Fuel-induced Insulin Release In Vitro from Insulinomas Transplanted into the Rat Kidney

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SUMMARY

We studied the release of insulin, glucagon, and somatostatin in response to glucose, glyceraldehyde (GA), and α-ketoisocaproate (KIC) from rat kidneys containing transplanted insulinomas. Kidneys were perfused about 11 wk after transplantation when the plasma glucose concentration of the fed animals had decreased from 180 ± 7 to 95.1 ± 9.9 mg/dl and plasma insulin concentrations had increased from 2.6 ± 0.5 to 14.2 ± 2.0 ng/ml. The insulin content of the tumor-containing kidney ranged from 40 to 679 μg; the glucagon and somatostatin concentrations ranged from undetectable levels to 3.7 μg and 248 ng, respectively. The average response to 30 mM glucose and 10 mM GA was a four- to fivefold increase in insulin secretion, whereas 30 mM KIC caused a 16- to 28-fold increase. In vitro stimulation of the insulinoma with 30 mM glucose primed the β-cell response to a second stimulus following a short rest period. Cytochalasin B did not enhance this primed glucose response. Diazoxide inhibited glucose, GA, and KIC-stimulated insulin release. Glucose, GA, and KIC stimulated glucagon release in 2 of 17 insulinomas studied here. Somatostatin release was not seen in any of the experiments. These findings show that this islet cell tumor transplanted under the kidney capsule releases insulin in response to physiologic and model fuel substances. Thus, this particular transplantable tumor offers an opportunity to study the biochemistry and biophysics that underlie fuel-stimulated insulin release. DIABETES 33:1–7, January 1984.

The study of islet cell secretory processes has been hampered by the difficulty in obtaining sufficient islet cell material. Islet cell tumors provide a greater quantity of secretory material than is obtainable by currently employed methods of separating islet tissue from the exocrine pancreas. These tumors therefore offer a potentially valuable system for studies of hormone secretion especially if they can be propagated by transplantation and if they retain functional characteristics similar to normal islet tissue. It is most important that the secretory responsiveness to glucose is preserved. However, the response of tumor tissue to fuel stimuli, especially to the major physiologic stimulus glucose, has been variable according to previous studies in which either tumor fragments have been used to investigate hormone release in vitro[1,2] or hormone secretion has been assessed in vivo.[3–6] This inconsistent and generally negative outcome could be due to inadequate technology.

We have therefore attempted to optimize the technique of tumor cell stimulation by establishing a system in which the physiology and biochemistry of tumor tissue could be studied in a perfusion setting that minimizes possibly harmful effects that could result from cell isolation procedures and avoids the vagaries of in vivo studies. Islet cell tumor tissue was transplanted under the kidney capsule and the tumor-bearing kidney was studied in vitro by vascular perfusion. The classic stimuli glucose, glyceraldehyde, and ketoisocaproate were delivered to the tumor tissue via vascular channels and hormone release was measured.

MATERIALS AND METHODS

Animals and transplantation procedure. A radiation-induced transplantable islet cell tumor[7] was maintained by serial subcutaneous transplantations in inbred albino NEDH rats (New England Deaconess Hospital, Boston, Massachusetts). For the transplantation of tumor material under the kidney capsule, a subcutaneous tumor was excised, placed into sterile Hanks solution, and finely minced with scissors. Fed male NEDH rats were used. The animals were anesthetized with ether and the abdominal cavity was opened with an incision in the right flank. The right kidney was exposed and a small incision was made in the kidney capsule. The

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right kidney was used because it is better suited for in vitro perfusion than the left kidney for anatomic reasons.[8,9] One or two small pieces of tumor tissue were then placed underneath the kidney capsule with a fine forceps. The abdominal cavity was closed with 2-0 chromic gut in a continuous suture pattern. The skin incision was closed with sterile autoclip wound clips (Fisher Scientific Company, Pittsburgh, Pennsylvania). All rats were allowed free access to Purina Rat Chow (Ralston Purina, St. Louis, Missouri) and water. At the time of the perfusion experiment the body weight of a group of 17 tumor-bearing rats used for this study was 387 ± 17 g.

Monitoring of plasma glucose. Blood for plasma glucose and insulin determination was obtained from the orbital sinus under light ether anesthesia in biweekly intervals during the first month and in weekly intervals thereafter. Rats were used for the kidney perfusion study when their nonfasting plasma glucose dropped below 120 mg/dl. Plasma glucose was measured by using a Beckman Autoanalyzer II (Beckman Instruments, Inc., Fullerton, California).

Reagents and perfusion protocols. The basic perfusion solution consisted of NaCl, 115 mM, KCl, 5 mM, $MgCl_2$, 1.0 mM, $NaHCO_3$, 24 mM, $CaCl_2$, 2.2 mM, bovine serum albumin (BSA), 0.25%, and dextran, 6%. The crystalline BSA used in the perfusion medium was from Armour (Reheis Co., Kankakee, Illinois). Dextran, D-glyceraldehyde (GA), sodium ketoisocaproate (KIC), cytochalasin B (CB), α-3-0-methyl-glucose (MG), sucrose, BSA (RIA grade), and ethylenediaminetetraacetic acid (EDTA) (tetrasodium salt) were obtained from Sigma Chemical Company (St. Louis, Missouri). Diazoxide was purchased from Schering (Kenilworth, New Jersey) and glucose was obtained from Pfanstiehl Laboratories (Waukegan, Illinois). To the basic perfusion medium were added 5 mM glucose for the initial equilibration period (0–30 min), and 30 mM glucose for the first stimulatory period (30–60 min). The tissue was then reequilibrated with basic perfusion medium (60–80 min). For a second stimulatory period (80–110 min), either 10 mM GA (N = 4), 30 mM KIC (N = 4), or 30 mM glucose, with (N = 2), or without (N = 2) 5 μg/ml CB, were used. In three experiments, the kidneys were perfused with 5 mM glucose for 80 min and with 30 mM KIC for 30 min. In three experiments, the kidneys were perfused with 5 mM glucose (0–30 min), 30 mM glucose, 30 mM KIC or 10 mM GA (30–90 min), and 1 mM diazoxide was added to either stimulus at 60 min for a 30-min period. In two experiments, the kidneys were perfused with 5 mM glucose (0–30 min), and 5 mM glucose plus either 25 mM MG or 25 mM sucrose (30–60 min). After a 20-min reequilibration period without fuels added (60–80 min) the kidneys were perfused with 30 mM glucose (80–110 min) or 30 mM KIC (110–125 min).

Perfusion system. The perfusion system was an open circuit system in which the kidney was supported outside the body. Perfusate was pumped at a constant flow rate of 10 ml/min monitored with an inline flowmeter (Brooks Sho-Rate, A. H. Thomas, Co., Philadelphia, Pennsylvania). The perfusate was oxygenated with a membrane oxygenator containing two parallel 25-ft coils of Medical Grade Silastic tubing (i.d. 0.058 in., o.d. 0.077 in., Dow Corning Corp., Midland, Michigan) wrapped around a cylindrical core heated by a constant-temperature water circulator (Haake FJ Circulator, Cole-Parmer, Inc., Chicago, Illinois) set to deliver perfusate to the kidney at 37°C. A humidified stream of 95% O_2-5% CO_2 was equilibrated with the medium across the silastic tubing bringing the arterial pO_2 to 500–600 mm Hg and the pCO_2 to 30–40 mm Hg and the pH to 7.35–7.45. Perfusion pressure was monitored with an anaeroid manometer. The perfusate was passed into a custom-made plexiglass bubble trap and was then delivered to the kidney through a glass cannula (~19 gauge). The volume of the perfusion system was 30 ml.

The technique for the isolated perfused rat kidney preparation was a modification of the method described by Bowman.[8,9] Eighteen rats were used for the study presented. They were anesthetized with sodium pentobarbital (50 mg/kg i.p.) (Abott Laboratories, North Chicago, Illinois). After blood was taken from the orbital sinus for plasma glucose and insulin determination, the rat was secured in dorsal recumbency and the abdomen was opened by a ventral midline incision. Heparin (200 U) was injected into the femoral vein. Briefly, the glass cannula was placed into the superior mesenteric artery after the flow in the perfusion apparatus was stopped with a pressure head of 140 mm Hg, by clamping the tubing leading to the cannula. The clamp was then released, the perfusion pump started, and the cannula manipulated into the renal artery without stopping renal perfusion. After the cannula was tied in place the kidney was removed from the body, trimmed of excess tissue, and placed over a glass container. The perfusion pressure was usually below 60 mm Hg.

The perfusate exited freely from the cut renal vein and 1-min samples of perfusate were collected on ice at suitable intervals in tubes containing 500 kallikrein-inactivating units of aprotinin (Trasylol) plus 1.2 mg EDTA/ml of perfusate and 0.25% BSA. Samples for measuring insulin, glucagon, and somatostatin were stored at −20°C until the time of the assay. Soon after the beginning of the perfusion of the right kidney, the left kidney was removed from the body, trimmed, cut sagitally, blotted on a paper towel, and weighed. At the end of the designated perfusion time, the perfusate flow was

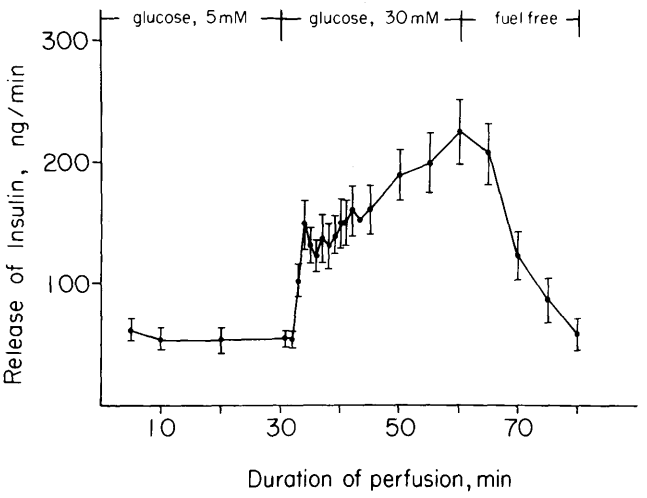

FIGURE 1. Insulin release from perfused insulinomas stimulated with high glucose. Rate of insulin release is recorded as mean ± SEM from 12 experiments.

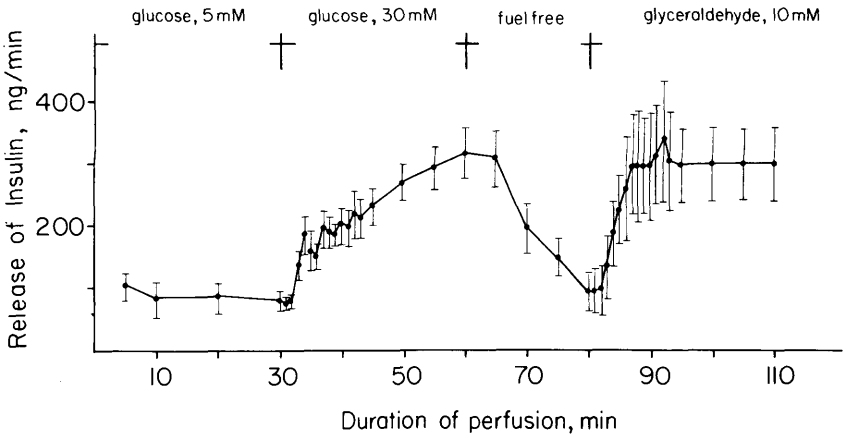

FIGURE 2. Response of perfused insulinomas to sequential stimulation with high glucose and glyceraldehyde (mean ± SEM; N = 4).

stopped. The right kidney was removed from the cannula, cut sagitally, blotted, and weighed. It was then frozen and stored at −80°C. Recovery of pancreatic hormones added in known concentrations to perfusate samples was tested in five experiments.

Calculation of tumor weight. In five separate experiments the weight of the tumor tissue was calculated as the difference between the weight of the perfused tumor-bearing right kidney and the unperfused left kidney. Portions of the unperfused left kidney and perfused right kidney were weighed and dried to constant weight in a drying oven. Normally, there are no significant differences between wet or dry weights of unperfused kidneys from the same rat (unpublished data). Perfusion leads to tissue swelling with an inherent increase in the tissue wet/dry (W/D) ratio. Assuming that renal and tumor tissue had parallel increases in wet weight during perfusion, the tumor wet weight normalized for tissue swelling was calculated as follows:

Normalized tumor weight = wet weight of perfused kidney, g/[(W/D ratio of perfused kidney)/(W/D ratio of unperfused kidney)] − wet weight of unperfused kidney, g.

For the perfusion conditions used in this study, the W/D ratio of the perfused kidney averaged 1.23 ± 0.06 (N = 5) times that of the unperfused kidney, which was 4.20 ± 0.08 (N = 5).

Hormone content of the perfused kidney. Insulin, glucagon, and somatostatin were extracted from the right kidney with acid ethanol (450 ml 100% ethanol, 39 ml distilled water, 15 ml concentrated HCl). The kidneys were placed in small glass containers and 10 ml acid ethanol (AE) was added to each kidney on dry ice. The kidneys were kept at −20°C for 24 h. Then they were finely minced with scissors and an additional 5 ml AE was added to each tube. The kidneys were then homogenized at 4°C with two 10-s pulses (Polytron, setting 7, Brinkman Instruments, Westbury, New York). The homogenates were kept at 4°C for 90 min and were then centrifuged at 1000 rpm for 15 min at 4°C. The supernatant fluid was aspirated and 5 ml AE was added again to the residue for repeated extraction as described above. The homogenates were kept at 4°C for 2 h and centrifuged again. The supernatants from both extractions were then combined and stored at −20°C until assayed. Recovery of pancreatic hormones added in known concentrations to normal kidneys using this extraction method was tested before it was applied to the experimental kidneys.

Radioimmunoassays. Immunoreactive insulin,[10] glucagon,[11] and somatostatin,[12] were assayed as described. Rat insulin provided by Dr. Mary A. Root (Eli Lilly, Indianapolis, Indiana) was used as standard for the insulin immunoassay; crystalline, beef-pork glucagon (Sigma Chemical Company, St. Louis, Missouri) and somatostatin (Calbiochem, San Diego, California) were used as standards for quantitating the other two hormones. Insulin antibodies came from Miles Laboratories, Inc. (Elkhart, Indiana) and the glucagon and somatostatin antibodies from Dr. Roger Unger (Southwestern University, Dallas, Texas).

Statistics. The significance of differences between means was determined by Student's *t* test and the significance of differences between paired data by the paired *t* test. All data are expressed as mean ± SEM unless otherwise stated. Best-fit lines between data points were determined by linear regression using least-squares analysis.

RESULTS

Comments on the procedures. The recovery of known quantities of pancreatic hormones added to perfusate samples was close to 100% for all hormones. In the kidneys, recovery was 92.6 ± 8.5% for insulin, 81.2 ± 8.8% for glucagon, and 60.7 ± 11.6% for somatostatin. The radioimmunoassay for insulin had a working range between 0.125 and 8 ng per tube, while the assays for glucagon and somatostatin were sensitive between 25 and 1000 pg per tube.

Plasma glucose and insulin concentrations. The plasma glucose concentration in normal fed rats anesthetized with ether was 180.5 ± 7.0 mg/dl (N = 26), while the insulin concentration was 2.6 ± 0.5 ng/ml. The plasma glucose concentrations in the tumor-bearing rats on the day of the kidney perfusion was 95.1 ± 9.9 mg/dl (N = 17) and the plasma insulin concentration was 14.2 ± 2.0 ng/ml (N = 17). Plasma glucose concentrations (x) correlated well with the logarithm of the insulin concentrations (y) ($y = -0.0064x + 1.6237$; $r = 0.94$; $P < 0.01$). The interval from the time of transplantation of tumor tissue to the time of perfusion was 77.2 ± 4.8 days.

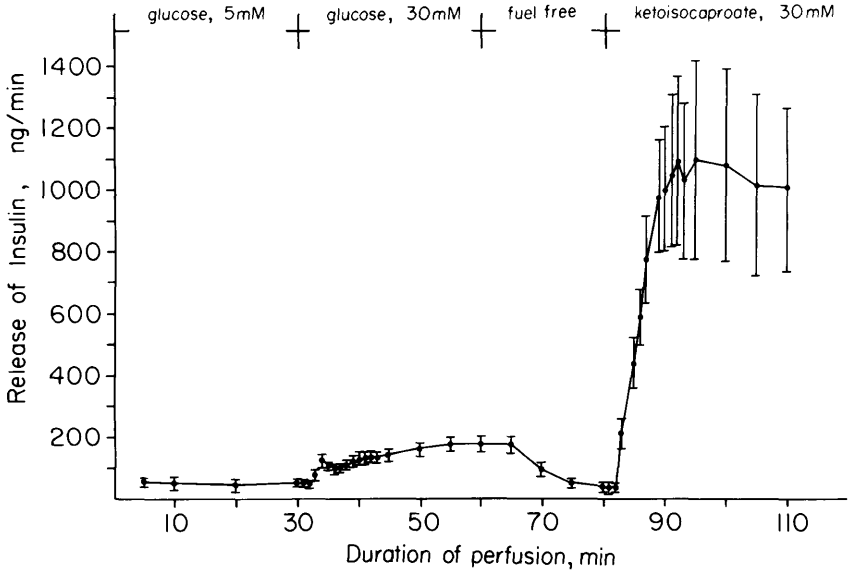

FIGURE 3. Release of insulin from perfused insulinomas stimulated sequentially with high glucose and ketoisocaproate (mean ± SEM; N = 4).

Weight and hormonal content of kidneys containing insulinomas. The average wet weight of the left kidneys was 1.40 ± 0.06 g (N = 17), and that of the perfused tumor-bearing right kidneys was 2.40 ± 0.11 g (N = 17). The approximate average wet tumor weight normalized for tissue swelling was 648 mg corresponding to 154 mg dry tumor weight. This value represents a minimal estimate, since it does not account for possible replacement of renal tissue by tumor tissue. The insulin concentration of the kidneys containing insulinomas was 285 ± 45.1 μg (N = 17) ranging from 40.3 to 679 μg. Glucagon was undetectable in 6 tumors, while the concentration in 11 tumors was 0.69 ± 0.37 μg ranging from 5.9 to 3.7 μg. It is noteworthy that 2 glucagon-secreting tumors had very high glucagon concentrations of 2.3 and 3.7 μg and relatively low concentrations of insulin (73.5 and 40.3 μg, respectively). Somatostatin was undetectable in 1 tumor; the concentration in 16 tumors was 58.8 ± 19.8 ng ranging from 1.6 to 248 ng.

B-cell responses to nutrients. Agonist concentrations in the maximal and supramaximal range (10 mM for GA and 30 mM for glucose and KIC) were chosen in order to increase the chances of uncovering any possible secretory responsiveness of islet tumor tissue to fuel stimulation.

Figure 1 shows the response of the tumor tissue to 30 mM glucose (N = 12). The biphasic increase in insulin secretion was followed by a rapid decline when buffer alone was used as the perfusion medium. The amount of insulin released during the 30-min stimulation period was 5.09 ± 0.1 μg (range 2.16–9.10 μg).

Figure 2 shows the response of insulinoma tissue to 30 mM glucose and 10 mM GA (N = 4). The amount of insulin released was 7.11 ± 1.0 μg using 30 mM glucose (range,

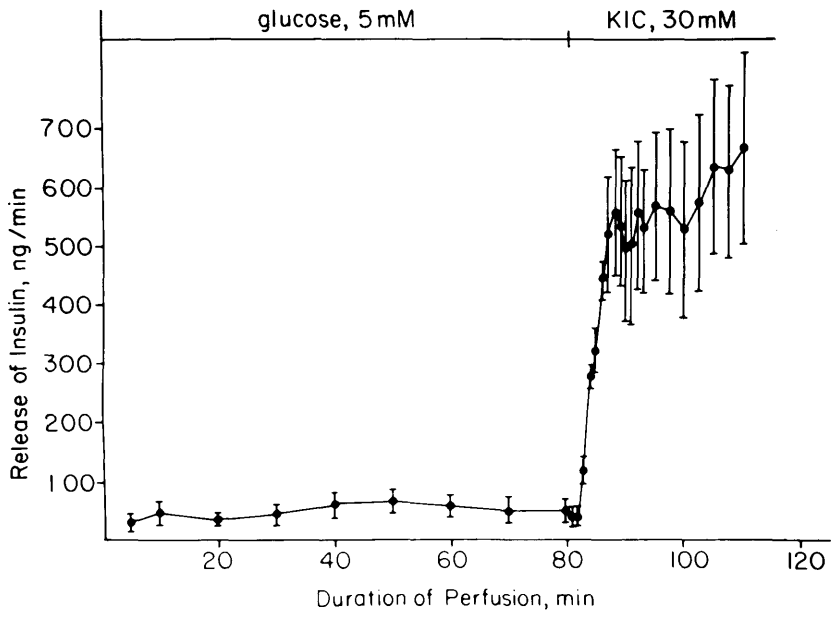

FIGURE 4. Insulin release from insulinomas perfused with low glucose, followed by stimulation with KIC (mean ± SEM; N = 3).

5.17–9.10 μg), and the amount secreted using 10 mM GA was 8.11 ± 2.08 μg (range, 5.88–14.3 μg). A fuel-free period was interposed to accelerate return to baseline release.

KIC was used as the second stimulus in four experiments (Figure 3). During the 30-min stimulation period using 30 mM glucose, 3.88 ± 0.44 μg of insulin (range, 2.84–4.62 μg) was secreted and 30 mM KIC stimulated the release of 25.6 ± 6.3 μg (range, 10.4–39.4 μg), which was markedly higher than the first response ($P < 0.05$). The amount of insulin released in response to glucose was 19.0 ± 5.5% (range, 9.1–33.4%) of that released in response to KIC. Perfusion of tumor tissue with 5 mM glucose for 80 min evoked no insulin release above baseline concentrations. However, 30 mM KIC in a subsequent 30-min perfusion period promptly stimulated secretion of insulin (Figure 4). These three experiments served as controls for most of the protocols employed in this study.

Another control experiment was required to check for possible osmotic effects that might have occurred with addition or withdrawal of 25 mM of solute. These controls clearly showed that minor hyperosmolarity as achieved here lacked effects on insulin release. Two tumor-containing kidneys were perfused with 5 mM glucose for 30 min and either 25 mM α-3-0-methylglucose or sucrose was then superimposed for an additional 30 min, followed by withdrawal of the sugars (i.e., in a totally fuel-free period). Neither addition nor withdrawal of these two sugars produced any effect. Subsequent stimulation with 30 mM glucose or KIC exhibited the usual positive insulin-release patterns (Figure 5). Another set of three controls (not shown here) was performed to test whether switching from fuel-free to 5 mM glucose-containing perfusate had any effect on insulin release. No response was seen in these three experiments, in which 30 mM glucose or KIC evoked typical secretory responses when added either before or following the test with low glucose described above.

Priming of insulin secretion by glucose. Priming of insulin secretion was apparent when the tumor tissue was stimulated sequentially for 30-min periods with 30 mM glucose. These periods were separated by a 20-min washout phase using buffer alone. The total amount of insulin released during the first period of stimulation was 6.06 and 6.27 μg (N = 2), and was 9.20 and 10.8 μg during the second period of stimulation. This was similar to the insulin secretion seen when 30 mM glucose plus 5 μg/ml of CB was used as the second stimulus, implying that CB did not add significantly to the glucose priming effect, as far as one can tell from these preliminary results. The total amount of insulin released during the first stimulatory period was 3.68 and 2.16 μg (N = 2) and 6.90 and 7.70 μg during the second stimulation period.

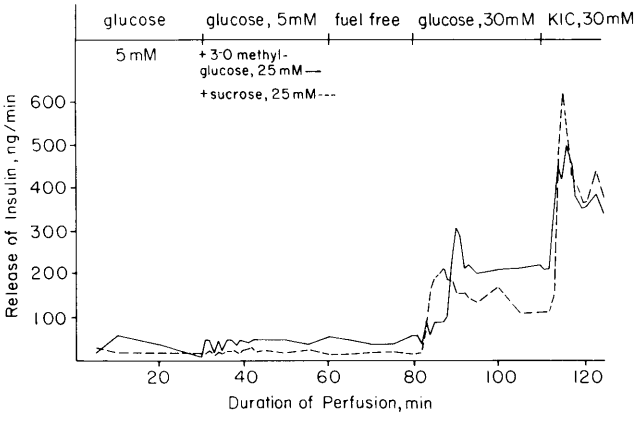

FIGURE 5. Lack of an osmotic effect on insulin release from perfused insulinomas.

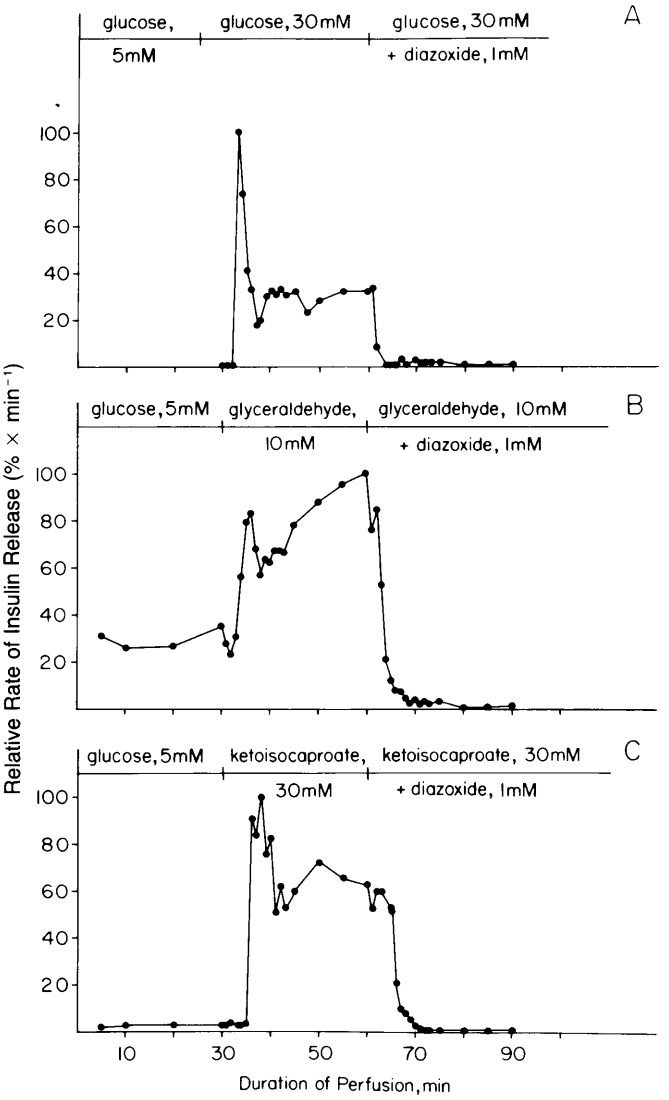

FIGURE 6. Inhibition by diazoxide of insulin release stimulated with glucose, glyceraldehyde, or ketoisocaproate. Rate of insulin release was calculated as percentage of the highest amount secreted in each experiment. The effect of diazoxide on each fuel stimulus was tested once and all results are shown in profiles A, B, and C.

Inhibition of insulin release by diazoxide. The addition of 1 mM diazoxide to 30 mM glucose, 30 mM KIC, or 10 mM GA completely inhibited insulin release stimulated by these secretagogues. Figure 6 shows the release of insulin depicted as a percentage of the highest amount secreted during each experiment. This method of presenting the data was chosen because of the great difference in the amount of insulin secreted between these three experiments. The

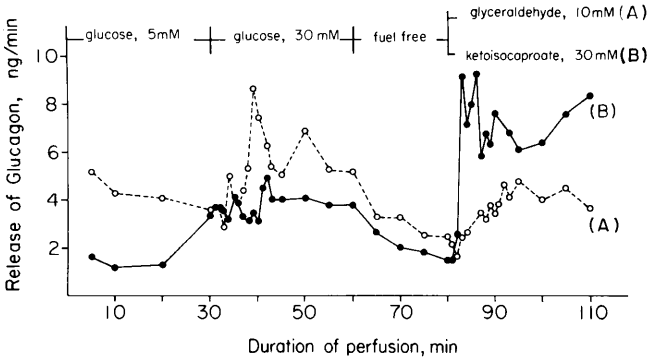

FIGURE 7. Glucagon release stimulated with glucose, glyceraldehyde, or ketoisocaproate. Individual experiments are shown.

total amount of insulin released when KIC was used as stimulus was 97.1 μg and this was the highest total amount released of all experiments performed here. When 30 mM glucose or 10 mM GA was used as a stimulus the total amount of insulin release was 3.98 and 7.99 μg, respectively.

Release of glucagon and somatostatin. In 2 of the 18 experiments using 30 mM glucose as the first stimulus and 30 mM KIC or 10 mM GA as the second, glucagon release was seen with all stimuli tested (Figure 7). In the other 16 perfusions glucagon release was undetectable. Release of somatostatin was not seen in any of the 18 experiments studied.

DISCUSSION

The distinguishing feature of insulinoma tissue grown under the kidney capsule is the ability to respond with insulin release to in vitro stimulation by high glucose, glyceraldehyde, and α-ketoisocaproate. In contrast, previous in vitro studies with the radiation-induced rat[7] and spontaneous Syrian hamster[13] transplantable insulinomas consistently failed to elicit secretory responses with glucose, whereas leucine in pharmacologic concentrations was found to stimulate insulin release by rat insulinoma tissue.[2] One recent publication[6] contains a suggestion that the transplantable radiation-induced rat insulinoma could respond to glucose in vivo. Still, there remains considerable uncertainty about the physiologic significance of these experiments because the tumor-bearing animals used in the quoted study were seriously hypoglycemic (with blood glucose concentrations of about 25 mg/dl). Such profound glucose deprivation of islet tumor cells might cause ATP deficiencies[14] and thus lead to blockade of basal insulin secretion and the replenishment with glucose during an IVGT test might then simply refuel the tumor cells, thus augmenting basal hormone secretion. It is therefore conceivable that the observed in vivo glucose response was unrelated to the physiologic stimulus-secretion process of normal β-cells during glucose stimulation. The absence of an in vitro glucose response of tumor fragments experienced in the same study is conspicuous; culturing of tumor tissue for 7 days led to results suggesting that these tumor cells have at least the potential for responding to glucose.

The preservation of glucose responsiveness in vitro clearly demonstrated in our work could be a consequence of the more suitable transplant site in the kidney, or it could be explained by the method of in vitro testing, i.e., the delivery of stimuli through the vasculature in a physiologic perfusion system. Detailed dose-dependency studies with glucose are required to fully assess the sensitivity of the rat insulinoma tissue to the physiologic stimulus glucose. Our previous finding that the activity of glucokinase, the proposed glucose sensor,[15–17] amounted to only 30% of the activity of normal rat islet tissue might suggest that the glucose sensitivity of the insulinoma cell is decreased compared with normal islet tissue.[18] This interpretation is also supported by our finding that KIC is a more potent stimulus than glucose, which is in contrast to the situation in normal islet tissue when prepared from fed animals. The high rate of secretion seen here with KIC further indicates that the release mechanism itself is not the rate-limiting factor for glucose-stimulated insulin release.

It needs to be appreciated that the magnitude of nutrient-stimulated insulin release observed with the isolated perfused insulinoma is less than in vitro release from normal islet tissue when expressed on the basis of tissue weight. This is best illustrated by comparing results typically obtained from the isolated perfused pancreas of adult rats with the present data from isolated perfused insulinoma tissue. The insulin release profiles with glucose as stimulus are comparable quantitatively and qualitatively; yet far more insulinoma tissue than normal islet tissue is required to achieve similar results. The average tumor wet weight was about 650 mg, approximately two orders of magnitude greater than the average islet mass (~10 mg, unpublished data). Could the reduced release be explained by cellular heterogeneity of the tumor with a small percentage of nearly normal cells and the remainder of unresponsive cells or are all cells equally ineffective? The absolute insulin content of the insulinomas studied here is similar to the insulin content of the whole pancreas of older rats (280 μg/tumor versus 250 μg/pancreas, unpublished data). However, the fractional insulin release from insulinoma tissue is comparable with that of normal β-cells, i.e., of the order of 5–20% of the stored insulin per hour depending on the nature of the stimulus. At this juncture it can only be speculated that the reduced capacity of insulinoma tissue for insulin release is due either to a reduction of cellular insulin pools or a diminished number of normal cells containing large stores of insulin.

In two tumors of this series glucagon was released in response to glucose, KIC, and GA, and both tumors also contained the highest amount of glucagon and the lowest amount of insulin in the tissue extracts. Originally it was reported that radiation-induced tumors contained insulin and somatostatin, which corresponds with the presence of β- and δ-cells.[7] More recently glucagon has also been detected.[6] Because of the heterogeneity of the cellular composition and because of the quantitatively variable endocrine response of the present tumor it is mandatory that tumor tissue be thoroughly characterized if it is to be employed for biochemical studies.

The present studies and similar previous studies were motivated by the desire to discover a plentiful source of β-cells for biochemical studies of the insulin release process as an alternative to working with the minute quantities of normal isolated islet tissue. The present demonstration that the in-

sulinoma responds with insulin release to the physiologic stimulus glucose and to the model substrates KIC and GA and the complementary report from our group that the proposed glucose sensor glucokinase is present in tumors from this line[18] raise our hopes that it might resemble β-cells closely enough to serve as a suitable model of β-cells.

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