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Research Article

Dynamic insulin secretion from perifused rat pancreatic islets

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Abstract. Insulin secretion from isolated pancreatic islets of 8- to 12-day-old rats was investigated in a dynamic in vitro (perifusion) system. The aims of the study were (i) to describe a carefully controlled in vitro method to study the mechanism of insulin secretion and to analyse the effects and dynamic interactions of bioactive compounds on isolated rat pancreatic islets, (ii) to validate the method by comparing fundamental data on the functions of the islets obtained with this method to those collected with other techniques; and (iii) to find novel features of the control of insulin secretion. The method was carefully designed to maintain the functional capacity of the explanted cells. A functional standardization system was elaborated consisting of (i) analysis of the changes in the basal hormone secretion of the cells; (ii) evaluating responses to a standard, specific stimuli (50 mM glucose for 3 min); (iii) determining the alteration of the momentary size of the hormone pool with responses to KCl; and (iv) direct determination of the total intracellular hormone content from the extract of the column. The technique provides accurate quantitative data on the dynamic responses to biologically active compounds that act directly on the pancreatic islets. The islets maintained their full responsiveness for up to 7 days, and responses as close as in 1-min intervals could be distinguished. A linear dose-response relationship was found on the glucose-induced insulin release in case of 3-min stimulation with 4 and 500 mM of glucose (lin-log graph). Utilizing this method, we showed that no desensitization to glucose-induced insulin release can be observed if the responsiveness of the cells is properly maintained and the parameters of the stimulation are carefully designed. Exposure of the explanted islets to 10 μM acetylcholine or 30 mM arginine (Arg) induced a transitory elevation of insulin release similar in shape to that experienced after glucose stimulation. Norepinephrine (NE), dopamine (DA) and somatostatin (SS) did not induce any detectable alteration on the basal insulin secretion of the islets. However, 100 nM SS given together with 50 mM glucose, 30 mM Arg or 10 μM acetylcholine significantly reduced the insulin-releasing effect of these substances (by 75.5, 71.5 and 72.5%, respectively). At the same time, SS did not alter the insulin response of the islets to 100 mM elevation of K^+ concentration. SS also inhibited glucose-induced insulin release in a dose-dependent way ($ED_{50} = 22$ nM). A similar dose-dependent inhibitory effect on glucose-induced insulin release was found with NE ($ED_{50} = 89$ nM) and DA ($ED_{50} = 2.2$ μM). γ-Aminobutyric acid (GABA) did not influence insulin release under similar circumstances.

Key words. Pancreatic islets; insulin; perifusion; secretion kinetics.

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Understanding the control and mechanism of insulin secretion from the pancreatic islets is of increasing importance both theoretically and in clinical practice. The regulation of pancreatic islets is based on a coordinated interplay of several factors, including various nutrients, gastrointestinal and pancreatic hormones as well as the autonomic nervous system and its transmitters.

The potential therapeutic utilization of isolated pancreatic islet transplant also calls for a better understanding of the islet physiology. In vivo observations and experiments provided invaluable contribution to our knowledge of the physiology of the pancreatic islets. However, metabolic feedback, unknown mechanisms of metabolism and absorption of test compounds prevent studying the direct effects and interactions of various bioactive compounds on the cells of the pancreatic islets. For instance, glucose has a stronger effect on the insulin release if it is taken per os in comparison to its parenteral application [1, 2]. The physiological, pulsatile stimulation and delicate timing of the dynamic responses are also difficult to follow under in vivo circumstances. Although, based on observation of pituitary cells, the response time of the release of hormones from peptide-secreting endocrine cells is expected to be well within 1 min [3], most of the experiments targeting the functions of pancreatic islets are based on much longer expected response times.

Dynamic in vitro bioassays meet these requirements. One form of this method is the perifusion technique, which utilizes isolated tissues perifused with a continuous stream of fresh tissue culture media. This system is especially suitable for the study of the direct effect of the various substances and their combinations on hormone release. A carefully controlled, well-designed system, described earlier by Csernus and Schally [3] in detail to study anterior pituitary cells, had to be adjusted to the conditions of pancreatic islets. To check the validity of the perifusion system in studying isolated rat pancreatic islets, our results were compared with results collected from in vivo or static in vitro investigations (batch technique).

The islets of Langerhans are integrated into the neuroendocrine system. Several components of this system affect insulin release by either enhancing or inhibiting it. Controversial data were found in the literature on the specific role of several bioactive compounds. These data were based on in vivo observations or experiments with perifused pancreatic slices [4], islets [5–7] or isolated B cells [8–10]. In particular, the dose dependency of glucose [11] or arginine [6, 8, 12]-stimulated insulin release, biphasic insulin release [11, 13], somatostatin inhibition of glucose-induced insulin release [14, 15] and insulin desensitization [16, 17] were investigated. Several inconsistent data were published as well on the cysteamine depletion of somatostatin from the D cells of the pancreatic islets [18], the pulsatile nature of the insulin secretion [19, 20] as well as on the effects of acetylcholine [15, 21–25], dopamine [26], norepinephrine [21, 27–34] and GABA [35] on the insulin secretion. Some of these discrepancies stem from neglected differences in the experimental setup. It has been shown that, depending on the circumstances in culture, profound alteration of the inositol phosphate-related intracellular signalling system can occur in the islet cells [15, 36]. Our goals were (i) to describe a carefully controlled in vitro bioassay with proper, functional standardization that provides accurate, quantitative data on the dynamic responses of explanted pancreatic islets, (ii) to investigate the possibilities and limitations of this method, (iii) to check the validity of the results obtained from this system by comparing them with data of other in vivo or static in vitro investigations and (iv) to study the direct effects of biologically active compounds on isolated pancreatic islets investigated in a well-defined environment.

Materials and methods

Chemicals. Medium-199 (M-5017), BSA (A-7906), Sephadex G-10 (G-10-120) and Gentamycin (G-3632) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Collagenase was a product from Serva (Heidelberg, Germany, no. 17449). Hanks' bovine serum albumin (BSA) solution contained 1 g/l BSA (Sigma A-7906) in Hanks' solution. Trasylol was produced by Bayer (Leverkusen, Germany). Other organic and inorganic chemicals were obtained from Merck (Darmstadt, Germany; purity p.a.). All solutions were made with pyrogen-free water prepared in a Barnstead EASYpure RF system.

Islet preparation. Eight- to twelve-day-old Wistar rats of both sexes were used. The animals were born in the animal facility of our laboratory and kept in a standard environment (L:D = 12:12, lights on 7 a.m.; temperature 21 ± 1 °C; food: Altromin 1316 and water were available ad libitum). Animals were sacrificed by decapitation, and pancreata of four animals were removed, cut into approximately 1-mm pieces and transferred to 5-ml glass tube containing 1 mg/ml collagenase in 2 ml of ice-cold Hanks/BSA. The tubes were hand-shaken vigorously for 5 min. The suspension was transferred to 30 ml of ice-cold Hanks/BSA. The islets were sedimented for 5 min and resuspended in 30 ml of Hanks/BSA. The sedimentation was repeated twice. The islets, free from exocrine tissue pieces, were collected with a glass capillary pipette under a preparation microscope.

Perifusion. Perifusion analysis of isolated rat pancreatic islets was performed in a system described earlier in detail [3]. Briefly, about 300 isolated pancreatic islets

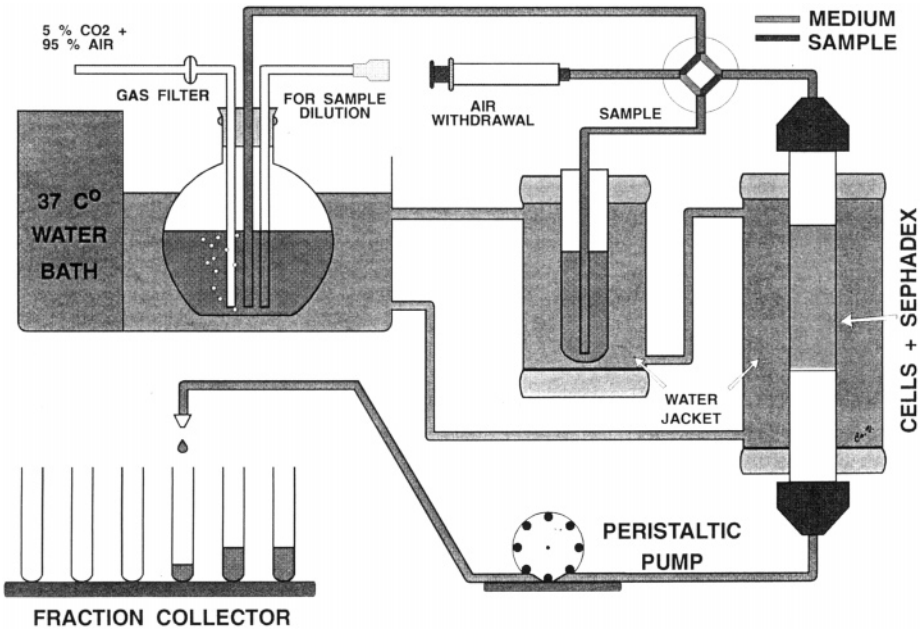

Figure 1. Schematic representation of the superfusion apparatus.

were packed into 6.6-mm glass columns together with Sephadex G-10 (fig. 1). Through the column, a Medium-199 based tissue culture medium, supplemented with 2.22 g/l of sodium hydrogen carbonate, 1.75 g/l of BSA and 80 mg/l of gentamycin was passed through the columns, at a flow rate of 1 ml/3 min. The medium was kept at 37 °C and equilibrated with a mixture of 95% air and 5% carbon dioxide. The dead volume of the system was set to 1 ml. During an overnight recovery period, no sample was collected. The test substances were diluted with 5 ml of medium from stock solutions immediately before application and entered into the column through a four-way valve.

The timing of the stimulation was synchronized with the movements of the fraction collector. The system was standardized with 3-min exposures to 50 mM glucose and 100 mM potassium chloride at the beginning and at the end of each experiment. The concentration of the stimulations mentioned in the text refers to alterations of the concentration of the substance in the medium: e.g. stimulation with 50 mM glucose means elevation of the glucose concentration of the medium (1 g/l = 5.56 mM) by 50 mM, resulting a total of 55.56 mM glucose concentration in the perifusion columns. In the experiments, 100 to 400 fractions were collected, 1 ml for 3 min each. In some cases, the parameters of the experiment were modified as indicated in the figure legends. At the end of each experiment, the total immunologically detectable hormone content of the cells was also determined from extracts made with 10 mM hydrochloric acid passed through the column.

Radioimmunoassay. Insulin levels were measured from 200 µl of the collected medium using Coat-A-Count kits from H. Biermann, Bad Nauheim, Germany. For standardization, rat insulin (Novo BioLabs, Bagsvaerd, Denmark, no. 7354118) was used in a seven-step dilution in a range from 0.25 to 50 ng/ml. The sensitivity limit of the assay was at 0.25 ng/ml. The intraassay and interassay variations were 6.6 and 9.4%, respectively.

Statistical analysis. Quantitative analysis of the superfusion results was carried out using a special computer program written for the purpose by one of the authors (Csernus). The principles of the mathematical analysis were described earlier [3]. To compare data obtained from experiments at different times, the results were standardized to the insulin response of the first potassium exposure (or first glucose stimulation). In the response plots, the signs for the sample administration were corrected with the dead volume of the system. As a consequence of this, the time lags between the sample administrations and the responses represent only the delays in the responses of the cells. In the figures, data collected in one representative experiment are plotted since after standardization, the variation between the identical experiments was in most cases negligible (invisible on plots). Further evaluations are based on the NET INT (net integral) values (the area of the peaks). Examples of statistical calculations have been published by the authors [37].

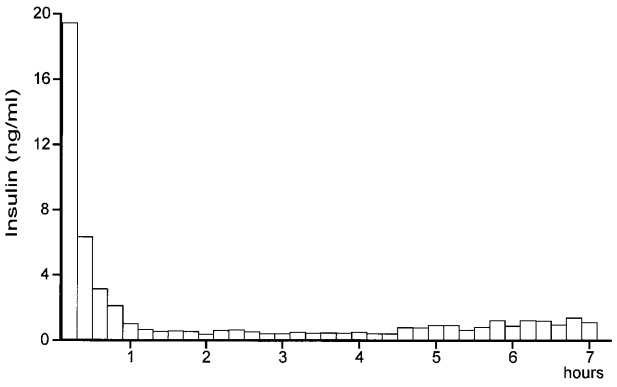

Figure 2. Basal insulin secretion from isolated rat pancreatic islets in vitro. Twelve-minute fractions were collected. Collection started immediately after column preparation. Virtually identical curves were obtained in 11 similar experiments.

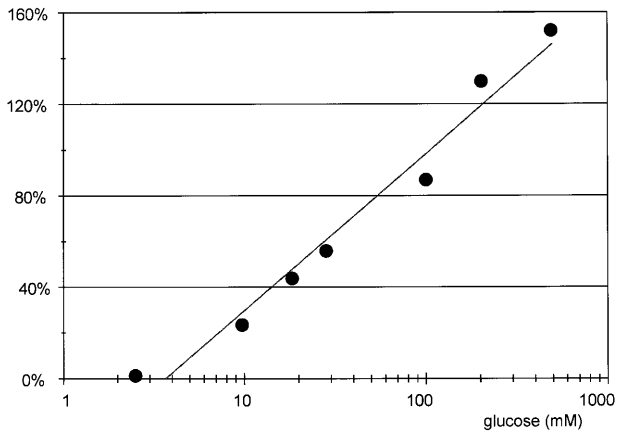

Figure 4. Dose-response curve of glucose-stimulated insulin release from isolated rat pancreatic islets in vitro. The results are based on net integral value of insulin release following 3-min stimulation with various concentrations of glucose relative (in %) to similar values measured after 3 min of stimulation with 100 mM KCl. Data are calculated from results of experiments similar to that plotted on figure 3.

Results

Baseline. To follow the basal secretion of the explanted pancreatic islets, 12-min fractions were collected for 7 h without stimulation, beginning immediately after the column preparation. A typical response curve of this type of experiment is seen in figure 2. The high insulin concentration (20 ng/ml) found in the first fraction declined rapidly, and in between 1 and 2 h it reached a steady baseline. When no stimulation occurred, a similar level was maintained for several days (up to 7 days in our experiments, data not shown).

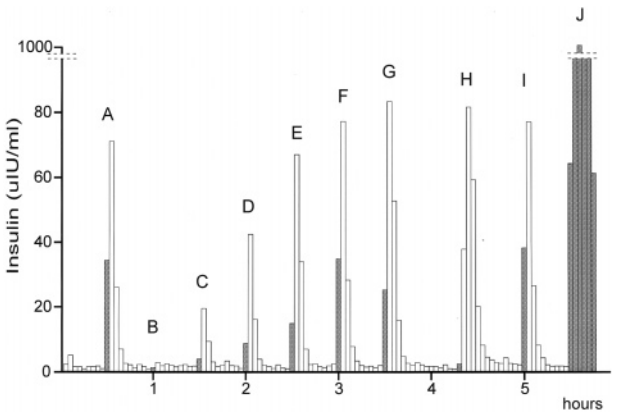

Figure 3. Insulin responses from rat pancreatic islets in vitro in a perifusion system. The islets were stimulated with 3-min exposures to 100 mM KCl (responses A, F and I) or to glucose at 2.5 mM (B), 10 mM (C), 20 mM (D), 100 mM (E), 200 mM (G) or 500 mM (H) concentrations (glucose concentrations always added to the 5.56 mM glucose of the culture medium). Peak 'J' was a result of extraction of the islets on the column with hypotonic solution (10 mM HCl). Similar responses were obtained from experiments with different sequences of the glucose concentrations (the results were reproduced in four experiments). Three-minute fractions were collected. Dark columns label the duration of stimulation.

Insulin response to pulsatile stimulation with glucose. Three-minute stimulation with elevated glucose concentration (an increase in the glucose concentration of the medium by between 2.5 and 500 mM) resulted in an immediate increase in the insulin concentration of the effluent medium. The elevated insulin concentration also dropped quickly after the stimulation had been stopped and reached the basal level in 9 to 12 min (fig. 3). When responses to randomly applied stimulations are plotted against the glucose concentration, a linear dose-response relationship resulted in the range of 4 to 500 mM of glucose concentration (with 3 min exposure time, net integral response plotted against the logarithm of dose, fig. 4). The median effective dose (ED_{50}) was at 45 mM (fig. 4). Based on these results, a 3-min stimulation with 50 mM elevation of glucose concentration was selected for standardization of the responsiveness of the islets. As an effect of this standard stimulation, only 0.48% of the insulin content of the B cells is discharged (net integral value of the response vs. the total hormone content of the cells calculated from the insulin content of the hypotonic HCl extraction of the columns at the end of the experiment).

Insulin response to prolonged stimulation with glucose. Prolonged (90-min) stimulation with glucose (3 to 20 mM) resulted in a biphasic insulin response. The stimulation induced a sharp increase in the insulin concentration followed by a transient drop. In about 20 to

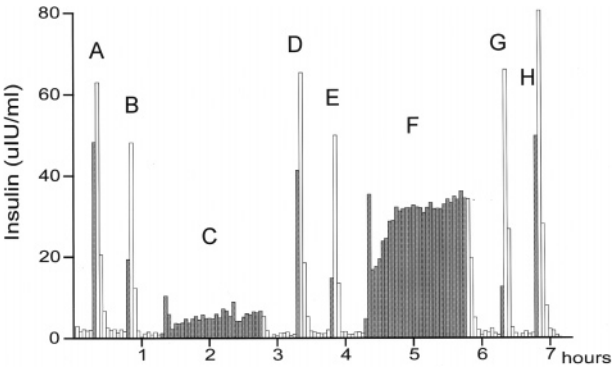

Figure 5. Insulin secretion from isolated rat pancreatic islets in vitro. The islets were stimulated with 100 mM KCl for 3 min (responses A, D and H), and with glucose: 50 mM for 3 min (B, E and G), 5 mM for 90 min (C) and 20 mM for 90 min (F) (glucose concentrations always added to the 5.56 mM glucose of the culture medium). Dark columns label the duration of stimulation. Similar results were obtained from experiments when 3 mM ($n = 30$), 4 mM ($n = 2$), 6 mM ($n = 6$), 9 mM ($n = 2$) and 15 mM ($n = 9$) were added to the 5.56 mM glucose in the culture medium.

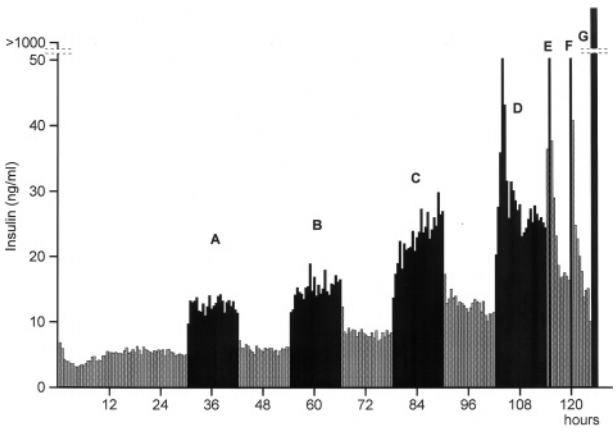

Figure 7. Insulin release from rat pancreatic islets. The glucose concentration of the medium was elevated by 3 mM at the beginning of the experiment (to 8.56 mM). The islets were also stimulated for 12 h with an additional 3 mM elevation of glucose concentration (to 11.56 mM, responses A, B, C and D) and 3-min exposure to 100 mM KCl (response E) and 50 mM glucose (response F). The last (G) peak represents insulin content of the hypotonic HCl extract of the islets (peak = 6000 ng/ml). Thirty-minute fractions were collected (two similar experiments were performed).

25 min, the insulin concentration gradually returned to and stayed at a permanent high level until the end of the stimulation. Data from a representative experiment are plotted in figure 5 (responses C and F). Similar results were obtained when the glucose concentration of the medium was elevated by 3 mM through a 5-day period.

After the transitory peak, the elevated insulin secretion was maintained until the end of the experiment (fig. 6). No sign of desensitization was experienced; the insulin concentration actually showed a constant, slightly increasing tendency. Under this condition, the islets maintained their responsiveness until the end of the experiment for both specific (50 mM glucose) and non-specific (100 mM KCl) pulsatile stimuli (responses A

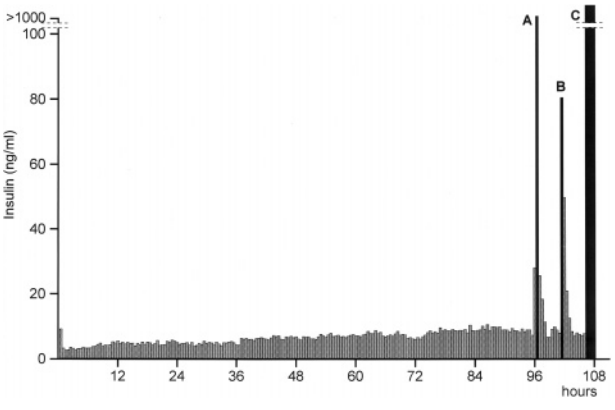

Figure 6. Insulin release from rat pancreatic islets exposed to elevated glucose concentration: the glucose concentration of the medium was elevated by 3 mM (to 8.56 mM) at the beginning of the experiment. Three-minute stimulations with 100 mM KCl (A) and 50 mM glucose (B) were also applied. Peak 'C' represents insulin content of the HCl extract of the column (peak = 15000 ng/ml). Thirty-minute fractions were collected (10 identical experiments were performed).

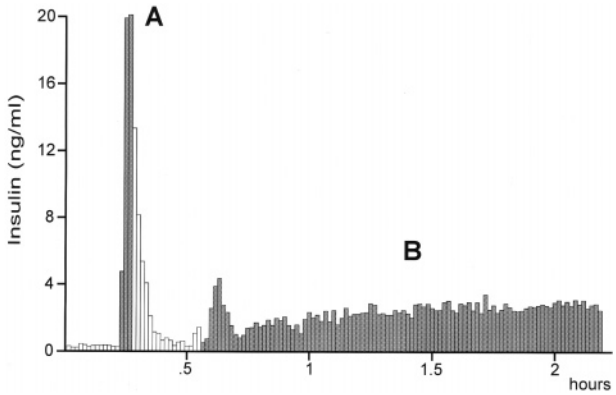

Figure 8. Insulin release from isolated rat pancreatic islets in vitro following stimulation with glucose: 50 mM (total 55.56 mM) for 3 min (A) and 7 mM (total 12.56 mM) for 100 min (B). One-minute fractions were collected.

and B in fig. 6). When in a similar experiment the cells were also stimulated with an additional 3 mM elevation of glucose concentration (i.e. 5.56 mM of the culture medium plus 3 mM = 8.56 mM) repeatedly for 12 h at 12-h intervals, consistent, slightly increasing responses were obtained (fig. 7).

Dynamics of insulin release following glucose stimulation. To analyse the dynamics of insulin release from isolated rat pancreatic islets, insulin content of tissue culture medium collected at 1-min intervals was also determined (fig. 8). There was a high insulin response to a 3-min stimulation with 50 mM glucose even in the first, 1-min fraction during the stimulus. The release reached the highest level in the second 1-min fraction. Following cessation of the stimulation, the insulin concentration in the fractions decreased rapidly, reaching the baseline 8 min after the stimulus had been stopped (fig. 8, response A). Stimulating the islets with 7 mM glucose (5.56 mM glucose in the medium was elevated to 12.56 mM) for 100 min resulted in an approximately 2-min delay in the onset of the insulin response. Contrary to continuous stimulation, after a 4-min initial high insulin level, there was a transient decrease in the insulin response for about 6 min followed by a continuously high, slightly increased concentration of the insulin release during the entire stimulation (fig. 8, response B).

Insulin response to transient elevation of K^+ concentration. Three-minute elevation of the potassium concentration of the medium (addition of 100 mM KCl) induced a temporary, sharp increase in the release of insulin from the perifused islets similar to that which followed glucose stimulation. Based on the data of HCl extraction, this stimulation liberated about 1% of the total insulin content of the cells. (fig. 3, responses A, F and I; fig. 5; responses A, D and H; fig. 9, responses A and M). The stimulation apparently does not significantly influence the responsiveness of the cells to consecutive stimuli repeated in 30-min intervals.

Direct effects of bioactive compounds on the insulin release. Exposure of the explanted islets to 10 μM acetylcholine (Ach) or 30 mM arginine (Arg) induced a transitory elevation of insulin release similar in shape to that experienced after glucose stimulation (fig. 9, responses E and I). Norepinephrine (NE) and dopamine (DA) in 10 μM concentration as well as 100 nM somatostatin (SS) did not induce any detectable alteration on the basal insulin secretion of the islets (fig. 9, responses C, D and F). However, 100 nM SS given together with 50 mM glucose, 30 mM Arg or 10 μM Ach significantly reduced the insulin-releasing effect of these substances (by 75.5, 71.5 and 72.5%, respectively) (fig. 9, responses H, J and L). At the same time, SS was unable to alter the insulin response of the islets to 100 mM elevation of K^+ concentration (fig. 9, responses M and N). SS also inhibited the glucose (50 mM for 3 min)-induced insulin release in a dose-dependent way ($ED_{50} = 22$ nM, fig. 10). A similar dose-dependent inhibitory effect was found with NE ($ED_{50} = 89$ nM) and DA ($ED_{50} = 2.2$ μM) (figs 10 and 11) on glucose-

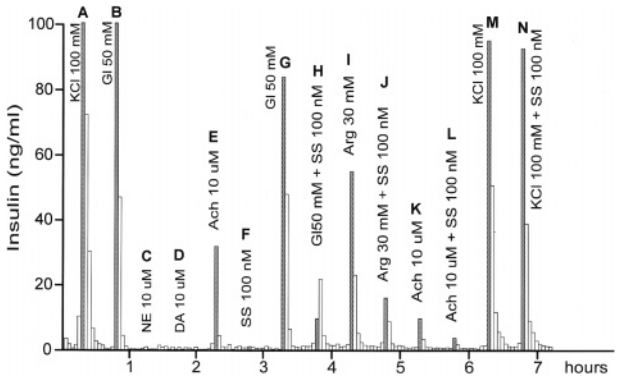

Figure 9. Insulin release from isolated rat pancreatic islets in vitro. All the stimulations lasted for 3 min. The stimulants and their concentrations are labelled in the body of the figure. (Gl, glucose; Ach, acetylcholine; DA, dopamine; NE, norepinephrine; Arg, arginine; SS, somatostatin). Nine similar experiments were performed.

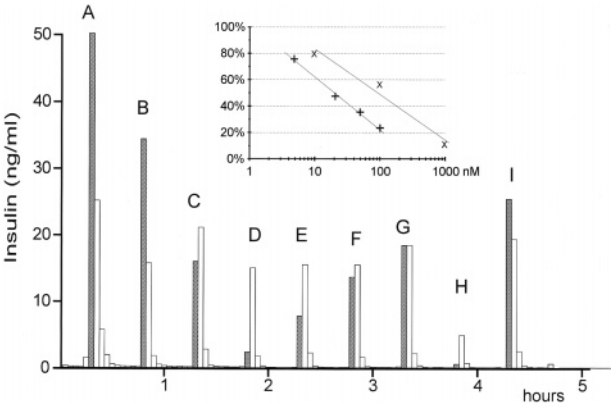

Figure 10. Effects of SS and NE on glucose-induced insulin release from isolated rat pancreatic islets in vitro. The islets were stimulated for 3 min with 100 mM KCl (response A) or 50 mM glucose (responses B–I) for standardization. Combined with 50 mM glucose (final concentration 55.56 mM glucose), each of the following substances was applied for 3 min: SS in concentration of 5 nM (C), 50 nM (D) and 20 nM (E), or NE in concentration of 100 nM (F), 10 nM (G) or 1000 nM (H). In the inset, the effects of SS (+) and NE (×) on glucose-induced insulin release are plotted. The values represent the total amount of insulin (NET INT value) released from the islets as a result of combined stimulation relative to that after glucose stimulation only. Five similar experiments were performed.

induced insulin release. GABA, however, did not influence insulin release under similar circumstances (fig. 11).

Discussion

The perifusion system proved to be suitable for analysing the control and dynamics of insulin release from isolated rat pancreatic islets in vitro. The islets maintain their responsiveness for up to 7 days (our observations, data not shown). During the initial hours after preparation of the perifusion column, high, then gradually decreasing insulin concentration in the effluent medium is detected (fig. 2). Similar observations have been described [27]. During this period, most of the insulin is liberated from cells that were damaged or mechanically irritated during the preparation of the column. Since in this phase also the responsiveness of the cells is strongly affected (own observation, data not presented), no reliable experimental data can be obtained in this period. Similar conclusions were drawn from in vitro experiments with anterior pituitary [3] or pineal cells [38]. In the perifusion system, the time needed to achieve a steady baseline is about 90 min (fig. 2). To regain full responsiveness even more time is required (data not shown). That is why we suggest an overnight recovery period before the first stimulation when using in vitro bioassay. Although similar waiting periods are also recommended by others [20, 32, 39, 40], this important step is sometimes neglected.

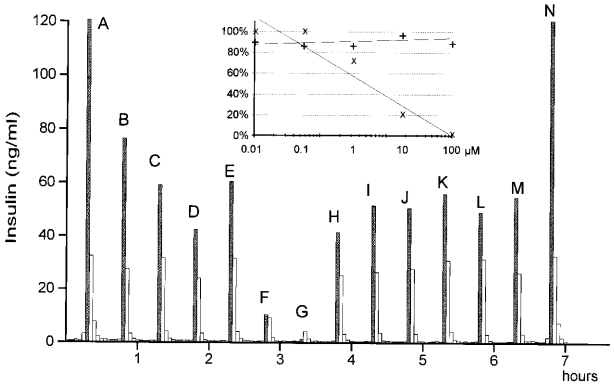

Figure 11. Effects of DA and GABA on glucose-induced insulin release from isolated pancreatic islets in vitro. The islets were stimulated with 100 mM KCl (responses A and N), or 50 mM glucose (responses B–M) for standardization. Combined with 50 mM glucose (final concentration 55.56 mM glucose), each of the following substances was applied for 3 min: DA in concentrations of 10 nM (C), 1 μM (D), 100 nM (E), 10 μM (F) and 100 μM (G) or GABA in concentrations of 10 nM (H), 1 μM (I), 100 nM (J), 10 μM (K) and 100 μM (L). In the inset, the effects of DA (×) and GABA (+) on glucose-induced insulin release are plotted. The values represent the total amount of insulin (NET INT value) released from the islets as a result of combined stimulation relative to that after glucose stimulation only (two experiments were performed).

Most laboratories utilize morphological criteria (tissue mass, number of cells, number of living cells or number of special type of cells) for standardization of the in vitro bioassay. These data, however, do not reflect several factors that significantly alter the responsiveness of the system rendering the standardization unreliable. Most of the tissues consist of multiple types of cells. Tissue mass or total number of cells provides only an unreliable estimation of the number of target cells. Even the number of specific cells (determined by counting immunofluorescent labelled cells) might be far from the quantity of cells actually participating in the experiment, since during the preparation of the perifusion column a significant, undetermined portion of the cells are damaged. Moreover, morphological criteria do not provide information on the functional state of the cell and its alteration during the experiment. This is why we utilized functional criteria for standardization of the perifusion method and give morphological data (number of organs) for reference.

The specific stimulator of the B cells, glucose, initiates a series of intracellular events resulting in insulin release. Glucose first internalizes into the B cells. This step is facilitated by specific subtypes of glucose transporters (like GLUT2). Then it becomes phosphorylated by glucokinase, a specific enzyme that is expressed only in cells which are closely related to the regulation of glucose metabolism, such as liver and pancreatic B cells. Thus, in certain aspects, glucokinase can be considered as 'glucose receptor'. The phosphorylated glucose initiates a partially known series of intracellular events resulting in both secretion and synthesis of insulin. The components of this process include G proteins, cyclic guanosine monophosphate (cGMP), bivalent cations (Ca^{2+}) and various other factors [14, 41].

Modification of any of the elements of this complex cascade results in an altered insulin response. When the intracellular mechanisms are studied, it should be determined which element of this cascade is responsible for the altered responsiveness. One of the most important components of responsiveness is the quantity of releasable intracellular insulin pool. We routinely determine the total hormone content at the end of each experiment by disrupting the cells with hypotonic solution. In our experience, passing 10 mM HCl for 9 to 12 min through the columns will extract virtually all the soluble hormones from the cells. The total amount of intracellular hormone pool can only be directly determined once at the end of the experiment, since it includes the destruction of the cells.

There is another way, however, to obtain data on the releasable hormone pool while maintaining the responsiveness of the cells. Any event that increases the intracellular Ca^{2+} concentration in peptide hormone-secreting cells results in a fusion of membranes of the cell and the secretory granules, and initiates a secretory response [34]. One of the most direct ways to induce an increase in intracellular Ca^{2+} concentration is transitory elevation of the extracellular K^+ concentration that opens the voltage-dependent Ca^{2+} channels by evoking depolarization and also facilitates Ca^{2+} liberation from intracellular Ca^{2+} reserves. This can be done easily in the perifusion system. Pulsatile elevation of potassium concentration induces a transient, repeatable, distinct increase of insulin secretion from the islets. The response is well reproducible, and the size of it is highly independent of the state of the specific hormone-releasing mechanism. Unlike glucose-, arginine- or acetylcholine-induced insulin release, KCl-induced insulin release cannot be inhibited with simultaneous addition of SS (fig. 9, response N), NE or DA (own data, not shown). These compounds apparently affect insulin release 'proximal' to Ca^{2+} liberation. The lack of effect of somatostatin on K^+-induced insulin release can also be explained by suggestion of Sharp [34] that depolarizing KCl concentrations may interact with the somatostatin receptor, reducing its inhibitory signal. Generally, we use 100-mM elevation of the KCl concentration for 3 min for standardization. A response to this stimulation induces a discharge of about 1% of the insulin content of the cells. The size of the response shows good correlation with the intracellular hormone pool (HCl extraction after the response) at any functional state of the cells. This stimulation is repeatable and does not alter the subsequent responsiveness of the cells.

Based on these observations, the functional standardization of the described method includes four fundamental parameters: (i) analysis of the changes in the basal hormone secretion of the cells, (ii) response to a standard, specific stimulus (50 mM glucose for 3 min), (iii) determining the alteration of the momentary size of the releasable hormone pool with response to KCl and (iv) direct determination of the total intracellular hormone content from the extract of the column.

Pulsatile stimulation (3 min) with elevated glucose concentration resulted in a dose-dependent, distinct insulin response. For functional standardization of the system, 50-mM elevation of the glucose concentration for 3 min was selected. This concentration is higher than the (average) physiological glucose level in the peripheral blood, but in case of short (3-min) pulsatile stimulation, it is close to the middle of the dose-response curve in the system. The response to this stimulation is repeatable and well reproducible. Based on data of the total insulin content of the cells determined with hypotonic extraction of the islets at the end of the experiment, less than 1% of the total insulin content of the B cells is discharged from the cells as a response to this type of stimulation. These data suggest that stimulation of the islets with transitory elevation of the cells with 50 mM glucose for 3 min will not profoundly affect the physiological state of the cells and is suitable for functional standardization of the perifused islets.

The lowest pulsatile elevation (3 min) of glucose concentration resulting in a significant increase in insulin release in our system was at 2.5 mM (an increase of glucose concentration in the medium from the standard 5.56 to 8.06 mM). Increasing the concentration of the pulsatile glucose stimulus increased the insulin response accordingly. A linear dose-response relationship was found in the range of 4 and 500 mM when the net integral values of the response were plotted against the logarithm of the elevation of the glucose concentration. The ED_{50} was at 45 mM.

The ED_{50} in our experiment is somewhat higher than that published [21, 42]. This difference may be due to several factors. One may be the different way we calculate the dose-response curve. We used the net integral value (the area below the curve but above the calculated baseline) as raw data for the dose-response curve and not the amplitude (the concentration of the highest fraction) or just the plain area below the response curve. The net integral value shows the total hormone release (increase of secretion) as a consequence of a stimulation; thus it describes more accurately the rate of the altered activity of the cell than the amplitude. The amplitude, unlike the net integral value, may be deeply affected by the parameters of the experiment (volume and timing of the fractions, the flow rate of the medium, the size and volume of the perifusion chamber, etc.), and still the 'traditional' calculation of the area below the response curve ignores the basal secretion of the cells, resulting in an overestimation of the responses to low stimuli (compare data in figs 3 and 4).

The larger ED_{50} may also be due to the relatively higher functional capacity of the cells in our system. In our experiments, we took great pains to preserve the function of the cells insofar as possible. Besides the careful preparation of the tissues, avoiding all unnecessary steps, we allowed adequate recovery time for the cells before collecting data and also used a highly sophisticated tissue culture medium (Medium-199) with a balanced composition of about 60 ingredients for our experiment. When the cells have not recovered from the stress of the preparation of the in vitro system or are ill-fed, they cannot 'perform well', reaching the maximal response at a lower dose of stimulant, resulting in a lower apparent ED_{50}. The effects of high osmolality on the insulin release (in stimulations higher than 50 mM) cannot be excluded either. Since the response to even

500 mM glucose was repeatable, and the shape of the response curve (response fractions) fitted well to similar responses in systems, where the effect of osmolality could be excluded (e.g. response of anterior pituitary cells to luteinizing hormone-releasing hormone (LHRH) or growth hormone-releasing hormone (GHRH) [3]) we believe that the effects of the osmolality have limited if any role in response. Response of the pancreatic islets to glucose stimulation proved to be quite fast. Significant insulin release was measured in the first minute after 50-mM glucose stimulation. In the second minute the insulin release had already reached the highest level (fig. 8). These results are in accordance with experiments detecting dynamic alterations of intracellular Ca^{2+} concentrations with fluorescent dyes [40, 43, 44]. When the islets were stimulated with lower glucose concentrations (e.g. 7 mM at response B in fig. 8), the onset of insulin release was consistently delayed by 1 to 2 min. This finding indicates that alternative routes (more sensitive, but slower, and less sensitive, but faster) of the insulin-releasing mechanism may be functioning.

Prolonged stimulation with glucose (for 20 min to several days in our experiments) resulted in a lasting, dose-dependent increase of insulin release from the isolated islets. After a sharp onset, there was a characteristic, transient drop of the insulin secretion in the first half hour at any tested insulin concentration (1 to 50 mM). This transient drop has also been observed by other authors [11, 13]. Subsequently, insulin secretion was maintained at a high level during the entire range of the experiment (7 days in our longest experiment [45]). No trend to desensitization could be detected under our experimental conditions. Moreover, a tendency toward a slight, continuous elevation was experienced even when the stimulus lasted for several days (figs 6 and 7). During and after these stimuli, the islets maintained or slightly increased their responsiveness to pulsatile stimulus. No detectable periodic alteration of the insulin response was found under any of our experimental conditions.

The lack of desensitization in our experiment, in contrast to some other observations [11, 16, 39], can be due to several factors. These include the maintained functional capacity of the cells, the carefully designed stimuli and the age of the animals we used.

Tissues from young animals generally adapt better to new (in this case in vitro) environments and show higher functional capacity. We used younger rats for our experiments in comparison to others [39], primarily because the islet preparation technique called for it. Using this method for islet preparation, unfortunately, does not work with older animals, so, up till now, we have no comparative data with them.

'Desensitization', that is reduced responsiveness of the cells, can be not only a result of downregulation of the receptors or other components of the intracellular signal transduction system but also a consequence of a depletion of the intracellular reserves, including that of the hormone ready to be released in the cells by forcing the cell to release more hormone than can be substituted by synthesis. Tissues kept under improper conditions have a reduced capacity for hormone resynthesis; thus they may show false symptoms of early 'desensitization'. With proper maintenance of the in vitro conditions, this phenomenon can be reduced. Desensitization described by others [16, 39] thus may be due to differences in the culture media they used. Additionally, it is possible that limited responsiveness of the cells in experiments started after a too short recovery period [11, 16] was evaluated as 'desensitization'.

Depletion of the hormone reserve from cells also depends on the parameters of the stimulation. For prolonged stimulation, we selected a concentration of the stimulant (in our case glucose) which did not induce a higher rate of hormone release than the intracellular hormone synthesis can keep pace with. In our experiments similar to the one plotted in figure 8, prolonged stimulation with a nondepleting concentration of glucose, a gradual increase in both insulin release and intracellular insulin reserve were actually observed due to the stimulation of intracellular insulin synthesis. Although depolarization-induced hormone secretion following a rapid increase of extracellular K^+ requires the functionality of some elements of intracellular Ca^{2+} metabolism, the depletion of intracellular hormone reserves could be well monitored with response to K^+. We consider 'real' desensitization only if reduced responsiveness to a specific stimulant concurred with maintained responsiveness to pulsatile stimuli with K^+.

Based on our results we believe that no desensitization to glucose-induced insulin release can be observed if the experimental conditions of the cells are properly maintained and the parameters of the stimulation are carefully designed. Further details of stimulant desensitization relationships have been discussed elsewhere [3].

Exposure of the islets to acetylcholine or arginine stimulate insulin secretion [6, 8, 12, 22–24], while DA, NE and GABA inhibit glucose-stimulated insulin release [26–28, 30–34, 35]. These results were obtained in in vitro (perifusion and batch technique) and in vivo investigations. In our experiments, following stimulation with 10 μM acetylcholine or 30 mM arginine, the shape and timing of the response curve was similar to that induced by glucose (fig. 9). The response could also be inhibited by SS (fig. 9). These data suggest that these two compounds act upon the islets directly. The physiological role of these compounds still remains to be elucidated. Since the effective concentrations are much higher than the physiological level, this response can be considered pharmacodynamic. Although neither 100

nM SS nor NE or DA in 10 μM concentration induced any detectable alteration in basal insulin secretion of the islets (fig. 9), they all showed a dose-dependent inhibition of insulin release to pulsatile stimulation with glucose (figs 10 and 11), with ED_{50} of 22, 89 and 2.2 μM, respectively [30]. These concentrations are within the physiological range and may have a physiological role in the control of insulin secretion.

In contrast to studies of others [35], GABA did not act upon insulin release in our experiments (fig. 11). This difference may be caused by the different experimental protocols: perfused pancreas vs. perifused isolated islets, they used larger GABA concentrations (up to 1 mM) and prolonged stimulation with 16.7 mM vs. our 3-min pulsatile exposure with 55.6 mM glucose.

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