The Enteroinsular Axis in Dipeptidyl Peptidase IV–Negative Rats

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Evidence has accumulated that the incretins glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-$1_{(7\text{-}36)}$ amide) are degraded and rendered biologically inactive in plasma by the enzyme dipeptidyl peptidase IV (DPIV). A strain of Fischer rats lacking the DPIV enzyme were used in the current investigation as a model for examining the enteroinsular axis under conditions in which normal inactivation of GIP and GLP-$1_{(7\text{-}36)}$ does not occur. This was assessed by comparing GIP and GLP-$1_{(7\text{-}36)}$ responses following oral glucose in normal versus DPIV-deficient Fischer rats, and by comparing the insulinotropic potency of both peptides in the perfused pancreas of both groups. The insulin response to an oral glucose challenge was decreased slightly in DPIV-negative rats compared with control animals. Of the two incretins, the GIP response to oral glucose was reduced by 50% compared with controls, whereas GLP-$1_{(7\text{-}36)}$ release in response to glucose was unchanged. A decrease of 30% in the sensitivity of the perfused pancreas of DPIV-negative rats to GIP was observed, whereas the insulin response to $GLP_{(7\text{-}36)}$ was identical in both groups. Incubation of both peptides in plasma from DPIV-positive and -negative rats was performed to determine the effect of the presence or absence of DPIV on the insulinotropic activity of GLP-$1_{(7\text{-}36)}$ and GIP in the isolated perfused rat pancreas. Incubation in plasma from DPIV-positive rats resulted in a 65% decrease in insulinotropic activity of both incretins compared with incubation in plasma from DPIV-deficient rats. It was hypothesized that the reduced GIP response and decreased sensitivity of the pancreas to GIP are compensatory mechanisms that maintain insulin and glucose levels within a normal range despite abnormal degradation of GIP. An explanation of the lack of effect of the absence of DPIV on the GLP-$1_{(7\text{-}36)}$ response to oral glucose and insulinotropic action of this peptide must await further study.

DIPEPTIDYL PEPTIDASE IV (DPIV) is an enzyme that circulates in plasma and is associated with the plasma membrane of a variety of cells, including capillary endothelial cells, enterocytes, hepatocytes, and cells of the kidney brush border.[1] This enzyme preferentially cleaves peptides and proteins having either X-Pro, X-Hyp, or X-Ala at the *N*-terminus,[2] and to a lesser extent, X-Ser, X-Thr, and X-Val.[3] In 1986, Frohman et al[4] observed that growth hormone–releasing hormone $GRF_{(1\text{-}44)}$ was cleaved to $GRF_{(3\text{-}44)}$ by an enzyme in plasma, and subsequently identified DPIV as the enzyme responsible.[5] Furthermore, $GRF_{(3\text{-}44)}$ resulting from enzyme degradation was biologically inactive, leading the researchers to speculate that DPIV serves as a mechanism for inactivating this peptide in plasma. Since GRF belongs to the VIP/secretin/glucagon superfamily of peptides, it could be predicted that this enzyme would act on other members of the family with similar *N*-terminal sequences. Mentlein et al[6] reported that incubation of glucose-dependent insulinotropic polypeptide (GIP) or glucagon-like peptide-1 (GLP-$1_{(7\text{-}36)}$ with either serum or purified DPIV resulted in the production of $GIP_{(3\text{-}42)}$ or GLP-$1_{(9\text{-}36)}$. The intact peptides serve as substrates for DPIV, since they both have alanine residues in the penultimate *N*-terminal position. It is significant that removal of the *N*-terminal two amino acids of both $GIP^{7\text{-}9}$ and GLP-$1_{(7\text{-}36)}$[10] renders these peptides biologically inactive. Subsequent studies by our group[11] indicated that both ^{125}I-GIP and ^{125}I-GLP-$1_{(7\text{-}36)}$ are rapidly degraded in vivo (50% in <2 minutes) to ^{125}I-$GIP_{(3\text{-}42)}$ and ^{125}I-GLP-$1_{(9\text{-}36)}$, respectively. Evidence was also provided by Deacon et al[12] that GLP-$1_{(7\text{-}36)}$ degradation to GLP-$1_{(9\text{-}36)}$ in vitro in human plasma involves DPIV and that the biologically inactive *N*-terminally truncated metabolite, GLP-$1_{(9\text{-}36)}$, is a major circulating peptide in humans. Analysis of serum-incubated GIP and GLP-$1_{(7\text{-}36)}$ by matrix-assisted laser desorption/ionization–time of flight mass spectrometry indicated that both peptides were cleaved to their *N*-terminal truncated forms by DPIV, with only secondary degradation due to other serum protease activity.[13] These findings support the contention that DPIV degradation is the primary, if not exclusive, mechanism for the physiological inactivation of these two important insulin-releasing hormones (incretins) involved in the enteroinsular axis.

A strain of Fischer 344 (F344) rats originating from Japan has been shown to lack the enzyme DPIV.[14] These rats have been used in studies on the role of DPIV in the intestine and kidney,[15] and provide a model system for examining the enteroinsular axis under conditions in which normal inactivation of GIP and GLP-1 does not occur, ie, a potentially exaggerated enteroinsular axis.

The present study was undertaken to assess the role of DPIV in the enteroinsular axis in DPIV-negative rats by comparing GIP and GLP-1 release following an oral glucose challenge with that in normal Fischer rats, and the insulinotropic potency of GIP and GLP-1 in the perfused pancreas of both groups. The effect of incubating both incretins with plasma from Fischer control versus DPIV-negative rats on insulinotropic activity was also assessed.

MATERIALS AND METHODS

Animals

Japanese DPIV-negative F344 rats (250 to 300 g) were obtained from a breeding colony maintained in the Physiology Department

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Submitted November 15, 1994; accepted June 10, 1996.

Supported by research grants from the Medical Research Council of Canada and the British Columbia Health Research Foundation.

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0026-0495/96/4511-0005$03.00/0

at our institution. This colony was established with breeding pairs kindly provided by Dr F.H. Leibach (Medical College of Georgia). Aged matched F344 DPIV-positive rats were obtained from Charles River (St Constant, Quebec). Using the assay method described by Matumura,[16] plasma levels of the enzyme were measured, verifying the absence of DPIV in DPIV-negative rats used in this study.

Oral Glucose Tolerance Test

Following an overnight fast, animals were administered oral glucose by syringe and feeding tube (1 g/kg) as a 40% solution (wt/vol). Blood samples (200 μL) were collected from the tail vein of conscious unrestrained rats into heparinized capillary tubes at 0, 10, 20, 30, and 60 minutes following the glucose load. Samples were kept in ice-chilled tubes containing 250 KIU aprotinin (Miles Canada, Etobicoke, Ontario) per 0.5 mL blood and centrifuged at 4°C for 30 minutes, and the plasma was stored at −20°C until analysis for glucose or peptides. Glucose levels were measured using the glucose oxidase procedure (Beckman, Mississauga, Ontario).

Pancreas Perfusion

The surgical and perfusion procedures were performed essentially as described previously.[17] Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg, Somnotol; MTC Pharmaceuticals, Cambridge, Ontario). A midline abdominal incision was made, the aorta was located, and the superior mesenteric artery and left renal artery and vein were ligated. The duodenum was sectioned, and a drainage tube was inserted distal to the pancreas at the ligament of Treitz. The colon was ligated, and the spleen, stomach, and large intestine were removed. The aorta was cannulated distal to the celiac and superior mesenteric arteries with heparinized saline-filled PE-160 tubing, and perfusion was achieved via this route. A cannula was inserted into the portal vein for collection of venous drainage.

The perfusate consisted of Krebs bicarbonate buffer containing 0.2% bovine serum albumin–radioimmunoassay (RIA) grade (Sigma, St Louis, MO) and 3% dextran (clinical grade; Sigma) gassed with 95% O_2 and 5% CO_2 to achieve a pH of 7.4. Portal venous outflow was collected at 1-minute intervals at a rate of 4 mL/min following a 15-minute equilibration period with basal glucose (4.4 mmol/L). Two perfusion protocols were used. When the stimulus was glucose alone, perfusate containing 16 mmol/L glucose was perfused from time 5 minutes until the termination of the experiment. In experiments in which peptides were involved, perfusate containing 16 mmol/L glucose was introduced at time 5 minutes along with a GIP or GLP-$1_{(7\text{-}36)}$ gradient. This was accomplished by use of a gradient apparatus consisting of two connected perfusate flasks of equal cross-section, with the flask distal to the pancreas containing perfusate with a GIP or GLP-1 concentration of 1 nmol/L. That this apparatus delivers a linear gradient has previously been established.[17,18]

Plasma Incubation Studies

Incubation studies involved addition of 2 μg synthetic human GIP or GLP-$1_{(7\text{-}36)}$ to 1 mL plasma from overnight-fasted F344 (DPIV-positive) or DPIV-negative rats. Peptide-containing plasma samples were incubated for 5 hours at 37°C, after which plasma samples were flash-frozen with dry ice in acetone and stored at −70°C for future bioassay. At the time of bioassay in the perfused rat pancreas, the peptide preparations were added to the gradient apparatus to yield a calculated gradient of 0 to 1 nmol/L GIP or GLP-$1_{(7\text{-}36)}$ based on the original peptide concentrations in the plasma samples.

Peptides

Synthetic porcine GIP and GLP-$1_{(7\text{-}36)}$ were purchased from Peninsula Laboratories (Belmont, CA).

RIAs

Insulin level was measured by RIA as described by Pederson et al,[17] using rat insulin as standard and a guinea pig anti–human insulin serum (GPO1).

The GLP-1 antiserum (Ab 9871) (rabbit) is specific for the C-terminus of GLP-$1_{(7\text{-}36)}$ amide. Cross-reactivity of the antibody with GLP-$1_{(7\text{-}37)}$, GLP-$1_{(7\text{-}35)}$, GLP-$1_{(7\text{-}34)}$, and GLP-$1_{(7\text{-}38)}$ is 0.5% or less. Synthetic GLP-$1_{(7\text{-}36)}$ amide was used as standard and for iodinization. Radiolabeled GLP-$1_{(7\text{-}36)}$ amide (Novo Nordisk, Copenhagen, Denmark) was prepared by iodination with ^{125}I-Na using H_2O_2/lactoperoxidase at a neutral pH, and purified by reverse-phase HPLC as previously described for insulin analogs.[19]

GIP levels were measured by the RIA method of Morgan et al.[20] Synthetic porcine GIP (Peninsula) was used for standard and for iodination. A specific anti–porcine GIP serum (LMR34) was kindly supplied by Dr Linda Morgan (University of Guildford, Surrey, UK). This antibody is also C-terminally directed. Negligible cross-reactivity of this antibody was demonstrated with glucagon, GLP-$1_{(7\text{-}36)}$, secretin, and VIP.

Data Analysis

Significant differences in fasting levels of glucose, insulin, GIP, and GLP-$1_{(7\text{-}36)}$ and increases over fasting levels in vivo as well as differences between insulin levels in pancreas perfusion experiments, were analyzed by a factorial or repeated-measures ANOVA, as appropriate, followed by a Scheffé F test with a significance level of .05.

RESULTS

Oral Glucose Tolerance Test

Glucose, insulin, immunoreactive (IR)-GIP, and IR-GLP-$1_{(7\text{-}36)}$ responses to an oral glucose challenge in DPIV-positive rats were compared with those in DPIV-negative rats. For each group, n = 10. Figure 1 shows glucose, insulin, IR-GIP, and IR-GLP-$1_{(7\text{-}36)}$ responses at 10, 20, 30, and 60 minutes following glucose administration, and Fig 2 shows the integrated responses of these four parameters over the 60 minutes of the oral glucose tolerance test (OGTT). Plasma IR-GIP levels were lower in DPIV-negative versus DPIV-positive rats both in the fasting condition and at all time points sampled after glucose administration (Fig 2C). Fasting insulin levels were significantly lower in DPIV-negative animals with concomitant mild fasting hyperglycemia (Fig 1A and B). Insulin levels were also significantly lower in DPIV-negative rats at 30 and 60 minutes following oral glucose, whereas glucose levels in the same group were significantly higher at only the 10-minute time interval. Although mean IR-GLP-$1_{(7\text{-}36)}$ levels were lower at all time points for DPIV-negative versus -positive rats, differences were not significant at any time point. The integrated data indicate that only the GIP response to oral glucose differs in DPIV-negative rats compared with DPIV-positive controls (reduced by 50%).

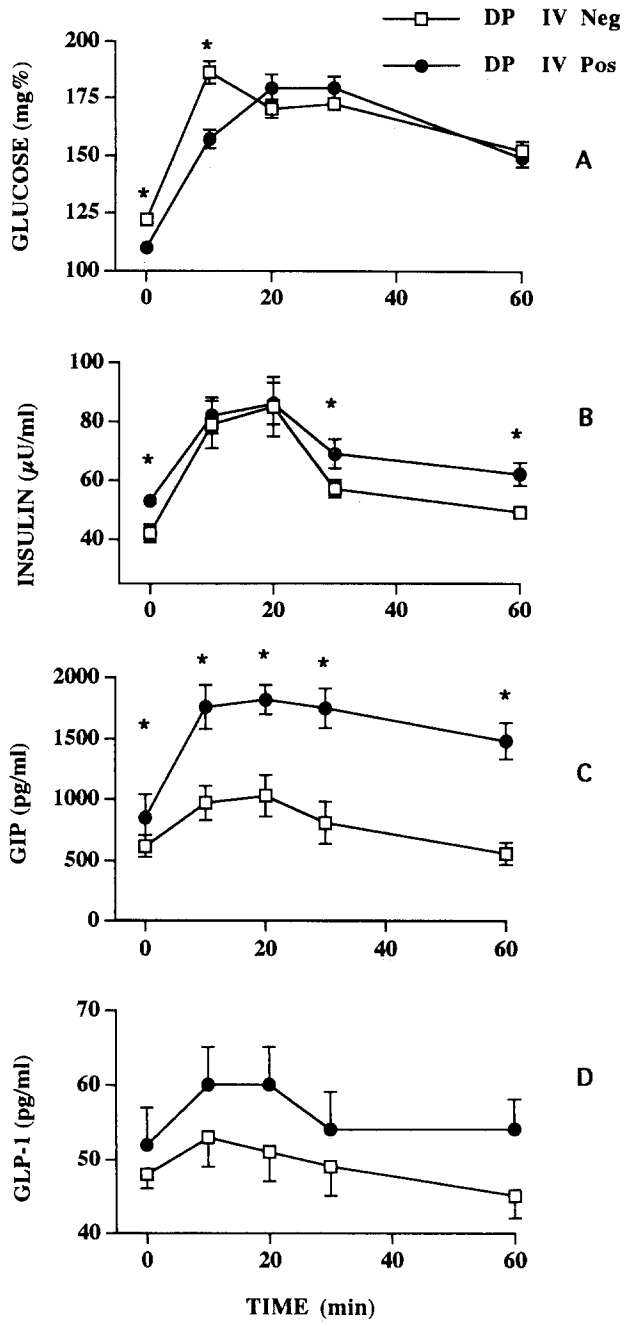

Fig 1. Plasma glucose (A), insulin (B), IR-GIP (C), and IR-GLP-$1_{(7\text{-}36)}$ (D) levels in response to 1 g/kg oral glucose in DPIV-negative (□) and positive (●) rats (n = 10 per group). In this and subsequent figures, *$P \leq .05$.

Pancreas Perfusions

Glucose + 0- to 1-nmol/L GIP or GLP-$1_{(7\text{-}36)}$ gradients

To compare the maximal effects of GIP and GLP-$1_{(7\text{-}36)}$ on insulin secretion in the two groups of rats, peptides were presented to the pancreas as a gradient of 0 to 1 nmol/L in the presence of a strong hyperglycemic stimulus (16 mmol/L). The insulin secretory response to GLP-$1_{(7\text{-}36)}$ did not differ in the two groups, with maximum values of 10.91 ± 0.78 mU/min for DPIV-negative and 11.09 ± 1.82 mU/min for DPIV-positive rats (Fig 3A). This is reflected in the integrated insulin responses of both groups compiled from the perfusion data (Fig 4A). In contrast, peak insulin values from the DPIV-negative group (8.08 ± 0.18 mU/min) in response to GIP were 30% lower than corresponding values

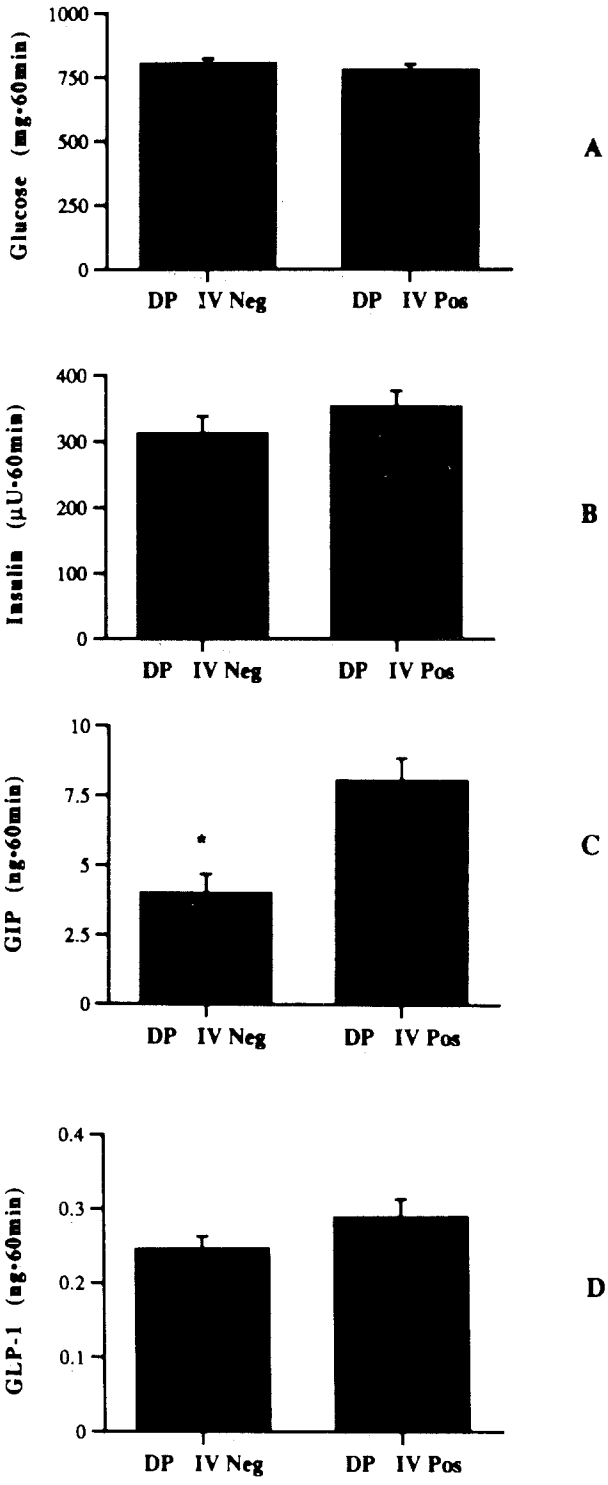

Fig 2. Integrated glucose (A), insulin (B), IR-GIP (C), and IR-GLP-$1_{(7\text{-}36)}$ (D) responses to an oral glucose challenge in DPIV-negative and -positive rats. Data were compiled from the data presented in Fig 1.

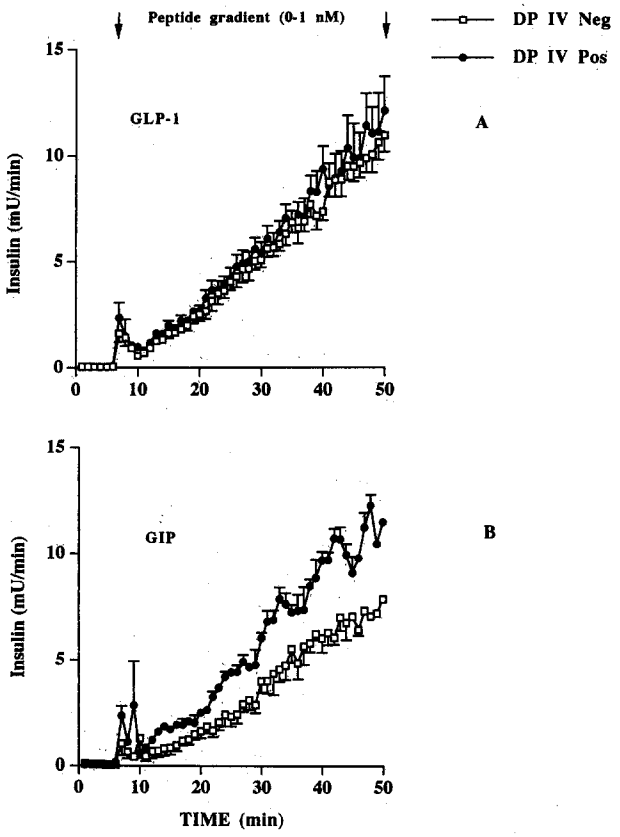

Fig 3. Insulin responses to 16 mmol/L glucose plus a gradient of 0 to 1 nmol/L GLP-$1_{(7-36)}$ (A) and GIP (B) in isolated pancreas of DPIV-negative (□) and -positive (●) rats.

for DPIV-positive controls (11.75 ± 0.20 mU/min). Figure 4B indicates that the integrated insulin response to GIP in DPIV-negative pancreas perfusions was also 30% lower than in control animals. These data also indicate that GIP and GLP-$1_{(7-36)}$ were equipotent as insulinotropic agents in the control pancreas perfusions (DPIV-positive rats), yielding similar maximum and integrated insulin responses when compared on a molar basis.

Glucose alone. Figure 4C shows integrated insulin responses from the perfused pancreas of DPIV-negative and -positive rats in response to high glucose alone. In the absence of GIP or GLP-$1_{(7-36)}$, insulin responses to glucose of the two groups were not different.

Glucose + GIP or GLP-$1_{(7-36)}$ incubated in plasma from DPIV-positive or -negative rats. Figure 5 shows the effects of incubating GIP or GLP-$1_{(7-36)}$ in plasma from rats possessing or lacking DPIV on the insulinotropic activity in the perfused pancreas. Figure 5A indicates that GLP-1 incubated in plasma from DPIV-negative rats retains the majority of its insulinotropic potency (versus Fig 4A), whereas the insulin-releasing activity of GLP-$1_{(7-36)}$ incubated in plasma from normal (DPIV-positive) rats was reduced by 66%. A similar relationship exists for GIP, with a reduction of insulinotropic potency of 64% following incubation in DPIV-positive plasma versus plasma from DPIV-deficient rats (Fig 5B).

DISCUSSION

GIP is released into the circulation in response to the ingestion of glucose, fat, and some amino acids.[21-24] We have recently demonstrated dose-dependent GIP release by glucose from isolated canine GIP cells maintained in short-term culture,[25] indicating that glucose acts directly on the GIP cell. In vivo studies have shown that GIP stimulates

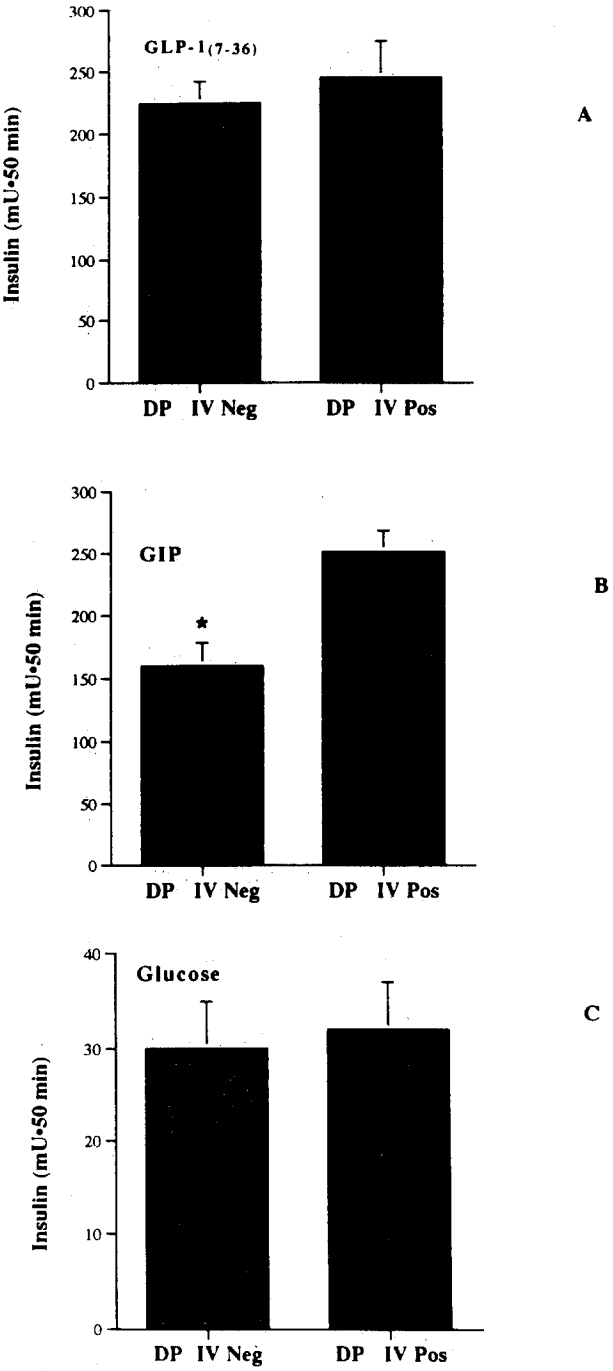

Fig 4. Integrated insulin responses to 16 mmol/L glucose plus a gradient of 0 to 1 nmol/L GLP-$1_{(7-36)}$ (A) and GIP (B) in isolated pancreas of DPIV-negative and -positive rats. Data were compiled from the data presented in Fig 3. Integrated insulin response to 16 mmol/L glucose alone is shown in C.

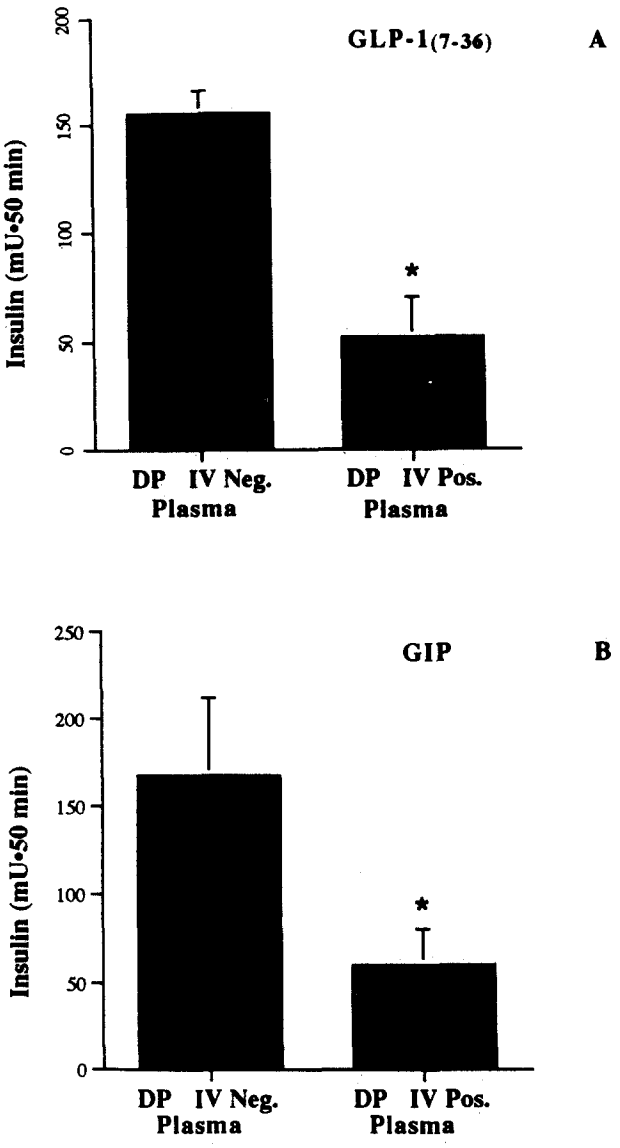

Fig 5. Integrated insulin responses to 16 mmol/L glucose plus a gradient of 0 to 1 nmol/L GLP-$1_{(7-36)}$ (A) and GIP (B) incubated in plasma of DPIV-negative and -positive rats.

insulin secretion during hyperglycemia in man[26,27] and dog.[23] There is a large volume of data demonstrating the glucose dependence of GIP action,[28,29] and the threshold was suggested to be approximately 4.4 mmol/L.[30-32] The glucose-dependent insulinotropic peptide GLP-$1_{(7-36)}$ is also secreted by the distal small intestine in response to glucose or fat and following a meal,[33-34] but the peak levels are considerably lower than those of GIP.[33-36] GIP has been shown to release glucagon-related peptides both in vivo[37] and from isolated intestinal endocrine cells,[38] suggesting that it may contribute to an enteroendocrine feed-forward pathway in which GIP releases GLP-$1_{(7-36)}$ from the distal bowel,[37] at least in the rat. It was proposed that this may account for the early increases in GLP-$1_{(7-36)}$ in the distal small bowel that occur in response to nutrient ingestion.

Most estimates of the circulating half-life values for GIP and GLP-$1_{(7-36)}$ have been calculated by RIA determination of circulating levels of peptides following an infusion. Using these methods, an average value for the half-life of IR-GIP has been reported to be 20 minutes,[30,39-41] whereas for IR-GLP-$1_{(7-36)}$ the range is 4 to 11 minutes.[33,42] It therefore appears that exogenously administered GIP and GLP-$1_{(7-36)}$ are both cleared relatively slowly from human plasma when measured by RIA. However, immunoreactivity may not be equivalent to biological activity, since proteases in plasma can render a hormone biologically inactive while preserving immunoreactivity. This is particularly relevant in light of studies by ourselves and others of the rapid inactivation of $GIP_{(1-42)}$ and GLP-$1_{(7-36)}$ to biologically inactive $GIP_{(3-42)}$ and GLP-$1_{(9-36)}$ by the circulating peptidase DPIV.[6,11,12] In this regard, we have recently shown that 50% of ^{125}I-GIP and ^{125}I-GLP-$1_{(7-36)}$ are converted to their *N*-terminally truncated forms within 2 minutes in vivo in the rat.[11]

Most, if not all, published RIAs for both peptides use C-terminally directed antisera, and thus would not distinguish between intact and *N*-terminally truncated (biologically inactive) forms of either peptide.[28,29,43] These data emphasize the need for RIAs using *N*-terminally directed antisera that distinguish between circulating levels of intact and *N*-terminally truncated forms of GIP and GLP-$1_{(7-36)}$.

Fischer DPIV-negative rats lack the DPIV enzyme. When ^{125}I-GIP and ^{125}I-GLP-$1_{(7-36)}$ were infused into these animals, the *N*-terminally truncated forms of both peptides were not produced,[11] adding support to the contention that DPIV cleavage is the principal route of metabolism of GIP and GLP-$1_{(7-36)}$ to biologically inactive $GIP_{(3-42)}$ and GLP-$1_{(9-36)}$ in the circulation. Taken together, the existing evidence strongly suggests that DPIV is the principal metabolizing enzyme for the incretins GIP and GLP-$1_{(7-36)}$, and that animals lacking this enzyme should exhibit an exaggerated insulin response to physiological stimulants of these two peptides, ie, ingested glucose. Figure 1 indicates that aside from mild fasting hypoinsulinemia and mild fasting hyperglycemia, the insulin response to an oral glucose challenge in DPIV-negative rats is normal, with a small decrease in the insulin response to oral glucose as compared with control animals. The evidence presented herein suggests that both GIP and GLP-$1_{(7-36)}$ would remain intact and biologically active for a longer period in DPIV-negative rats compared with normal controls. Since the integrated insulin and glucose responses to OGTT are the same in the two groups of animals, DPIV-negative animals must have compensated for the prolonged half-life of GIP and GLP-$1_{(7-36)}$. Thus, it might be predicted that secretion of the two incretins normally inactivated by DPIV would be appropriately reduced when compared with control levels. The circulating IR-GIP response to an oral glucose challenge was reduced in DPIV-negative rats by approximately 50% compared with controls. Surprisingly, no such reduction was observed in the IR-GLP-$1_{(7-36)}$ response in DPIV-deficient rats following glucose administration (Figs 1 and 2). A second mechanism that might be postulated to ensure appropriate insulin release in the presence of long-acting incretins in DPIV-negative rats is desensitization of the pancreas to GIP and GLP-$1_{(7-36)}$. This was assessed by measuring GIP- and GLP-$1_{(7-36)}$-induced insulin release

from the isolated perfused pancreas of DPIV-negative and control rats. Total insulin release from the perfused pancreas in response to GIP was decreased by approximately 30% in DPIV-negative versus DPIV-positive rats (Fig 3 and 4), whereas the insulin response to GLP-$1_{(7\text{-}36)}$ was identical in both groups. Reduced GIP release might be effected by an alteration in feedback inhibition of GIP release by insulin.[44] In this study, it was shown that in the rat hyperinsulinemia but not hyperglycemia decreased the GIP response to an OGTT. Homologous desensitization of GIP receptors on pancreatic B cells may also account for the reduced in vitro insulin response from DPIV-negative rats. This has previously been observed when insulinoma cells were subjected to a preperfusion of GIP.[45] Thus, the attenuated insulin response to GIP in DPIV-negative rats could be an adaptation to the elevated half-life of bioactive $GIP_{(1\text{-}42)}$. Figure 3C indicates that the integrated insulin response to elevated glucose (in the absence of either incretin) is normal (not different from controls), indicating that the perfused pancreas of DPIV-negative rats responds normally to secretagogues other than GIP. The absence of change in either IR-GLP-$1_{(7\text{-}36)}$ release or sensitivity of the pancreas to GLP-$1_{(7\text{-}36)}$ in DPIV-negative rats is noteworthy. The possible explanation that DPIV is less effective in degrading GLP-$1_{(7\text{-}36)}$ is not supported by our findings and those of others that the enzyme in plasma appears equally effective in metabolizing GIP and GLP-$1_{(7\text{-}36)}$.[6,11] To test for this possibility, we assessed the insulin-releasing potency of GIP and GLP-$1_{(7\text{-}36)}$ incubated in plasma from both groups of animals. Figure 5 indicates that an equal reduction in the insulinotropic potency of both peptides resulted from incubation of GIP or GLP-$1_{(7\text{-}36)}$ in plasma containing the normal component of DPIV as compared with DPIV-deficient plasma. These data also support the contention that DPIV degradation is the principal route of metabolism/inactivation for both hormones. It is possible that an overactive enteroinsular axis is avoided by alternate pathways for biological inactivation of GLP-$1_{(7\text{-}36)}$ in the DPIV-negative strain of rats after a meal. However, no evidence to support this hypothesis was found in our previous investigation in which the in vivo degradation of ^{125}I-GLP-$1_{(7\text{-}36)}$ was measured in these animals.[11] Also in the current study, DPIV-positive plasma inactivated GIP and GLP-$1_{(7\text{-}36)}$ to a similar degree (Fig 5). The compensatory mechanism(s) for the long-acting insulinotropic action of GLP-$1_{(7\text{-}36)}$, if present in DPIV-deficient rats, is not evident from the experimental methods used in this study. However, this investigation does not rule out other possibilities, eg, increased rates of clearance of biologically active GLP-$1_{(7\text{-}36)}$ from the circulation of DPIV-negative rats. In summary, this investigation provides further support for the hypothesis that the circulating enzyme DPIV is the primary mechanism for inactivation of the incretins GIP and GLP-$1_{(7\text{-}36)}$, and that in the absence of the enzyme compensatory changes occur in GIP release and insulinotropic potency. Evidence was provided that the Japanese F344 (DPIV-negative) strain of rat is a valuable model for assessing the metabolism of GIP and GLP-$1_{(7\text{-}36)}$, as well as other hormones bearing proline or alanine in the *N*-terminal sequences.

ACKNOWLEDGMENT

We would like to thank Leslie Checknita for excellent technical assistance.

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