentration of the A_2-cell. This action, however, was without effect on the total sum of adenine nucleotides in the A_2-cell. The simultaneous increase of glucagon release confirms the findings of Edwards and Taylor [11] who demonstrated that metabolic inhibitors stimulated the glucagon release from guinea pig islets. On the basis of these observations it was suggested that ATP may be of direct regulatory significance for glucagon secretion [10,11]. In further support of this notion Hahn and Ziegler found that iodoacetamide enhanced glucagon release from isolated rat islets [24].

In order to obtain a sufficient number of islets from one animal for each perifusion experiment, the perifusions were performed with islets isolated from normal guinea pigs. Since the A_2-cell has previously been shown to be sensitive to insulin [1—6], it must be considered whether the present effect of metabolic inhibitors on glucagon release from normal islets is a direct effect on the A_2-cell or an effect mediated via influences on the insulin release. Unfortunately, insulin could not be assayed since no suitable antiserum to guinea pig insulin was available. The use of guinea pigs in this study is, however, justified by the fact that islets from these animals treated with streptozotocin are more easily isolated and are more suitable for metabolic evaluation than are the corresponding islets from other species. It is well known that 2,4-dinitrophenol [25] and iodoacetate [24,26—29] inhibit glucose-stimulated insulin release in other species, though the latter compound has been shown to stimulate insulin release at very low concentrations [28,29]. Malonate, at a concentration higher than that used in the present study, does not, however, seem to affect insulin release [25]. Thus, it seems unlikely that the observed alterations in glucagon release induced by the inhibitors should be attributed directly to an inhibition of the rate of insulin secretion.

The present experimental design permits a comparison between the dynamics of glucagon release and the temporal changes of the ATP concentration of the islets. In addition, the various patterns of release may be interpreted in the light of present knowledge of the specific action of each of the different inhibitors. Thus malonate, which acts mainly by inhibiting succinate dehydrogenase, thereby leading to a decreased endogenous respiration and an enhanced glycolytic rate by the Pasteur effect [30], reduced the ATP concentration by only 67 percent of the initial control value. Concomitantly, the glucagon release was stimulated as a single, short-lived peak after a lag period of about three minutes. Iodoacetate, at the concentration used in the present study, is

known to block anaerobic glycolysis by at least 90 percent by inhibiting 3-phosphoglyceraldehyde dehydrogenase [31]. It may also, at least in part, block aerobic glycolysis and cell respiration [31], and influence certain membrane bound mechanisms [28,29]. 2,4-Dinitrophenol, an uncoupler of the oxidative phosphorylation, and iodoacetate reduced the initial ATP level during the 30 min incubation by approximately 95 percent. Indeed, these two latter inhibitors created a more pronounced increase in the release of glucagon as compared to that exerted by malonate, suggesting a role of ATP in the regulation of glucagon release. However, 2,4-dinitrophenol seemed to cause irreversible damage to the secretory mechanisms of the A_2 cell, as shown by the continued uncontrolled glucagon release following its omission from the perifusion medium.

The overall observation of an increased glucagon release shortly after exposure of A_2-cells to metabolic inhibitors indicates an inverse relationship between the ATP concentration and the secretory rate of the A_2-cell. This notion is reinforced by our recent observation that glucose, in combination with a high concentration of insulin, causes a reduced glucagon secretion as well as an increase in glucose utilization and ATP concentration of A_2-cells [10]. Furthermore, it is possible that, as suggested by the demonstrated differences between effects of malonate and iodoacetate on glucagon release, the inhibition of the second phase of glucagon release is more closely coupled to the cellular energy supply than is the first phase. Our findings therefore conform to the idea that regulation of glucagon release, at least by glucose and probably also by glyceraldehyde [24], is mediated via energy-yielding reactions of the A_2-cell.

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