Miniglucagon, the COOH-terminal (19-29) fragment processed from glucagon, is a potent and efficient inhibitor of insulin secretion from the MIN 6 β-cell line. Using the rat isolated-perfused pancreas, we investigated the inhibitory effect of miniglucagon on insulin secretion and evaluated the existence of an inhibitory tone exerted by this peptide inside the islet. Miniglucagon dose-dependently inhibited insulin secretion stimulated by 8.3 mmol/l glucose, with no change in the perfusion flow rate. A concentration of 1 nmol/l miniglucagon had a significant inhibitory effect on a 1 nmol/l glucagon-like peptide 1 (7-36) amide-potentiated insulin secretion. A decrease in extracellular glucose concentration simultaneously stimulated glucagon and miniglucagon secretion from pancreatic α-cells. Using confocal and electron microscopy analysis, we observed that miniglucagon is colocalized with glucagon in mature secretory granules of α-cells. Perfusion of an anti-miniglucagon antiserum directed against the biologically active moiety of the peptide resulted in a more pronounced effect of a glucose challenge on insulin secretion, indicating that miniglucagon exerts a local inhibitory tone on β-cells. We concluded that miniglucagon is a novel local regulator of the pancreatic islet physiology and that any abnormal inhibitory tone exerted by this peptide on the β-cell would result in an impaired insulin secretion, as observed in type 2 diabetes. Diabetes 51:406–412, 2002

Proglucagon, the 160–amino acid hormone precursor, undergoes posttranslational processing in the pancreatic α-cells, leading to glucagon (1–4). Glucagon is further processed into the COOH-terminal (19-29) fragment, miniglucagon, through a cleavage at the Arg9–Arg18 basic doublet by a partially characterized protease (5) referred to as “miniglucagon-generating endopeptidase” (MGE). This process occurs both from the circulating hormone on glucagon target tissues (5) and from glucagon inside the islet α-cells (6). In both cases, this processing is partial, leading to concentrations of miniglucagon, which accounts for a few percent of the glucagon concentrations present at the processing site (4–6). The resulting miniglucagon concentration, which, comparatively, may appear as relatively low, is compensated more than enough by the extremely high potency (below picomolar) of the shorter peptide, which is two to three orders of magnitude higher than that of glucagon. Miniglucagon modulates the action of glucagon through an effect mediated by cholera toxin-sensitive G protein on the hepatic plasma membrane calcium pump (7,8). Miniglucagon is a very potent (ID50 close to 0.1 pmol/l) and efficient inhibitor of secretagogue-induced insulin release from the MIN6 β-cell line (9). Its mechanism of action is mediated by a pertussis toxin-sensitive G protein linked to a pathway that involves potassium channel opening and resulting membrane repolarization (9). The presence of miniglucagon in islets of Langerhans at significant levels (3.7% of the glucagon content) (6), together with its high potency and efficacy in inhibiting insulin secretion (9), led us to hypothesize that miniglucagon secretion from pancreatic α-cells might directly influence the activity of neighboring insulin-secreting β-cells. To address this issue, we used the isolated-perfused pancreas from normal rats, a model that features many of the complex physiological regulatory mechanisms, including intra-islet cell-to-cell relationships.

We show that miniglucagon, from 1 pmol/l to 1 nmol/l, dose-dependently exerts an inhibitory effect on glucose and on glucose plus glucagon-like peptide 1 (GLP-1) (7–36) amide (tGLP-1)–stimulated insulin secretion. Miniglucagon, located in glucagon-containing secretory granules of pancreatic α-cells, is simultaneously secreted with glucagon in response to a decrease in extracellular glucose concentration. Perfusion of an anti-miniglucagon antibody that recognizes the NH2-terminal moiety, which is crucial for the biological effectiveness of the peptide, significantly increased glucose-induced insulin secretion. We conclude that miniglucagon released from α-cells exerts a tonic inhibitory control on the neighboring β-cells via a paracrine pathway. This newly discovered mechanism might be of importance in the pancreatic islet physiology and/or pathologies that will now deserve further attention.
Animals were fasted overnight (16–18 h) before preparation for pancreatic perfusion.

Isolated-perfused pancreas model. The surgical and perfusion procedures used were described by Loubatieres et al. (10). Briefly, the pancreases were isolated and perfused at 37°C through the arterial system. The perfusion medium was Krebs-Ringer bicarbonate (KRB) containing 2 g/l BSA and various concentrations of glucose and test agents. Perfusate, adjusted to 2.5 ml/min, was bubbled with 95% O2/5% CO2 to maintain pH 7.4 and warmed at 37°C. Total perfusion time, including a 45-min equilibration period, did not exceed 120 min. To test the effect of miniglucagon on stimulated insulin secretion, 8.3 mmol/l glucose was perfused during the experiments, including a 45-min equilibration period, followed by miniglucagon perfused with or without 1 mmol/l 1GLP-1. To study the glucagon and miniglucagon secretion, the glucose concentration was switched from 11 to 3 mmol/l after a 45-min stabilization period, and the peptides secreted were measured by radioimmunoassay.

Radioimmunoassays. Insulin was measured by radioimmunoassay as previously described (11). Glucagon was measured using an antibody directed toward the centro/N-terminal moiety of the molecule and 125I-glucagon as the tracer. Miniglucagon was measured as previously described (4,12) using a rabbit anti-miniglucagon antiserum (12) that specifically recognizes the NH2-terminal epitope present in miniglucagon and masked in glucagon, the mother molecule (4,12). A very low cross-reactivity (0.02%) exists with glucagon (4,12) in the miniglucagon assay. This NH2-terminal antiserum was anticipated to suppress the biological effectiveness of the peptide because the integrity of the NH2-terminal moiety of miniglucagon is a prerequisite for its biological activity (S.D., G.F., A.-D.L., L.L., R.G., G.R., M.D., L.B., D.B., unpublished observations). High-performance liquid chromatography (HPLC) separation of the peptides was performed as previously described (6) on rat pancreas extracts obtained by the heat-coagulation/acid extraction method (13).

Isolation of islets. Islets were isolated from adult male Wistar rats using collagenase digestion and collected after centrifugation on a Ficoll density gradient as previously described (14). For electron microscopy studies, islets were incubated for 1 h at 37°C in KRB, pH 7.4, containing 1 g/l BSA and 2.8 mmol/l glucose. For immunofluorescence experiments, islets were cultured in RPMI-1640 containing 5.6 mmol/l glucose, 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/l glutamine for 24 h.

Immunofluorescence. Dissociated islets from adult male Wistar rats were separated on a poly-L-lysine–coated (Sigma-Aldrich Saint Quentin Fallavier, France) Lab-Tek Chamber Slide System. After a 7-day culture period, they were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min and permeabilized by a 5-min incubation in 0.1% Triton X-100. After washing, a rabbit anti-insulin antibody (diluted 1:100) or with a rabbit anti-glucagon antibody directed against the centro/NH2-terminal glucagon epitope (dilution 1:200; Biogenesis, Poole, England) and a rabbit NH2-terminal anti-miniglucagon antibody (dilution 1:100). After washing, a fluorescein isothiocyanate (FITC)-conjugated anti-sheep or -goat antibody (diluted 1:100; Vector Laboratories, Burlingame, CA) and a Texas Red–conjugated anti-rabbit antibody (dilution 1:100, Vector Laboratories) were separately applied to the cells for 1 h. Cells were finally mounted in Citifluor (Citifluor LTD, London) and observed with a Zeiss (Oberkochen, Germany) dual photon confocal microscope. For insulin staining, cells were incubated with guinea pig anti-insulin antibody (dilution 1:1600; ICN, Costa Mesa, CA) and a FITC-conjugated antibody (dilution 1:100; Vector Laboratories).

Electron microscopy. Isolated islets were fixed with 2.5% paraformaldehyde/0.1% glutaraldehyde in 100 mmol/l phosphate buffer (pH 7) for 1 h at room temperature. The islets were washed in 50 mmol/l NH4Cl-PBS, postfixed in 1% osmium tetroxide for 2 min, dehydrated in an ascending series of ethanol, and embedded in LR White (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin (60-nm) sections were cut using a Reichert ultramicrotome (Ultracut H-7100; Hitachi, Duesseldorf, Germany). The specificity of the immune reaction, for both immunofluorescence and electron microscopy, was tested by incubating the sections with only the secondary antibody or by saturating the primary antibodies with synthetic miniglucagon (100 μg) or glucagon (100 μg) before incubation of the cells or the sections.

Peptides and chemicals. [Nleu27]miniglucagon was synthesized in our laboratory (15). The presence of a norleucine (Nleu) residue instead of a methionine does not introduce any difference into the biological characteristics of the peptide. [Tyrβ29]glucagon (19–25), used as the tracer in the miniglucagon assay, was synthesized as previously described (5). 125I-insulin was purchased from Amersham (Amersham, UK). Rat insulin, for radioimmunoassay standards, was obtained from Novo Nordisk (Bagvaerd, Denmark). Synthetic 3GLP-1 was obtained from Peninsula Laboratories (San Carlos, CA).

Statistical analysis. Insulin secretion data were expressed as nanograms per minute. All results are presented as mean values ± SE together with the number of individual determinations (n). We used the trapezoidal rule for calculation of areas under the curve (AUCs). The statistical significance was assessed by ANOVA using Stat simple analysis program from Nidus Technologies (Oakville, Canada).

RESULTS

Effect of miniglucagon on glucose-stimulated insulin secretion from isolated perfused pancreas. Insulin output from the isolated perfused rat pancreas was stimulated by a rise in glucose concentration from 0.5 to 8.3 mmol/l glucose. In control experiments, 8.3 mmol/l glucose-stimulated insulin output was stable throughout the perfusion period.

In Fig. 1 it can be observed that miniglucagon induced a prompt and significant inhibition of glucose-stimulated insulin secretion. The maximal inhibitory effect of miniglucagon observed during the peptide infusion was 43, 50, 54, and 35% at 1, 10, 100, and 1,000 pmol/l, respectively (Fig. 1). Figure 1 (inset) shows the dose-response curve computed from the data obtained in the first phase of inhibition (maximally observed at minute 3 of peptide perfusion). The inhibitory effect of 1,000 pmol/l was lower than that of 100 or 10 pmol/l (Fig. 1, inset). Such a bell-shaped dose-response curve was already observed for the miniglucagon effect in other biological models (8), with the conclusion that concentrations >100 pmol/l are supra-physiological. Miniglucagon did not significantly modify the perfusion flow rate, suggesting a lack of...
vascular effect of the peptide that would otherwise have interfered with the results (data not shown).

Effect of miniglucagon on glucose plus tGLP-1-stimulated insulin secretion from isolated perfused pancreas. The addition of 1 nmol/l tGLP-1, a major incretin peptide (16–18), strongly increased the 8.3 nmol/l glucose-stimulated insulin secretion, with a typical biphasic effect on insulin output (Fig. 2).

tGLP-1 induced a 10-fold increase during the first phase and a 8.7-fold increase during the second plateau phase, resulting in overall 15- and 12-fold increases in the secretion rates above the 8.3 nmol/l glucose basal secretion, respectively. A concentration of 1 nmol/l miniglucagon inhibited the 1 nmol/l GLP-1-induced potentiation of insulin secretion by 35.4% for the first phase and by 28.3% for the second phase. The miniglucagon-induced inhibition of tGLP-1-stimulated insulin secretion was biphasic over time (Fig. 2, inset), an observation that provides clues for further insights into the cellular and molecular mechanisms underlying the dynamics of miniglucagon action on insulin release. At the end of the tGLP-1/miniglucagon administration, insulin output progressively returned to basal values (Fig. 2), indicating a perfect reversibility of the action of the peptides.

**Glucagon and miniglucagon secretion from isolated perfused pancreas.** We next determined whether miniglucagon is secreted from pancreatic α-cells and whether this potential secretion occurs simultaneously with glucagon. We studied the glucagon and miniglucagon secretion in response to a decrease in extracellular glucose concentration, a known stimulus of glucagon secretion (19–21).

As shown in Fig. 3A, a gradual and monophasic increase in glucagon release from the perfused rat pancreas was observed when glucose concentrations were decreased from 11 to 3 mmol/l glucose, as observed by Weir et al. (22). Under these conditions, we observed a simultaneous miniglucagon secretion, and the computed amount of miniglucagon secreted corresponded to 3.48% of the amount of glucagon that was released (Fig. 3B). These findings fit perfectly with the observation that, as shown in Fig. 3 (left panel), miniglucagon is present in rat pancreatic α-cells at 3.7% of the glucagon content (6) and suggest that miniglucagon is located in the same secretory granules as glucagon. Figure 3 (left panel) shows the lack of recognition of the glucagon peak in the miniglucagon radioimmunoassay.

**Immunofluorescence and electron microscopy.** Next, we investigated the presence of miniglucagon in pancreatic α-cells and its subcellular localization. We used the anti-miniglucagon antibody (12), which specifically recognizes the NH₂-terminal epitope present in miniglucagon and masked in glucagon, the mother molecule (4), and an anti-glucagon antibody directed against the centro/NH₂-terminal moiety of glucagon that recognizes both glucagon and its precursors (including proglucagon) and does not cross-react with miniglucagon.

As shown in Fig. 4G, miniglucagon is absent from insulin-secreting β-cells and is only present in glucagon-secreting α-cells (Fig. 4A–F). The glucagon immunoreactivity (Fig. 4A and D) is punctuated and distributed in the entire cytoplasm of the α-cell, indicating that the antibody stains the secretory granules at different stages of maturation as well as the rough endoplasmic reticulum and the Golgi apparatus. This observation agrees with the previous demonstration that glucagon arises from the conversion of a large precursor (proglucagon) in the α-cell during the maturation of the secretory
granules (1–4,23–26). Miniglucagon immunofluorescence (Fig. 4B and E) is also punctuated and strongly colocalizes with the glucagon secretory granules (Figs. 4C and F) at the α-cell periphery. Miniglucagon staining is faint in a perinuclear crescent-shaped zone, probably corresponding to the Golgi apparatus and immature granules, and the fluorescence progressively increases from this region to mature secretory granules. Saturation of the anti-miniglucagon and -glucagon antibodies with the corresponding peptides resulted in an inhibition of the fluorescent signal (data not shown).

We then performed electron microscopy with the miniglucagon antibody (12) on sections of isolated islets. Pancreatic α-cell secretory granules are morphologically distinctive. They vary in appearance from species to species but in general have an electron-dense central core and a less dense outer portion of the granule, producing the appearance of a small halo (24). The central core contains fully processed glucagon with its COOH-terminal moiety available for reaction with COOH-terminally directed anti-glucagon antibody, and the halo region does not react with such an antibody. The halo region contains proglucagon, the biosynthetic precursor of glucagon (24), giving a positive signal with anti-glucagon antibodies directed against the centro/NH₂-terminal moiety of the molecule, an epitope present in proglucagon (23,25,26).

As shown in Fig. 5, the miniglucagon reactivity is mainly

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**FIG. 4.** Localization of miniglucagon in pancreatic α-cells by double immunofluorescence. Pancreatic α-cells were double-immunostained with anti-glucagon and -miniglucagon antibodies as described in RESEARCH DESIGN AND METHODS. Glucagon and miniglucagon stainings appear, respectively, as green (A and D) and red (B and E) fluorescence. Coincidence of both fluorosciences results in the appearance of a yellow color (C and F). The scale bars are 10 μmol/l. G: double staining with anti-insulin and the anti-miniglucagon antibodies. H: negative control for both fluorosciences.
miniglucagon antibody perfusion area curve value 599
insulin secretion (control area curve value 394
fold factor over control the amount of glucose-stimulated
miniglucagon antiserum significantly increased by perfusion of the anti-miniglucagon antiserum. 
mainly the second phase of insulin secretion was in-
P during the 36-min period after the glucose challenge, 
ments were conducted with a nonimmune rabbit serum 
peptide. The antibody dilution was chosen according to in 
mature granules. Electron microscopy was performed as described in 
the glucagon-producing 
protease (5). Evidence that the peptide was also present in 
miniglucagon arises from the partial conversion of gluca-
nded and tGLP-1–potentiated insulin secretion. The inhibitory effect of miniglucagon over time on tGLP-1–
potentiated insulin secretion displays a biphasic behavior that compares well with the biphasic effect of the secretagogue but is delayed by ~2 min. Because the two peptides were perfused at the same time, these interesting 
dynamics are best interpreted in terms of complex interactions between the stimulatory and the inhibitory pathways, possibly occurring at the level of the β-cell voltage-sensitive calcium channel, although other mechanisms may also have been involved (for a review of mechanisms involved in inhibition of insulin release, see reference 28).

Second, we wanted to determine whether miniglucagon is localized with glucagon in the same secretory granules and whether it is secreted. Using electron and confocal microscopy, we found that glucagon and miniglucagon are colocalyzed in mature secretory granules. By qualitative analysis of immunofluorescence micrographs, we observed an increase in miniglucagon immunoreactivity from the Golgi apparatus area to mature secretory granules. As miniglucagon arises from the partial conversion of glucagon, it is logical that the COOH-terminal fragment appears 

Miniglucagon was initially discovered (4,7,8) as a second-
ary processing product of circulating glucagon within the 
localization in the electron-dense core of the mature α-secretory 
granules (Fig. 5, small arrows), which were identified 
according to the morphologic description by Orci (27). On 
the other hand, less mature granules and the halo region of 
mature granules were barely stained (arrow heads).

**Effect of a perfusion of miniglucagon antiserum on the glucose-induced insulin secretion.** To test the hypothesis that locally released miniglucagon might induce a negative tone on the β-cells, we used an antibody directed toward the NH₂-terminal miniglucagon moiety that is known to be crucial for the biological activity of the peptide. The antibody dilution was chosen according to in vitro titration experiments (not shown). Control experiments were conducted with a nonimmune rabbit serum used at the same dilution.

Figure 6 shows that the perfusion of the rabbit anti-
miniglucagon antiserum significantly increased by a 1.5-
fold factor over control the amount of glucose-stimulated 
insulin secretion (control area curve value 394 ± 56, 
miniglucagon antibody perfusion area curve value 599 ± 59 during the 36-min period after the glucose challenge, 
P < 0.05 vs. control area curve value). We observed that 
mainly the second phase of insulin secretion was 
increased by perfusion of the anti-miniglucagon antiserum.

**DISCUSSION**

Miniglucagon was initially discovered (4,7,8) as a secondary 
processing product of circulating glucagon within the 
glucagon target tissues via the action of a cell surface protease (5). Evidence that the peptide was also present in 
the glucagon-producing α-cells in the islets (6) prompted us to determine whether the peptide might display an effect on the neighboring β-cells. Using a homogeneous β-cell population, the MIN6 cell line, we showed that miniglucagon is a highly potent and efficient inhibitor of insulin release by closing, via hyperpolarization, voltage-
depending Ca^{2+} channels through a pathway that involves a pertussis toxin-sensitive Gi or Go GTP-binding protein (9).

First, we wanted to confirm our findings (9) using the 
isolated-perfused rat pancreas (10), which involves all 
types of islet cells, normal cell-to-cell interactions within the 
islets through vascular and paracrine pathways, exocrine/endocrine interactions, and interactions between 
islets and vascular or intrinsic nervous systems without 
the drawbacks of in vivo models. We confirm that picomolar concentrations of miniglucagon inhibit glucose-induced and tGLP-1–potentiated insulin secretion. The inhibitory effect of miniglucagon over time on tGLP-1–
potentiated insulin secretion displays a biphasic behavior that compares well with the biphasic effect of the secretagogue but is delayed by ~2 min. Because the two peptides were perfused at the same time, these interesting 
dynamics are best interpreted in terms of complex interactions between the stimulatory and the inhibitory pathways, possibly occurring at the level of the β-cell voltage-sensitive calcium channel, although other mechanisms may also have been involved (for a review of mechanisms involved in inhibition of insulin release, see reference 28).

Second, we wanted to determine whether miniglucagon is localized with glucagon in the same secretory granules and whether it is secreted. Using electron and confocal microscopy, we found that glucagon and miniglucagon are colocalyzed in mature secretory granules. By qualitative 
analysis of immunofluorescence micrographs, we observed an increase in miniglucagon immunoreactivity from the Golgi apparatus area to mature secretory granules. As miniglucagon arises from the partial conversion of glucagon, it is logical that the COOH-terminal fragment appears 
later in the protein export processing and is preferentially located in mature secretory granules. Thus, from the
condensing material in Golgi cisternae to the mature α-granules, glucagon first arises from the conversion of proglucagon (1–4,22–24) and then undergoes a partial proteolytic cleavage at the dibasic Arg17-Arg18 processing site, leading to the production of the COOH-terminal fragment. Miniglucagon is present in α-cells at 3.7% of the glucagon content (6), making it possible that miniglucagon acts physiologically as a modulator of the glucagon action on insulin release. In accordance with the fact that glucagon and miniglucagon are located in the same secretory granules, we observed a miniglucagon release concomitantly to the glucagon secretion. Thus, both glucagon and miniglucagon appear to be present in the neighborhood of the β-cell in response to a fall in glucose concentration. Miniglucagon is released at a rate that makes it present at concentrations that are ∼25 times less than that of the mother hormone, whereas the fragment acts at concentrations (∼10^{-11} mol/l) that are at least two orders of magnitude lower than the active doses of glucagon (∼10^{-9} mol/l) (4–9). Altogether, this means that the observed miniglucagon release is biologically significant and that the naturally released miniglucagon may entirely suppress the possible insulinitropic effect of glucagon via its functional receptors coupled to insulin secretion (29). When glucagon is released from α-cells every time an increase in blood glucose is necessary, an insulin release triggered by glucagon from the fringe of β-cells that surround α-cells would be sufficient to abolish the hyperglycemic effect of glucagon. A release of miniglucagon, together with glucagon, is thus likely to represent a suppressive mechanism for this undesirable side effect of glucagon. Whether the miniglucagon-to-glucagon proportion is regulated under physiological or pathological conditions, as a consequence of a change in the cellular expression of the MGE, it will be an important issue to address. According to the present data and that obtained on a β-cell line (9), it appears that the major effect of miniglucagon on insulin secretion is related to a direct action on the β-cell. This fits well with the islet architecture (30–32), in which 36 and 53% of the β-cells in the rat and human islet, respectively, have direct contacts with the α-cells. Moreover, studies of the morphology of pancreatic islet microcirculation showed that the direction of microcirculation is from cortex to core in 66% of the islets and from core to cortex in 44% (33–35). Thus, the paracrine regulation may occur via hormonal secretion into intercellular spaces or vascular hormonal delivery to adjacent cells in the islet (33–35). On the other hand, we cannot exclude additional actions of miniglucagon, such as an inhibition of glucagon secretion or an action via intrinsic nervous output, that deserve further investigation.

The third issue that we want to address is whether locally released miniglucagon might exert an inhibitory tone on the neighboring β-cells under physiological conditions. We show that immunoneutralization of miniglucagon increases the capacity of glucose to stimulate insulin secretion by 50%, strongly suggesting that there is indeed an inhibitory miniglucagonergic tone on the β-cell and, accordingly, a biologically significant miniglucagon secretion under normal physiological conditions. Because glucagon and miniglucagon are synthesized and secreted synchronously by α-cells, it will be of interest to investi-gate whether changes in the secretory ratio between the two peptides exist under specific conditions, suggesting a differential control as shown for insulin and islet amyloid polypeptide release from β-cells (36).

Thus, whether this tone is permanent (basal secretion) or whether it may vary depending on the physiological and/or pathological conditions will require further investigations. However, giving the remarkably high potency of miniglucagon, any abnormally high inhibitory tone exerted by this peptide on the β-cell would result in an impaired insulin secretion, such as that observed in type 2 diabetes. In consequence, it appears that miniglucagon is a new piece in the insulin-regulatory processes puzzle that now has to be taken into account during future investigations on islet cell physiology and pathology.

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