Intra-arterial Calcium Stimulation Test for Detection of Insulinomas: Detection Rate, Responses of Pancreatic Peptides, and Its Relationship to Differentiation of Tumor Cells

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The selective intra-arterial calcium stimulation test has greatly facilitated the precise regionalization of insulinomas smaller than 2 cm, which noninvasive techniques (ultrasound [US], computed tomography [CT], magnetic resonance imaging [MRI]) often fail to localize. This study examined not only the role of the test in the localization of insulinomas, but also the responsiveness of 3 β-cell peptides (insulin, C peptide, and proinsulin) and their relationship to the degree of differentiation of the tumor cells, using percentage decrease of both proinsulin/insulin (P/I) and proinsulin/C peptide (P/C) ratios after stimulation as indices. Ten consecutive surgically proven insulinoma patients each received an injection of calcium into the arteries supplying the pancreas after standard selective angiography and β-cell peptide levels were measured in samples taken from the right hepatic vein before and 30, 60, 90, 120, and 180 seconds after each injection prior to operation. After surgery, the expressions of the calcium sensing receptor (CaSR) on the resected tumors were assessed by immunohistochemistry. Intra-arterial calcium stimulation with sampling either for insulin or for C peptide correctly predicted the site of insulinoma in 8 of 9 patients or in 7 of 8 patients if the 2 big malignant insulinomas were excluded; thus, the detection rate of this test was 89% and 88%, respectively. Calcium administration stimulated a marked and prompt release of insulin and C peptide simultaneously. Both peaked within 30 to 60 seconds, then declined gradually thereafter, remaining above the baseline at 180 seconds. The magnitude of increase correlated well with the corresponding percentage decrease of P/I and P/C ratios. The response of proinsulin was much less. Immunohistochemistry demonstrated variable membraneous staining for CaSR in normal pancreatic islets and in about 9% of the total normal β cells, whereas staining in tumor cells was only minimally detectable. We conclude that selective intra-arterial calcium stimulation with hepatic venous sampling either for insulin or for C peptide is a highly sensitive method for the preoperative localization of small insulinomas. Calcium injection stimulates a brisk response of insulin, C peptide, and proinsulin simultaneously and the magnitude of increase of both insulin and C peptide appears to be correlated well with the degree of differentiation of the tumor cells. The exact mechanism by which calcium provokes the release of β-cell peptides is less clear and whether the CaSR is involved in the mechanism of its action requires further study.

THE SUCCESSFUL resection of insulin-producing tumors (insulinomas) of the pancreas is greatly facilitated by precise preoperative localization.[1] Although insulinomas can usually be diagnosed biochemically by measuring insulin and C peptide at the termination of a prolonged fast,[2] the localization of biochemically proven insulinomas smaller than 2 cm remains a problem, despite recent developments of more sophisticated cross-sectional imaging techniques, such as computed tomography (CT), magnetic resonance imaging (MRI), and ultrasonography (US).[3] In several highly specialized referrals centers, the sensitivities of these noninvasive methods were 17% to 33% for CT, 38% to 50% for MRI, and 9% to 30% for US.[3-6] On the other hand, of the invasive localization techniques, pancreatic arteriography and percutaneous transhepatic portal venous sampling were reported to have sensitivities of 36% to 56% and 55% to 100%, respectively.[3-7] However, new modalities of imaging techniques, such as dual phase spiral CT,[8] fast spin-echo, fat saturation, and dynamic contrast-enhanced MRI[9,10] have much improved its sensitivity for the detection of small insulinomas.

Imamura et al used secretin for selective intra-arterial stimulation and hepatic venous sampling to localize gastrinomas in 1987.[11] Doppman et al in 1991 reported 4 cases of successful localization of insulinomas using calcium as the secretagogue.[12] In 1995, investigators from the same institution reported successful localization of an additional 21 cases by this method and had a sensitivity of up to 88%.[3] Kato et al proposed that the mechanism by which calcium stimulates insulin release should be mediated by the calcium sensing receptor (CaSR).[13] Until now, however, there are no data available concerning the responses of C peptide and proinsulin nor any possible correlation with the differentiation or expression of CaSR of tumor cells. For this purpose, we have preoperatively determined both the responses of plasma insulin, C peptide, and proinsulin and the percentage changes of both poinsulin/insulin (P/I) and proinsulin/C peptide (P/C) ratios to calcium stimulation in 10 patients with functional insulinomas. After the operation, we investigated the expression of CaSR by immunohistochemistry on the resected tumors. Our results showed that the magnitude of the responses of β-cell polypeptides to calcium stimulation correlates well with the degree of differentiation, but not with the expression of CaSR, of tumor cells.

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Table 1. Clinical Features

		Patient No.									
		1	2	3	4	5	6	7	8	9	10
Symptoms of neuroglycopenia		+	+	+	+	+	+	+	+	+	+
Plasma glucose during spontaneous symptomatic episode (mmol/L)		ND	1.6, 1.4	1.4	ND	ND	ND	2.6	ND	ND	ND
Evidence for endogenous hyperinsulinemia during fasting or 48-h fast	Diagnostic criteria										
Plasma glucose (mmol/L)	≤2.8	2.4	2.6	2.3	1.5	2.65	2.3	2.6	2.7	2.3	1.7
Plasma insulin (pmol/L)	≥36	77	217	78	154	279	847	289	486	133	705
Plasma C peptide (pmol/L)	≥200	332	1496	616	374	877	1499	829	ND	652	1770
CT/MRI of pancreas		+	−	+	+	+	−	−	−	+	+
Celiac axis angiography		+	−	+	+	−	+	−	−	+	+
Surgery											
Site		Tail	Neck	Uncinate	Head	Body	Uncinate	Head, Tail	Tail	Head	Body, Tail
Size (cm)		1.5	2.0	1.0	1.0	1.5	3.0	0.3, 2.0	2.5	5.0	15.0

Abbreviations: +, presence; −, absence; ND, no data.

MATERIALS AND METHODS

Patients

From June 1997 to August 2002, 10 consecutive patients, 4 men and 6 women, were studied. All patients had symptomatic hypoglycemia (plasma glucose < 2.8 mmol/L) during fasts or a prolonged fast with inappropriately elevated insulin (>36 pmol/L) and C-peptide (>200 pmol/L) levels.[14]

Patient 1. A 55-year-old woman had recurrent episodes of diaphoresis and confusion for 6 months. Hyperinsulinemic hypoglycemia was confirmed by the finding of a plasma glucose level of 2.4 mmol/L and simultaneous plasma insulin and C peptide levels of 77 pmol/L and 332 pmol/lL, respectively, during a prolonged fast. CT scan of the abdomen disclosed a 1.5-cm tumor in the tail of the pancreas (Table 1). A selective intra-arterial calcium injection demonstrated an 18-fold increase in insulin levels in the splenic artery (SA) (Fig 1) and angiography indicated a tumor blush in the tail of the pancreas. At surgery, an insulinoma of 1.5 cm in diameter was removed from the tail of the pancreas.

Patient 2. A 38-year-old woman had recurrent neuroglycopenic attacks with confusion and weight gain of 15 kg over 4 years. During a spontaneously occurring spell, plasma sugar was 1.6 mmol/L, but plasma insulin and C peptide levels were not determined. Hyperinsulinemic hypoglycemia was confirmed by the finding of a plasma glucose level of 2.6 mmol/L and simultaneous plasma insulin and C peptide levels of 217 pmol/L and 1496 pmol/L, respectively, during fasting. MRI and angiography of the pancreas were negative (Table 1). A selective intra-arterial calcium injection revealed a 10.2-fold increase of insulin levels in the gastroduodenal artery (GDA) (Fig 1), suggesting the presence of an insulinoma in this area. At subsequent surgery, a solitary insulinoma of 2 cm in diameter was removed from the neck of the pancreas.

Patient 3. A 65-year-old man had an episode of loss of consciousness and had a plasma sugar level of 1.4 mmol/L. Hyperinsulinemic hypoglycemia was confirmed by the finding of plasma sugar 2.3 mmol/L, plasma insulin 78 pmol/L, and plasma C peptide 616 pmol/L during a prolonged fast. A CT scan of the pancreas disclosed a 1-cm tumor in the head of the pancreas (Table 1). A selective intra-arterial calcium injection demonstrated a 11.5-fold increase of insulin levels in the GDA (Fig 1) and angiogram indicated a hypervascular tumor in the head of the pancreas. At surgery, a solitary insulinoma of 1 cm in diameter in the uncinate process adjacent to superior mesenteric artery (SMA) and superior mesenteric vein (SMV) was removed.

Patient 4. A 69-year-old man had recurrent neuroglycopenic attacks with loss of consciousness for 2 years. Hyperinsulinemic hypoglycemia was proven by the finding of plasma sugar 1.5 mmol/L, plasma insulin 154 pmol/L, and plasma C peptide 374 pmol/L during a prolonged fast. A CT scan of the pancreas disclosed a 0.8-cm hypervascular tumor in the head of the pancreas (Table 1). A selective intra-arterial calcium injection revealed 10.8- and 2.9-fold increases of insulin levels in the GDA and SA, respectively (Fig 1) and angiogram indicated a hypervascular tumor in the head of the pancreas. At surgery, a solitary insulinoma of 1 cm in diameter was removed from the head of the pancreas.

Patient 5. A 37-year-old woman had recurrent episodes of consciousness disturbance, cold sweating, and a weight gain of 7 kg over 2 years. Hyperinsulinemic hypoglycemia was documented by the finding of an inappropriately elevated plasma insulin level (279 pmol/L) and plasma C peptide level (877 pmol/L), with a plasma sugar of 2.65 mmol/L during fasting. A MRI of the abdomen disclosed a 1-cm tumor in the body of the pancreas, but angiogram was negative (Table 1). A selective intra-arterial calcium injection demonstrated a 2.7-fold increase of insulin levels in the SA (Fig 1), suggesting the presence of an insulinoma in this area. At surgery, a solitary insulinoma of 1.5 cm in diameter was removed from the body of the pancreas.

Patient 6. A 63-year-old woman had recurrent neuroglycopenic attacks of bizarre behavior, cold sweating, and a weight gain of 35 kg over 5 years. Hyperinsulinemic hypoglycemia was documented by the finding of an inappropriately elevated plasma insulin level (847 pmol/L), plasma C peptide (1,499 pmol/L), and plasma sugar of 2.3 mmol/L during fasting. An angiogram of the pancreas failed to show a tumor blush. An exploratory laparotomy was performed 4 years before, based on the finding of a 1.1 × 1.0 × 1.4-cm tumor in the head of pancreas by dynamic CT scan; unfortunately the laparotomy failed. Repeated MRI of the pancreas was negative, but angiogram disclosed a hypervascular tumor in the uncinate process of the pancreas (Table 1). The tumor receives blood supply from both the GDA and the SMA. A selective intra-arterial calcium injection revealed 7.6- and 5.2-fold increases of insulin levels in the GDA and the SMA, respectively (Fig 2), suggesting the presence of an insulinoma in the right area of the SMA. At surgery, a solitary insulinoma of 3 cm in diameter was removed from the uncinate process of the pancreas.

Patient 7. A 41-year-old woman had recurrent neuroglycopenic episodes with bizarre behavior, seizure attacks, and diaphoresis over 2 years. The diagnosis of an insulinoma was made after the finding of

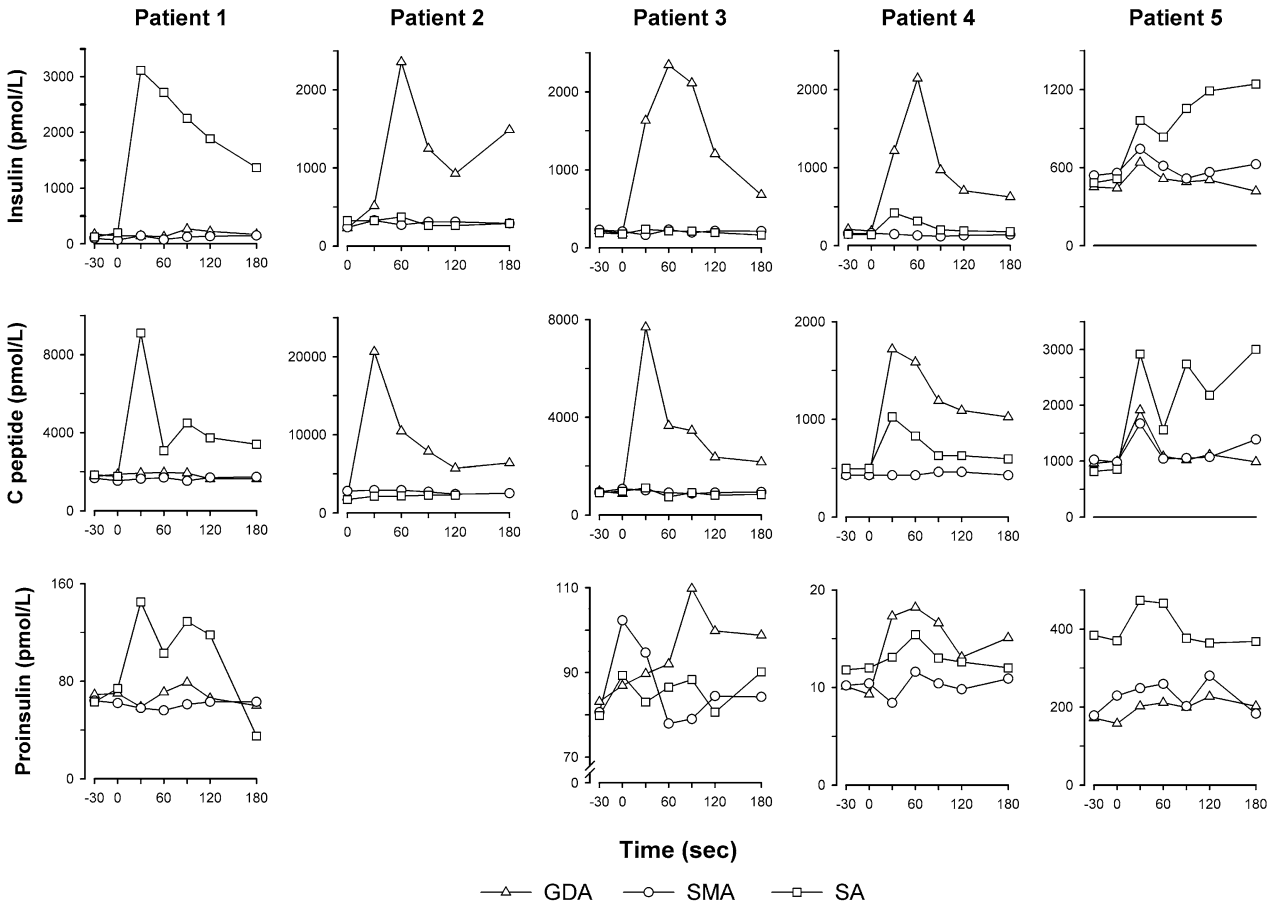

Fig 1. Responses of serum insulin, C peptide, and proinsulin to selective intra-arterial calcium stimulation in patients no. 1 to 5.

inappropriately elevated serum insulin and C peptide levels (plasma glucose of 2.6 mmol/L and simultaneous plasma insulin and C peptide of 289 pmol/L and 829 pmol/L, respectively) during a hypoglycemic episode. Laboratory evaluations disclosed serum calcium 2.65 mmol/L, intact parathyroid hormone (PTH) 74 pg/mL, and prolactin 35 ng/mL. A sonogram of the parathyroid revealed a hypoechoic nodule over the right inferior aspect of the thyroid, suggesting a parathyroid adenoma. MRI of the sella disclosed a 0.5-cm microadenoma over the right pituitary. MRI and CT scan of the pancreas were negative (Table 1). A selective intra-arterial calcium injection demonstrated a 7.5-fold increase of insulin levels into the SMA and a 6.5-fold increase into the SA (Fig 2). Under the impression of multiple endocrine neoplasia, type 1 (MEN 1), an exploratory laparotomy was performed and at surgery a 2-cm solid tumor in the tail and another two 0.3-cm small tumors in the head and neck, respectively, of the pancreas were found. A subtotal pancreatectomy, enucleation of the pancreatic head and neck tumors, and a right inferior parathyroidectomy were subsequently performed. Insulinomas and parathyroid adenoma were confirmed by pathology.

Patient 8. A 24-year-old woman had a family history of MEN 1 and had recurrent neuroglycopenic attacks with poor concentration, fatigability, and hunger sensation. She was found to have an inappropriately elevated insulin level (486 pmol/L) with a plasma sugar level of 2.7 mmol/L during fasting, suggestive of hyperinsulinemic hypoglycemia. Laboratory evaluations disclosed serum calcium 2.68 mmol/L, and intact-PTH 23.8 pg/mL. A sonogram of the parathyroid revealed a 0.9 cm hypoechoic nodule behind the left thyroid gland, suggesting a parathyroid adenoma. CT scan and angiogram of the pancreas were negative (Table 1). A selective intra-arterial calcium injection revealed a 12-fold increase of insulin levels in the SA (Fig 2), suggesting the presence of an insulinoma in this area. Under the impression of MEN 1, she received a right superior and a left superior parathyroidectomy, and parathyroid hyperplasia was confirmed by pathology. Distal pancreatectomy was subsequently performed and at surgery a solitary insulinoma of 2.5 cm in diameter between the body and the tail of the pancreas was found.

Patient 9. A 69-year-old man had a history of a proven pancreatic head tumor of 4 cm in diameter by CT scan for 6 years and began to experience episodes of diaphoresis, palpitation, and queasy feelings for the last 2 months before admission to the hospital. Hyperinsulinemic hypoglycemia was confirmed by the finding of a plasma sugar level of 2.3 mmol/L, plasma insulin of 133 pmol/L, and plasma C peptide of 652 pmol/L during a prolonged fast. MRI of the pancreas revealed a 6.0 $\times$ 5.5 $\times$ 4.2-cm tumor in the uncinate process of the pancreas (Table 1). A selective intra-arterial calcium injection demonstrated a 3.5-fold increase of insulin levels into the GDA and angiogram indicated a 5- to 6-cm hypervascular tumor in the uncinate process of pancreas (Fig 2). At surgery, a tumor of 5 cm in diameter in the head of the pancreas was removed by a modified Whipple's operation. Pathological examinations of the resected tissues confirmed the diagnosis of malignant insulinoma with capsular invasion and local lymph node metastasis.

Patient 10. A 56-year-old man had recurrent episodes of diaphoresis, confusion, and hand tremors for 10 years and was proven to have hyperinsulinemic hypoglycemia (a plasma sugar level of 1.7 mmol/L,

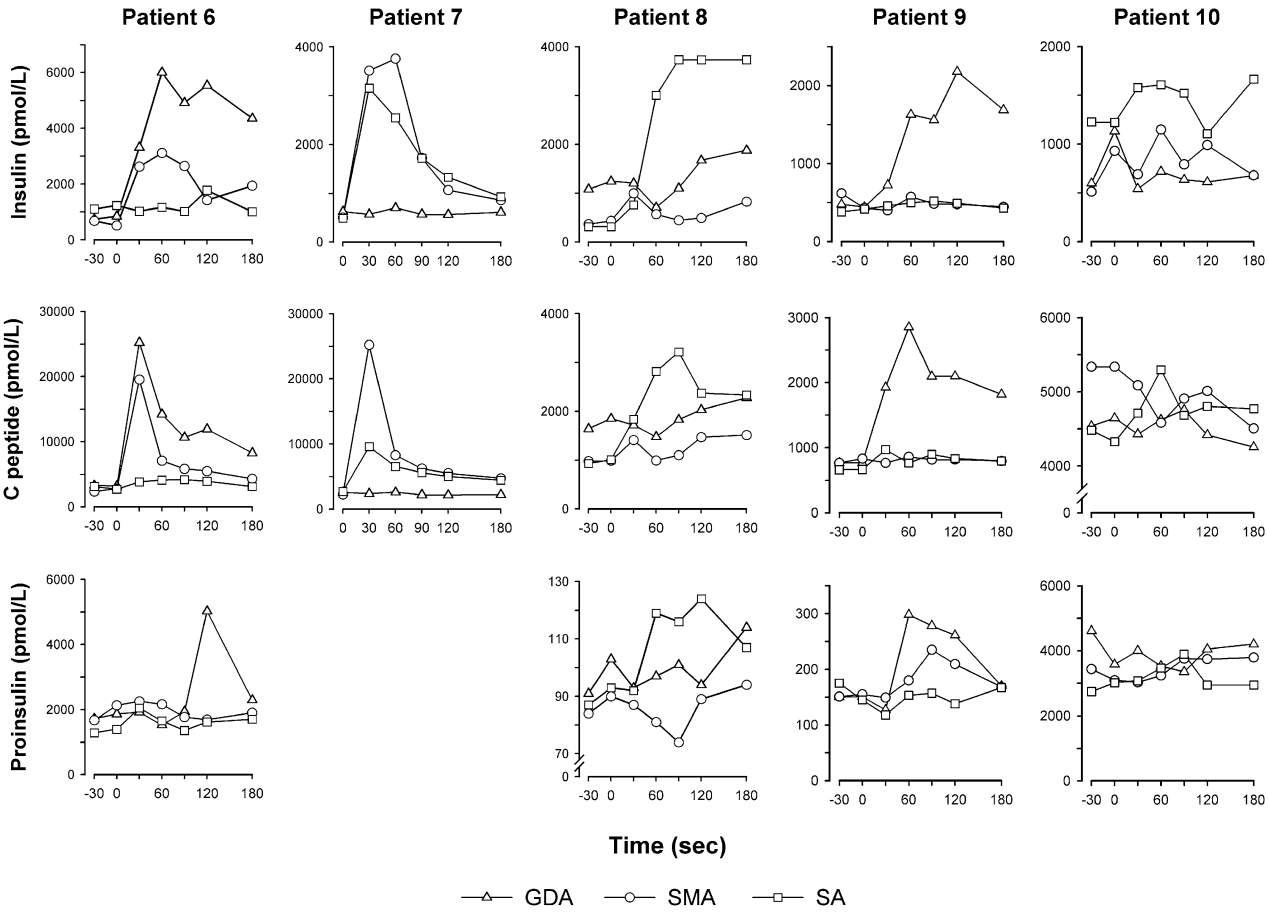

Fig 2. Responses of serum insulin, C peptide, and proinsulin to selective intra-arterial calcium stimulation in patients no. 6 to 10.

plasma insulin 705 pmol/L, and plasma C peptide 1,770 pmol/L during a prolonged fast) for 6 years. Both CT scan and angiography of the abdomen disclosed a huge mass, 10 × 13 × 7 cm in size, in the body and tail of the pancreas (Table 1). A selective intra-arterial calcium injection revealed a 1.3-fold increase of insulin levels in the SA (Fig 2). At surgery, a 15 × 10 × 5-cm tumor in the tail of the pancreas was removed by distal pancreatectomy and splenectomy. Pathological examinations confirmed the diagnosis of malignant insulinoma with soft tissue and spleen invasion

Procedures

Forty-eight–hour fast. The procedures were performed by a modification of the protocol of Service et al[14] and plasma glucose, insulin, and C peptide levels were measured every 4 hours instead of every 6 hours. The diagnostic criteria for hyperinsulinemia is a plasma insulin level greater than 36 pmol/L at the time of hypoglycemia (plasma glucose < 2.8 mmol/L).[14] A recent study suggests that a 48-hour fast is sufficient for the diagnosis of insulinoma, with a sensitivity of 95%.[15]

Selective intra-arterial calcium stimulation test. The procedures were performed as originally described[12] and later modified.[3] Briefly, a catheter was placed in the right hepatic vein via a femoral vein puncture. After catheterization of the femoral artery, standard pancreatic arteriography was performed by selectively injecting non-ionic contrast medium into the GDA, SA, and SMA. Following each angiogram, calcium gluconate 10%, diluted with saline to a volume of 5 mL, was injected as a bolus into the artery at a dose of 0.025 mmol calcium/kg body weight. Blood samples were taken from the right hepatic vein before and 30, 60, 90, 120, and 180 seconds after calcium injection. Specimens were placed on ice, centrifuged, and stored at −20°C until insulin, C-peptide, and proinsulin levels were measured by radioimmunoassay (RIA) and samples from the same patient were assayed simultaneously to avoid interassay variation. A 2-fold or more increase in insulin levels 30 to 120 seconds after the injection of calcium was considered indicative of a tumor in the vascular territory of the artery studied.[3,16] In general, the GDA supplies the superior pancreatic head and neck, the SMA supplies the inferior pancreatic head and uncinate process, and the SA supplies the pancreatic body and tail.[12] When a positive response was found both in the GDA and SMA, the insulinoma was presumed to be located to the right of the SMA.[3] All patients gave informed consent for these studies, which were approved by the institutional review board of the hospital.

Analytical Methods

RIAs. Plasma insulin was determined by a home-made RIA using anti-insulin serum B1. The B1 antiserum was raised in a guinea pig against porcine insulin (crystalline insulin Lot 615-07J-256, a gift of Eli Lilly, Indianapolis, IN). The antiserum reacts fully with human insulin and porcine insulin and cross-reacts 17.5% with proinsulin, 0.14% with glucagon, and 0.025% with C peptide, but does not react with guinea pig insulin, secretin, or somatostatin. The sensitivity of the assay was 18 pmol/L and the intra- and interassay coefficients of variation were 7% and 9%, respectively.[17] Plasma C peptide was determined by RIA

(KPED2, Diagnostic Products Corp, Los Angeles, CA). The sensitivity of the assay was 61 pmol/L and cross-reacts 20% with proinsulin, but does not react with insulin, secretin, or glucagon. Plasma proinsulin was determined using a highly specific RIA (HPI-15K, Linco Research, St Charles, MO) with a sensitivity limit of 5 pmol/L. This assay showed no cross-reactivity (<0.1%) with insulin, C peptide, or 64-65-split proinsulin, but showed 95% cross-reactivity with 31-32-split proinsulin.

Correction for proinsulin cross-reactivity. Since insulinomas are known to hypersecrete proinsulin[18,19] and proinsulin cross-reacts both in the insulin and the C peptide assays, the concentration of insulin and C peptide in each sample might be influenced by the proinsulin component. Therefore, the total insulin and C peptide measured by the insulin assay and C peptide assay, respectively, were corrected for the proinsulin component in each sample as follows: first, proinsulin was determined by a specific assay. The proinsulin component of each total insulin and C peptide measurement was estimated using the concentration of proinsulin measured in each sample and the known cross-reactivity of proinsulin in the insulin assay and the C peptide assay, respectively. The proinsulin component thus calculated was then subtracted to give the estimated true insulin and C peptide value for each measurement, respectively. This method of correction has been described previously.[20,21]

Immunohistochemical Studies

Tumor specimens from patients were serially sectioned into 3-mm slices, fixed in buffered formalin, and embedded in paraffin. Immunohistochemical staining was performed using the labeled streptavidin-biotin method (LSAB 2 kit, DAKO, Carpinteria, CA). Briefly, 6-μmol/L thick paraffin sections were mounted on charged glass slides. After deparaffinization and rehydration, the slides were placed in citrate buffer (pH 6.0) and treated with three 5-minute cycles in a microwave oven at 650 W to ensure optimal retrieval of the antigen. Then, they were successively incubated for 15 minutes with normal goat serum (1:10 dilution, DAKO) and for 25 minutes with a polyclonal anti-CaSR antibody (1:100 dilution, PA1-934, Affinity BioReagents, Golden, CO). After washing, they were incubated for 25 minutes with anti-rabbit IgG-biotin conjugate and then with streptavidin-peroxidase conjugate. The peroxidase activity was demonstrated by incubation for 25 minutes in the dark with 3-amino-9-ethyl carbazole (AEC, DAKO).

We also analyzed the pancreatic tissue from six age-matched patients using double immunohistochemical staining as a control.[22] The subjects had all undergone surgery for benign pancreatic cystadenomas and had no evidence of endocrine disease. Briefly, immunostaining was performed first for CaSR using the streptavidin-biotin-peroxidase complex system as described above. After demonstration of the peroxidase activity with AEC, the slides were successively incubated for 25 minutes with 0.1N HCl and then for 15 minutes with normal goat serum (DAKO), followed by a polyclonal anti-insulin antibody (DAKO) for 25 minutes. After rinsing, they were incubated for 25 minutes with anti-rabbit IgG-biotin conjugate (Vector Laboratories, Burlingame, CA) and then with streptavidin-alkaline phosphatase conjugate (Vector). The activity of alkaline phosphatase was revealed by incubation for 25 minutes in the dark with vector blue (Vector). The percentages of immunoreactive cells for CaSR were calculated by counting 500 insulin staining-positive β cells at high magnification (400X) in each case by 2 independent observers. The results are expressed as the mean $\pm$ SD.

Statistical Analyses

To determine the possible relationship between the responses of pancreatic peptides to calcium stimulation and the degree of differentiation of tumor cells, we correlated the increase of insulin and C peptide after calcium in each patient with the corresponding percentage decrease (from basal level to peak response) in P/I ratio and P/C ratio. The rationale to use P/I and P/C ratios as indices of degree of tumor cell differentiation lies in the fact that insulin, C peptide, and the single-chain precursor of both peptides, proinsulin, are cosecreted[18] and that the percentages of proinsulin secreted tend to be higher in undifferentiated malignant insulinomas (a higher P/I or P/C ratio) than in differentiated benign ones (a lower P/I or P/C ratio).[19,23] Therefore, the greater the percentage decrease of P/I or P/C ratio after calcium, the more insulin or C peptide released and, thus, the more likely the tumor cells are to be differentiated. This concept was supported by the findings that after calcium injection, we found an 89% and 58% decrease of the P/I and P/C ratios in patient no. 1, an 89% and 86% decrease of P/I and P/C ratios in patient no. 6, and an 89% and 85% decrease of P/I and P/C ratios in patient no. 3. In all of them abundant typical dense-core endosecretory granules (insulin granules) were revealed by electron microscopy (Fig 3A-C). In contrast, in patient no. 10 we found a 7% and −12% decrease of P/I and P/C ratios after the calcium injection, and there were no typical dense-core endosecretory granules (Fig 3D). For calculation, we used the percentage decrease of both ratios, instead of their absolute values, to avoid interpatient variations since all of the samples were not assayed simultaneously.

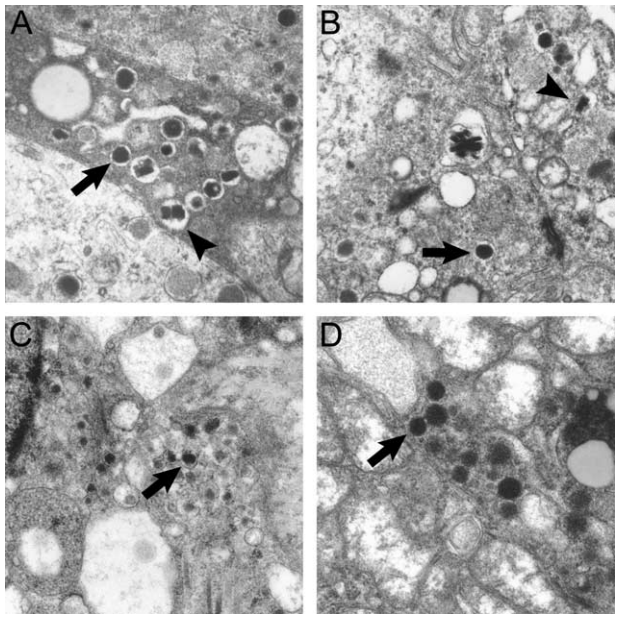

Fig 3. Ultrastructural appearance of insulinoma cells. (A) Typical well-granulated β cell with both crystalline-cored (arrowhead) and dense-cored (arrow) secretory granules (patient no. 1), 28,000X. (B) Typical well-granulated β cell with both crystalline-cored (arrowhead) and dense-cored (arrow) secretory granules (patient no. 6), 22,000X. (C) Typical well-granulated β cell with dense-cored (arrow) secretory granules (patient no. 3), 26,000X. (D) Unclassified β cell with atypical granules. The granules have a homogenous, relatively dense core and with a narrow space between the limiting membrane and core (arrow) (patient no. 10), 30,000X.

RESULTS

Detection Rate of Localization Methods

As shown in Table 1 and Figs 1 and 2, prior to operation, the CT/MRI and arteriography correctly localized the site of insulinoma in 5 of the 10 patients, thus, having a detection rate of

Table 2. Percentage Changes of Both P/I and P/C Ratios During Calcium Injection in 8 Patients With Insulinoma

Patient No.	P/I Ratio			P/C Ratio		
	Basal	Peak	ΔChange (%)	Basal	Peak	ΔChange (%)
1	0.428	0.046	↓89	0.038	0.016	↓58
3	0.416	0.047	↓89	0.092	0.014	↓85
4	0.050	0.009	↓83	0.023	0.010	↓57
5	0.876	0.312	↓64	0.450	0.162	↓64
6	2.260	0.253	↓89	0.550	0.076	↓86
8	0.290	0.031	↓89	0.093	0.036	↓61
9	0.327	0.137	↓58	0.196	0.102	↓48
10	4.010	3.740	↓7	0.654	0.733	↑12

50%. In patient no. 3 with an insulinoma in the uncinate process, the results of an insulinoma in the head of the pancreas by CT scan and angiogram were considered to be a false localization. A positive response of insulin to intra-arterial calcium stimulation occurred in 9 of the 10 patients. Intra-arterial calcium stimulation with venous sampling for insulin precisely regionalized the site of insulinoma in 8 of these 9 patients, thus, having a detection rate of 89%. In the patient with a false localization (patient no. 3), a positive response to the GDA injection occurred in the presence of an insulinoma in the uncinate process. If the 2 patients with big malignant insulinomas (patients no. 9 and 10) were excluded, the detection rates of CT/MRI, arteriography, and intra-arterial calcium stimulation were 38%, 38%, and 88%, respectively, and the mean size of the insulinomas was 1.8 cm, ranging from 1 to 3 cm.

If the criteria used for insulin, defined as a 2-fold increase or more above basal after calcium, were applied for C peptide, the results were identical to that of insulin; a positive response occurred in 9 of the 10 patients, and intra-arterial calcium stimulation with venous sampling for C peptide accurately predicted the site of insulinoma in 8 of 9 patients, thus having a detection rate of 89% and 88% (excluding the 2 patients with big malignant insulinomas). Patient no. 3, who had a false localization and an insulinoma in the uncinate process, had a response to the GDA injection. These results indicate that intra-arterial calcium stimulation with venous sampling either for insulin or for C peptide is a much more powerful localization method than CT/MRI imaging or arteriography for insulinomas smaller than 2 cm.

Detailed Kinetic Profile of Insulin, C Peptide, and Proinsulin Secretion in Response to Intra-arterial Calcium Stimulation

As previously reported, calcium, at the dosage used, stimulates an immediate insulin release from insulinoma cells but not from normal β cells, and its effect appears to be short-lived[3,12,16]; insulin concentration increases immediately after calcium injection, peaks within 30 to 60 seconds and declines gradually, remaining above the baseline at 180 seconds. Three patients did not have a peak within 60 seconds; patient no. 5 peaked at 120 to 180 seconds, patient no. 8 at 90 seconds, and patient no. 9 at 120 seconds.

The kinetic profile of the secretory response of C peptide, which was cosecreted with insulin at an equimolar basis,[23] to calcium stimulation was in general similar to that of insulin; C peptide level increased immediately after calcium injection, reached a peak within 30 to 60 seconds, and declined gradually, remaining above the basal level at 180 seconds. Patient no. 8 had a peak delayed to 90 seconds. The secretory pattern of proinsulin in response to calcium stimulation was roughly similar to that of insulin and C peptide, but the response was much less prominent; only 3 patients (patients no. 1, 6, and 9) had a 2-fold increase after the calcium injection.

The multiples of increase of insulin, C peptide, and proinsulin to calcium were 19.5, 5.3, and 2.1 for patient no. 1 in the SA; 11.5, 8.3, and 1.3 for patient no. 3 in the GDA; 10.8, 4.0, and 1.9 for patient no. 4 in the GDA; 2.7, 3.5, and 1.3 for patient no. 5 in the SA; 7.6, 7.8, and 2.8 for patient no. 6 in the GDA; 12.0, 3.3, and 1.4 for patient no. 8 in the SA; 4.8, 3.7, and 2.0 for patient no. 9 in the GDA; and 1.5, 1.2, and 1.4 for patient no. 10 in the SA. In the 2 patients whose proinsulin levels were not determined because the specimens were unavailable, the multiples of increase of insulin and C peptide were 10.0 and 10.0 for patient no. 2 in the GDA; and 6.5 and 3.6 in the SA and 7.5 and 11.2 in the SMA for patient no. 7 who had multiple insulinomas (Figs 1 and 2). The baseline, peak, and its percentage change of both P/I and P/C ratios during calcium stimulation in each patient are listed in Table 2. Administration of calcium usually results in a significant decrease in both the P/I and P/C ratios, suggesting that much greater amounts of insulin or C peptide compared with proinsulin are released by calcium.

Relationship Between the Responsiveness of β-Cell Peptides to Calcium Stimulation and the Degree of Differentiation of Tumor Cells

Previous studies suggest that the suppressibility of insulin secretion to the inhibitory action of somatostatin and diazoxide is related to the morphological characterisitics of insulinomas: the presence or absence of typical β granules, and diffuse or patchy insulin immunoreactivity, ie, the stages of differentiation of tumor cells.[24,25] We wondered whether there also exists a relationship between the magnitude of response of β-cell peptides to calcium stimulation and the degree of differentiation of tumor cells. Therefore, we correlated the fold increase of both insulin and C peptide after calcium with the corresponding percentage decrease of both P/C and P/I ratio in each patient. As shown in Fig 4A, the insulin and C peptide responses to calcium correlated well with the percentage decrease of P/I and P/C ratios ($r = 0.719$, $P = .002$). Actually, the

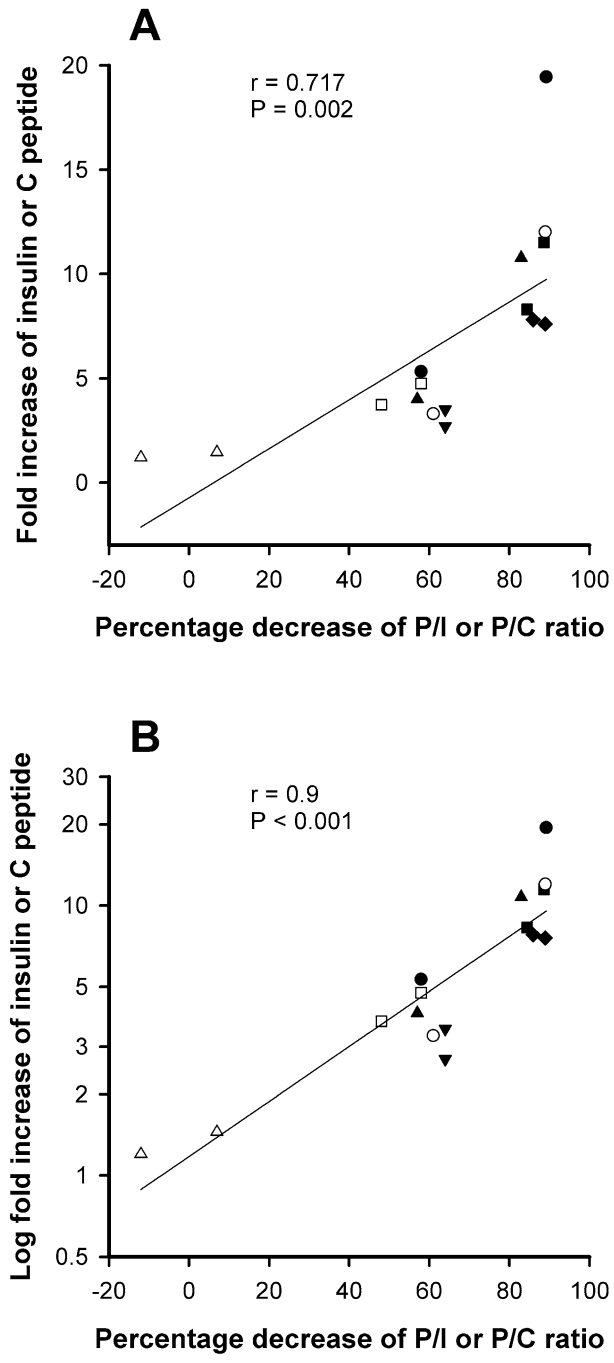

Fig 4. Correlation of percentage decrease of P/I or P/C ratio and fold increase (A) or log fold increase (B) of insulin or C peptide in response to calcium stimulation in 8 patients with functioning insulinomas. Each individual patient is represented by a different symbol.

percentage decrease in P/I and P/C ratios correlated more with the log fold increase of insulin and C peptide ($r = 0.9$, $P < .001$) (Fig 4B). Thus, it appears that the degree of differentiation of tumor cells is also an important factor in determining the responsiveness of β-cell peptides to calcium ions.

Immunohistochemical Staining for CaSR in Normal Pancreatic Islets and in Tumor Cells

The double immunostaining by insulin and CaSR antibodies permitted easy recognition of β cells, immunostained in blue, and the clear-cut distinction of CaSR-immunoreactive cells by their red color. In normal pancreatic islets, the majority of cells were immunoreactive for insulin, ie, insulin-secreting β cells. On the other hand, staining for CaSR showed positive staining in only a minority of cells, a fraction of which were also immunoreactive for insulin, and there was a complete absence of staining in the exocrine pancreas. The staining pattern of the CaSR was primarily membraneous, corresponding to the binding of the antibody to the receptor in the plasma membrane, although in cells, positive staining in the cytoplasm was also observed. The staining intensity varied, indicating variations in the quantities of the antigen in these cells. An approximately 9% fraction (46 ± 8 per 500 cells) of the total β cells were immunoreactive for CaSR (Fig 5A and B). In contrast, in tumor tissues, immunostaining for CaSR showed positive staining in only a few cells or even a complete absence of staining. The staining intensity varied; some cells exhibited strong red staining, whereas others were only weakly labeled. The poor staining intensity for CaSR was not due to technical problems, since

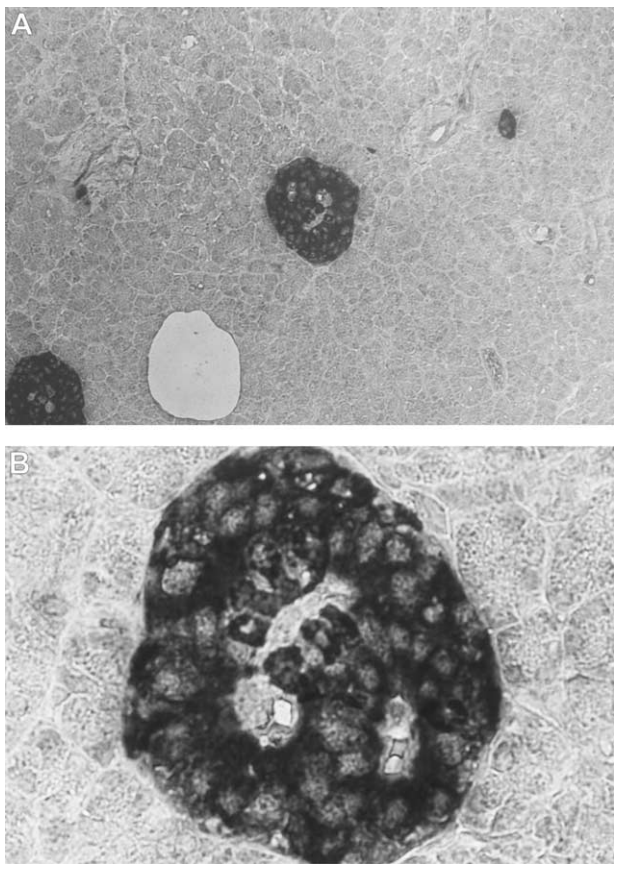

Fig 5. Double-labeling of the normal pancreatic islets showing cells with insulin and/or CaSR immunoreactivity. (Double label, insulin [Vector blue, blue], CaSR [AEC, red]; A, 100X; B, 400X).

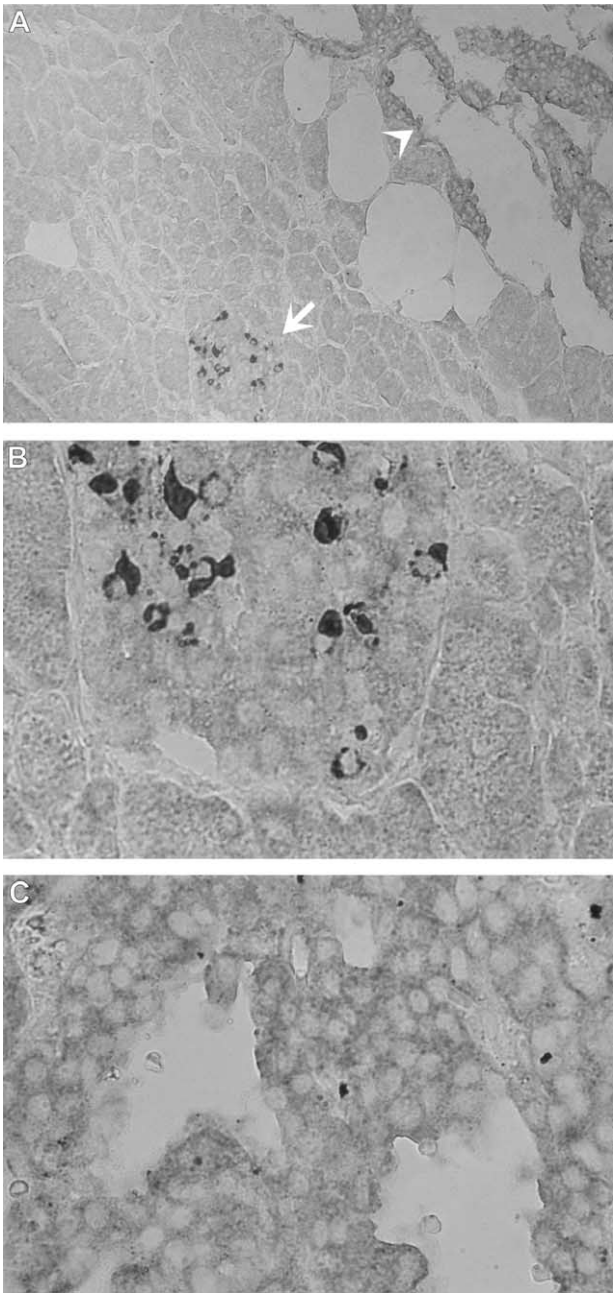

Fig 6. (A) Immunohistochemistry of the CaSR in normal islet (arrow) and insulinoma (arrowhead) (patient no. 6), 100X. (B) Higher power view of the same CaSR-stained islet (AEC), demonstrating the membraneous staining pattern, 400X. (C) Hgher power view of the insulinoma in the same specimen, demonstrating absence of CaSR staining, 400X.

a positive staining was usually found in the normal islets of the same specimen (Fig 6A-C).

DISCUSSION

In this series of 10 patients with surgically proven insulinomas, intra-arterial calcium stimulation with hepatic venous sampling for insulin appears to be the most sensitive preoperative localization method (8 of 9 [89%]) among the 3 most commonly and widely used localization techniques—CT/MRI, arteriography, and intra-arterial calcium stimulation test. This is also true if the 2 patients with large malignant insulinomas were excluded (7 of 8 [88%]). The detection rate of our results is generally similar to that reported by others,[3,4,6,16,26] ranging from 88% to 100%, and thus confirms the notion that the intra-arterial calcium stimulation test is a much more sensitive and accurate method in regionalizing insulinomas smaller than 2 cm.[3,4,6,16,26,27] Furthermore, because sampling for C peptide virtually has an identical detection rate as the insulin sampling and the fact that the combination of insulin and C peptide sampling does not improve the overall detection rate, we recommend that additional sampling for C peptide is not necessary for the localization of insulinomas. Moreover, it is interesting to find that in our series there were 2 patients (patients no. 3 and 6) who have an insulinoma in the uncinate process, an incidence (20%) apparently higher than previously reported (7%).[5] It is also worth noting that both of them had a positive response in the GDA, instead of the SMA, a situation rendering accurate regionalization of the tumors difficult or even impossible. We realize that an insulinoma in the uncinate process may receive blood supply from either the GDA or the SMA, as did patient no. 3, or sometimes from both of them, as did patient no. 6.

In this study, we have determined simultaneously the responses of insulin, C peptide, and proinsulin, attempting to characterize more fully the kinetic features of peptide secretion from insulinoma cells. Due to the difficulties of simultaneously measuring intact proinsulin, proinsulin intermediates, and insulin, earlier studies concentrated mainly on insulin responses alone.[3,6,16,26,27] However, since the development of highly specific proinsulin assays with virtually no human insulin cross-reactivity, as was used in this study, accurate measurement of both molecules is now possible.[28,29] In agreement with previous reports, when calcium is injected into the artery perfusing the islet cell tumor, it stimulates a marked, prompt release of insulin.[3,6,16,26,27] Insulin concentration usually peaks within 30 to 60 seconds, declines thereafter, but remains a little higher above the baseline at 180 seconds. This kinetic profile of insulin response suggests that calcium ion is a quite potent but short-lived secretagogue and obviously an agent of choice for this type of study if trying to avoid the unwanted complication of potential severe, or even life-threatening, hypoglycemia during the procedure. In our series, no symptomatic hypoglycemia occurred following the bolus injection of calcium at the dosage used.

The kinetic feature of the secretory responses of both C peptide and proinsulin to calcium is, in general, similar to that of insulin. Nevertheless, the response of proinsulin is much less pronounced as compared to those of both insulin and C peptide. The reason for this is less clear. Possible mechanisms might include a defective processing and/or storage, but not a defective release mechanism, of the peptide in tumor cells, as previously reported,[18] resulting in an increased basal concentration, a characteristic feature of those tumors,[19,23] and, consequently, an attenuated response. Moreover, although the absolute concentrations of C peptide are usually higher than those of insulin at every time point for each patient, this presumably reflects the preferential transhepatic clearance of

insulin alone.[30] However, C peptide sampling is not more advantageous than the insulin sampling, as both tests have virtually an identical diagnostic sensitivity. The reason for this discrepancy may be partly due to the fact that a fixed portion (~50%) of insulin released by calcium from the pancreas and delivered to the liver was extracted transhepatically, which apparently markedly attenuates the magnitude of increase of insulin response observed here, while the fold increase of insulin response was unaffected.

Previous studies have reported that the suppressibility of insulin secretion from insulinoma cells as induced by diazoxide and somatostatin correlates well with the stages of tumor cell differentiation.[24] Consistent with the idea, our results show that the better the responsiveness of insulin or C peptide, the greater the percentage decrease of the P/I or P/C ratio. This demonstrates clearly that the degree of differentiation of tumor cells per se is also an important factor in determining the responsiveness to calcium stimulation. Since the P/I or P/C ratio immediately after the acute stimulation with calcium would adequately reflect the P/I or P/C ratio in tumor cells, the magnitude of percentage decrease of both ratios after stimulation might be used as an index for the efficacy of the conversion of proinsulin to insulin and C peptide. Our findings suggest that the greater the responsiveness of β-cell peptides to calcium stimulation, the lesser the severity of conversion defects residing in tumor cells. In addition, despite the observation that a greater response is usually found in benign tumors than in malignant ones, 1 of the 2 patients with malignant insulinomas (patient no. 9) had a better response than the patient with a benign one (patient no. 5). It would thus appear that it is the degree of differentiation, rather than the benign or malignant nature of the tumor, that is responsible. However, since the number of patients with islet cell tumors is small, further studies are required to confirm our findings.

Calcium ions have already been shown to play an important role in the secretion of insulin from normal β cells,[31] yet the precise mechanism by which calcium ions stimulate insulin release from insulinoma cells remains poorly understood. Previous studies suggest that calcium ions may directly trigger the release of β-cell peptides by an as yet defined mechanism not shared by normal β cells.[32,33] A more recent in vitro study shows that it is the CaSR that mediates the calcium-evoked insulin release from cultured insulinoma cells and that the CaSR gene is expressed in insulinoma tissues.[13] In the present study, we used double immunostaining to examine the possible relationship between the expression of CaSR with the responsiveness of β-cell peptides to calcium. Consistent with previous studies,[34] the CaSR protein is expressed in normal pancreatic islets and specifically in approximately 9% of total normal β cells, but not in exocrine acinar cells. However, there is no evidence that in insulinoma cells the CaSR protein is overexpressed and actually the expression of CaSR protein in those cells is only minimally detectable or even completely undetectable. Based on the above findings and the fact that calcium at the dosage used has no effect on the release of β-cell peptides from normal β cells as previously reported[3,12,16] and observed here, we conclude that the calcium-stimulated peptide release from insulinoma cells might not be solely mediated by the CaSR. However, since the level of expression needed for detection by immunohistochemistry as employed in our studies is generally higher than that obtained when using molecular amplification strategies, the possibility that CaSR mRNA in insulinoma tissues can be detected by polymerase chain reaction (PCR) amplification but the protein could be missed by immunohistochemisty cannot entirely be excluded. Further studies assessing the functions and regulations of CaSR and calcium channels both in normal β cells and insulinoma cells are needed to elucidate the basis for this discrepancy.

Our results suggest that intra-arterial calcium stimulation with hepatic venous sampling either for insulin or for C peptide is a highly sensitive method for the preoperative localization of small insulinomas. Calcium injection stimulates a marked and prompt simultaneous release of insulin, C peptide, and proinsulin, and the magnitude of increase of both insulin and C peptide appears to be correlated well with the degree of differentiation of the tumor cells. This study thus provides additional evidence for the correlation between the functional properties and morphological features of functioning insulinomas. Finally, the exact mechanism by which calcium provokes the release of β-cell peptides is less clear and whether the CaSR is involved in the mechanism of its action requires further study.

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