Expression of islet inducible nitric oxide synthase and inhibition of glucose-stimulated insulin release after long-term lipid infusion in the rat is counteracted by PACAP27

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Chronic elevation of plasma FFA impairs the insulin-secreting response to glucose in both humans and animals (8, 31, 32, 34, 36, 45, 51), and this hyperlipidemia has been suggested to be closely linked to type 2 diabetes (24). We have previously reported that nutrient-stimulated insulin secretion was markedly disrupted after lipid infusion in rats (8, 31, 34–36, 39) and that this insulin secretory defect could be reversed by agents stimulating cyclic AMP production (34, 35, 39). We also found that islets of lipid-infused rats exhibited an increased nitric oxide (NO) production due to the induction of inducible NO synthase (iNOS) (34, 35).

In a recent study (39), we compared the closely related cyclic AMP-producing peptides pituitary adenylate cyclase-activating polypeptide (PACAP)38, PACAP27, and VIP with regard to their effects on the impairment of glucose-stimulated insulin release and islet cyclic AMP production in lipid-infused rats. Since we found that the insulin secretory response as well as islet cyclic AMP generation in lipid-infused compared with control rats was markedly increased by all three neuropeptides, although less so by VIP, we considered PACAP27 to be an interesting cyclic AMP-producing agent that was possibly capable of suppressing the deleterious action of iNOS expression and activity during the development of islet lipotoxicity. We (35) have shown previously that the incretin hormone glucagon-like peptide-1 (GLP-1) as well as cyclic AMP itself (dibutyryl cyclic AMP) counteracted the lipid-induced expression and activity of iNOS in islet tissue. Our study of the effects of GLP-1 on islet NOS activities in lipid-infused rats was performed by incubating isolated islets at high glucose (20 mmol/L), because the gut hormone GLP-1 is secreted in association with food intake when the plasma glucose levels are raised. In contrast, PACAP is localized to islet nerve fibers and to the islet secretory granules (11, 29, 48, 49) and is thought to be involved in neurally-induced islet effects also in the presence of lower and more basal plasma glucose concentrations just as, e.g., during the cephalic phase of insulin secretion as well as in other situations of increased neural activity in the islets. Moreover, the effects of both GLP-1 and PACAP seem to be strongly related to cyclic AMP generation, although they use different types of G protein-coupled receptors (11, 12, 17, 19, 43), and GLP-1 has been shown to activate a multitude of other signaling pathways in the β-cells (17), whereas the effects of PACAP are currently less well understood and more controversial (10–13, 19, 29, 41, 43, 50). In addition, GLP-1...
has been shown to inhibit glucagon secretion and exert a blood glucose-lowering effect, whereas PACAP increases glucagon secretion and induces hyperglycemia (12, 13, 17). In the present study, we wished to explore possible beneficial actions of PACAP27 in islets from lipid-infused rats in a situation where the islets were incubated at a glucose concentration within a normal, nonfasting range, with special regard to iNOS expression and activity in relation to insulin secretion and the cyclic AMP/PKA pathway.

Hence, the main questions to be answered were the following. 1) Is the previously reported expression of islet iNOS (Western blot) after lipid infusion (34) localized to the β-cells? 2) Is iNOS expression in islets from lipid-infused rats still evident after in vitro incubation of isolated islets at a physiological glucose concentration (8.3 mmol/l), and if so, is PACAP27 capable of counteracting and abrogating iNOS expression? 3) Are the effects of cyclic AMP on NOS activities dependent and/or independent of PKA, and how is the pattern of cyclic AMP vs. cyclic GMP related to PACAP27-induced insulin release in islets from lipid-infused rats? 4) Is there a role for the proteasome in islet iNOS regulation in relation to the effects of PACAP27?

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (B&K, Sollentuna, Sweden) weighing 200–225 g were used in all experiments. The rats intended for lipid infusion were anesthetized intraperitoneally with 5% chloral hydrate before operation. The neck of the rat was shaved and the operative field washed with iodine solution. The operation was performed under sterile conditions. A silicon rubber catheter was inserted into the right external jugular vein. The catheter was delivered to the skull subcutaneously and connected to a swivel via a protective coil. The skull subcutaneously and connected to a swivel via a protective coil attached to the skin of the skull. When the lipid-based solution composed for TPN was infused, the catheter was flushed with 100 U·kg⁻¹·day⁻¹ of low-molecular-weight heparin (Fragmin; Pharmacia, Uppsala, Sweden) every second day. The rats serving as freely fed controls underwent the same operative procedure (“sham” operation), but no catheter was inserted because the rats did not eat appropriately during continuous infusion of saline. A detailed description of the methodology used and the composition of the lipid-based (Intralipid; Pharmacia) TPN solution has previously been reported (8, 34–36). The animals were infused for 8 days, and there was no difference in body weights of control and lipid-infused rats at the end of the experiments. Furthermore, as recently shown (36), blood glucose levels are within the normal range and the basal plasma insulin levels slightly enhanced after 8 days of lipid infusion. The concentrations of FFA, triglycerides, and cholesterol in serum, however, were greatly increased by 150, 80, and 90%, respectively (36).

Chemicals. Bovine serum albumin was from ICN Biochemicals (High Wycombe, UK). PACAP27 was from Peninsula Europe (Uppsala, Sweden) and MG-132 from Calbiochem (La Jolla, CA). The radioimmunoassay kits for cyclic AMP and cyclic GMP measurements were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The radioimmunoassay kits for insulin determination was obtained from Diagnostika (Falkenberg, Sweden). All other drugs and chemicals were from Sigma Chemical (St. Louis, MO) or Merck (Darmstadt, Germany).

Isolation of pancreatic islets. Preparation of isolated pancreatic islets from the rat was performed with the Gotoh method, i.e., retrograde injection of a collagenase solution via the bile-pancreatic duct, as previously described (33). Islets were collected under a stereomicroscope at room temperature, thoroughly washed, and then used immediately for the different experiments. Insulin content in the islets was measured after acid ethanol extraction, as previously described (37).

In vitro experiments. The freshly isolated islets were preincubated for 30 min at 37°C in Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4, supplemented with 10 mmol/l HEPES, 0.1% bovine serum albumin, and 1.0 mmol/l glucose as previously described (16, 33). Each incubation vial contained 50 islets in 1.5 ml of buffer solution and was gassed with 95% O₂-5% CO₂ to obtain constant pH and oxygenation. After preincubation the buffer was changed to a medium supplemented with test agents, and the islets were incubated for 60 min. All incubations were performed at 37°C in an incubation box (30 cycles/min). Immediately after incubation, aliquots of the medium were removed for assay of insulin (33). As far as the authors are aware, the actual local concentration of PACAP in islet tissue is not known. Therefore, we used a concentration of PACAP27 (100 nmol/l) that was previously found to be the maximal insulin-releasing concentration during our present in vitro conditions (39).

Confocal microscopy. The freshly isolated islets as well as the incubated islets were fixed with 4% formaldehyde and permeabilized with 5% Triton X-100, and unspecific sites were blocked with 5% normal donkey serum (Jackson Immunoresearch Laboratories, West Grove, PA). iNOS was detected with the corresponding rabbit-raised primary antibody (BD Transduction Lab, San Jose, CA) in combination with Cy2-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories). For staining of insulin, islets were incubated with a guinea pig-raised anti-insulin antibody (Eurodiagnostica, Malmö, Sweden) followed by a Cy5-conjugated anti-guinea pig IgG antibody (Jackson Immunoresearch Laboratories). Fluorescence was visualized with a Zeiss LSM510 confocal microscope by sequentially scanning at (excitation/emission) 488/505–530 nm (Cy2) and 633/>650 nm (Cy5).

Assay of islet NOS activities. Preincubation and incubation of freshly isolated islets were performed as stated above, with the exception that each incubation vial contained 200 islets in 1.5 ml of buffer solution. After incubation, aliquots were removed for insulin determination. The islets were then thoroughly washed and collected in 200 μl of buffer solution containing 20 mmol/l HEPES, 0.5 mmol/l EDTA, and 1 mmol/l trithiothreitol and immediately frozen at −20°C. On the day of assay the islets were sonicated on ice, and for nNOS activity, the buffer solution was supplemented to also contain 0.45 mmol/l CaCl₂, 2 mmol/l NADPH, 25 U/ml calmodulin, and 0.2 mmol/l l-arginine. For the determination of iNOS activity, both Ca²⁺ and calmodulin were omitted (16). The homogenate was then incubated at 37°C under constant air bubbling, 1.0 ml/min for 2 h. Aliquots of the incubated homogenate (200 μl) were then passed through a 1-ml Amprep CBA cation exchange column for high-performance liquid chromatography analysis of the l-citrulline formed. The methodology has previously been described in detail (33). Since L-citrulline is created in equimolar concentrations to NO, and since l-citrulline is stable, whereas NO is not, l-citrulline is the preferred parameter when measuring NO production. Protein was determined according to Bradford (4) on samples from the original homogenate.

Western blot analysis. Approximately 250 islets (n = 4 in each group) were collected in Hanks’ buffer (100 μl) and sonicated on ice (3 × 10 s). Homogenate samples representing 20 μg of total protein from islet tissue were then run on 10% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes by electrotransfer (10–15 V, 60 min) (semidy transfer cell; Bio-Rad, Richmond, CA). The membranes were blocked in 9 mmol/l Tris-HCl (pH 7.4) containing 5% nonfat milk powder for 40 min at 37°C. Immunoblotting with rabbit anti-mouse iNOS (N-7782; Sigma) was performed for 16 h at room temperature. The membrane was washed twice and then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:10,000; Sigma) for 90 min. Antibody binding to iNOS was detected using 0.25 mmol/l CDP-Star (Tropix, Bedford, MA) for 5 min at room temperature. The chemiluminescence
Signal was visualized by exposing the membranes to Dupont Cronex X-ray films for 1–5 min. An appropriate standard, i.e., molecular mass markers, was run in all analyses.

Measurement of islet cyclic AMP and cyclic GMP. After incubation the islets were thoroughly washed in glucose-free KRB buffer and collected and stored in 500 µl of ice-cold 10% trichloroacetic acid (TCA) containing the phosphodiesterase inhibitor IBMX (0.2 mmol/l), followed by immediate freezing in a −70°C ethanol bath. Before assay 500 µl of H₂O was added, and the samples were sonicated (3 × 5 s) followed by centrifugation (1,100 g) for 15 min. As previously described (27, 35), the supernatants were then collected and extracted with water-saturated diethyl ether (4 × 2 ml). The aqueous phase was removed and freeze-dried, using a Lyovac GT 2 freeze dryer. The residue was then dissolved in 450 µl of Na-acetate buffer (50 mmol/l, pH 6.2). The amounts of cyclic AMP and cyclic GMP were quantified with 125I-cyclic AMP and 125I-cyclic GMP radioimmunoassay kits (Rianen; Du Pont, Boston, MA). [3H]cyclic GMP was added to the TCA islet homogenate to determine the recovery of cyclic AMP and cyclic GMP during the ether extraction. The mean recovery was 90%.

Statistics. Probability levels of random differences were determined by analysis of variance followed by Tukey-Kramer multiple comparison test. Results are expressed as means ± SE.

RESULTS

Expression of iNOS protein in islets from control and lipid-infused rats. In the first experiment we wished to study, by confocal microscopy, the influence of long-term lipid infusion on iNOS expression in the pancreatic islets. Insulin immunoreactivity is seen in Fig. 1, A and D. As shown in Fig. 1B, no iNOS immunoreactivity was observed in islets isolated from freely-fed control rats, whereas after lipid infusion a majority of islet cells showed a strong immunoreactivity for iNOS (Fig. 1E). Double immunolabeling of islets from lipid-infused animals showed that most iNOS-immunoreactive cells also expressed insulin immunoreactivity (Fig. 1F). The immunocytochemical findings after lipid infusion were confirmed by Western blot analysis showing that there was a marked expression of iNOS protein in these islets (Fig. 1G). The insulin content of islets isolated from control vs. lipid-infused rats was 6.75 ± 0.60 vs. 5.46 ± 0.18 nmol insulin/mg protein (not significant) in islets taken from six rats in each group.

Confocal microscopy of the effects of PACAP27 on iNOS expression in incubated islets from control and lipid-infused rats. We recently observed that raising islet cyclic AMP levels brought about a marked suppression of the increased iNOS activity seen in islets exposed to high glucose (16, 34, 35). Hence, the next series of experiments was designed to explore the effects of PACAP27 on the lipid-induced expression of islet iNOS after incubation at a modest physiological concentration of glucose (8.3 mmol/l), which by itself does not induce iNOS (30). The islets were isolated directly after stopping the lipid infusion. Figure 2 shows a confocal micrograph of the
islets after incubation in the presence of 8.3 mmol/l glucose with or without addition of PACAP27 (100 nmol/l). As shown in Fig. 2, B and E, no iNOS immunoreactivity was detected in control islets after incubation at 8.3 mmol/l glucose in either the absence (Fig. 2B) or presence (Fig. 2E) of PACAP27. In contrast, the expression of iNOS induced by lipid infusion was still evident in such islets after the incubation period (Fig. 2H). Addition of PACAP27 to the incubation medium suppressed the expression of iNOS in islets from lipid-infused rats (Fig. 2K). Double immunostaining of insulin and iNOS shows that insulin and iNOS immunoreactivities are confined to the same cells (Fig. 2I).

**Influence of PACAP27 on neuronal constitutive NOS and iNOS activities and glucose-stimulated insulin secretion in incubated islets from control and lipid-infused rats.** In the following experiments, we studied the influence of PACAP27 in the presence of 8.3 mmol/l glucose on islet neuronal constitutive (nc)NOS and iNOS activities in relation to insulin release. Figure 3A shows that PACAP27 (100 nmol/l) suppressed ncNOS activity in association with a concomitant potentiation of insulin release in islets taken from control rats and then incubated at 8.3 mmol/l glucose (Fig. 3C). No iNOS activity could be detected in the control islets (Fig. 3A). As illustrated in Fig. 3B, pancreatic islets taken from lipid-infused rats

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**Fig. 2.** Confocal micrographs of rat islets. Islets isolated from controls or lipid-infused rats were incubated for 60 min in the presence of 8.3 mmol/l glucose (A–C: controls; G–I: lipid infused) or glucose + pituitary adenylate cyclase-activating polypeptide (PACAP)27 (100 nmol/l) (D–F: controls; J–L: lipid infused). After incubation, the islets were double immunostained for insulin (red) and iNOS (green) and analyzed by confocal microscopy. *I:* colocalization of insulin-iNOS appears as yellowish fluorescence. Bars, 20 μm.
and then incubated in the presence of 8.3 mmol/l glucose showed a slight increase in ncNOS activity ($P < 0.05$) and a marked appearance of iNOS activity compared with islets from controls (Fig. 3, A and B). Hence, the total NO production in the islets was greatly elevated after lipid infusion (compare total NO in Fig. 3, A and B). The addition of PACAP27 to the incubated islets markedly suppressed the activity of ncNOS in control islets (Fig. 3A) and both ncNOS and iNOS and thus the total NO activity in lipid-infused rats (Fig. 3B). This suppressive effect of PACAP27 on iNOS and ncNOS activities was reversed, and the activities of both isoenzymes were even modestly increased when H-89 (an inhibitor of PKA), 2 $\mu$mol/l, was added to the incubation medium (Fig. 3B). In parallel to the suppressive effects of PACAP27 on islet iNOS and ncNOS activities in islets isolated after lipid infusion, the glucose-stimulated insulin secretion was markedly amplified (Fig. 3C). However, addition of H-89 together with PACAP27 to islets from lipid-infused rats did not completely reduce the PACAP-induced amplification of glucose-stimulated insulin release back to the “+PACAP” level (Fig. 3C). Hence, the “+PACAP + H-89” effect was significantly greater ($P < 0.01$) than “+PACAP” (Fig. 3C), and thus the inhibitory effect by H-89 constituted only ~80% of the “+PACAP” stimulatory effect on insulin release.

**Effect of PACAP27 on islet cyclic AMP and cyclic GMP production in relation to glucose-stimulated insulin secretion in control and lipid-infused rats.** To further study the influence of PACAP27 on lipid-induced NO production in relation to the cyclic AMP/PKA system and possible interaction with cyclic GMP, we measured cyclic AMP and cyclic GMP levels in incubated islets taken from control and lipid-infused rats after addition of PACAP27 to the incubation medium. The islets were thereby incubated in the presence of 8.3 mmol/l glucose in the absence or presence of PACAP27 (100 nmol/l). As shown in Fig. 4A, the insulin secretion was slightly lower ($P < 0.05$) and the cyclic AMP level slightly increased ($P < 0.05$; Fig. 4B), whereas the cyclic GMP level was greatly elevated ($P < 0.001$; Fig. 4C) in the presence of 8.3 mmol/l glucose in islets isolated from lipid-infused animals compared with the controls. Addition of PACAP27 to the incubation medium resulted in a marked suppression of cyclic GMP levels in the islets of lipid-infused rats. In contrast, PACAP27 did induce a much greater increase in cyclic AMP levels in islets of lipid-infused animals than in control islets. The insulin secretory response to PACAP27 in islets taken from lipid-infused rats was also much greater compared with control islets (Fig. 4A).

Confocal microscopy of the effect of the proteasome inhibitor MG-132 on iNOS expression in incubated islet from lipid-infused rats. Since PACAP27 through the action of the cyclic AMP/PKA system induced the loss of iNOS protein expression and activity in the islets from lipid-infused rats during incubation, we next wished to study the involvement of the proteosome system in this process since the proteasome has been suggested to modulate iNOS expression in other cell types (20). Figure 5 shows that the lipid-induced expression of iNOS (Fig. 5, A–C) was highly suppressed by PACAP27 (Fig. 5, D–F) in the β-cells of the incubated islets and that this suppression was unaffected after treatment with MG-132 (10 $\mu$mol/l; Fig. 5, G–J). Furthermore, and surprisingly, MG-132 itself induced a marked suppression of the lipid-induced expression of iNOS (Fig. 5, J–L).

**DISCUSSION**

**General observations.** The metabolic syndrome and diabetes are often associated with an abnormal lipid metabolism (24, 25, 45, 51), and most type 2 diabetic patients exhibit an elevated plasma level of FFA in addition to increased plasma glucose concentrations (24). Although the pathogenesis of type 2 diabetes is multifactorial in origin, the pancreatic β-cell plays a crucial role in the development of the disease (24, 25, 40, 45, 51). Chronic elevation of plasma levels of FFA has been attributed to β-cell dysfunction (24, 25, 40, 45, 51), and we have recently found (39) that PACAP is able to reverse the impaired glucose-stimulated insulin release in isolated islets from lipid-infused rats. We (34, 35) and others (40, 45) have hypothesized that an important contribution to such a β-cell dysfunction might be exerted by FFA-induced expression and activity of islet iNOS. We now show here by confocal microscopy that our previously reported data (34) on iNOS expression, as determined by Western blot in islets from lipid-infused rats, could be confined to the insulin-producing β-cells. We also show that the expression of β-cell iNOS persists after short-term incubation of isolated islets in a physiological non-
fasting glucose concentration (8.3 mmol/l) and that this iNOS expression is abrogated by addition of PACAP27 to the incubation medium. Furthermore, in parallel experiments we also show by biochemical determination of NOS activities that PACAP27 greatly suppressed both iNOS and ncNOS activities concomitant with increased insulin release and that the PKA inhibitor H-89 could reverse these effects, suggesting that the suppressive action of PACAP27 on the NOS activities is transduced mainly through the PKA pathway. Finally, we show that the effects of PACAP27 in isolated islets from lipid-infused rats are associated with greatly increased levels of cyclic AMP and greatly decreased levels of cyclic GMP and that the rapid loss of iNOS protein expression is less likely to be regulated by proteosomal activity.

Long-term lipid infusion in rats is considered a good model for investigating the effect of increased plasma levels of lipids on β-cell function and insulin secretion (8, 31, 34, 36, 39). We (36) have previously shown that lipid-infused animals are characterized by hyperlipidemia (FFA, triglycerides, and cholesterol), but at the same time they display normoglycemia, and there is no significant difference in blood glucose levels in these rats compared with freely-fed control rats. From these results, we have therefore suggested that, in our rat model, the increased NO production, exerted by a marked expression and activity of islet iNOS, and the associated reduction of the insulin-secretory capacity of the β-cell is a consequence of increased plasma lipids and not due to glucose or a combination of elevated levels of both glucose and FFA (28) that might be still more deleterious (28). In the present study, we could now show by confocal microscopy that the expression of iNOS was manifested throughout the entire islet and colocalized with insulin and thus was harbored in the β-cells. This further indicates that the observed biological effects in the islets upon induction of iNOS by lipid infusion might be a contributing factor to the insulin secretory defect seen in these animals. Since the islet insulin content was similar in both categories of rats, the observed changes in insulin secretion could not be readily explained by a difference in insulin storage. Notably, cyclic GMP seems to not primarily be a positive modulator of glucose-stimulated insulin release in lipid-infused rats, since we show here that an excessive increase in islet cyclic GMP content was associated with a significant reduction in insulin secretion. The slight increase in cyclic AMP content found in the islets of lipid-infused rats was probably exerted through the cyclic GMP-inhibitable cyclic AMP phosphodiesterase. Such an effect has previously been reported (42) in other tissues.

In contrast with the consistent experimental support of a critical role of iNOS for degenerative and apoptotic processes in β-cells, significant controversy exists with regard to the role played by ncNOS in the regulation of insulin secretion (16, 22, 34, 44). A clear indication that ncNOS-derived NO deserves consideration as a negative modulator of glucose-stimulated insulin release is coming from the findings that inhibition of ncNOS by different NOS inhibitors positively affects the insulin response to glucose (1, 14–16, 27, 30, 38, 44). Moreover, we (16) have recently shown that stimulation of ncNOS-derived NO inhibits glucose-stimulated glucose-stimulated insulin release is coming from the findings that inhibition of ncNOS by different NOS inhibitors positively affects the insulin response to glucose (1, 14–16, 27, 30, 38, 44). Moreover, we (16) have recently shown that stimulation of ncNOS-derived NO inhibits glucose-stimulated insulin release is coming from the findings that inhibition of ncNOS by different NOS inhibitors positively affects the insulin response to glucose (1, 14–16, 27, 30, 38).

Fig. 4. Insulin secretion (A) and islet accumulation of cyclic (c)AMP (B) and cyclic GMP (C) in the absence (open bars) or presence (black bars) of PACAP27 (100 nmol/l) in islets incubated with 8.3 mmol/glucose. Islets were isolated from control or lipid-infused rats and then incubated for 60 min. Means ± SE for 6–8 batches of islets/group, isolated from 6 rats, are shown. Asterisks denote probability level of random difference: *P < 0.05; ***P < 0.001.
targets for neuronally generated NO through S-nitrosylation processes (18). In contrast with the negative impact exerted by NO on glucose-stimulated insulin release, this gaseous messenger seems to have no appreciable effect on insulin secretory mechanisms elicited by secretagogues directly activating the cyclic AMP system (2, 3, 33–35), since such secretagogues apparently act independently of regulating thiol groups (3). In fact, NO may even amplify cyclic AMP-induced insulin release (2). Hence, the detailed mechanisms of action of NO on different signaling pathways in the β-cell will await further studies.

Effects of PACAP27. Quantitative biochemical analysis of iNOS and nNOS activities in incubated islets of lipid-infused rats showed that PACAP27 dramatically reduced the activities of both isoenzymes as well as the cyclic GMP production. Regarding iNOS, this effect was further supported by double immunostaining for insulin and iNOS, showing a marked PACAP-induced reduction of the iNOS fluorescence intensity in the β-cells. The glucose-stimulated insulin secretory response of islets isolated from lipid-infused rats was strikingly enhanced by PACAP27 and even greater than in PACAP27-treated control islets, confirming our previous data (35) showing that an increase in the β-cell cyclic AMP system elicited by addition of dibutyl-cyclic AMP has a compensatory effect on NO-induced impairment of glucose-stimulated insulin release in islets from lipid-infused rats. Furthermore, there is reason to believe that the great insulin response to PACAP27 in islets from lipid-infused rats is not only associated with inhibition of
NO generation but also to the pronounced increase in cyclic AMP, which per se is known to strongly amplify glucose-stimulated insulin release (7, 35).

Notably, recent observations (7) have shown that not only is the action of cyclic AMP in the secretory process elicited through activation of PKA but also that PKA-independent direct effects on distal stages in the stimulus-secretion coupling are operating. Hence, although our present data using the PKA inhibitor H-89 speak in favor of PKA being of decisive importance for regulating islet NOS activities and insulin release in lipid-infused rats, they also show clear indications for an additional PKA-independent mechanism in this respect. In this context it is worth mentioning that PACAP reportedly attenuates glutamate-induced NO production and cytotoxicity in rat pheochromocytoma PC12 cells and that such effects could be partially reversed by H-89 (26).

The implication of a proteasomal mechanism in the loss of iNOS protein during islet incubation with PACAP27 was examined by the use of the proteasome inhibitor MG-132 (20). Surprisingly, MG-132 did not affect the PACAP-induced suppression of iNOS expression. In contrast, MG-132 by itself induced a loss of iNOS protein in islets from the lipid-infused rats. Hence, the present data indicate that proteasome inhibition in the β-cell did not prevent but stimulated the degradation of iNOS, thus suggesting more complex control mechanisms regulating the cellular balance of this protein. A similar paradoxical effect of proteasome inhibition in the β-cell was previously reported with regard to (pro)insulin, the degradation of which was increased in the presence of the proteasome inhibitor lactacystin (21). A paradoxical effect of proteasome inhibition has also been reported (20) in rat vascular smooth muscle. These findings also raise the question of whether the basic mechanisms regulating the expression and degradation of iNOS might be circumvented by the lack of appropriate signaling due to the mere presence of a proteasome inhibitor or by other hitherto less known mechanisms possibly related to endoplasmic reticulum stress (47).

In conclusion, the present investigation reveals a novel pathway by which cyclic AMP, and hence PACAP27, may stimulate and reverse the reduced insulin response to glucose from control animals. The detailed biochemical mechanism of action of PACAP27 on islet NOS isoenzymes seems to be very complex and will certainly await further studies, since regulation of NOS enzymes has been described (18) at all levels from gene transcription to covalent modifications and allosteric regulation of the enzyme itself. It should be noted that there are some potential drawbacks of PACAP as therapeutic agent in the long term, especially with regard to type 2 diabetes, since PACAP is reportedly inactivated by dipeptidyl peptidase IV and stimulates the release of glucagon and epinephrine as well as hepatic glucose production (41, 50). On the other hand, PACAP has insulin-sensitizing properties and suppresses appetite (41). The effects of PACAP on carbohydrate metabolism are thus very complex and sometimes also biphasic in that stimulation at lower doses and inhibition at higher doses might take place (41), and unpredictable results have been obtained in animals with genetic obesity showing sometimes adverse effects on carbohydrate homeostasis (13, 41). Nevertheless, PACAP agonism might hopefully be considered a future potential therapy for type 2 diabetes, since it was recently reported (43) that a rather specific agonist for the important VPAC2 receptor on the β-cell could be used in the rat without appreciable side effects known to occur after systemic administration of PACAP itself (43). Such a PACAP agonist might also be useful and provide a rationale for attempting to restore the reduced insulin secretion seen in patients undergoing TPN treatment (46) by activating the cyclic AMP/PKA pathway in their β-cells.

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