Glucagon Release from Rat Pancreatic Islets

A COMBINED MORPHOLOGICAL AND FUNCTIONAL APPROACH

JEAN-LOUIS CARPENTIER, FRANCINE MALAISSE-LAGAE, WALTER A. MÜLLER, and LELIO ORCI, *Institut d'Histologie et d'Embryologie and Institut de Biochimie Clinique, Université de Genève, Genève, Switzerland*

ABSTRACT The release of glucagon induced in isolated rat islets by arginine or by calcium deprivation has been subjected to combined functional and morphological quantifications. Arginine-stimulated glucagon release was associated with a significant increase of morphological events linked to exocytosis. By contrast, the paradoxical release of glucagon provoked by calcium deprivation, although accompanied by a significant loss of granule stores, was not associated with an increase of morphologically detectable exocytosis.

INTRODUCTION

The ultrastructural characteristics of glucagon release by the A cells (or A_2 cells) of the islets of Langerhans are poorly understood. Several authors have suggested that release occurs through intracytoplasmic dissolution of the secretory granules (1–4), while others have proposed that granules are extruded by exocytosis (5–10).

In the present study, we have attempted to clarify this issue by simultaneously studying the functional and morphological events involved in glucagon secretion from isolated islets. This was accomplished with biochemical measurement, with morphometric quantification of thin section preparations, and by employing freeze fracturing, a technique particularly suitable for the study of exocytotic events (11).[1]

We have studied two conditions known to stimulate glucagon release: arginine that might be considered most representative of a physiological secretagogue and calcium deprivation which is a highly unphysiological condition that stimulates glucagon release (13–16). Since glucagon secretion is known to be calcium requiring in nearly all systems tested (17–20), it was of interest to test this paradoxical condition. Our study demonstrated that arginine-stimulated glucagon release occurred predominantly by an exocytotic process, but when glucagon was released by calcium deprivation no evidence for exocytosis was found.

METHODS

Animals and islet preparation. Fed Wistar rats weighing 250–300 g were used. Where indicated, the rats were pretreated with a total of five i.p. injections (0.5 mg each) of glibenclamide (a gift from Hoechst A.G., Frankfurt, W. Germany) given at 12-h intervals, the last dose being administered 1 h before sacrifice. The rats were killed by decapitation. Three pancreases were used for each experiment and the islets were isolated by collagenase digestion (21).

Incubation procedure. All incubations were carried out in a bicarbonate buffer (22) equilibrated with O_2/CO_2 (95:5 vol/vol) and adjusted to pH 7.4. Added to the incubation medium were: albumin (0.5% wt/vol) and Trasylol (1,000 KIU/ml, kindly donated by Bayer A.G., Wuppertal, W. Germany). The effect of 40 mM arginine (Merck, Darmstadt, W. Germany) was tested in the absence of added glucose in a medium with decreased NaCl (75%) content to maintain isoosmolarity. The effect of absence of calcium from the incubation medium (achieved by omitting calcium chloride and adding 1 mM EGTA [Fluka A.G., Buchs, Switzerland]) was studied at a glucose concentration of 16.7 mM.

In each experiment, 20–24 vials containing 20 islets in 1 ml of medium were incubated at 37°C for three successive periods. At the end of a first period of 20 min (equilibration period) the medium was discarded, and replaced by 1 ml of fresh medium. In the second or preincubation period (0–30 min) all vials contained the control medium. For the third or incubation period (30–60 min) half of the vials contained control medium, and the other half contained the test medium. Both preincubation and incubation media were collected and frozen for hormone assay.

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[1] Since in normal rat islets the different islet cell types cannot be identified in freeze-fracture replicas, this morphological approach was made with rats treated with glibenclamide. This drug produces a marked degranulation of the insulin-containing B cells and allows the identification of B cells separately from the remaining granulated cells of the islets, which are primarily A cells (12).

TABLE I

Secretion of Glucagon and Insulin by Isolated Islets

	Glucose, 0		Glucose, 16.5 mM	
	0–30 min	30–60 min	0–30 min	30–60 min
	ng/ml			
Glucagon				
Normal rats	1.49±0.14	2.04±0.13	0.99±0.06	1.24±0.10
	$n = 33$	$n = 31$	$n = 46$	$n = 44$
	$P < 0.01$		$P < 0.05$	
Glibenclamide-treated rats	1.26±0.13	1.85±0.18	0.84±0.09	1.15±0.07
	$n = 36$	$n = 36$	$n = 49$	$n = 46$
	$P < 0.01$		$P < 0.01$	
Insulin				
Normal rats	11.2±1.1	34.0±3.9	107.5±6.0	151.1±6.8
	$n = 34$	$n = 36$	$n = 48$	$n = 46$
	$P < 0.001$		$P < 0.001$	
Glibenclamide-treated rats	2.8±0.4	3.9±0.3	5.6±0.5	7.8±0.6
	$n = 36$	$n = 33$	$n = 36$	$n = 37$
	$P < 0.05$		$P < 0.02$	

Secretion of glucagon and insulin by isolated islets (20 islets/vial) with either no glucose present or 16.5 mM glucose present during both the preincubation (0–30 min) and the incubation (30–60 min) time in normal and glibenclamide-treated rats. At 30 min, the medium was removed and replaced by fresh medium.

At the end of the incubation period, the islets were either kept for the determination of insulin and glucagon content or fixed in 2.5% glutaraldehyde in cacodylate buffer for morphological examination. For freeze-fracture studies, the islets were centrifuged into a pellet before fixation.

Determination of insulin and glucagon in islets and in medium. The extraction of the islets was accomplished by sonication (40 s at 50 W, Branson Sonic Power Co., Danbury, Conn.) in 2 ml of glycine buffer (pH 8.8) kept in an ice bath. Insulin and glucagon were determined by radioimmunoassay (23, 24) with charcoal for separation of free from antibody-bound hormone. Insulin antiserum was kindly donated by Dr. P. H. Wright (University of Indiana, Indianapolis, Ind.) and the glucagon antiserum (30K) was a gift of Dr. R. H. Unger (University of Texas, Dallas, Tex.).

Presentation of data. Each experiment consisted of 10–12 vials incubated under a given test condition and a similar number of control vials. These experiments were repeated at least three times. Mean values obtained in each one of these experiments were similar. The n value used in statistical tests constituted therefore the total number of vials incubated under particular test condition. Two-tailed t tests were used, according to Snedecor and Cochran (25).

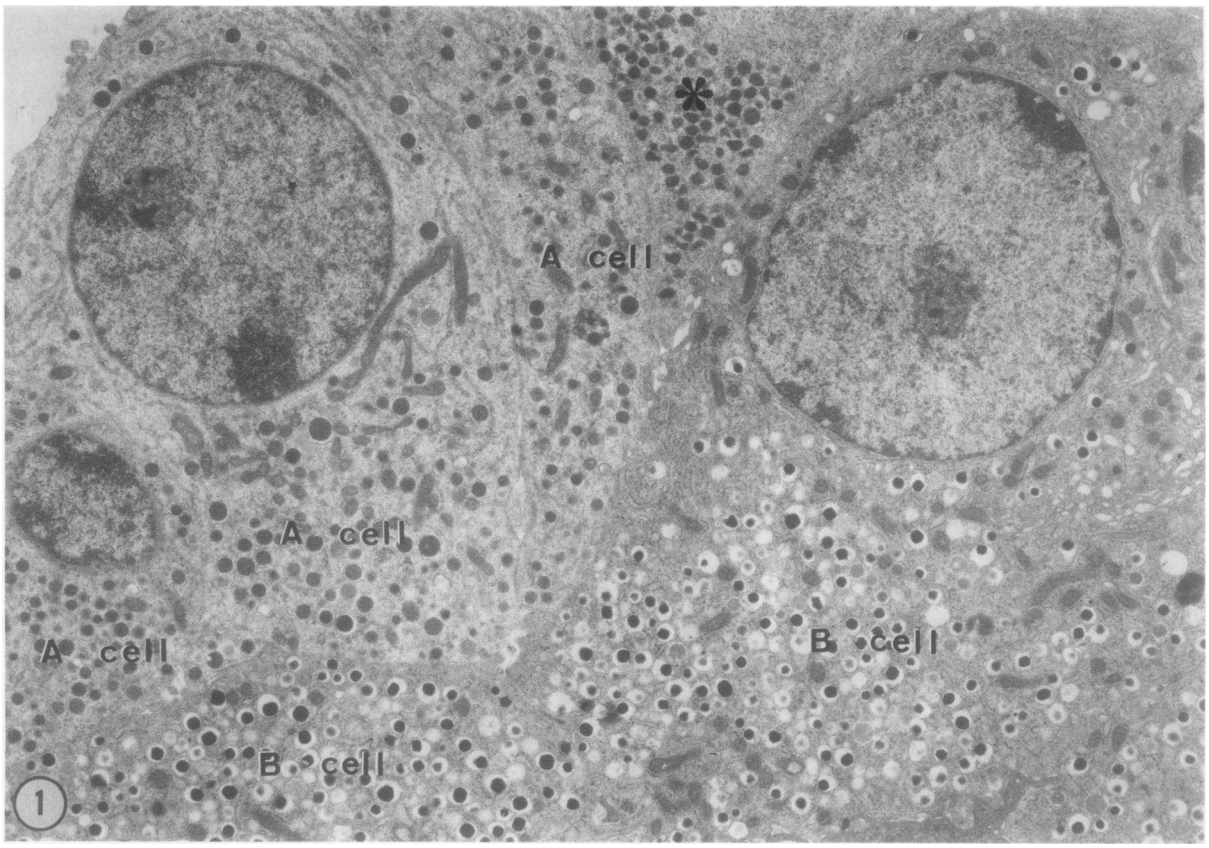
Morphologic examination. For conventional electron microscopy, the islets were fixed for 2 h, washed in cacodylate buffer, postfixed for 2 h in 2% cacodylate-buffered OsO_4, then dehydrated in graded concentrations of ethanol, and embedded in Epon (Shell Oil Co., Houston, Tex.). Thin sections stained with uranyl acetate and lead citrate were examined in a Philips EM 300 electron microscope (Philips Industries, Eindhoven, The Netherlands). Magnifications were calibrated with a reference grid (Ernest F. Fullam, Inc., Schenectady, N. Y.: 2,160 lines/mm). For freeze-fracturing procedure, pellets of islets were fixed for 2 h and then immersed in 30% glycerol buffered with 0.1 M phosphate buf-

TABLE II

Effect of Arginine (40 mM) in the Absence of Glucose and of the Absence of Calcium (αCa^{++}) in the Presence of Glucose on the Secretion of Glucagon and Insulin by Isolated Islets

	Glucose, 0	Arginine 40 mM, Glucose 0	Glucose, 16.5 mM	αCa^{++} Glucose, 16.5 mM
	% of content			
Glucagon	$P < 0.001$		$P < 0.001$	
Normal rats	5.1±0.4	8.3±0.5	3.6±0.3	9.9±0.9
	$n = 29$	$n = 29$	$n = 42$	$n = 42$
	$P < 0.005$		$P < 0.001$	
Glibenclamide-treated rats	4.7±0.6	7.5±0.8	3.0±0.2	13.6±1.4
	$n = 30$	$n = 34$	$n = 45$	$n = 37$
Insulin	$P < 0.05$		$P < 0.001$	
Normal rats	1.8±0.2	2.7±0.4	8.5±0.8	2.8±0.4
	$n = 36$	$n = 32$	$n = 21$	$n = 20$
	$P < 0.001$		NS	
Glibenclamide-treated rats	1.7±0.2	6.1±0.7	2.7±0.4	2.5±0.5
	$n = 30$	$n = 37$	$n = 36$	$n = 32$

The data are expressed as percent of hormone content released during 30 min of incubation (time 30–60 min according to Table I), after a control period of 30 min (times 0–30 min according to Table I, data not shown. Glucagon release from islets of normal and glibenclamide-treated rats differed only ($P < 0.05$) in the absence of calcium.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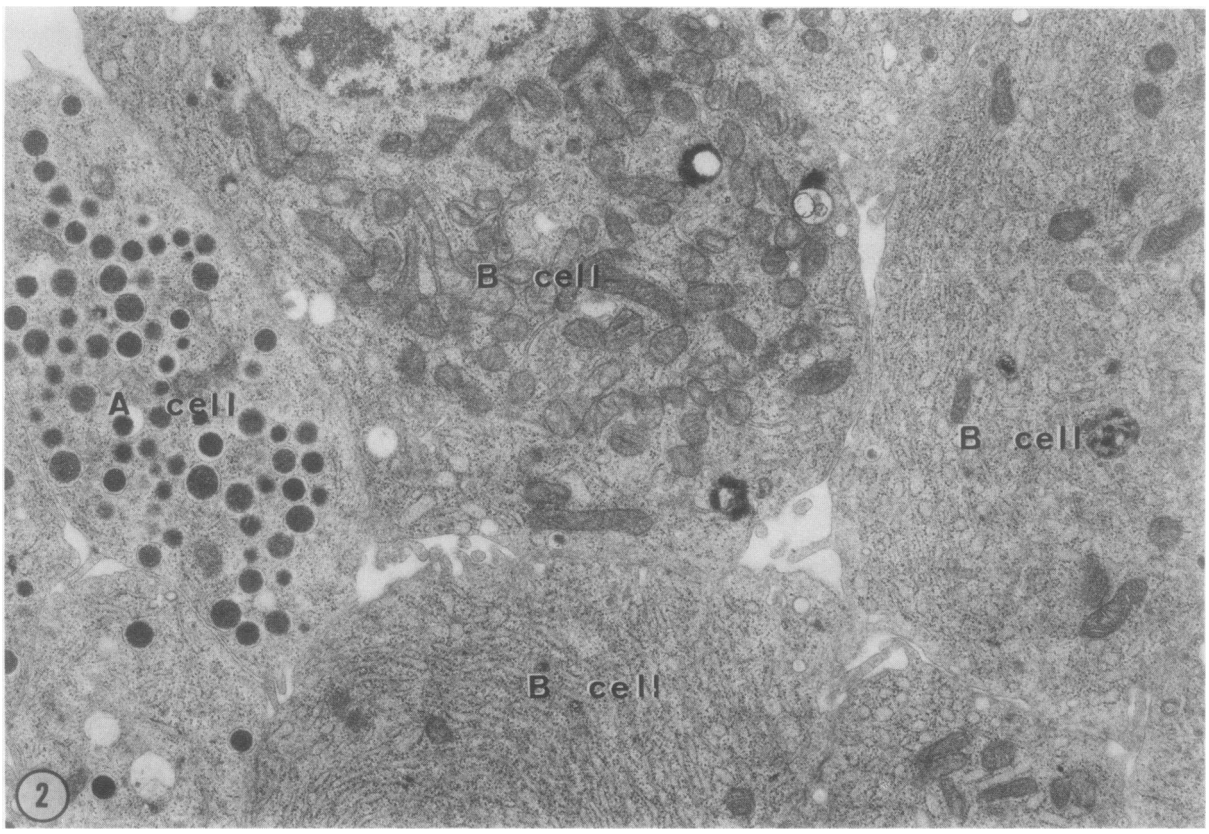
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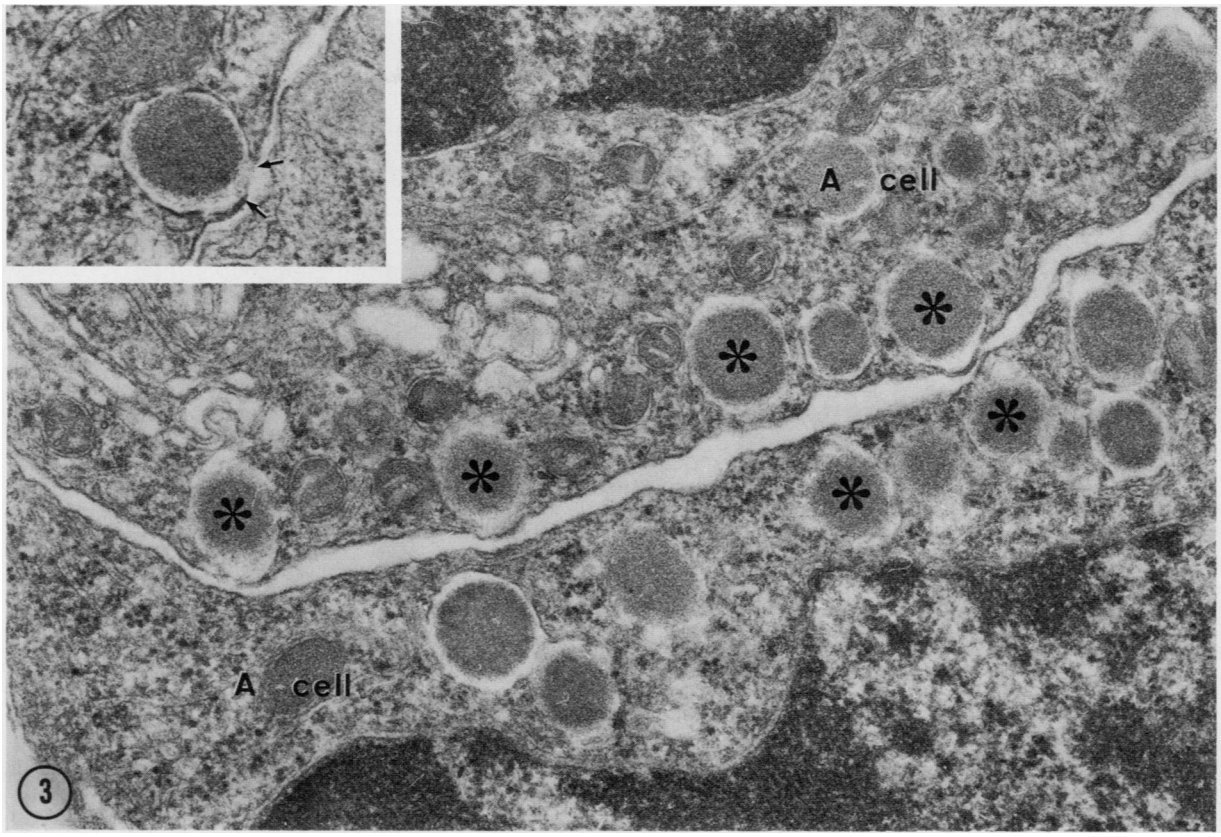

FIGURE 3 Portions of two A cells from isolated islets incubated for 30 min in a medium added with 40 mM arginine. Secretory granules are seen in close contact with the plasma membrane (*). In the inset, the arrows indicate an interruption of the coalesced granule-limiting and plasma membranes. Magnification ×39,000; inset, ×50,000.

fer (pH 7.4) for at least 30 min. The islets were then rapidly frozen in Freon 22 cooled in liquid nitrogen, fractured and shadowed in a Balzers BAF 301 apparatus (Balzers High Vacuum Corp., Balzers, Liechtenstein) according to the technique of Moor and Mühlethaler (26). The freeze-fracture replicas were cleaned in sodium hypochlorite for 2 h followed by dimethyl formamide (Merck, Darmstadt, W. Germany) for 20 min. The replicas were then rinsed in distilled water, mounted on copper grids and examined in a Philips EM 300 electron microscope.

Morphometric evaluation in thin section preparations. Ultrathin sections from 12 selected islets from three different experiments (4 islets per experiment) were examined for changes caused by each incubation condition. Six micrographs of A cells chosen at random were prepared from each of these islet sections (total: 6 × 12 = 72) and evaluated at two initial magnifications: ×5,100 and 9,200. Morphometric determinations were made with the "multipurpose test system" used on a table projection unit (27).

The volume density, V_v, of granules was determined at an initial magnification of ×5,100 by point counting (27): $V_v = P_p$ granules/P_p cytoplasm. P_p represents the test points enclosed by a given profile. The numerical density, N_v, of granules was evaluated at an initial magnification of ×5,100 by $N_v = N_i/P_T \cdot \sqrt{3}/2 \cdot d^2 \cdot \bar{D}$ (27), where N_i = number of secretory granules enclosed by a given profile, P_T = number of the test points enclosed by the same given profile, d = length of the test line; and $\bar{D}$ = secretory granule diameter.

To check the number of granules in close contact with the plasma membrane (margination), we have exmained the rim of cytoplasm adjacent to this plasma membrane. The number of granules in close contact with the plasma membrane has been counted in the layer of cytoplasm within 480 nm of the membrane. The formula used was the same as that used for N_v where N_i = number of secretory granules in close contact with the cell plasma membrane and P_T = number of test points enclosed by a half-test line.

The diameters of the central core and of the external envelope of the secretory granules were determined on prints (final magnification: ×27,600). A minimum of 600 granules

FIGURE 1 Untreated rat. Part of the periphery of an isolated islet containing A cells as well as granulated B cells and one non A, non B cell (*). Magnification ×6,000.

FIGURE 2 Glibenclamide-treated rat. Peripherically located A cells are well granulated whereas the neighboring B cells are conspicuously deprived of secretory granules. Magnification ×12,000.

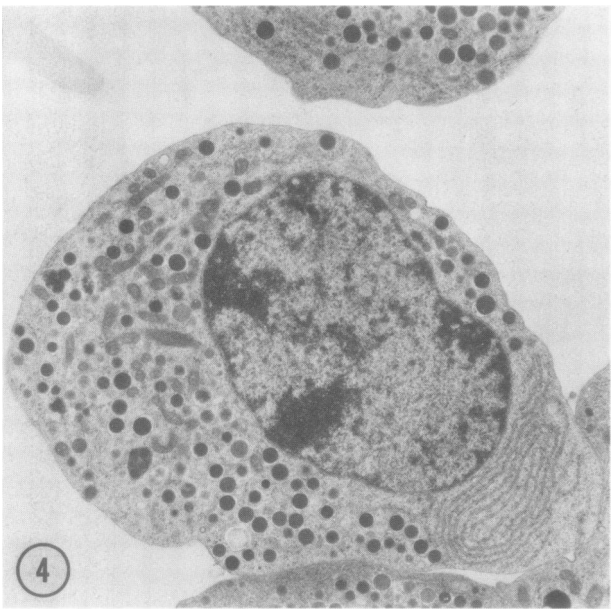

FIGURE 4 A cell from an isolated islet incubated for 30 min in a medium deprived of calcium. The extracellular space is markedly enlarged but the cells maintain focal areas of contact. Magnification ×6,000.

was measured for each experimental condition. Mean values of these apparent diameters were corrected by $4/\pi$ to obtain true diameters (27).

Quantitative evaluation on freeze-fracture replicas. Membrane faces of 25–30 different granulated islet cells from glibenclamide-treated rats were studied for each experimental condition (minimum of six preparations). The pictures were studied at a final magnification of ×18,900. The membrane surface was measured by planimetry and the number of exocytotic sites was counted. The total membrane surface evaluated in each experimental condition represented at least 180 μm^2.

RESULTS

Hormone content and secretion. The amount of glucagon recovered (2.12±0.11 ng/islet of untreated rat, $n = 142$) remained unaffected by glibenclamide treatment (2.11±0.10 ng/islet of treated rats, $n = 149$). In contrast, glibenclamide treatment resulted in a marked depletion of insulin content (132.9±4.5 ng/islet from untreated rats vs. 18.9±1.6 ng/islet from treated rats).

Since glibenclamide treatment apparently did not influence the hormone content of the pancreatic A cells, it remained to be established that the function of these cells, i.e., glucagon secretion, remained intact.

Table I shows that high glucose concentration (16.5 mM) reduced the secretion of glucagon ($P < 0.005$) and stimulated that of insulin ($P < 0.005$). The secretions of the two hormones were not constant over time; during the incubation period (30–60 min), there was always significantly more hormone measured than during the preincubation period (0–30 min). This was observed regardless of glucose concentration in the medium or of glibenclamide pretreatment in vivo. Hence for the comparison between test and control condition, only results obtained from the incubation period (30–60 min) were taken into account. Hormone release was expressed as a percentage of the calculated content at the onset of the incubation period (hormone content of the islets at the end of the incubation plus hormone released during incubation).

Both stimuli for glucagon secretion employed, i.e., arginine and the absence of calcium in the medium, significantly stimulated A-cell secretion (Table II). Glibenclamide pretreatment in vivo did not alter these effects significantly, except that the absence of calcium was a more potent stimulus (expressed in percentage of content) for islets derived from glibenclamide-treated rats (Table II). Therefore, the secretory function of A cells from isolated islets, as far as tested, remained virtually unaffected by glibenclamide treatment in vivo.

The secretion of insulin is reported in Tables I and II for comparison. It is apparent from data in Table I that glucose-induced insulin release is reduced in islets degranulated by glibenclamide treatment in vivo when compared to release from islets of untreated rats. Another difference observed was that, although low, glucose-induced insulin release (expressed in percentage of the content) from "glibenclamide islets" could not be further decreased by the absence of calcium, whereas a marked attenuation was

TABLE III

Morphometric Data of Secretory-Granules of A Cells under the Influence of 40 mM Arginine or the Absence of Calcium (αCa^{++}) in Normal and Glibenclamide-Treated Rats

	Margination index	Volume density of granules	Numerical density of granules
Normal			
Glucose, 0	1.43±0.14	26.8±1.4	7.82±0.51
Arginine, 40 mM	3.42±0.28*	26.1±2.0	9.27±0.63
Glucose, 0			
Glibenclamide			
Glucose, 0	1.10±0.06	30.2±1.3	8.57±0.45
Arginine, 40 mM	2.66±0.27*	29.1±1.8	9.62±0.66
Glucose, 0			
Normal			
Glucose, 16.5 mM	0.83±0.13	27.9±0.9	8.80±0.69
αCa^{++}	0.98±0.12	22.1±1.4*	7.20±0.38‡
Glucose, 16.5 mM			
Glibenclamide			
Glucose, 16.5 mM	0.97±0.10	26.6±1.3	8.22±0.54
αCa^{++}	0.68±0.07‡	22.5±0.9§	6.81±0.33‡
Glucose, 16.5 mM			

For each value, n = number of islets = 12.
* $P < 0.005$.
‡ $P < 0.05$.
§ $P < 0.02$.

TABLE IV

*Secretion of Glucagon and Insulin and Morphometric Data of Secretory Granules of A Cells after a 10-min Incubation in the Absence of Calcium**

	Glucagon	Insulin	Margination index	Volume density of granules	Numerical density of granules
	ng/ml	*ng/ml*			
Glucose, 16.5 mM	0.56±0.06	4.14±0.33	1.43±0.10	24.9±1.2	9.3±0.6
	$n = 20$	$n = 24$	$n = 9$	$n = 9$	$n = 9$
	$P < 0.05$	$P < 0.001$	$P < 0.001$	$P < 0.05$	$P < 0.02$
αCa^{++} Glucose, 16.5 mM	0.81±0.09	2.67±0.19	0.83±0.09	21.2±1.2	7.1±0.5
	$n = 22$	$n = 23$	$n = 9$	$n = 9$	$n = 9$

For biochemical data, n = number of vials (20 islets/vial); for morphometric determinations; n = number of islets.

* Islets were obtained from glibenclamide-treated rats. The experimental protocol is the same as that reported in Methods except that the incubation time is reduced to 10 min.

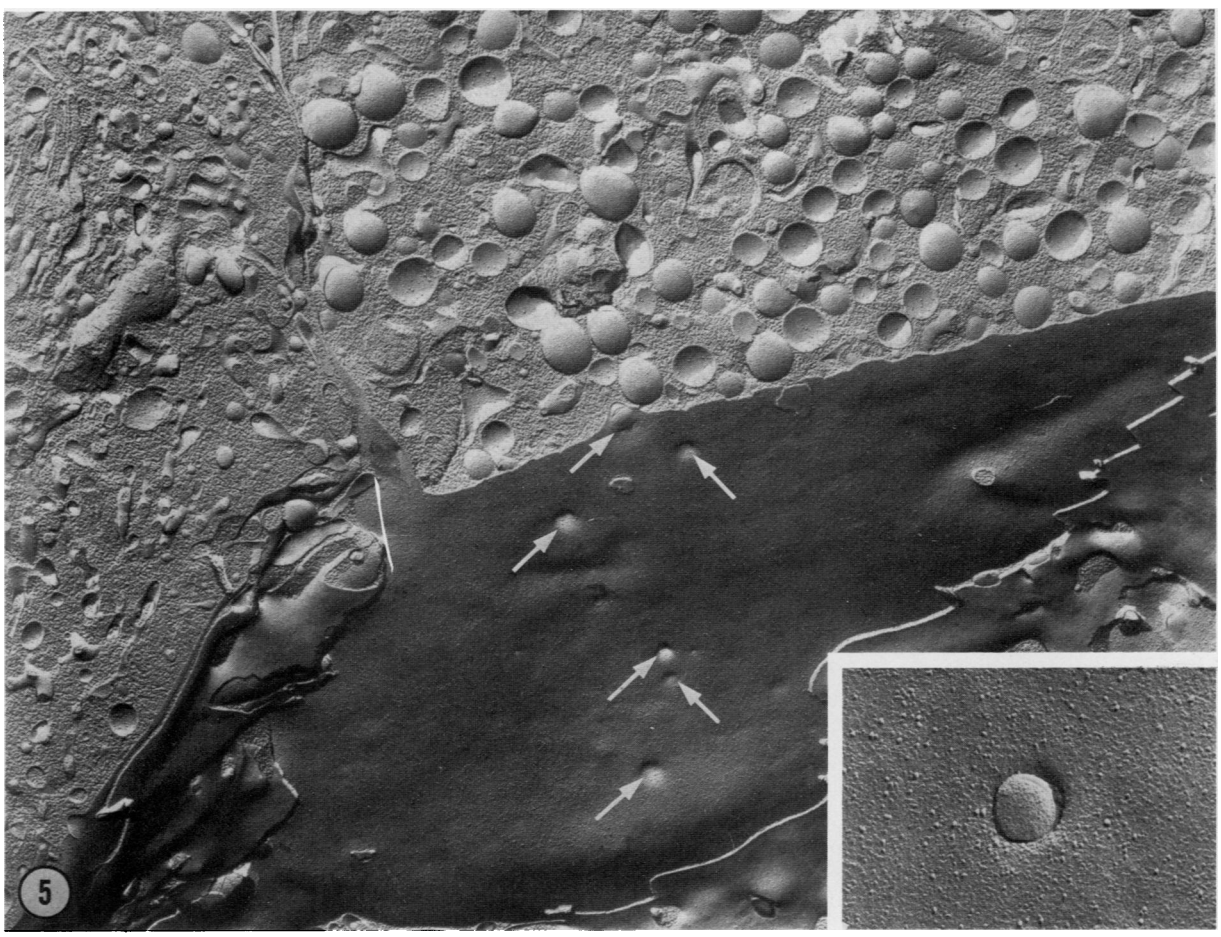

FIGURE 5 Freeze-fracture replica of the periphery of an isolated islet incubated for 30 min in the presence of arginine. The fracture process has split two neighboring cells tentatively identified as B and A cells on the basis of their content in secretory granules. The exposed face of the plasma membrane of the granulated cell exhibits several depressions or exocytotic stomata indicated by the arrows. The inset shows at high magnification a stage of exocytosis where the secretory granule of a granulated cell is in process of being extruded. Magnification ×19,000; inset, ×52,000.

TABLE V

Quantification of the Number of Exocytotic Events per Total Surface of Granulated Cells Available and Examined in Each Experiment

Exp. no.	Exocytosis/ surface (μm^2)	Exocytosis/ 100 μm^2	Exocytosis/ surface (μm^2)	Exocytosis/ 100 μm^2
	Control		Arginine	
I	6/94.0	6.4	10/29.5	33.9
II	1/5.0	20.0	6/15.5	38.7
III	28/164.8	17.0	16/63.3	25.3
IV	2/31.3	6.4	28/91.0	30.8
V	5/30.0	16.7	17/48.5	35.1
Mean		13.3±2.9		32.8±2.3‡
	Control		Deprivation of calcium	
VI	15/76.0	19.7	4/33.8	11.8
VII	5/70.5	7.1	2/15.5	12.9
VIII	13/52.8	24.6	9/63.0	14.3
IX*	4/34.3	11.7	2/30.8	6.5
X*	1/7.5	13.3	10/37.5	26.7
Mean		15.3±3.1		14.4±3.3

* Unpaired experiments.

‡ $P < 0.001$ vs. control group.

observed in untreated islets (Table II). By contrast, arginine seemed to be a relatively more potent insulin-releasing agent in islets from glibenclamide-treated rats than in islets from untreated animals.

Electron microscopy of A cells. Electron microscopic appearance of the A and B cells from islets isolated from untreated rats (Fig. 1) corresponded to that reported in islets examined in the whole pancreas (9, 28). Glibenclamide treatment resulted in a marked degranulation of the B cells (Fig. 2) but did not affect the general appearance of the A cells (Fig. 2).

After 30 min incubation with 40 mM arginine, A-cell secretory granules were often seen in close contact with the cell plasma membrane (Fig. 3) and some of them were found in the process of being extruded into the extracellular space. Although the general appearance of the A cell was normal, the nuclear and cytoplasmic matrixes of the A cells were more dense.[2]

As has been previously described (29), the incubation of islets in a medium devoid of calcium (Fig. 4) resulted in an enlargement of the extracellular space. The intracellular organelles of A cells appeared unaffected however and secretory granules were rarely seen in close contact with the cell membrane.

[2] These changes appeared even when lower concentrations of arginine (5 or 10 mM) were used (data not shown).

Morphometry of A-cell secretory granules. Morphometric measurements related to A-cell secretory granules are reported in Table III. They were unaffected by glibenclamide treatment. After a 30-min incubation in the presence of arginine, the quantity of granules stored in the A cell was unchanged as determined by their volumetric (V_v) and numerical (N_v) density. By contrast, over the same incubation period, calcium deprivation significantly decreased both the numerical and volumetric density of the granules (Table III). The size of granules was not significantly affected by either arginine or the absence of calcium (data not shown).

The "margination index" of secretory granules was significantly increased in A cells exposed to arginine, but was unaffected (untreated islets) or even slightly decreased (glibenclamide islets) after 30 min of incubation in a medium deprived of calcium. To test the possibility that a change in margination index could have occurred early in the 30-min incubation period in calcium-deprived media, we performed a comparable morphometric study on islets exposed for 10 min to a calcium-deprived medium. As shown in Table IV, the results obtained under these circumstances gave the same indications as those obtained after 30 min.

Freeze fracturing. Since in freeze-fracture replicas it is not possible to distinguish the different islet cell types, the numerically predominant B cells were degranulated by glibenclamide. Therefore, degranulated cells can be recognized as B cells while well-granulated cells are considered to be predominantly A cells. The examination of the exposed membrane of granulated cells revealed easily identifiable exocytotic events (Fig. 5).

Under both control conditions, an average of 13.3 and 15.3 exocytotic events per 100 μm^2 were counted (Table V). When the islets had been incubated in the presence of arginine (40 mM), a highly significant increase to 32.8 exocytotic events per 100 μm^2 was observed. It is of particular interest that in each experiment analyzed, an increased number of exocytotic events is noted under the influence of arginine. In contrast, when glucagon release was stimulated by the absence of calcium, no change in the number of exocytotic events could be found at the surface of the granulated cells.

DISCUSSION

The present study utilizes three simultaneous approaches to investigate A-cell secretion: (*a*) measurements of glucagon secretion by radioimmunoassay; (*b*) quantitative assessment of the morphological events by conventional electron microscopy; (*c*) quantification of exocytotic events by freeze-fracture techniques. Two

conditions were used to stimulate glucagon release: arginine, widely used in vitro (30–32) and in vivo (30); and the paradoxical influence of the absence of calcium previously studied in vitro (13–16).

In both conditions, the release of glucagon was significantly increased. Moreover, and although tested in unpaired experiments, the presence of glucose clearly suppressed A-cell secretion as generally observed in vivo and in vitro (22).

The freeze-fracturing techniques used in this study required a preparation rich in A cells in which this cell type could be easily distinguished from the more frequent insulin producing B cells. Islets were therefore obtained from rats treated with the B-cell depleting agent, glibenclamide, which did not substantially affect A-cell function. The model was, therefore, accepted as satisfactory to perform the morphologic analysis of A-cell secretion.

Under the influence of arginine, the granular store of A cells, measured ultrastructurally by the volumetric and numerical density of granules, remained statistically unchanged in spite of increased glucagon release. The data presented here do not allow to determine whether this is due to a lack of sensitivity of the morphometric technique or whether glucagon biosynthesis was stimulated to an extent which compensated for the released hormone.

Our results show that arginine stimulates exocytotic glucagon release since the "margination index" of secretory granules and the number of exocytotic stomata on A-cell membrane were both increased. Furthermore, the magnitude of increase of glucagon secretion in response to the arginine stimulus was approximately of the same order of magnitude as the increase in the two morphologic parameters of exocytosis (Tables II, III, and V). This indicates that exocytosis represents a significant mechanism of glucagon secretion secondary to arginine stimulation.

The second experimental condition studied, i.e., a medium deprived of calcium, was a potent stimulus in isolated islets. To our surprise, however, this glucagon release, associated with a significant reduction of granule stores, was not accompanied by an increase in the morphologic parameters linked to granule extrusion. In view of these results, we have analyzed the influence of calcium deprivation on exocytosis after a shorter period of incubation (10 min). The results obtained followed the same pattern as those reported for a longer period of incubation. These data suggest therefore that the morphologic events involved in glucagon secretion are different from those seen with an arginine stimulus. We have not observed either qualitative or quantitative changes of the α-granules after exposure to a medium deprived of calcium. In particular, we found no evidence for intracytoplasmic dissolution of α-granules as reported by others (1–4).

Since it seems that calcium could play a role in the stabilization of the membrane (33), it could theoretically be possible that glucagon release under this highly unphysiologic stimulus, i.e., deprivation of calcium, is due to a leakage through a more permeable membrane. If present, this change in membrane permeability affected A- and B-cell membranes in a diametrically opposed manner; indeed B-cell secretion was dramatically decreased while A-cell secretion was simultaneously increased in calcium-deprived media. In this context, it seems worthwhile to recall that calcium deposits observed by the pyroantimonate precipitation technique appear on different sites of A- and B-cell plasma membrane (29).

It appears, therefore, that differences exist, with respect to the mode of action, between stimulation by arginine or by calcium deprivation. These differences emphasize the fact that if exocytosis is an important mode of glucagon release by the A cell, as suggested by our results and in agreement with previous non-quantitative data (5–10), this does not exclude the possibility that other mechanisms of release exist (1–4) especially in the case of highly artificial conditions such as calcium deprivation.

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