INCREASE OF GAP JUNCTIONS BETWEEN PANCREATIC B-CELLS DURING STIMULATION OF INSULIN SECRETION

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ABSTRACT

The development of gap junctions between pancreatic B-cells was quantitatively assessed in freeze-fracture replicas of isolated rat islets under different conditions of insulin secretion. The results show that in resting B-cells, gap junctions are small and scarce but that these junctions increase when insulin secretion is stimulated. Both a short (90 min) stimulation by glucose in vitro and a prolonged (2.5 d) stimulation by glibenclamide in vivo raise the number of gap junctions; in addition, the glibenclamide stimulation causes an increase in the size of individual gap junctions. As a consequence, the total area occupied by gap junctions on the B-cell membrane and the ratio of this area to the cell volume were found significantly increased in the latter condition. The slight increase of these values observed after the glucose stimulation did not reach significance. These data indicate a change of gap junctions during the secretory activity of the pancreatic B-cells. The possibility that the coupling of the cells is affected by the treatment is discussed.

KEY WORDS islet of Langerhans · B-cells · gap junctions · glibenclamide · freeze-fracture · electron microscopy

Indirect evidence indicates that islets of Langerhans are not aggregates of independent endocrine cells but, on the contrary, may represent precisely ordered assemblies in which endocrine cells interact so as to maintain an optimal hormonal output. Morphologically, interactions are suggested by the precise numerical and topographical relationships shown by the four main types of islet cells (30) and by the occurrence, between each islet cell type, of specialised regions of cell-to-cell contact (14). Physiologically, interrelationships between islet cells are suggested by the finding that normal concentrations of either insulin, glucagon, or somatostatin can influence the secretion of at least one of the other islet hormones (1, 13, 33, 38, 39) and by the observation that insulin itself may directly act on the B-cells to regulate its own release (32). Among the several mechanisms which might be responsible for cellular interrelationships in the islet, one could be cell-to-cell communication mediated by gap junctions (18). These junctions, which are well represented between all islet cell types (27, 28, 31), may account for the synchronised electrical activity recorded in neighbouring B-cells of microdissected islets (20). In the present study, we have carried out a quantitative evaluation of gap junctions between pancreatic B-cells under various conditions of insulin secretion as a first approach to establish whether a link exists between gap junctions and islet functioning. Our results show that gap junctions are modified during the secretory activity of the pancreatic B-cells. Preliminary results of this work have been reported in abstract form (19).

MATERIALS AND METHODS

Animals

Adult female Wistar rats, weighing 250–350 g, were used throughout.

Isolation of Islets and Dissociation of Islet Cells

For each experiment, islets of Langerhans were isolated by a collagenase digestion (15) from three rat pancreases.

When single islet cells were needed (see below), the isolated islets were further incubated for 90 min at 37°C in a bicarbonate-buffered medium, without Ca^{++} but containing 1 mM EGTA (Fluka AG, Buchs, Switzerland) and 4 μg/ml pronase (Pronase B grade; Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.). This procedure dissociated single islet cells which appeared spherical and floated free among the connective and vascular framework of the dissociated islets.

Experimental Procedure

Stimulation of insulin secretion was carried out both in vitro and in vivo.

For in vitro studies, islets isolated from control rats were collected at room temperature in a medium containing 0.5 mg/ml glucose. After 45 min, the collection medium was discarded and replaced by fresh incubation medium. To stimulate the insulin secretion, the islets were incubated for 90 min at 37°C in the presence of a high (3.0 mg/ml) glucose concentration. Control islets were incubated for the same period of time in the presence of a low (0.5 mg/ml) glucose concentration at 37°C. Both the collection and the incubation of the islets were performed in a bicarbonate-buffered medium (Krebs-Ringer solution), equilibrated with O_2/CO_2 (95/5 vol/vol), adjusted to pH 7.4 and supplemented with 5 mg/ml of albumin. The islets were fixed at the end of the incubation period.

In vivo stimulation was performed by injecting rats twice a day, i.p., with 0.2 mg/100 g body weight of glibenclamide (a gift from Hoechst A. G., Frankfurt, W. Germany) for 2.5 d. Control rats received saline only. The islets were isolated from treated and control rats 2 h after the last injection. They were collected as described above and fixed 45 min after the end of the isolation procedure.

Preparation of Tissue

FREEZE-FRACTURE: At the end of each experiment, batches of 150–200 islets were packed into pellets by centrifugation, fixed in 2% glutaraldehyde buffered with 0.1 M phosphate buffer, pH 7.4, for 60 min at room temperature, and subsequently rinsed several times in phosphate buffer. The pellets were then soaked for 60 min in a 30% solution of glycerol also buffered with phosphate, rapidly frozen in Freon 22 cooled in liquid nitrogen, and finally fractured and shadowed in a Balzers BAF 301 apparatus (Balzers High Vacuum Corp., Balzers, Liechtenstein) according to Moor and Mühlethaler (23). The replicas were cleaned in a sodium hypochlorite solution, rinsed in distilled water, and mounted on copper grids.

CONVENTIONAL ELECTRON MICROSCOPY: Glutaraldehyde-fixed islets or pellets of dissociated islet cells were postfixed in 2% phosphate-buffered osmium tetroxide, dehydrated in graded ethanols, and embedded in Epon. Semithin sections were examined in a phase-contrast microscope (Carl Zeiss, Oberkochen, W. Germany). Thin sections, stained with uranyl acetate and lead citrate, and freeze-fracture replicas were examined in Philips EM 300 and EM 301 electron microscopes. Magnifications were calibrated with a reference grid (2,160 lines/mm; E. F. Fullam, Inc., Schenectady, N. Y.).

Quantitative Evaluation

FREEZE-FRACTURE REPLICAS: For each experimental condition, 6–10 islets were randomly selected in at least three different replicas, each obtained from a different experiment. To restrict our evaluations to the insulin-containing cells, we have studied the central region of each islet, which in the rat contains virtually only B-cells (9). All the fracture faces of plasma membranes exposed in the islet center were considered, except those facing connective or vascular tissues. Gap junctions were identified by their characteristic appearance on fractured membranes (18). However, in some cases, due to the very small size of the junctions or to a linear rather than macular arrangement of their constituent particles, the following additional criteria were used for identification. We considered as a gap junction (*a*) any tight aggregate of at least three uniformly-sized particles (Fig. 1); (*b*) any linear assembly of particles in continuity with a characteristic gap junction aggregate (Fig. 5). In this case the aggregate and its attached linear array were considered to form a single gap junction. (*c*) Any isolated linear assembly of particles merging, in a P-E fracture face transition, into a linear array of pits on the E-face. When pits were not visible (P-fracture face only) the isolated linear assemblies of particles were not taken into consideration. Since, at present, the possible significance of a different gap junction shape is unknown, no attempts were made to evaluate separately the rare linear arrays and the more familiar gap junction aggregates. Three different evaluations were made to assess the development of gap junctions: (*a*) the number of gap junctions per membrane area, (*b*) the number of particles in each gap junction, and (*c*) the area of gap junctions per membrane area. The areas of plasma membranes and of gap junctions were measured with a planimeter (Aristo No. 1130L; Dennert-Pape, Hamburg, W. Germany) on × 19,000 and on × 92,000 enlarged micrographs, respectively.

SEMITHIN SECTIONS: To express some of the numerical data on a "per cell" basis, the radius of dissociated islet cells was measured on phase-contrast micrographs of semithin sections with a × 1,125 magnification. The values obtained were corrected according to the Wicksell procedure (43) to account for sectioning artefacts, and the corrected radius was used to calculate the mean cell surface and volume.

Presentation of Data

Results are expressed as mean ± SEM with n representing the number of islets evaluated in each experimental condition. Statistical comparisons of mean values were carried out using a Student's unpaired t-test.

The distributions of gap junction sizes, based on the number of particles contained by each junction, were analysed by a Kolmogorov- Smirnov nonparametric test. Because these distributions were highly asymmetric, the median numbers of gap junction particles were also used as representative of gap junction size and compared by the median test.

RESULTS

Qualitative Observations

The general organization of B-cells corresponded to that described extensively in previous studies (24, 29). In control and glucose-stimulated islets, most of the B-cells appeared well granulated. By contrast the B-cells from glibenclamide-treated islets showed very few secretory granules. The other islet endocrine cell types appeared unaffected.

In freeze-fracture replicas of islets, the B-cell plasma membranes showed variably-shaped particle aggregates on the P-face (Fig. 1) and aggregates of pits on the E-face (Fig. 2). Such aggregates, interpreted as gap junctions (see Materials and Methods), were also visible on P-E fracture face transitions at regions of close apposition between two plasma membranes (Fig. 3).

On B-cells, gap junctions were frequently grouped in clusters and associated with tight junctional fibrils (or grooves) (Figs. 1–3). Approx. 10% of the gap junctions, however, did not show association with other junctional elements (Fig. 4). The number, size, and shape of gap junctions varied to a great extent from one B-cell membrane to another. Gap junctional aggregates were usually of round or polygonal shape but some of them took the form of linear assemblies of particles with corresponding rows of pits on E-faces (Fig. 5).

Quantitative Evaluation

Table I summarizes the primary data used to compute, in each experimental group: (*a*) the percent of exposed plasma membrane occupied by gap junctions, (*b*) the total area of gap junctions per B-cell, (*c*) the number of gap junctions per 100 μm^2 of membrane, (*d*) the number of gap junctions per B-cell, (*e*) the mean number of particles per gap junction, (*f*) the median number of particles per gap junction, and (*g*) the ratio of the total gap junction area to the B-cell volume (Table II). Fig. 6 shows the distribution of gap junctions on the basis of the number of their constituent particles.

A general feature observed was the large variability of the data from one islet to another, which is reflected in the rather large standard deviations.

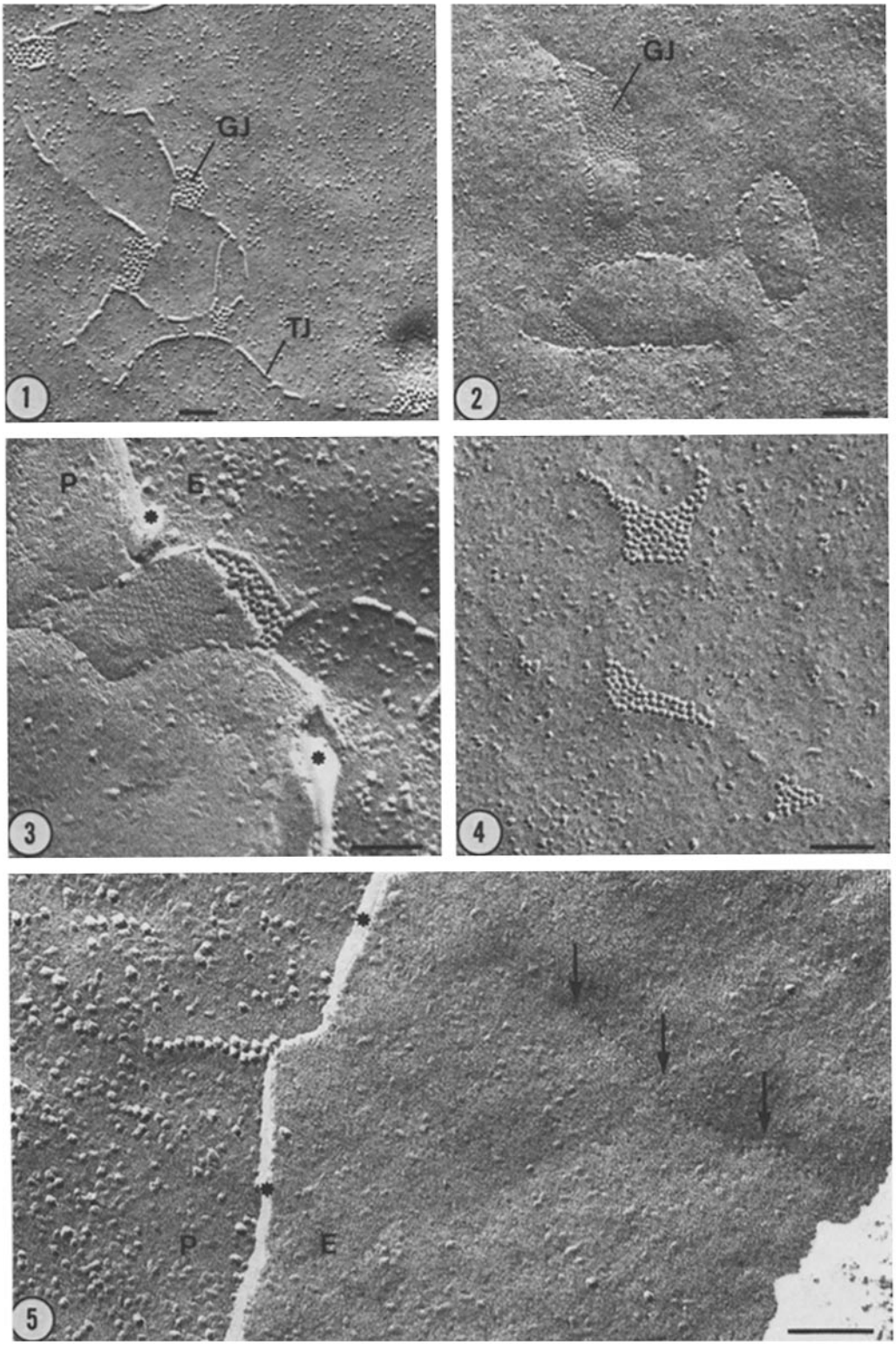
CONTROL ISLETS: In control islets, gap junctions occupied an average of 0.019% of the exposed membrane area (relative gap junction area) or 0.08 μm^2 of the B-cell surface (Table II). The ratio of the total area occupied by gap junctions on a B-cell to the cell volume was $9.3 \cdot 10^{-5}$ μm^{-1} (Table II). These low values resulted both from the scarcity of gap junctions and from their small size. In control islets, there was an average of 5–6 gap junctions per 100 μm^2 of membrane or ~24 gap junctions per B-cell (Table II). The distribution curve of gap junctions according to the number of their constituent particles (Fig. 6) showed that the mean and median numbers of gap junction particles were 26 and 17, respectively, and that 85% of gap junctions were formed by <40 particles.

INCUBATED ISLETS (LOW GLUCOSE): No significant change in any parameter characterizing gap junction development was found in islets incubated for 90 min in the presence of 0.5 mg/ml of glucose, as compared to non-incubated control islets (Table II and Fig. 6).

INCUBATED ISLETS (HIGH GLUCOSE): A 90-min incubation of islets in the presence of 3.0 mg/ml of glucose induced a significant 1.7-fold increase in the number of gap junctions per 100 μm^2 of membrane (Table II). The mean number of particles per gap junction appeared unchanged, but a significant ($p < 0.005$) decrease in the median number of gap junction particles was detected (Table II).

The increase of the percent of membrane area occupied by gap junctions and the increase of the ratio of the total gap junction area to the cell volume did not reach significance (Table II).

GLIBENCLAMIDE-TREATED ISLETS: The glibenclamide treatment induced a significant increase of all values characterizing gap junction development: gap junctions were 2.1 times more



frequent and contained 1.5 more particles than in control islets (Table II). The latter change was reflected in the distribution curve of gap junctions (Fig. 6). In glibenclamide-treated islets, this distribution was significantly different ($p < 0.001$) from that observed in control islets and showed that large (>40 particles) gap junctions were 2.2 times more frequent in glibenclamide-treated islets than

TABLE I

Primary Data Collected to Estimate Gap Junction Development in Resting and Stimulated B-Cells

Group	Total membrane Area measured	Total gap junction Area measured	No. of gap junctions	B-cell surface	B-cell volume
	(μm^2)	(μm^2)		(μm^2)	(μm^3)
Control	9,549	1.62	422	442.6*	901.4*
	n = 6	n = 6	n = 6		
Glibenclamide	11,054	6.74	1,224	377.3	798.4
	n = 8	n = 8	n = 8		
Glucose	9,451	1.33	470	442.6	901.4
0.5 mg/ml	n = 10	n = 8	n = 10		
Glucose	10,719	3.17	1,013	416.9	836.9
3.0 mg/ml	n = 10	n = 8	n = 10		

* This value was taken from the 0.5 mg/ml glucose group. n = No. of islets investigated.

TABLE II

Quantitative Estimation of Gap Junction Development in Resting and Stimulated B-Cells

Group	Percent of membrane area occupied by gap junctions	Gap junction area per B-cell	No. of gap junctions per 100 μm^2 of plasma membrane	No. of gap junctions per B-cell	Mean No. of gap junction particles	Median No. of gap junction particles	Ratio of the gap junction area to the B-cell volume
		μm^2					$10^{-5}\mu m^{-1}$
Control	0.019 ± 0.005	0.08	5.48 ± 1.47	24	26.07 ± 2.72	17.44	9.28 ± 2.40
	n = 6		n = 6		n = 6		n = 6
	$p < 0.05$		$p < 0.05$		$p < 0.005$	$p < 0.0005$	$p < 0.01$
Glibenclamide	0.068 ± 0.011	0.24	11.49 ± 1.84	43	44.74 ± 2.98	25.48	30.45 ± 5.00
	n = 8		n = 8		n = 8		n = 8
Glucose	0.022 ± 0.007	0.09	5.80 ± 1.25	25	27.60 ± 3.70	18.92	11.31 ± 3.50
0.5 mg/ml	n = 8		n = 10		n = 8		n = 8
	N.S.		$p < 0.05$		N.S.	$p < 0.005$	N.S.
Glucose	0.035 ± 0.008	0.13	9.74 ± 1.30	40	22.49 ± 1.80	16.03	17.36 ± 3.50
3.0 mg/ml	n = 8		n = 10		n = 8		n = 8

Data are mean ± SEM; n = No. of islets investigated.

FIGURES 1–5 Examples of the gap junctions evaluated quantitatively.

FIGURE 1 Characteristic aspect of gap junction aggregates (*GJ*) and tight junction fibrils (*TJ*) associated on the P-face of a B-cell plasma membrane. Bar, 0.1 μm. × 63,000.

FIGURE 2 Characteristic aspect of aggregated pits (*GJ*) associated with tight junctional furrows on the E-face of a B-cell plasma membrane. Bar, 0.1 μm. × 69,000.

FIGURE 3 P-E fracture face transition showing aggregated particles on the P-face (*P*) with corresponding pits on the E-face (*E*) of the neighbouring B-cell. The step across the intercellular space is indicated by asterisks. This step is low at the level of the gap junction. As in Figs. 1 and 2, the gap junction illustrated here is associated with tight junctional elements. Bar, 0.1 μm. × 115,000.

FIGURE 4 P-fracture face of a B-cell plasma membrane showing gap junction aggregates not associated with tight junctional fibrils. Such "free" aggregates represent only 10% of all gap junctions evaluated. Bar, 0.1 μm. × 104,000.

FIGURE 5 P-E fracture face transition showing a linear form of a gap junction aggregate on the P-face (*P*) and a corresponding linear array of pits (arrows) on the E-face (*E*). The fracture step across the intercellular space (asterisks) is low at the level of the gap junctional aggregate. Bar, 0.1 μm. × 132,000.

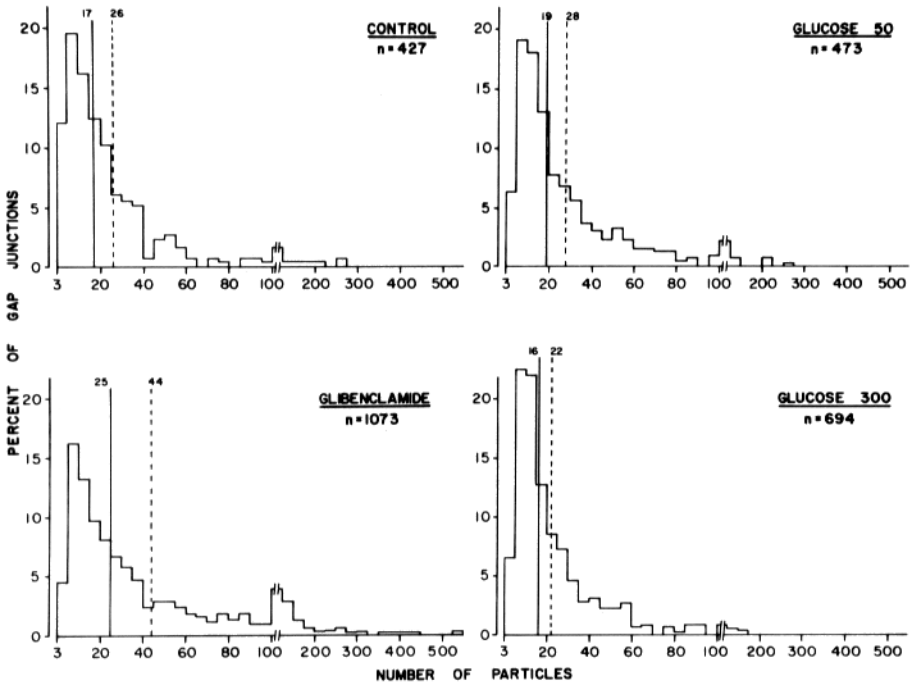


FIGURE 6 Size distribution curve of gap junctions based on the number of particles contained by each junction. The vertical solid line represents the median, the vertical dotted line, the mean value of the number of particles forming the gap junctions. n = No. of gap junctions evaluated.

in the control group. The median number of gap junction particles was also significantly higher ($p < 0.0005$) than that of controls (Table II).

As a result of the gap junction changes and of the slight decrease of the cell volume, both the total area occupied by gap junctions in the B-cell membrane and the ratio of this area to the cell volume were found increased three times by the glibenclamide treatment (Table II).

DISCUSSION

Our quantitative evaluation shows that the gap junctions of pancreatic B-cells are scarce and very small in resting islets (the ratio of the gap junction area to cell volume and the relative gap junction area are among the lowest reported so far in the literature) (37, 41), but that the number of these junctions increases in two experimental conditions widely used by different groups of workers to augment the release of insulin from the pancreatic B-cells in vivo or in vitro (15, 17, 29). In vivo, glibenclamide results in a marked depletion of islet insulin content which, after 2 d of treatment, averages ~15% of the control value (4) (P. Meda, unpublished data). In vitro, the incubation of isolated islets prepared in the same way as ours in the presence of 3.0 mg/ml of glucose significantly increases the amount of insulin released in the medium and decreases the insulin cell content (4, 26). Before attempting to interpret the observed changes, one was confronted with the problem of identifying the junctions to be evaluated quantitatively. In most cases, the typical morphology of particle aggregates on the P-face or of aggregates of pits on the E-face rendered this task easy but there were instances where the nature of the aggregates could not be definitely ascertained: this was especially the case for linear aggregates of intramembrane particles, which might represent an unusual form of gap junction (2, 36), a step in the formation of tight junctional fibrils (22) or one in the interconversion between the two types of junctions as suggested in (8).[1] In spite of these interpretative uncertainties, we have considered as

[1] In this context, it is worth mentioning that stimulation of insulin release by glucose determines also an increase of the tight junction length as measured by morphometry on islet cell plasma membranes (25).

a gap junction any linear assembly of particles in continuity with a characteristic gap junction aggregate and/or merging with an array of aligned pits in the E-face of a P-E fracture face transition.

Another possible experimental bias was that the mild protease (collagenase) treatment used to isolate the islets might have altered cell junctions (35, 42). While such an effect would be important to characterize if one were to assess the absolute development of gap junctions, it was considered unlikely to alter significantly the comparison of data obtained in our different groups of islets since they all have been isolated in the same way using the same collagenase batch.

Functionally, the presence of gap junctions has been correlated in several systems with the demonstration of low resistance pathways which probably accounts for the ionic and metabolic coupling of adjacent cells (18). The total area of gap junctions and the ratio of this area to the cell volume have been proposed as indirect estimates of the capability for electrical and nonelectrical communications between cells sharing gap junctions (40, 41). These values were not significantly altered by the glucose stimulation (in spite of an increase in the number of gap junctions) while they showed a significant augmentation after the stimulation of insulin release by glibenclamide. In the latter situation, this suggests that the transfer of ions and small molecules, i.e., coupling, between stimulated B-cells may be increased as compared to resting cells. At present, the reason for the difference in the effects of the two types of stimulation used is not known. Work in progress with glibenclamide-treated islets suggests that the development of gap junctions varies with the insulin content of the B-cells (P. Meda, unpublished data); the glucose stimulation does not deplete the B-cell insulin content as extensively as glibenclamide, and this may be one reason for the lesser effect of the former stimulation.

Beside the total area of gap junctions and its ratio to the cell volume, the arrangement and packing of the junctional particles were shown in other systems to be related to the degree of coupling (34). In the present study, we have not attempted to distinguish between the "uncoupled" and "coupled" appearance of B-cell gap junctions.

In summary, our data show that a physiologic stimulus of insulin release (high glucose) of relatively short duration induces an increase in the frequency of small-sized gap junctions while a prolonged pharmacological stimulation (glibenclamide) is associated with both a numerical increase and an enlargement of gap junctions. The latter result implies that new gap junctions must form between the B-cells. Although the mechanism of formation (insertion of new junctional particles or recruitment of already present particles or both) is unknown, it is of interest to note that rare formation plaques (12) were found in the glibenclamide-treated islets but were not detected in the control and in the glucose-stimulated ones.

In view of the increasing evidence of the hormonal modulation of gap junctions (3, 6, 7, 10, 11, 21) and of intercellular communication (5), it would be most interesting to know (*a*) whether the gap junctions of the islet are under the control of islet hormones and (*b*) assuming that islet gap junctions allow intercellular transfer of molecules, which molecules are exchanged and how these molecules participate in the secretory response of insulin-containing cells. Recent experiments, carried out in other coupled systems, show that cyclic AMP could be a mediator (16).

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Note added in proof: While this work was submitted, further experiments were carried out to determine the morphology of gap junctions in islets treated with uncoupling agents (DNP, elevated CO_2). Freeze-fracture replicas of such islets showed that gap junction particles were definitely more tightly and regularly packed than those forming the gap junctions evaluated in the present study. We therefore surmise that the gap junctions evaluated do not correspond morphologically to uncoupled junctions.

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