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Miniglucagon (Glucagon 19–29), a Potent and Efficient Inhibitor of Secretagogue-induced Insulin Release through a Ca^{2+} Pathway*

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Stéphane Dalle, Paul Smith‡§, Philippe Blache, Dung Le-Nguyen, Laurence Le Brigand, François Bergeron, Frances M. Ashcroft‡, and Dominique Bataille¶

From INSERM U376, CHU Arnaud-de-Villeneuve, 34295 Montpellier, France and the ‡University Laboratory of Physiology, Oxford University, Parks Road, Oxford OX1 3PT, United Kingdom

Using the MIN6 B-cell line, we investigated the hypothesis that miniglucagon, the C-terminal (19–29) fragment processed from glucagon and present in pancreatic A cells, modulates insulin release, and we analyzed its cellular mode of action. We show that, at concentrations ranging from 0.01 to 1000 pM, miniglucagon dose-dependently (ID_{50} = 1 pM) inhibited by 80–100% the insulin release triggered by glucose, glucagon, glucagon-like peptide-1-(7–36) amide (tGLP-1), or glibenclamide, but not that induced by carbachol. Miniglucagon had no significant effects on cellular cAMP levels. The increase in $^{45}Ca^{2+}$ uptake induced by depolarizing agents (glucose or extracellular K^+), by glucagon, or by the Ca^{2+}channel agonist Bay K-8644 was blocked by miniglucagon at the doses active on insulin release. Electrophysiological experiments indicated that miniglucagon induces membrane hyperpolarization, probably by opening potassium channels, which terminated glucose-induced electrical activity. Pretreatment with pertussis toxin abolished the effects of miniglucagon on insulin release. It is concluded that miniglucagon is a highly potent and efficient inhibitor of insulin release by closing, via hyperpolarization, voltage-dependent Ca^{2+} channels linked to a pathway involving a pertussis toxin-sensitive G protein.

Like many other polypeptide hormones (1–3), glucagon is processed from a large precursor, the 160-amino acid proglucagon produced in the A-cells of the islets of Langerhans, in the L cells of the intestinal mucosa, and in specialized neurons of the central nervous system present mainly in the hypothalamus and in the medulla oblongata (4). Glucagon is known for its hyperglycemic activity through its action on liver via a seven-transmembrane domain receptor linked to adenylyl cyclase via a GTP-binding protein of the Gs sub-type (5). At the level of its target tissues such as the liver, glucagon is partially processed through a cleavage at the Arg^{17}-Arg^{18} basic doublet by a cell surface protease referred to as "miniglucagon-generating endopeptidase" (MGE)[1] (6) leading to the production of a C-terminal (19–29) fragment called "miniglucagon" (7–9). Miniglucagon, which does not interfere with the adenylyl cyclase activity, inhibits at picomolar concentrations the hepatic plasma membrane calcium pump (10). On cultured cardiac myocytes, miniglucagon was shown to potentiate at nanomolar concentrations the positive inotropic effect of glucagon, whereas, when used alone at picomolar concentrations, it displayed a negative inotropic effect on myocyte contraction (11). These observations suggested a new role for glucagon as a prohormone and a biological role for miniglucagon as a daughter hormone that modulates the effects of the mother hormone (12). On the other hand, pancreas is the only known tissue in which miniglucagon is present in a stored form, at molar concentrations in the range of 2–5% of that of glucagon (13).

In view of preliminary results suggesting that miniglucagon is able to inhibit glucose- and glucagon-induced insulin release (12), we studied the ability of miniglucagon to modulate secretagogue-induced insulin release using the MIN6 cell line which displays characteristics that compare well with that of normal β cells (14), in particular a response to glucose in the physiological range. We have also explored the intracellular pathway through which miniglucagon inhibits insulin release.

We show here that miniglucagon, in a concentration range (starting at 10^{-14}–10^{-13} M) which fits with the amount of peptide presumably present in the extracellular medium within the islets, is able to suppress by 80–100% the insulin release stimulated by molecules known to open voltage-dependent calcium channels such as glucose, glucagon, tGLP-1, or glibenclamide, but not the insulin release stimulated by carbachol, a molecule known to increase $InsP_3$ and cytosolic Ca^{2+}. Miniglucagon had no effect on the cellular cyclic AMP levels but suppressed secretagogue-induced calcium entry. Miniglucagon induced a hyperpolarization of the membrane potential and thus probably inhibited the action of all those secretagogues *via* an indirect inhibition of the voltage-dependent L-type calcium channels. The miniglucagon action on insulin release was suppressed after pre-treatment of the cells with pertussis toxin. It is proposed that miniglucagon, present in pancreatic A-cells, acts as a local inhibitory regulator of insulin release by turning off the main external calcium source for β cells via a specific receptor linked, through a pertussis toxin-sensitive GTP-binding protein, to ion channels that control the cell polarity.

EXPERIMENTAL PROCEDURES

Peptides and Chemicals

Nle^{27} miniglucagon was synthesized in our laboratory (15). Synthetic glucagon-like peptide-1-(7–36) amide (GLP-1-(7–36) amide) was obtained from Peninsula Laboratories (San Carlos, CA), glucagon from Novo Research Institute (Bagsvaerd, Denmark), glibenclamide from Guidotti Spa (Pisa) Laboratory, and somatostatin from Neosystem. Radioimmunoassay of insulin was performed using ^{125}I-porcine insulin, rat insulin (Novo, Denmark) as standard, and the guinea pig anti-porcine insulin antibody 41 previously described (16). ^{45}Ca was ob-

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¶ To whom correspondence should be addressed. Tel.: +33 4 67 41 52 20; Fax: +33 4 67 41 52 22; E-mail: bataille@u376.montp.inserm.fr.

[1] The abbreviations used are: MGE, miniglucagon-generating endopeptidase; tGLP-1, glucagon-like peptide-1-(7–36) amide; BSA, bovine serum albumin; IBMX, 3-isobutyl-1-methylxanthine; VDCC, voltage-dependent Ca^{2+} channels; $InsP_3$, inositol trisphosphate.

tained from NEN Life Science Products (France). Bay K-8644 was purchased from Calbiochem-Novabiochem (La Jolla, CA), and nifedipine was from Sigma.

Methods

Cell Culture—MIN6 cells were originally obtained from Dr. H. Ishihara (Tokyo, Japan). The cells were grown in Dulbecco's modified Eagle's medium containing 25 mmol/liter glucose (DMEM, Life Technologies, Inc.) supplemented with 15% fetal calf serum (Life Technologies, Inc.), 100 μg/ml streptomycin (Life Technologies, Inc.), 100 units/ml penicillin sulfate (Life Technologies, Inc.), and 75 nM β-mercaptoethanol (Sigma), equilibrated with 5% CO_2, 95% air at 37 °C. DMEM was changed every 48 h of culture. When reaching 80% confluence, the cells were detached by treatment with 2.5% trypsin, 0.5 mM EDTA. MIN6 cells used in the present study were harvested at passages 15 to 25.

Insulin Release—MIN6 cells were plated in 1 ml of DMEM (25 mM glucose) in 24-well plates at a density of 1×10^6 cells per well for 3–5 days. Insulin release was determined using a static incubation method in 5% CO_2, 95% air at 37 °C with cells in exponential growth. The medium culture was changed 18 h before the experiments. Insulin secretion from MIN6 cells monolayers was performed in HEPES-balanced Krebs-Ringer bicarbonate buffer (119 mmol/liter NaCl; 4 mmol/liter KCl; 1.2 mmol/liter KH_2PO_4; 1.2 mmol/liter $MgSO_4$; 2.5 mmol/liter $CaCl_2$; 20 mmol/liter HEPES, pH 7.5) containing 0.5% BSA (KRB buffer).

The day of the experiment, the medium was removed and the cells were washed twice with 500 μl of KRB buffer. Cells were preincubated for 1 h in 500 μl of KRB buffer containing 1 mmol/liter glucose in 5% CO_2, 95% air at 37 °C. This buffer was removed, and MIN6 cells were then preincubated for 2 h in 500 μl of KRB buffer containing varying concentrations of glucose and other test agents. At the end of the incubation period, media were collected, and floating cells, if any, were eliminated by centrifugation at 1000 rpm for 5 min.

Radioimmunoassay of Insulin—Insulin in supernatants was measured by radioimmunoassay as described previously (16), using ^{125}I-insulin, rat insulin standard, and anti-insulin porcine antiserum. Briefly, the assay was performed in a final volume of 500 μl of 0.025 M borate buffer containing 0.5% BSA. After a 4-day incubation at 4 °C, separation of free insulin was realized using activated charcoal (5 g/100 ml in 0.1 M borate buffer) after addition of 100 μl/sample horse serum. After dilution and centrifugation at 3000 rpm for 5 min, the pellet was counted in a γ-scintillation counter (LKB-Wallac).

Determination of cAMP Production—MIN6 cells were grown in 24-well plates for 3–5 days under the same conditions as for insulin release. The medium was changed 1 day before the experiments. On the day of the experiment, cells were washed twice with DMEM containing 4.5 mmol/liter glucose without fetal calf serum before the addition of 500 μl of DMEM buffer supplemented with 1% BSA and 1 mM IBMX as an inhibitor of cyclic AMP phosphodiesterase and containing the test substances. After a 15-min incubation at room temperature, the cells were extracted using 60% perchloric acid, the sample was neutralized with 9 N KOH succinylated to increase the sensitivity of the assay (17), and cyclic AMP was quantified by radioimmunoassay.

Measurement of $^{45}Ca^{2+}$ Influx—24 h before the experiment, the culture medium was changed. On the day of the experiment, the cells were washed twice with 500 μl of KRB buffer and preincubated for 30 min at 37 °C in 250 μl of KRB buffer containing 1 mmol/liter glucose in 5% CO_2, 95% air. The preincubation solution was then replaced by 250 μl of KRB containing 8 μCi/ml $^{45}CaCl_2$ (Amersham Pharmacia Biotech, UK; 5–50 mCi/mg Ca) and the test agents. The reaction, developed at 37 °C, was stopped by aspiration of the medium. The cells were rapidly washed four times with ice-cold buffer (135 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM lanthanium chloride, 10 mM HEPES). The cells were then solubilized in 1 ml of KRB containing 0.1% Triton X-100 for 1 h at room temperature. An aliquot of the solution (100 μl) was then assayed for $^{45}Ca^{2+}$ content in a β-counter after addition of a liquid scintillation medium (Complete Phase Combining System, Amersham Pharmacia Biotech).

Electrophysiology—Cells were plated onto plastic Petri dishes and maintained in tissue culture prior to use. For whole-cell voltage clamp, cells were chosen that were rounded and apparently single. For current-clamp recording, both single cells and clusters of cells were used. To maintain cell metabolism and second-messenger systems intact, membrane currents and potential were recorded using the perforated-patch whole-cell technique as described previously (18, 19). Ca^{2+} currents were measured using voltage clamp with a bath solution that contained (in mM) 108 NaCl, 30 TEACl, 5.6 KCl, 1.2 MgCl2, 2.6 CaCl2, 10 HEPES (pH 7.4 with NaOH), and 10 glucose and 0.1% w/v bovine serum albumin. The pipette solution contained (in mM) 76 Cs_2SO4, 10 KCl, 10 NaCl, 1 MgCl2, and 10 HEPES (pH 7.2 with CsOH). Perforation was obtained by the addition of 0.2 μg ml^{-1} amphotericin B to the pipette solution and was considered adequate when the series conductance was > 40 nS (mean 69 ± 8 nS, n = 10). Inward Ca^{2+} currents were elicited by stepping the membrane to potentials positive to −60 mV for 250 ms at a frequency of 0.1 Hz. The holding potential was −70 mV. Currents flowing because of leak conductances and uncompensated capacitance were removed by subtracting the scaled average of currents elicited by voltage steps to −60, −80, and −90 mV. To control for variation in cell size, currents have been normalized to the cell capacitance (mean 7.3 ± 0.5 picofarad, n = 10). Membrane potential was monitored using current clamp with the same solutions used for the measurement of Ca^{2+} currents except that TEACl in the bath was replaced with NaCl, glucose was added as required, and Cs^+ in the pipette was replaced with K^+. Currents and potentials are referenced to the pipette in the bath. No corrections have been made for liquid junction potentials (<4 mV) or series resistance errors (<2 mV). All experiments were conducted at 32 ± 1 °C.

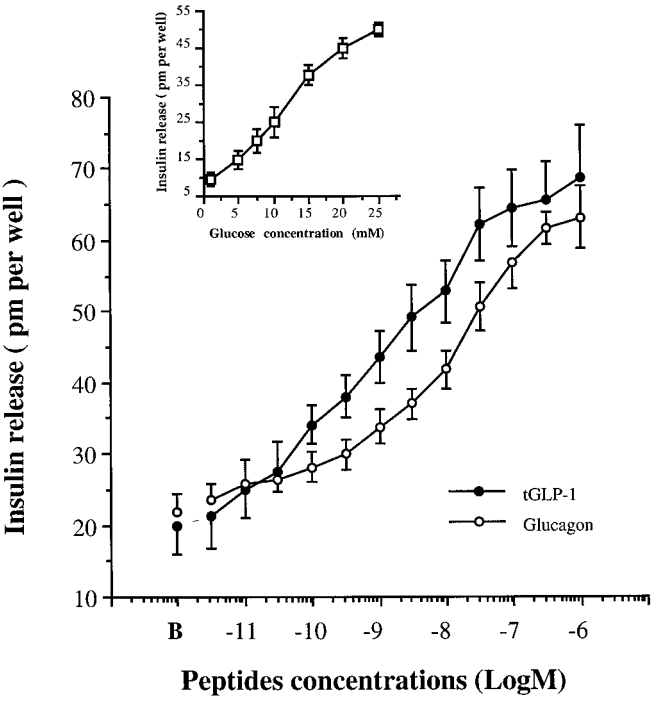

FIG. 1. **Glucose-, glucagon-, and tGLP-1-stimulated insulin release from MIN6 cells.** Insulin release from MIN6 cells in culture was measured after incubation with the corresponding secretagogue as described under "Experimental Procedures." Data are means ± S.E. for fifteen determinations (*inset*) or twelve determinations.

Statistical Analysis—Results were analyzed by Student's t test for unpaired data.

RESULTS

Effects of Glucose, Glucagon, and tGLP-1 on Insulin Release—We first analyzed the response of our batches of MIN6 cells to classical secretagogues, namely glucose (an indirect depolarizing agent), glucagon, and tGLP-1 (potentiators of glucose-induced insulin release *via* a cyclic AMP-dependent pathway), and the sulfonylurea glibenclamide (a blocker of ATP-dependent potassium channels). These molecules increased, at concentrations similar to that observed in other biological models, insulin release from this beta cell line: glucose, at 20 mM, induced a 6-fold increase in this parameter as compared with base line measured at 1 mM glucose, the half-maximal response being achieved at around 10 mM (Fig. 1, *inset*). As shown in Fig. 1, insulin release was also dose-dependently stimulated by glucagon (EC_{50} = 10.1 ± 0.15 nM) and tGLP-1 (EC_{50} = 1.32 ± 0.2 nM), as well as (data not shown) by the sulfonylurea glib-

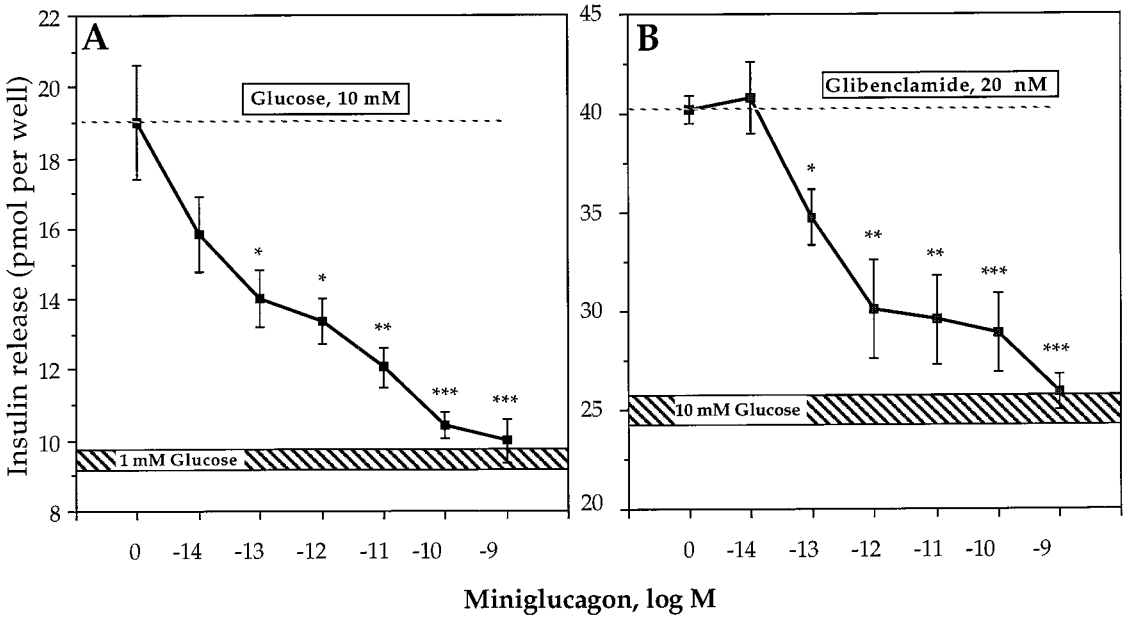

FIG. 2. **Effects of miniglucagon on glucose- and sulfonylurea (glibenclamide)-stimulated insulin release.** *A*, dose-response curve of miniglucagon on 10 mM glucose-stimulated insulin release. Data are means ± S.E. of three to four experiments, each performed in triplicate. *B*, dose-response curve of miniglucagon on 10 mM glucose and 20 nM glibenclamide. Data are means ± S.E. of two experiments, each performed in triplicate. Statistical significance was determined by comparing the data obtained in the presence and in the absence of miniglucagon. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

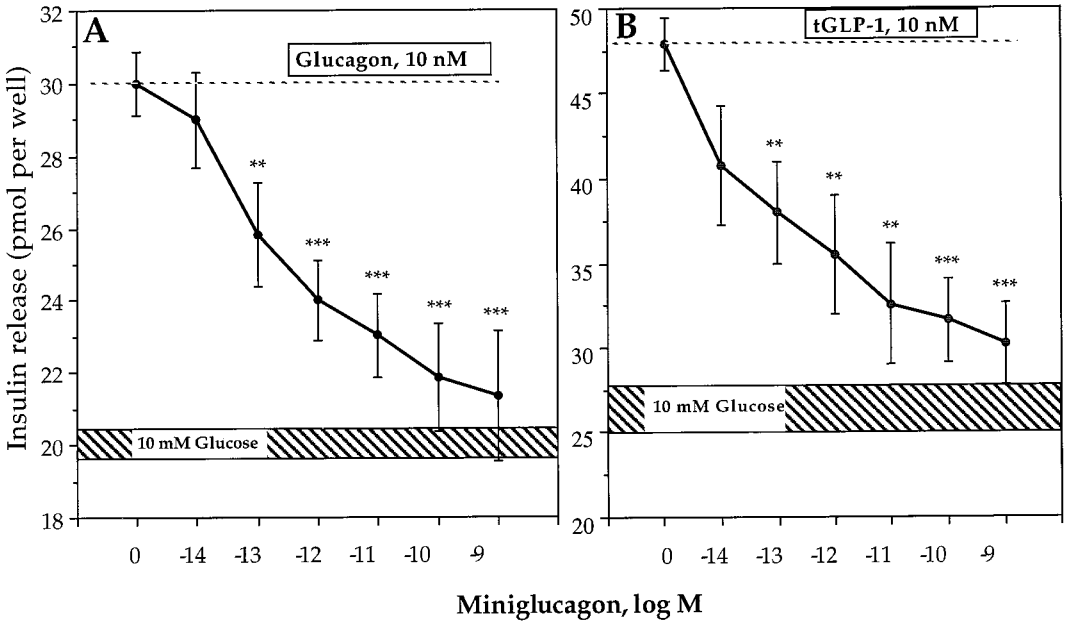

FIG. 3. **Effect of miniglucagon, glucose+, glucagon−, or glucose + tGLP-1-stimulated insulin release.** Shown is the dose-response curve of miniglucagon on 10 mM glucose + 10 nM glucagon-stimulated insulin release (*A*) and on 10 mM glucose + 10 nM tGLP-1-stimulated insulin release (*B*). Data are means ± S.E. of three to four experiments, each performed in triplicate. Statistical significance was determined by comparing the data obtained in the presence and in the absence of miniglucagon. **, $p < 0.01$; ***, $p < 0.001$.

enclamide ($EC_{50} \approx 20$ nM), all experiments being run in the presence of 10 mM glucose.

Effects of Miniglucagon on Glucose, Sulfonylurea-stimulated Insulin Release—As shown in Fig. 2*A*, miniglucagon dose-dependently inhibited the 10 mM glucose-induced insulin release. The threshold dose for the miniglucagon effect was observed between 0.01 and 0.1 pM, EC_{50} was around 2 pM, and a virtually complete inhibition was noted at 1 nM miniglucagon. In Fig. 2*B*, it may be seen that miniglucagon also dose-dependently inhibited 20 nM glibenclamide-induced insulin release in a range starting in the sub-picomolar concentrations with an IC_{50} around 0.3 pM.

Effects of Miniglucagon on Glucagon- and tGLP-1-stimulated Insulin Release—In Fig. 3, *A* and *B*, it may be seen that miniglucagon also dose-dependently inhibited 10 nM glucagon-induced or 10 nM tGLP-1-induced insulin release, again at threshold doses between 0.01 and 0.1 pM. Half-maximal effects were noted at concentrations close to 1 pM, and again, a very large proportion (around 90%) of the stimulated secretion was suppressed at 1 nM miniglucagon (Fig. 3, *A* and *B*).

Because glucagon and tGLP-1 are known to stimulate insulin release *via* a cyclic AMP-dependent pathway (20), we deter-

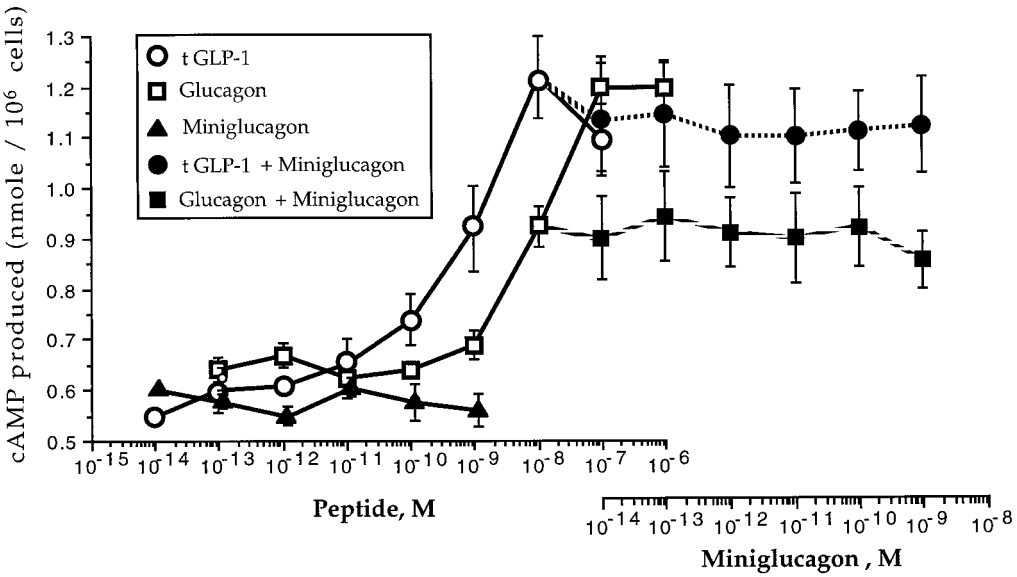

FIG. 4. **Effects of miniglucagon on basal, glucagon-, or tGLP-1- stimulated cyclic AMP production in MIN6 cells.** Data are means ± S.E. of three experiments, each performed in triplicate.

mined whether miniglucagon might modify the B-cell cyclic AMP content.

Effect of Miniglucagon on Glucagon- and tGLP-1-stimulated Cyclic AMP Production—Both tGLP-1 and glucagon stimulated by a 2-fold factor cyclic AMP production in MIN6 cells in a concentration-dependent manner (Fig. 4). As expected from the data obtained when insulin release was studied, glucagon (EC_{50} = 3.17 ± 0.05, n = 4) was less potent than tGLP-1 (EC_{50} = 1.34 ± 0.1, n = 4) (Fig. 4). In contrast, miniglucagon did not display any effect on basal or on glucagon- or tGLP-1-stimulated cyclic AMP production. These results clearly indicate that miniglucagon inhibits both glucagon- and tGLP-1- stimulated insulin release without interfering with the cyclic AMP pathway.

Effect of Miniglucagon on Cholinergic Potentiation of Insulin Release—Because glucose, sulfonylureas, glucagon, and tGLP-1 have the common ability to induce calcium entry, we determined whether miniglucagon might also inhibit insulin release stimulated by a secretagogue known to act mainly via a calcium release from intracellular stores. For that purpose, we used the cholinergic agonist carbachol, known to potentiate glucose-induced insulin release. As shown in Fig. 5, miniglucagon was unable to modify the 10 μM (Fig. 5*A*) or the 1 μM carbachol-induced (Fig. 5*B*) insulin release in the range of 0.01 to 100 pM, which was shown to deeply affect the stimulatory effect of glucose, sulfonylurea, glucagon, or tGLP-1. However, a significant inhibition was noted of the highest (1 nM) miniglucagon dose both at 1 and 10 μM carbachol.

It is apparent from all these data that miniglucagon acts on the beta cell through a pathway that is shared by glucose, glucagon, tGLP-1, and sulfonylureas but not by carbachol. Because calcium influx through L type voltage-dependent Ca^{2+} channels (VDCC) has a key role in secretagogue-induced insulin release (29), VDCC are a good candidate for being this common step. We therefore tested the modulating effect of miniglucagon on Ca^{2+} influx triggered by the above secretagogues.

Effect of Miniglucagon on Stimulated Ca^{2+} Influx—As shown in Fig. 6*A*, miniglucagon dose-dependently inhibited 10 mM glucose-induced Ca^{2+} influx with an IC_{50} around 1 pM. Similarly, miniglucagon totally suppressed the potentiating effect of 10 nM glucagon plus 10 mM glucose-induced Ca^{2+} influx (Fig. 6*B*). Miniglucagon displayed significant effects at

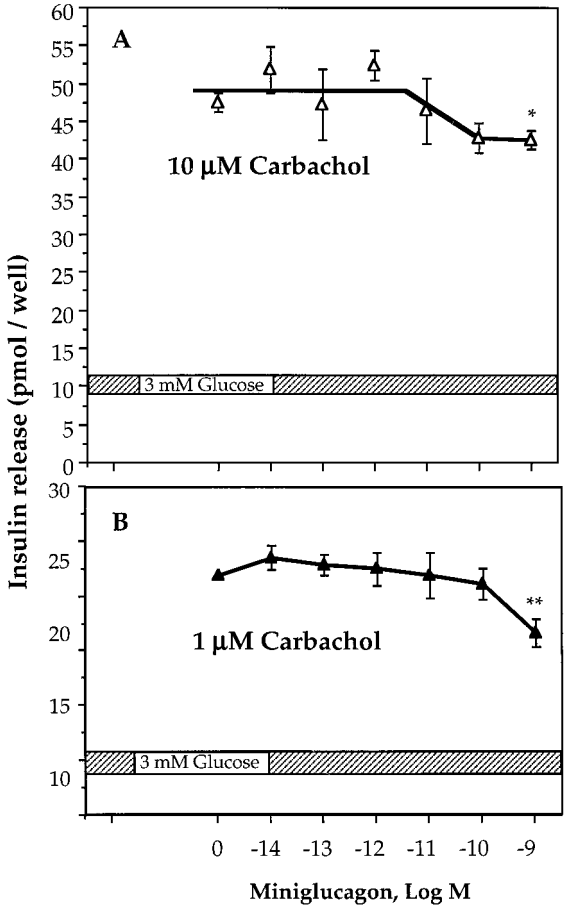

FIG. 5. **Effect of miniglucagon on 10 and 1 μM carbachol-stimulated insulin release.** Dose-response curve of miniglucagon on 10 μM carbachol-stimulated insulin release (*A*) and on 1μM carbachol-stimulated insulin release (*B*), in the presence of 3 mM glucose. Data are means ± S.E. of two experiments, each performed in triplicate. Statistical significance was determined by comparing the data obtained in the presence and in the absence of miniglucagon. *, $p < 0.05$; **, $p < 0.01$.

doses as low as 0.1 pM on these Ca^{2+} influx. Taken together, our findings strongly suggested that miniglucagon inhibits insulin release by blocking the secretagogue-induced Ca^{2+} influx into

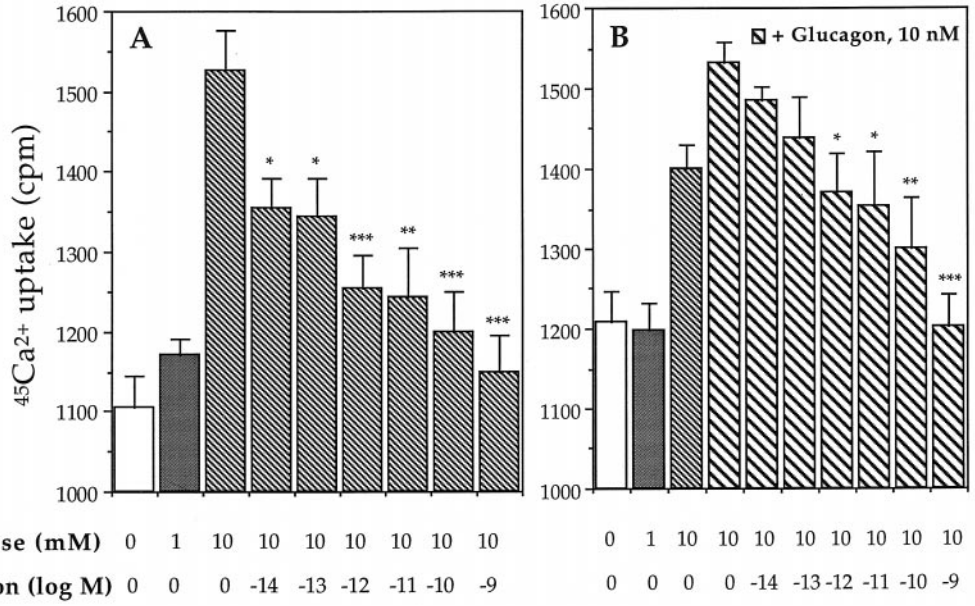

FIG. 6. **Effects of miniglucagon on glucose- and glucagon-stimulated calcium uptake from MIN6 cells.** $^{45}Ca^{2+}$ uptake was measured as described under "Experimental Procedures." The test agents were present simultaneously. *A*, 10-min incubation with 10 mM glucose and with or without various concentrations of miniglucagon; *B*, 10-min incubation with 10 nM glucagon in the presence of 10 mM glucose. Data are means ± S.E. of nine determinations (*A*) and six determinations (*B*). Statistical significance was determined by comparing the data obtained in the presence and in the absence of miniglucagon. **, $p < 0.01$; ***, $p < 0.001$.

the beta cells.

Given the importance of VDCC in secretagogue-induced insulin release (23, 29), we hypothesized that miniglucagon might affect the behavior of this particular channel. To address this issue, we designed the following series of experiments. First, an opener of L type Ca^{2+} channels, Bay K-8644 (24), was used to open the channel under conditions of 10 mM glucose stimulation. As shown in Fig. 7*A*, miniglucagon was able in the range 0.01 pM to 1 nM to totally suppress the Bay K-8644-induced Ca^{2+} influx, displaying significant effects at doses as low as 0.01 pM. For comparison, 1 nM miniglucagon was as effective as 2 μM nifedipine (Fig. 7*A*), a direct inhibitor of L type Ca^{2+} channels (25). As additional proof of the identity of the type of Ca^{2+} channel involved in the miniglucagon action and because opening of voltage-sensitive Ca^{2+} channels may be obtained by membrane depolarization, we analyzed whether miniglucagon was able to suppress a potassium-induced Ca^{2+} influx similar in size to that induced by physiological insulin secretagogues. As shown in Fig. 7*B*, miniglucagon, in the 0.01 pM to 1 nM dose range, almost totally suppressed the 10 mM potassium-induced Ca^{2+} influx.

The above data suggest that the inhibitory effect of miniglucagon on insulin release is because of closure of L type voltage-dependent Ca^{2+} channels. To address the hypothesis of a direct or an indirect effect of the miniglucagon on these calcium channels, we tested the effect of miniglucagon 1) on L type Ca^{2+} currents and 2) on the MIN6 cell electrical activity that controls Ca^{2+} influx (26–29), using the whole-cell patch-clamp method.

Electrophysiology, Ca^{2+} Currents—Inward Ca^{2+}currents were elicited at potentials positive to −60 mV. They were characterized by a rapid rising phase that peaked and then slowly inactivated to a steady state level (Fig. 8*A*). Both the peak- and steady state current-voltage relationships are bell-shaped. The maximum inward current occurs at approximately +10 mV and reverses at potentials positive to +40 mV (Fig. 8*C*). The relationship between integrated Ca^{2+} entry and voltage mirrors that of current with voltage. Maximum Ca^{2+} influx occurred at approximately +10 mV (Fig. 8*D*).

After 5 min of perfusion of 10^{-10} M miniglucagon, a concentration that inhibits insulin secretion by 93% (Fig. 2*A*), the Ca^{2+} currents remained unchanged in seven cells tested (two examples displayed in Fig. 8*B*). Neither the peak nor the steady state current-voltage relationship were affected by the hormone (Fig. 8*C*). The integral Ca^{2+} entry was also unaffected by the peptide (Fig. 8*D*). A lack of effect of 10^{-10} M miniglucagon on Ca^{2+} currents was also observed in single beta cells isolated from normal mice (data not shown, $n = 4$). These data indicate that miniglucagon does not reduce Ca^{2+} influx and insulin secretion from MIN6 cells by a direct block of the Ca^{2+} channels. Somatostatin, at a concentration (10^{-9} M) that inhibits insulin secretion by 45% was also without effect on Ca^{2+} currents (data not shown, $n = 2$), as previoulsy shown in beta cells isolated from normal mice (30).

Electrophysiology, Membrane Potential—We next investigated the effect of the peptide on the membrane potential and electrical activity induced by glucose. The electrical activity of the beta cell results from the complex interplay of several different ionic conductances (29). Therefore, changes in electrical activity can be used as a sensitive detector of the effect of drugs and hormones on ion channels. Furthermore, glucose-induced insulin secretion is directly controlled by the Ca^{2+} influx that results from the Ca^{2+}-dependent action potential activity induced by this secretagogue. Therefore, any minor change in ion channel behavior and electrical activity will have major effects on insulin secretion.

In the absence of exogenous metabolite, the membrane potential of the MIN6 cell was electrically silent with a mean value of −65 ± 1 mV ($n = 18$). In 19 cells tested, the membrane potential began to depolarize within 33 ± 13 s ($n = 11$) of the addition of 10 mM glucose. After 51 ± 4 s, the membrane potential was sufficiently depolarized (−48 ± 2 mV, $n = 8$) to evoke electrical activity (Fig. 9*A*). In the majority of cases, this consisted of continuous firing of action potentials. However in three instances, the clustering of action potentials into burst-like electrical activity was observed. These were reminiscent of

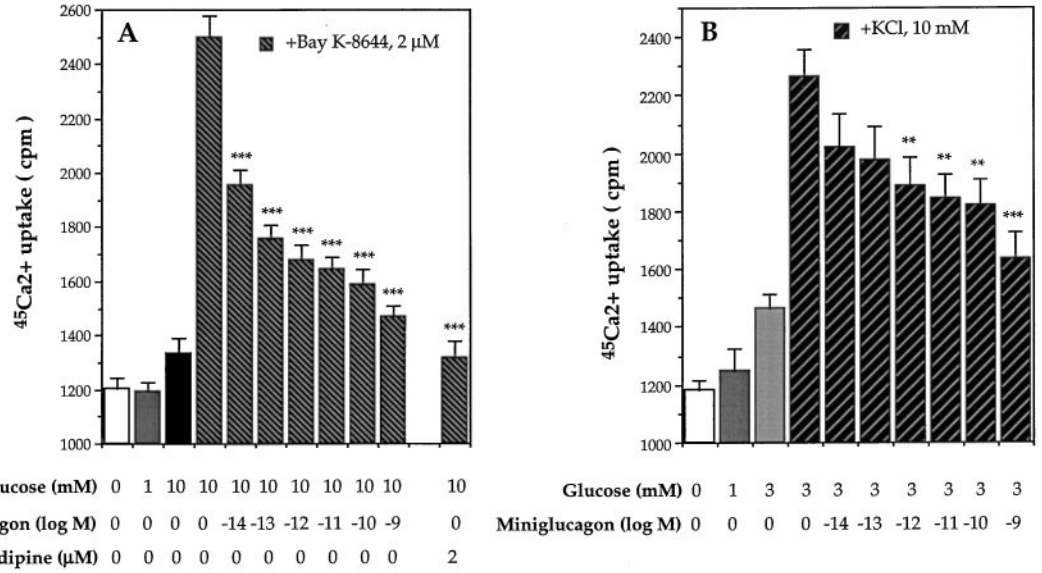

FIG. 7. **Effects of miniglucagon on $^{45}Ca^{2+}$ uptake triggered by opening of voltage-dependent Ca^{2+} channels.** $^{45}Ca^{2+}$ uptake was measured as described under "Experimental Procedures." The test agents were present simultaneously. *A*, 3-min incubation with 10 mM glucose and a fixed (2 μM) concentration of Bay K-8644 with or without various concentrations of miniglucagon or 2 μM nifedipine. Data are means ± S.E. of nine determinations. *B*, 3-min incubation with 3 mM glucose and 10 mM KCl with or without various concentrations of miniglucagon. Data are means ± S.E. of six determinations. Statistical significance was determined by comparing the data obtained in the presence and in the absence of miniglucagon (*panels A* and *B*). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

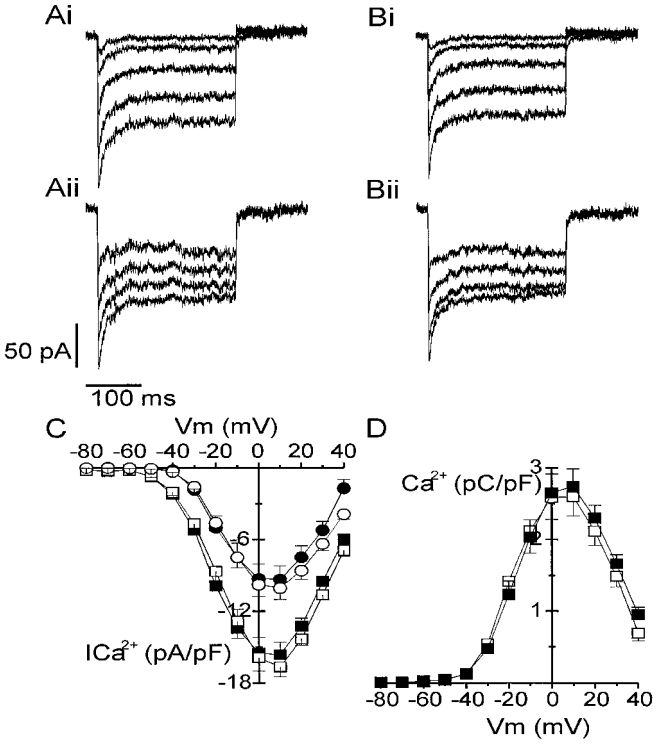

FIG. 8. **Calcium currents.** *Panels A* and *B*, representative perforated patch whole-cell recordings of Ca^{2+} currents from single MIN6 cells. Currents were elicited by 250-ms pulses from a holding potential of −70 mV to potentials of: −40, −30, −20, −10, and 0 mV (i); +10, +20, +30, and +40 mV (ii). *A*, Ca^{2+} currents recorded in control. *B*, Ca^{2+} currents recorded after 5 min in 10^{-10} M miniglucagon. *C*, mean peak (*squares*) and steady-state (*circles*) current-voltage relationships for Ca^{2+} currents recorded in control (*filled symbols*) and after 10^{-10} M miniglucagon (*open symbols*) ($n = 7$). *D*, mean integral Ca^{2+} entry recorded in control (*filled symbols*) and after 10^{-10} M miniglucagon (*open symbols*) ($n = 7$).

the typical bursting behavior observed in the intact islet (data not shown) (29, 31). In three of nine cells tested, 10^{-10} M miniglucagon reduced the frequency of action potential firing. In four cells tested, 10^{-9} M miniglucagon consistently caused the membrane potential to hyperpolarize, which terminated the electrical activity. The membrane potential hyperpolarized to −64 ± 4 mV, a value close to that found in the absence of glucose (−68 ± 2 mV for the same cells). The hyperpolarization was also associated with a decrease in voltage noise and a reduction in the input resistance of the cell. Taken together these data are consistent with miniglucagon activating a potassium conductance. A 10-fold high dose of miniglucagon (10^{-8} M) had similar effects.

In three cells tested, 10^{-7} M somatostatin produced very similar effects to 10^{-9} M miniglucagon: hyperpolarization of the membrane potential and abolition of the electrical activity. These effects of somatostatin are very similar to those that have been previously reported for beta cells, isolated from normal mouse using a similar concentration of peptide (30, 31).

Effect of Pertussis Toxin Pretreatment of MIN6 Cells on Miniglucagon Action—It is known from other studies (32) that miniglucagon is active through a specific receptor linked to at least one type of GTP binding protein (G protein). To determine the type of G protein involved in the action of miniglucagon on beta cells, we analyzed whether pretreating MIN6 cells with pertussis toxin would modify the effectiveness of miniglucagon on secretagogue-induced insulin release. First, both control and toxin-pretreated MIN6 cells (200 ng/ml, overnight) responded to an elevation of glucose from 1 to 25 mM with an increased rate of insulin release, although toxin-pretreated cells consistently showed a higher secretory rate at glucose 10 mM (data not shown). An overnight pretreatment of the cells with 200 ng/ml pertussis toxin completely suppressed the inhibitory effect of 0.1 μM somatostatin and 1 nM miniglucagon on 10 mM glucose-induced insulin release (Fig. 10). Because it was shown that adrenaline may activate, via the α2 receptors, potassium channels in a G protein-sensitive manner (38), we have verified that yohimbine, an α2-receptor blocker, did not interfere with the miniglucagon action. Indeed, 10 mM glucose-induced insulin release by MIN6 cells was inhibited by 0.1 nM miniglucagon to the same extent in the absence and in the presence of 10 μM yohimbine (data not shown).

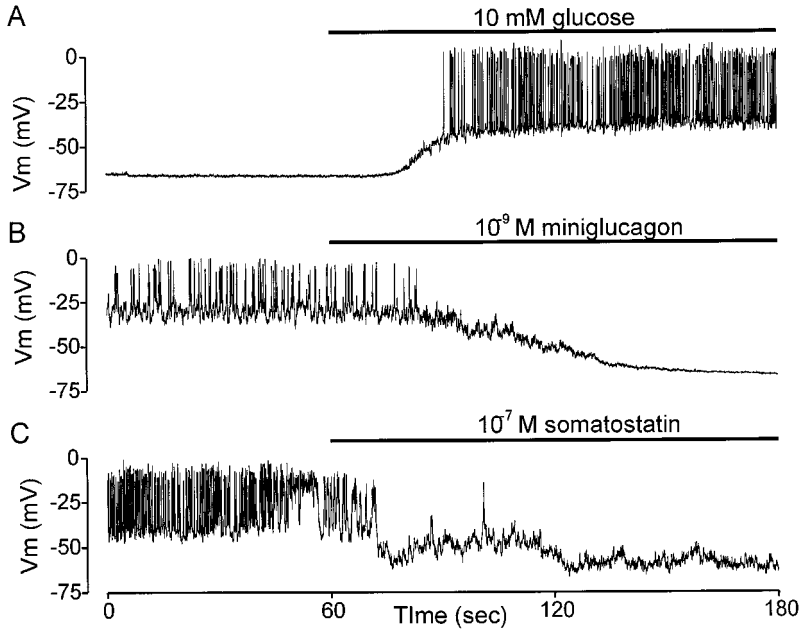

FIG. 9. **Membrane potential.** Representative perforated patch current-clamp recordings of the membrane potential of a MIN6 cell. *A*, response to 10 mM glucose; *B*, response to 10^{-9} M miniglucagon in the continued presence of 10 mM glucose; *C*, response to 10^{-7} M somatostatin in the continued presence of 10 mM glucose. All three records are from the same cell. Because of the slow time course of recovery, only the onset of the responses are illustrated.

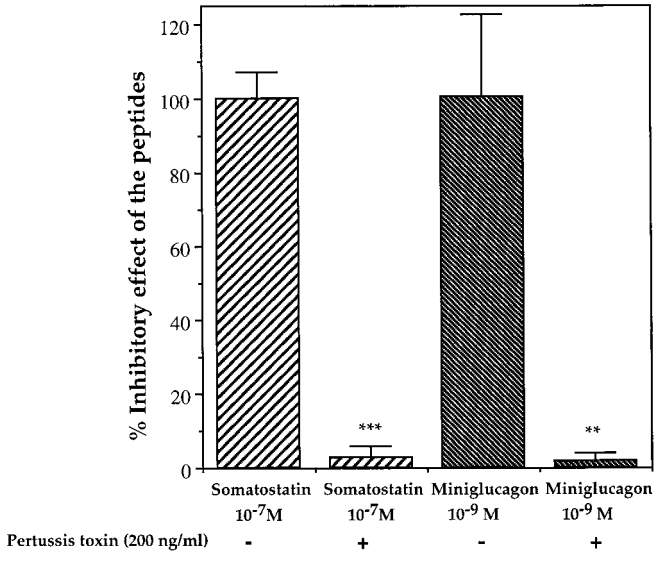

FIG. 10. **Reversal of somatostatin and miniglucagon inhibition of insulin release from MIN6 cells by pertussis toxin.** MIN6 cells were incubated overnight in culture medium containing 200 ng/ml *Bordetella* pertussis toxin or in standard culture medium. Statistical significance was determined by comparing the data obtained in the presence and in the absence of *Bordetella* pertussis toxin. **, $p < 0.01$; ***, $p < 0.001$.

DISCUSSION

Within the general mechanisms of proglucagon processing leading to various peptide fragments with different biological roles (7), processing of glucagon into miniglucagon at its Arg^{17}-Arg^{18} doublet displays many singularities. (i) The site is not a typical processing site for prohormone convertases (PCs) which use mostly Lys-Arg or Arg-Lys doublets (33, 34) or more complex combinations such as the R-*X*-K/R-R used by furin (35). On the other hand, we showed that glucagon is cleaved at its dibasic site by an original protease referred to as MGE, which was isolated from liver membranes (6) and characterized as a 100-kDa protein (6). The C-terminal product of the reaction, glucagon 19–29 or "miniglucagon," displays original features, in particular it modulates the hepatic plasma membrane calcium pump (10) and plays a role in the inotropic and chronotropic action of glucagon (11). Its activity is mediated by a specific receptor that remains to be characterized but that is already known to be linked to G proteins (32). (ii) The miniglucagon action is observed at picomolar concentrations, that is 2 to 3 orders of magnitude lower than the active concentrations of glucagon, the mother hormone. (iii) The strikingly high clearance rate of the peptide from circulation, mostly because of its very rapid degradation by the liver (8, 13), precludes any hormonal status. Accordingly, a role as a "daughter peptide", released locally from circulating glucagon and modulating the action of the mother hormone just before being degraded, was established (7, 12). On the other hand, the presence of miniglucagon in pancreas (13) and in a pancreatic alpha cell line[2] at molar concentrations ranging from 2 to 5% of that of glucagon suggested a role for miniglucagon in islet physiology. Our study was designed in light of this background.

As an *in vitro* model of pancreatic beta cell, we used the MIN6 cell line which displays most of the features of authentic beta cells, in particular the graded release of insulin in response to physiological concentrations of glucose (14) and to several other insulin-secretagogues. Here we show that miniglucagon is a highly potent and efficient inhibitor of insulin release. In view of the miniglucagon concentrations in the alpha cells (2 orders of magnitude lower than that of glucagon) and if we suppose, as a working hypothesis, that glucagon and miniglucagon are released at the same rate, then it is logical to expect an effect of miniglucagon in the 10^{-11} M range because glucagon acts on beta cells, and on other target cells, in the nanomolar range (36). The threshold effects of miniglucagon observed in the 10^{-13} to 10^{-12} M range gives miniglucagon the status of a possible local regulator of insulin release even if the peptide is released at a much lower rate than glucagon. Further studies are required, however, to analyze the conditions under which miniglucagon is released from the alpha cells to get further insight in the precise role of miniglucagon in islet physiology.

Miniglucagon has the characteristics of a very potent and efficient inhibitor of insulin release triggered by secretagogues known to act largely via calcium influx through voltage-dependent calcium channels. These channels may be activated by membrane depolarization induced either by changes in extracellular K^+ concentration or via closure of K_{ATP} channels (*e.g.*

[2] P. Blache and M. Dufour, unpublished data.

glucose and sulfonylurea), by cyclic AMP-dependent protein kinase phosphorylation (*e.g.* glucagon and tGLP-1), or by direct action of a pharmacological agent (*e.g.* Bay K-8644). We report here that miniglucagon is able to completely inhibit calcium uptake elicited by glucose, high extracellular K^+, glucagon, or tGLP-1 and Bay K-8644. It was thus of particular importance to determine how miniglucagon acts on calcium channels. First, as miniglucagon is unable to modify cyclic AMP levels, we can conclude that the peptide does not modulate L-type voltage-sensitive Ca^{2+} channels via cyclic AMP-dependent protein kinase phosphorylation. Another possibility was that miniglucagon acts directly on Ca^{2+} channels via a G protein, as shown for acetylcholine (37). However, from electrophysiological experiments, we were able to determine that the peptide does not have a direct effect on the beta cell Ca^{2+} channels. Third, miniglucagon was able to alter transmembranous cationic fluxes, as manifested by hyperpolarization, and to decrease the incidence of spike activity elicited by glucose. This provides a strong indication that miniglucagon acts by opening potassium channels, triggering a membrane hyperpolarization and thus suppressing the ability of secretagogues to open voltage-sensitive Ca^{2+} channels. It is noteworthy that, similarly to somatostatin, galanin or adrenaline (30, 38), the miniglucagon receptor is linked to its main effector (probably the potassium channels) via a pertussis-sensitive G protein. On the other hand, one of the originalities of miniglucagon is that it has no effect on cyclic AMP, indicating that a peptide may trigger a deep and long term inhibition of insulin release without modifying this parameter and suggesting that the miniglucagon receptor interacts with a G protein, or a set of G proteins that differ from that interacting with somatostatin and galanin receptors.

Determining the precise type of G protein involved in the miniglucagon action, characterizing the beta cell miniglucagon receptor linked to this G protein and analyzing the physiological conditions of miniglucagon release inside the islets, in a dependent or independent manner as compared with that of glucagon, are new issues in the development of this research on this newly discovered potential physiological mechanism by which an islet cell may control insulin release. Analysis of this mechanism in pathological states may also shed a new light on particular aspects of diabetes mellitus.

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