Identical Biological Effects of Pancreatic Glucagon and a Purified Moiety of Canine Gastric Immunoreactive Glucagon

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ABSTRACT Because in the dog, the gastric fundus contains the largest amount of glucagon immunoreactivity (IRG), the IRG of mucosal scrapes of 105 canine stomachs was extracted by acid-ethanol and then precipitated by ether-ethanol. The IRG recovered was measured by antisera 30K, specific for glucagon and K-4023, which cross-reacts with glucagon-like immunoreactivity. Extracts of mucosa of stomach fundus were further purified by gel filtration on Bio-Gel P-30 in 3M acetic acid. One pooled fraction corresponding to marker pancreatic glucagon in its elution volume was then gel-filtered on Bio-Gel P-30 in 0.05 M NH_4HCO_3 and yielded one IRG peak, which, however, showed three immunoreactive components on polyacrylamide disc gel electrophoresis in urea. In addition, antiserum K-4023 reacted more strongly with that peak than antiserum 30K indicating the presence of glucagon-like immunoreactivity in this fraction. Subsequent ion-exchange column chromatography on DEAE-Sephadex A-25 and then CM-Bio-Gel A allowed purification to a single protein band on disc gel electrophoresis reacting equally to both antisera 30K and K-4023. 1.5 μg of purified gastric glucagon was obtained and its biological effects were compared to those of pancreatic glucagon in isolated rat hepatocytes. When immunoequivalent amounts (300–2,500 pg/ml) of either type of glucagon were used, the same biological responses with respect to glycogenolysis and gluconeogenesis as well as urea, lactate, and pyruvate production were observed. Liver cyclic AMP was also raised to the same extent by either one of these hormones. We conclude that this moiety of gastric IRG is apparently identical to pancreatic glucagon because (*a*) their molecular weights, elution properties in ion exchange chromatography, and their electrophoretic mobility are indistinguishable and (*b*) both hormones elicited identical biological effects in isolated rat hepatocytes.

INTRODUCTION

Immunoreactive glucagon (IRG)[1] of extrapancreatic origin is most abundant in the canine stomach (1–3) and its physiochemical and immunological characteristics cannot be distinguished from those of purified pancreatic glucagon (2–5). Despite these structural similarities, the control of secretion of gastric IRG differs from that of the pancreatic hormone when studied in depancreatized dogs (6–8) or in isolated stomach preparations (9). Secretion of gastric IRG is prominent only in diabetic conditions (10); in acutely depancreatized dogs its secretion rate is 0.25 ng/kg per min and it rises to 1 ng/kg per min during arginine stimulation (11). In the dogs, the significance of extrapancreatic IRG is further affirmed by the fact that it is still secreted 5 yr after pancreatectomy (12) and that chronic diabetes can lead to hyperplasia of gastric A cells (13). Partially purified extracts of gastric mucosa yielded a number of immunoreactive peaks classified according to their molecular weights. In isolated liver

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[1] *Abbreviations used in this paper*: GLI, glucagon-like immunoreactivity; IRG, immunoreactive glucagon.

preparations, two of these peaks, $IRG^{3,500}$ and $IRG^{6,500}$, had the same glycogenolytic activity as pancreatic glucagon. Whereas $IRG^{6,500}$ had the same effect as pancreatic glucagon in stimulating adenylate cyclase, the $IRG^{3,500}$ moiety was many times more active (3). These data would indicate that either gastric $IRG^{3,500}$ is biologically much more active or that it has not been sufficiently purified and that some unknown contaminants might be responsible for the augmented effect. The sensitivity of isolated liver preparations apparently required pharmacological rather than physiological concentration of IRG to be used (3).

The aim of the present work was to purify gastric $IRG^{3,500}$ until immunological homogeneity had been achieved and to collect sufficient amounts of the material to study a wide range of biological responses of liver to physiological concentrations of IRG. This was possible because isolated hepatocytes were found to be very sensitive to glucagon. The effects of pancreatic and gastric glucagon were then compared with respect to the production of glucose, urea, lactate, and pyruvate, and with respect to gluconeogenesis and cyclic AMP content.

METHODS

Extraction and purification of gastric IRG

Acid ethanol extraction. The extraction procedure of Kenny (14), modified by Okuno et al. (15), was used to extract IRG from the fundic mucosa of the dog as described previously (2). Mucosal scrapes were obtained from the fundus and corpus of dog stomachs that had been stored frozen at −76°C. Five stomachs were used for each extraction, giving ≅100 g wet wt of mucosal scrapes. The dry precipitates obtained from the final step of the ether-ethanol precipitation were kept at 5°C in a dessicator over $CaCl_2$.

Further purification was carried out by gel filtration (in acid and then in alkaline pH) and by ion-exchange chromatography (DEAE-Sephadex, Pharmacia Fine Chemicals Inc., Piscataway, N. J. and CM-Bio-Gel, Bio-Rad Laboratories, Richmond, Calif.). Gel-filtration has been described in detail previously (2). One immunoreactive fraction had the same molecular weight as pancreatic glucagon, but was not immunologically pure as confirmed by electrophoresis. Therefore, this fraction was further purified by two successive ion-exchange chromatography procedures that will be described in detail. In eluates from all columns, protein content was estimated by measuring optical density of the samples. This permitted an estimate of relative specific immunoreactivity to be made and to thereby assess the relative purity of the IRG extract during successive purification procedures.

Gel-filtration column chromatography. The ether-ethanol precipitates from each extraction dissolved in 15 ml of 3 M acetic acid were chromatographed on a column (5.0 × 100 cm) of Bio-Gel P-30 (Bio-Rad Laboratories) equilibrated to 3 M acetic acid. As molecular weight markers, ^{125}I-insulin, ^{125}I-glucagon, albumin, and blue-dextran were used routinely. Fractions of ≅8 ml each were collected into tubes that contained a concentrated solution of benzamidine so that the final concentration of benzamidine was 0.01 M in each fraction. Fractions that contained IRG, as determined by radioimmunoassay (16), and that corresponded in elution pattern to ^{125}I-glucagon were combined, reduced in volume by rotary evaporation *in vacuo* at room temperature, and then lyophilized. The lyophilized material was dissolved in 4 ml of 0.05 M ammonium bicarbonate and chromatographed on a column (2.5 × 100 cm) of Bio-Gel P-30 equilibrated to 0.05 M ammonium bicarbonate. Fractions of ≅8 ml each were collected in tubes that contained benzamidine. Fractions that contained IRG and corresponded to elution position of the glucagon marker were combined and concentrated by ultrafiltration with the Amicon UM-2 membrane (Amicon Corp., Scientific Sys. Div., Lexington, Mass.). The concentration was lyophilized. The immunological dilution curves of this eluate were parallel to those of standard pancreatic glucagon (2).

Ion-exchange chromatography on DEAE-Sephadex. The lyophilized material from gel filtration was purified by ion-exchange column chromatography on a column (1.5 × 30 cm) of DEAE-Sephadex A-25 equilibrated to 0.01 M Tris-HCl buffer, pH 8.6, that contained 7 M urea. The Tris-HCl urea buffer was freshly prepared and treated with charcoal before use. The lyophilized material was dissolved in 2 ml of the buffer and applied to the column. The column was eluted by a salt gradient generated by the addition of 0.3 M NaCl in the urea buffer to a reservoir of 250 ml of the starting buffer. Fractions of ≅5 ml each were collected at a flow rate of 15 ml/h. Fractions with glucagon immunoreactivity that corresponded to the elution position of the glucagon marker were combined. The urea and salt in the combined pool were removed by repeated concentration and dilution in an Amicon Diaflo Ultrafilter (Amicon Corp.) with an UM-2 membrane. The concentrated material was then lyophilized.

Ion-exchange chromatography on CM-Bio-Gel A. Materials from DEAE-Sephadex ion-exchange chromatography of 21 extractions, representing the processing of 105 stomachs, were combined for further purification on a column (1.5 × 30 cm) of CM-Bio-Gel A, equilibrated to 0.01 M sodium acetate-HCl buffer, pH 4.5, that contained 7 M urea. Elution was achieved by a salt gradient generated by the addition of 0.3 M sodium acetate-HCl buffer with urea to a reservoir of 250 ml of the starting buffer. Fractions of ≅5 ml each were collected at a flow rate of 15 ml/h. Fractions with IRG immunoreactivity were combined and dialyzed for 2 h in a Biofiber-20 beaker (Bio-Rad Laboratories) with running deionized water and reduced in volume by ultrafiltration and lyophilization. Desalting of the final preparation was performed on a column (1.5 × 30 cm) of Sephadex G-15 equilibrated to 0.05 M ammonium bicarbonate. Final yield of purified glucagon was 1.5 μg.

Polyacrylamide disc gel electrophoresis. To determine immunological purity after the various purification steps, disc electrophoresis (17) was carried out in 15% gel that contained 4 M urea at pH 8.7. The gel was stained with Coomassie Blue. Unstained gel was cut into 1.5-mm sections, which were eluted with the immunoassay buffer by repeated freezing and thawing. Aliquots of the eluate were immunoassayed for glucagon (30K) and glucagon-like immunoreactivity (GLI) (K-4023) to find out whether the final eluate contains only IRG or GLI as well.

Tests of biological activity

Preparation and incubation of rat hepatocytes. 6-wk-old male albino rats derived from a Wistar strain were used. They weighed between 165 and 185 g, and were fed ad libitum with a standard laboratory chow. Rats were anaesthetized by intraperitoneal injection of pentobarbital (55 mg/kg body wt). Liver cells were isolated by the collagenase method described previously for mouse hepatocytes (18), with a few modifications. Livers were first perfused *in situ* with a nonrecir-

culating, calcium-free, phosphate buffer (130 ml, pH 7.6), gassed with pure oxygen, then perfused for 6 min with identical recirculating medium. Perfusion was continued for 20–25 min with a recirculating, calcium-free, phosphate buffer that contained collagenase (50 mg/100 ml), bovine serum albumin (2 g/100 ml), and glucose (150 mg/100 ml). Livers were removed, and after gently hand stirring in a Petri dish that contained a calcium-free, phosphate buffer with albumin (0.5 g/100 ml, pH 7.4), isolated cells thus obtained were further incubated for 3 min at 37°C in a shaking incubator. They were filtered through a nylon gauze, centrifuged, and washed three times with this buffer. Cells were then preincubated under an atmosphere of O_2:CO_2 (95:5 vol/vol) for 45 min in a Krebs-Ringer bicarbonate buffer (pH 7.4, 37°C) that contained glucose (100 mg/100 ml), bovine charcoal-treated serum (25%), and bovine defatted albumin (1.5 g/100 ml) prepared as described elsewhere (19). After preincubation, cells were centrifuged, resuspended in the same medium, and distributed as 1.0- or 1.5-ml aliquots into 10-ml Erlenmeyer flasks. Cell viability by trypan blue exclusion was routinely 90–98%. Incubations were stopped by transferring cell suspensions into conical centrifuge tubes placed in ice water. Tubes were centrifuged at 4°C (3,000 *g* for 5 min), pellets and supernates thus obtained were used for measurements of various metabolic indices. The biological activity of the purified gastric IRG was compared to pork pancreatic glucagon. The concentrations of the gastric IRG and the pork pancreatic glucagon used in each assay vial were determined by immunoassay in duplicate with antiserum 30K. Experiments to compare the biological activity were repeated at least twice and each comparison was carried out in triplicate or quadruplicate. All results are expressed per gram of wet weight of hepatocytes ±SEM, measured at time zero of incubation. All of the comparative experiments reported in this paper were always performed with the same preparation of hepatocytes.

Biochemical determination. Biochemical determinations were carried out after 30 min of incubation and expressed as the differences from values at time zero. Aliquots of supernates were used for the following measurements: 0.01 ml for glucose (20) and 0.1 ml for urea (20). Lactate and pyruvate (cell plus medium content) were measured from 0.5 ml cell suspension. These metabolites were determined on the neutralized perchlorate extract (20). [^{14}C]lactate conversion to [^{14}C]glucose (i.e., gluconeogenesis) was determined from 1-ml cell suspension (cell plus medium) as described by Exton and Park (21). Absolute cyclic AMP content of hepatocytes was measured after 4 min of incubation from 0.5-ml cell suspension by the protein binding assay of Gilman (22), after purification of samples on Dowex 50 (Dow Corning Corp., Midland, Mich.) (23). The total amount of purified gastric IRG obtained from 105 stomachs (≅2 kg of mucosa) was 1.5 μg as determined by immunoassay, with the 30K antiserum. Pancreatic pork glucagon and dog gut IRG were added to the incubation vials as 100 times concentrated solutions. Concomitantly, the same amounts of concentrated hormone solutions were added to tubes (4°C) that contained a solution of human serum albumin (0.5 g/100 ml) and NaCl (0.9 g/100 ml) so that the final concentrations of hormones in tubes were the same as those of incubation vials. Aliquots of these solutions were used for measurements of immunoreactive glucagon, with the 30K antiglucagon serum, to give the concentration of the respective hormones added to time zero.

Chemicals

All organic and inorganic chemicals were purchased from E. Merck (Darmstadt, West Germany), Fluka A. G. (Buchs, Switzerland), Sigma Chemical Co. (St. Louis, Mo.,), and were of analytical grade. Labeled compounds were secured from The Radiochemical Centre (Amersham, Buckinghamshire, England). Collagenase (CLS IV) was obtained from Worthington Biochemical Corp. (Freehold, N. J.); glucagon used was twice recrystallized, highly purified pork glucagon of Novo Research Institute (Copenhagen, Denmark). Insulin contamination was 0.002%. Radioiodinated glucagon was purchased from Novo Research Institute. Specific antiserum 30K was obtained from Dr. Unger (Dallas, Tex.). In addition, GLI was measured by another antibody, K-4023, which cross-reacts strongly with a preparation of GLI (from L. Heding, Novo Research Institute).

RESULTS

Purification of stomach IRG. 21 extractions of dog stomach mucosa were carried out to purify immunologically homogeneous IRG in amounts sufficient for extensive study of its biological activity. Five stomachs were used for each extraction, giving 100 g wet wt of mucosal scrapes. The starting total mucosal weight was over 2 kg. The average concentration of IRG in mucosal extracts was 2 μg/g mucosa as measured by antiserum 30K, and 2.4 μg/gm when measured by nonspecific antiserum K-4023. Thus, IRG and GLI constituted 84 and 16%, respectively of the total immunoreactivity. We could confirm our previous findings (2) that gel-filtration of the acid-ethanol extract of the fundic mucosal scrapes of the dog yielded several fractions of protein having glucagon immunoreactivity. One of these fractions was similar to pancreatic glucagon in its elution volume through Bio-Gel P-30 equilibrated to 3 M acetic acid. Other components reacted more strongly with nonspecific antiserum K-4023 suggesting that stomach extracts contain not only IRG, but also GLI. Rechromatography of the IRG peak through Bio-Gel P-30, but equilibrated with 0.05 ammonium bicarbonate yielded only one peak. However, polyacrylamide gel electrophoresis of this material in urea showed the presence of three components which were reactive towards a specific antiglucagon serum (30K) and more strongly towards a nonspecific antiserum K-4023. One of these three immunoreactive components was electrophoretically identical to pancreatic glucagon, but the presence of GLI components was still evident. Further purification procedures were thus necessary to obtain a material that was immunologically homogeneous. Therefore, the IRG peak from the Bio-Gel P-30 column (ammonium bicarbonate) was subjected to ion-exchange chromatography on DEAE-Sephadex A-25, as shown in Fig. 1.

This material was resolved into two immunoreactive components, one of which was eluted at an ionic concentration where pancreatic glucagon was eluted under similar conditions. Based on ultraviolet absorption and immunoreactivity, this peak was purified 400-fold over the materials obtained after gel filtration. On polyacrylamide gel electrophoresis, staining revealed two

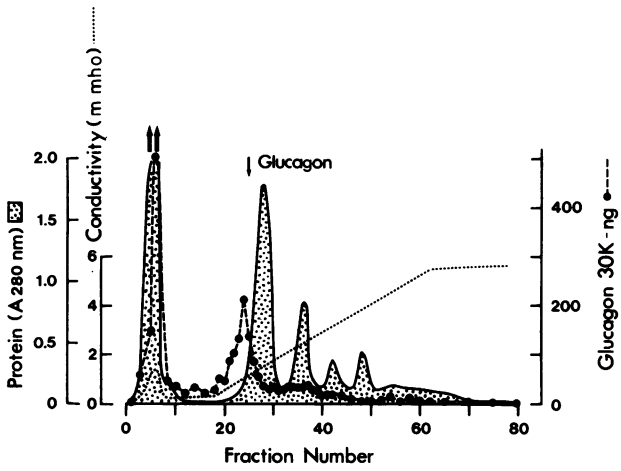

FIGURE 1 DEAE-Sephadex ion-exchange column chromatography of immunoreactive material pooled from three extractions after gel filtration sequentially on Bio-Gel P-30 in 3 M acetic acid in 0.05 M ammonium bicarbonate. The arrow indicates the elution position of porcine pancreatic glucagon. Immunoreactive fractions corresponding to this position were pooled.

protein bands. Ion-exchange chromatography on CM-Bio-Gel A was then used to purify this component further. As shown in Fig. 2, the immunoreactive peak was eluted from the ion-exchange column at an ionic strength required for the elution of pancreatic glucagon under these conditions. This step gave a further twofold purification. Polyacrylamide disc gel electrophoresis of this immunoreactive component in urea established its immunohomogeneity in that it showed only one distinct immunoreactive component electrophoretically similar to pancreatic glucagon, which was equally reactive towards both the specific and the nonspecific antisera (Fig. 3A). On staining of the disc

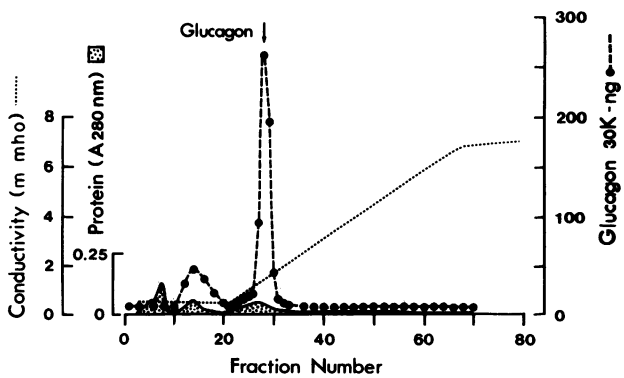

FIGURE 2 CM-Bio-Gel A ion-exchange chromatography of pooled immunoreactive fractions from DEAE ion-exchange chromatography of gel-filtered materials from 21 extractions. The arrow indicates the elution position of porcine pancreatic glucagon. m mho, milli reciprocal ohms.

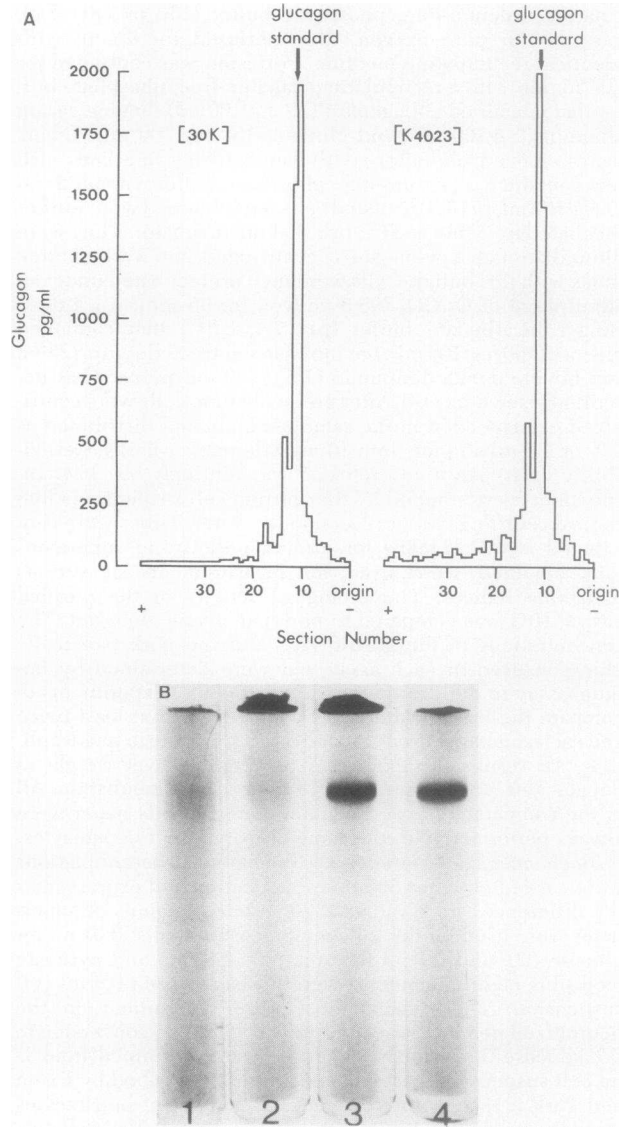

FIGURE 3 Polyacrylamide disc gel electrophoresis of an aliquot of the purified immunoreactive material obtained after ion-exchange chromatography. Electrophoresis was carried out in a 15% gel that contained 4 M urea at pH 8.7. Standard porcine glucagon was electrophoresed in parallel in a separate gel and stained. (A) The unstained gel that contained the immunoreactive material was cut into 1.5-mm sections and eluted with 0.1 ml of the immunoassay diluent. The eluate was assayed for IRG with antisera 30K and K-4023 as indicated. (B) Gel stained with Coomassie Blue: 1 and 2, purified stomach glucagon; 3 and 4, standard porcine pancreatic glucagon.

gel only a faint band corresponding to pancreatic glucagon was evident (Fig. 3B). The amount of immunoreactive material recovered after CM-Bio-Gel chromatography from a total of 21 extractions (≅2 kg of mucosa from 105 stomachs) was about 1.5 μg as determined by immunoassay with antiserum 30K. Because the

average IRG content of stomach mucosa was 2 μg/g mucosa, the amount of IRG recovered was 0.04%.

Biological activities of pancreatic and stomach glucagon. As shown in Fig. 4, the stimulatory effect of immunologically equivalent quantities of pancreatic and gastric IRG upon glucose output (i.e., mostly glycogenolysis under these conditions) was the same and concentration dependent. Marked effects of both glucagons could be clearly demonstrated in physiological and pharmacological ranges of concentrations. Urea output in the medium gave similar results although this process was less sensitive to the effect of the hormones (Table I). Lactate and pyruvate production were decreased in a dose-related fashion by the presence of immunologically equivalent amounts of the hormones (Table I). Gluconeogenesis, measured in hepatocytes from fed rats by the conversion of [^{14}C]-lactate to [^{14}C]glucose was stimulated to the same extent by analogous concentrations of either pancreatic or gastric IRG (Fig. 5). These findings were corroborated by the measurement of tissue cyclic AMP that was raised to the same extent by either one of these hormones (Fig. 6).

DISCUSSION

These studies extend our previous observations (2), as well as those of others (3) that oxyntic mucosa of the dog contains a number of immunoreactive moieties cross-reacting with antiserum 30K. One IRG peak cannot be distinguished from pancreatic glucagon with respect to immunoreactivity, apparent molecular size, elution properties on both cation and anion ion-exchange chromatography, and electrophoretic mobility on polyacrylamide gel. Previously, when stomach mucosal scrapes were subjected to Bio-Gel chromatography and rechromatographed, although only one single immunoreactive peak was apparent, that peak was shown not to be immunologically homogeneous when subjected to disc gel electrophoresis in urea. Lack of homogeneity was demonstrated because three immunoreactive peaks were visible

TABLE I

Effects of Pancreatic and Gastric Glucagon on Urea, Lactate, and Pyruvate Production by Isolated Rat Hepatocytes

Addition	IRG	Urea production	Lactate production	Pyruvate production
	pg/ml		*μmol/g wet wt*	
None	0	4.30±0.05	15.44±0.98	5.22±0.03
Pancreatic	270	4.75±0.08	12.39±0.48	4.41±0.13
Gastric	322	4.76±0.05	12.19±0.36	4.31±0.13
Pancreatic	555	4.96±0.05	9.07±0.48	3.84±0.19
Gastric	572	5.07±0.07	9.14±0.18	3.65±0.02
Pancreatic	2,912	6.26±0.06	0.01±0.41	1.66±0.13
Gastric	2,450	6.10±0.08	0.76±0.48	1.81±0.02

Experimental conditions as in Fig. 4. Each value is the mean of one experiment with SEM calculated from triplicate tubes that contained hepatocytes.

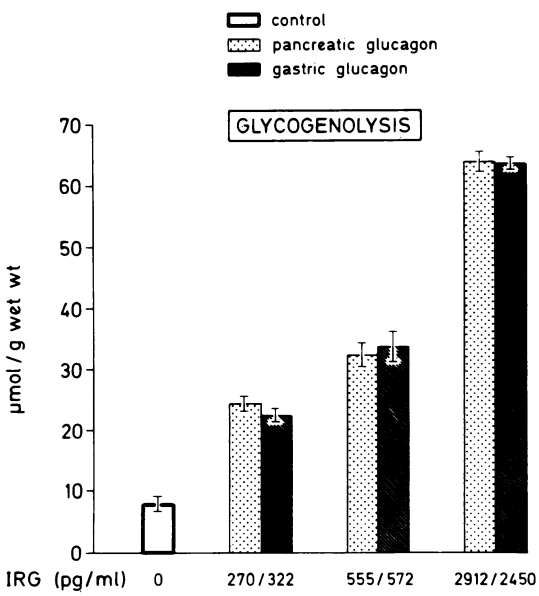

FIGURE 4 Effect of pancreatic and gastric IRG upon glycogenolysis of isolated rat hepatocytes. The ordinate represents unlabeled glucose output. Hepatocytes from fed rats were incubated for 30 min in 1 ml Krebs-Ringer bicarbonate buffer (pH 7.4) that contained bovine charcoal-treated serum (25%), bovine serum albumin (1.5 g/100 ml), and glucose (100 mg/100 ml). Each figure is the mean of one experiment with SEM calculated from triplicate tubes that contained hepatocytes.

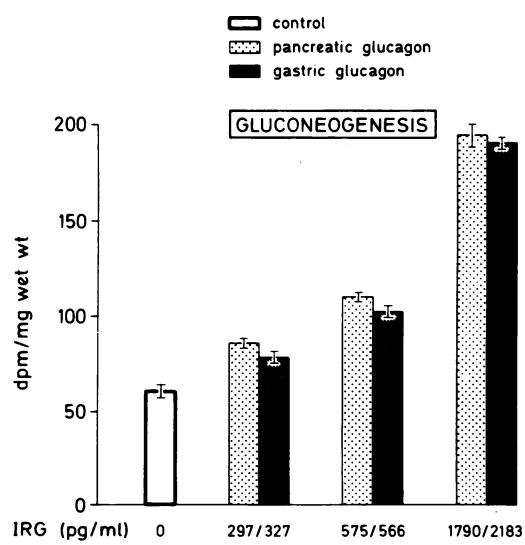

FIGURE 5 Effect of pancreatic and gastric IRG upon [^{14}C]-lactate conversion to [^{14}C]glucose by isolated hepatocytes from fed rats. Experimental conditions are as in Fig. 4 (without glucose); lactate, 5.0 mM.

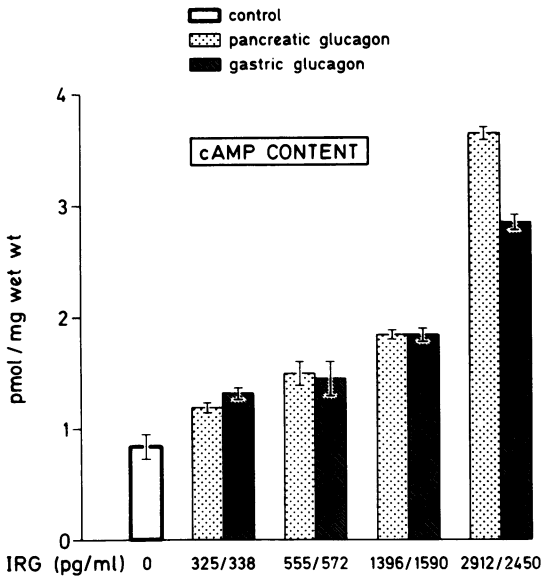

FIGURE 6 Effect of pancreatic and gastric IRG on cyclic AMP content of isolated rat hepatocytes measured after 4 min of incubation. Experimental conditions are as in Fig. 4. Each bar is the mean of three values ±SEM.

when assayed either with specific antiserum 30K or nonspecific antiserum K-4023. The stronger cross-reactivity with K-4023 indicates presence of GLI in stomach mucosa, as reported also by others (3).

Further purification on ion-exchange chromatography with DEAE-Sephadex and then on CM-Bio-Gel A yielded a peak of high specific immunoreactivity, which was proven to be immunologically homogeneous upon electrophoresis. One single distinct peak was observed with the use of both 30K and K-4023 antisera. This peak, thus, did not contain any GLI contaminants and staining of the gel indicated only one protein band. The yield of purified gastric IRG was 1.5 μg, sufficient for extensive biological testing, but not for further characterization of its chemical purity. The biological activity of this IRG was compared to biological activity of pancreatic glucagon in incubated isolated hepatocytes. It was shown that the metabolic parameters investigated were sensitive to physiological concentrations of IRG, and were dose dependent. Stomach and pancreatic "glucagon" activity of immunologically equivalent quantities were the same with respect to glycogenolysis, gluconeogenesis, lactate and pyruvate production, and [^{14}C]lactate conversion to [^{14}C]glucose (a measure of gluconeogenesis). These findings were corroborated by the observation that tissue cyclic AMP was raised to the same extent by either one of these hormones. Srikant et al. (3) have recently obtained a partially purified fraction of IRG^{3,500} from dog stomach mucosa. This IRG had the same glycogenolytic activity in perfused rat livers. However, in rat liver membranes it was three times more potent in activating adenylate cyclase, but not in its receptor binding activity. Their observation raised the possibility that gastric IRG^{3,500} could be much more potent than pancreatic glucagon. This is contrast to our present findings. These discrepancies may be caused by different degrees of homogeneity between our purified IRG and Srikant's. Although our purified IRG was immunologically homogeneous, such homogeneity was not demonstrated by Srikant et al. (3). In their fractions after isoelectrofocussing, some GLI contaminants were still present and these fractions were 0.1 pH unit lower than pancreatic glucagon in its isoelectric point. Our final preparation represents a 800-fold purification over the material obtained from gel filtration, compared to a 30-fold purification obtained by Srikant et al. (3), and we could test biological effects of purified stomach IRG^{3,500} at physiological concentrations.

It is clear from Fig. 1 that the immunoreactive component eluting like pancreatic glucagon represent ≅30% of the total IRG recovered from the ion-exchange column. The other 70% was that of an immunoreactive component not retarded by the ion exchanger. The nature of this nonretarded component is not known, although its chromatographic behavior would suggest that it is similar to glucagon in size but more basic.

We conclude that one moiety of gastric IRG is most likely identical to pancreatic glucagon because its molecular weight, elution properties on cation, and anion ion-exchange chromatography and electrophoretic mobility cannot be distinguished from the pancreatic hormone. In addition, its biological activity related to its immunoreactive concentration is identical to that of pancreatic glucagon with respect to glycogenolysis, gluconeogenesis, ureogenesis, production of lactate and pyruvate, and activation of cyclic AMP.

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