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The characterization of radioimmunoassay for rat pancreatic polypeptide in serum

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Summary

A radioimmunoassay for the measurement of rat pancreatic polypeptide (RPP) in serum or plasma has been developed and characterized using a new guinea-pig anti-rat-PP antibody. The assay provides a high degree of sensitivity and lacks cross-reactivity (CR < 0.01%) to neuropeptide Y and peptide YY. It also does not interact with PPs of other species or peptide hormones namely, amylin, glucagon, human insulin, human-PP, human-proinsulin, rat C-peptide and rat insulin. The assay employs synthetic rat PP as standards from concentrations of 21–2100 pg/ml (i.e., 5–500 pM) and produces a sensitivity limit of 19 pg/ml (4.5 pM) PP at ± 3 S.D. The intra- and inter-assay % coefficient of variations are 6.4% and 5.9%, respectively. The % recovery of RPP added to rat serum samples ranges from 98% to 103%. Assay of serum volumes ranging from 25 μl to 100 μl does not significantly alter the expected RPP level. The migration patterns of rat serum PP and that of a synthetic RPP are identical by Sephadex G-50 chromatographic analysis. The mean values of fasting and a 2 h post-feeding plasma RPP levels in normal rats are 40 ± 2 and 80 ± 10 pg/ml (9.5 pM and 19.0 pM), respectively. Rat-PP release during insulin induced hypoglycemia in conscious rats rises from 38 ± 5 pg/ml to 261 ± 34 pg/ml (9.0 to 62.1 pM, $P < 0.005$) by 30 min. Additionally, the antibody used in this study cross-reacts well with mouse-PP as determined by linear serum dilution curves, thus making it useful in the measurement of murine-PP.

In conclusion, we have developed and validated a sensitive and specific rat-PP assay. This assay provides a new tool for the reliable measurement of PP in physiologic studies using rat and mouse animal models.

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Introduction

Pancreatic polypeptide (PP) is a hormone secreted by F-cells of pancreatic islets of Langerhans [1,2] with little inter-mammalian species variation in the primary structures [3–6]. The similarity in PP primary structure among most mammals has previously justified use of non-isologous antibodies (e.g., anti-human-PP) and bovine-PP tracer ^{125}I(tyr^{0}27) bPP$_{1\text{-}36}$ in radioimmunoassays (RIA) to determine PP concentrations in tissue and plasma. Availability of RIA techniques for PP in human and other vertebrates has made possible studies which have uncovered physiologic roles for PP including, the inhibition of stimulated pancreatic exocrine secretion [5] and the augmentation of insulin inhibited hepatic glucose production [6–8]. These actions of PP are mediated by specific receptors which have been identified and characterized in various vertebrate tissue and plasma membranes [9–13]. Pancreatic polypeptide receptor studies and the recent novel findings that PP circulates in heterogeneous forms in human plasma [14] have rekindled interest in the physiologic role of this hormone.

Levels of PP in human and many other vertebrates are readily determined by specific RIA techniques, however, there was previously no reliable radioimmunoassay for rat PP due largely to the high degree of heterology between rat PP and PPs of other mammalian species. This heterology occurs in a region of PP structure which results in very low crossreactivity with commonly employed antibodies to human or bovine PPs. A sensitive and specific rat-PP immunoassay capable of detecting minute concentrations in plasma is desirable since the rat is a widely used animal model for the studies of pancreatic islet secretory function. The present report describes and validates a specific radioimmunoassay for rat-PP which provides a high degree of sensitivity and specificity. The cross-reactivity of PPs of other vertebrate species (except mouse) and various other peptide hormones is negligible. This RIA will provide a reliable assay for PP levels in the rat and mouse plasma samples.

Materials and Methods

Materials

Synthetic rat pancreatic polypeptide was obtained from Peninsula Laboratories, Inc. Guinea-pig anti-rat-PP serum (lot RPP64), goat anti-guinea-pig IgG serum (GP020P) and normal guinea-pig carrier were obtained from Linco Research, Inc., 16217 Westwoods Business Park, St. Louis, MO 63021, U.S.A. Other reagents, namely, buffer salts, EDTA, bovine serum albumin (BSA, radioimmunoassay grade), ethylmercurithiosalicylic acid (EMTSA) sodium salt, trasylol and leupeptin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Procedure

(i) Rat serum preparation. Male Sprague-Dawley rats weighing 350–400 g (Washington University Office of Laboratory Animal Care) were used in these studies. Sera were obtained from blood collected under light diethyl ether anesthesia via cardiac puncture and/or carotic arterial sampling catheter. Trasylol (1000 KIU/ml serum) was

added before storing serum at – 20 °C for later assay. All serum samples were assayed within 3 weeks of collection following a single freeze-thaw cycle.

(ii) Rat-PP release during glycemic studies. (a) Insulin induced hypoglycemia. To validate the applicability of our rat-PP assay in an experimental situation, hypoglycemia was induced in fasted (conscious) male Sprague–Dawley rats (350–400 g) by administering regular porcine insulin (1–2 units per kg body weight) i.v. through a jugular cannula. After an initial blood sample was drawn and placed in an EDTA tube to determine base line glucose level, blood samples for measurement of glucose and PP levels were drawn again 30 min after insulin injection. Plasma was separated for glucose and PP analyses.

(b) Meal stimulated rat-PP release. In the experiments designed to measure fasting and postfeeding levels of rat serum PP, male rats (350–400 g) had carotid arterial sampling catheters (ID 0.016in, OD 0.029in, Teleflex, Inc., New York) implanted 3 days prior to the study and were fasted overnight for 18 h. At 9 am, a blood sample (1 ml) was drawn and the animals were immediately fed (ad libitum) normal laboratory chow (Purina Feed Company). Blood samples were drawn again after 1, 2 and 4 h postfeeding. Plasmas were separated for glucose and PP analyses and was stored at – 20 °C until hormone analyses were completed.

(iii) Radioiodination of rat-PP. Rat-PP was radiolabeled by a lactoperoxidase-hydrogen peroxide method [15]. The following reagents were added in the order listed at room temperature to the reaction vessel (glass culture tube 10 × 75 mm): rat-PP (5 μg in 200 μl of 0.2 M phosphate buffer, pH 7.6), 30 μl lactoperoxidase (145 μg/ml), 30 μl of iodine-125 (carrier-free sodium salt; 3 mCi/30 μl, Amersham Corporation). The reaction was initiated by two-interval additions of 30 μl of 0.015% hydrogen peroxide. Each addition of hydrogen peroxide was followed by 5 min waiting interval. The reaction was stopped by adding 100 μl of 7.5 M urea. Free iodine was removed by Sephadex G-50 column chromatography using phosphate 0.05 M with 0.1% BSA, pH 7.6) as eluting buffer. Immunoreactive labeled rat-PP migrated as a single peak at approx. $K_{av} \sim 0.7$. Sufficient tracer was diluted to 15,000 cpm/100 μl to perform all assays anticipated over a 6-week period. The dilution factor was recorded for future reference and the 'assay-ready' tracer was kept frozen at – 20 °C until used. This tracer retained stable immunoreactivity for 8 weeks.

(iv) Rat-PP radioimmunoassay. Assay set-up consisted of total radioactive tubes (TOTAL), non-specific binding tubes (NSB), rat-PP standards (range 21–2100 pg/ml), quality controls and unknowns. Phospho/saline buffer (0.05 M) was employed as assay buffer containing 0.025 M EDTA, 1000 KIU/ml trasylol, 25 μg/ml leupeptin and 1.0% BSA (RIA grade) at pH 7.4. Assays were performed as follows: rat-PP standard (100 μl of 0, 21, 42, 84, 210, 420, 840, 2100 pg/ml) were pipetted in triplicate into borosilicate glass tube (12 × 75 mm). Quality controls (100 μl) and unknowns (100 μl) were pipetted in duplicate. Assay buffer was added to NSB (300 μl) and to the remainder of assay tubes (100 μl), except TOTAL. Rat-PP antiserum, diluted to obtain 35–45%, B/B_0 binding, was pipetted into all tubes except TOTAL and NSB. The tubes were gently shaken, covered with aluminum foil and incubated 72 h at 4 °C.

After the first 72 h incubation, ^{125}I-rat-PP tracer, diluted when freshly iodinated to obtain 15,000 cpm per 100 μl, was pipetted to all tubes. The tubes were again shaken,

covered and incubated for an additional 24 h at 4 °C. Radiolabeled rat-PP bound to antibody was separated from free ligand by second antibody precipitation during a 2 h incubation (4 °C). Immediately prior to centrifugation, 1 ml assay buffer without BSA was added to all tubes except TOTAL tubes to reduce non-specific counts associated with precipitate. The tubes, were shaken, centrifuged (3000 *g*) for 20 min at 4 °C. The supernatant was decanted and the pellet counted in an automated gamma counter (Apex Automatic Gamma Counter, Micromedic Systems, Inc.). The calculations for each sample were performed by the automated gamma counter with a data reduction system using log/logit transformation.

(v) Limits of test procedure. The assay was rejected if one reference quality control fell outside of two standard deviations of the mean obtained from 10 previous assays. Unknown rat-PP levels were reassayed if the difference between duplicates was > 10% coefficient of variation. Any PP value less than the lowest standard (21 pg/ml) when using 100 µl sample was recorded as < 21 pg/ml and any value greater than the highest standard (2100 pg/ml) was diluted and reassayed.

Results

Assay performance characteristics-reference curve

An assay standard curve for rat-PP (RPP) is presented in Fig. 1. Each point represents mean ± S.E. (n = 20) of percent binding of known RPP standard concentrations over zero binding ($\%B/B_0$). The $\%B/B_0$ observed for each standard was plotted on the ordinates against the log of concentration of standard on the abscissa. The ED_{50} was 290 ± 9 pg/ml, while ED_{20} and ED_{80} were 960 ± 13 pg/ml and 88 ± 3 pg/ml, respectively. Sensitivity limit of rat-PP assay calculated at ± 3 S.D. was 19 pg/ml. Sensitivity is defined as the smallest amount of unknown ligand that can be distinguished from no ligand.

Preliminary data revealed a decrease in serum PP concentration following freezing and thawing of samples. This was attributed to degradation of PP by serum proteinases. Since proteolytic degradation is generally considered to be a greater potential problem when using serum as compared to plasma samples, we used serum to test the protective property of various protease inhibitors. Leupeptin (25 µg/ml) and benzamidine (1 mg/ml) afforded 94% and 87% protection, respectively, when tested on the degradation of rat-PP tracer in serum samples during 24 h incubation at 4 °C. The high concentration of benzamidine (1 mg/ml, however, significantly decreased % bound over zero binding (B/B_0). Trasylol (1000 KIU per ml) was minimally effective while chloromercuriphenylsulfonic acid (PMCB 200 µg/ml), chymostatin (125 µg/ml) and trypsin inhibitor (625 µg/ml) were ineffective. Therefore, leupeptin (25 µg/ml) and trasylol (1000 KIU/ml) were added in all subsequent RPP assays to prevent proteolytic degradation of PP. Leupeptin was also added to all serum or plasma samples anticipated for storage prior to assay.

Intra-assay and between-assay variation

Intra-assay variation is shown in Table Ia. Five rat serum samples spiked with

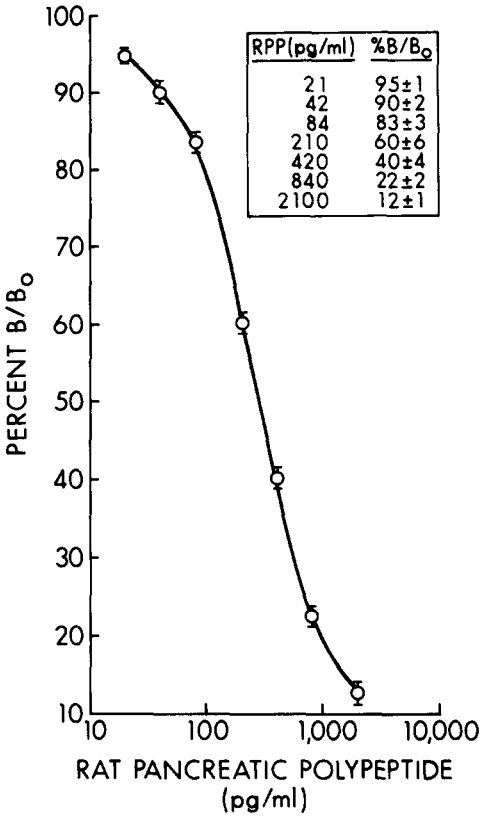

Fig. 1. Rat-PP assay standard curve. Each point represents mean ± S.E. (n = 20) of percent binding of known RPP standard concentrations over reference binding. ED_{80} = 88 ± 3 pg/ml, ED_{50} = 290 ± 9 pg/ml, ED_{20} = 962 ± 13 pg/ml. Sensitivity limit at ±3 S.D. = 19 pg/ml. PP values on x-axis are expressed in log concentration.

varying concentrations of RPP were assayed duplicate in 10 separate assays. Intra-assay variation of the five samples in 10 assays ranged from 5.9 to 9.4%. Percent coefficient of variations (%CV) were within acceptable range regardless of the concentration of RPP in the serum sample. To determine variation between assays (Table Ib), four rat serum samples containing a wide range concentrations of RPP were assayed in 12 duplicate tubes in 10 separate assays. Between-assay %CV ranged between 2.7% and 8.6% with the expected higher %CV encountered at both ends of the standard curve.

Spiking recovery of rat-PP and parallelism of serum dilution

The recovery of RPP at different concentrations in serum is shown in Table IIa. Varying concentrations of RPP were added to five rat serum samples and the RPP level in each sample was measured in 20 separate assays. Recovery was similar whether high

TABLE I

Intra-assay variation and between assay variation

Intra-assay variation (a) (mean ± S.D.)			Between assay variation (b) (mean ± S.D.)		
sample No.	RPP (pg/ml)	% C.V.	sample No.	RPP (pg/ml)	% C.V.
1	159 ± 15	5.4	1	169 ± 13	5.7
2	286 ± 19	3.0	2	276 ± 7	2.7
3	509 ± 30	6.9	3	524 ± 32	6.4
4	986 ± 79	8.0	4	1069 ± 93	8.6
5	1446 ± 123	8.5			

Intra-assay variation (a) was performed in 10 duplicate tubes from five rat serum samples containing varying concentrations of RPP in ten separate assays. Mean ± S.D. and % C.V. of ten observations are shown. Between assay variation (b) was determined in 12 duplicate tubes in 10 separate assays from four rat serum samples containing varying concentrations of rat-PP. Mean ± S.D. of means and % C.V. are shown.

TABLE II

Spiking recovery of rat pancreatic polypeptide (a) and effect of serum dilution on rat pancreatic polypeptide assay (b)

(a)	Rat-PP added (pg/ml)	Observed (pg/ml)	Expected (pg/ml)	Percent recovery
	0 (basal)	47 ± 6	–	–
	84	128 ± 14	131 ± 9	98 ± 5
	100	150 ± 4	147 ± 4	102 ± 4
	210	262 ± 11	257 ± 8	102 ± 6
	420	492 ± 25	477 ± 7	103 ± 3
	840	899 ± 24	882 ± 40	102 ± 8
(b)	Serum dilution effect: serum volume			
	100 µl	627 ± 40	–	–
	50 µl	654 ± 17	627 ± 40	104 ± 3
	25 µl	668 ± 14	627 ± 40	107 ± 9

(a) Varying concentrations of RPP were added to five rat serum samples and the RPP content was determined by RIA. Mean ± S.D.M. of the observed concentrations for 20 separate assays are shown. % recovery was calculated on the observed vs. expected. (b) Aliquots of pooled rat serum containing high RPP levels were added to assays in volumes indicated. Dilution factor of 1, 2 and 4 representing 100 µl, 50 µl and 25 µl, respectively, were applied in calculating observed concentrations. Mean ± S.D.M. of RPP concentration and % recovery for 20 separate assays are shown.

(840 pg/ml) or low (84 pg/ml) concentrations of RPP was tested indicating recovery efficiency throughout the standard curve range. Average percent recovery range from 98% to 103%.

Effect of varying serum volumes (25 µl, 50 µl and 100 µl) was tested in each of

20 separate assays to determine the effect of serum on the expected concentrations of RPP, Table IIb. The assay produced similar observed values and % recoveries regardless of volume of serum in the assay. Hence, varying the volume of rat serum in the assay from 25 μl to 100 μl will produce no significant alteration of the observed RPP level.

Specificity

The specificity of rat-PP antibody used in this study was tested in assays designed to determine percent cross reactivity (CR) of the rat-PP antibody to other ligands likely to cocirculate with PP in blood under various experimental situations. As shown in Table III, amylin (100 ng/ml), glucagon (33 nM), human insulin (32 nM), human-PP (1.7 nM), human proinsulin (1.7 nM), neuropeptide Y (430 ng/ml), peptide YY (430 ng/ml), rat C-peptide (32 nM) and rat insulin (32 mM), respectively, did not produce measurable displacement of rat-PP tracer.

Rat serum-PP migration of Sephadex chromatograph

The authenticity of assay measurements as native PP was ascertained by column chromatography. 4 ml of rat serum was collected after insulin-induced hypoglycemia to insure high levels of PP (255 pg/ml). The serum was lyophilized, reconstituted with 1.5 ml of water and applied to a Sephadex G50 column (0.9 × 55 cm, 1.2 ml/fraction) using phospho/saline (0.05 M, plus 1% BSA, pH 7.4) as eluting buffer. Rat-PP was measured in each column fraction and plotted in Fig. 2. The migration pattern of rat

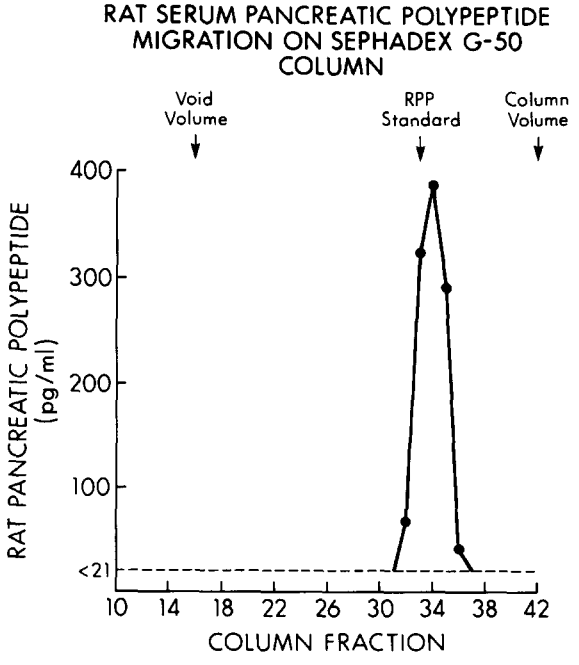

Fig. 2. A representative rat serum PP migration in a Sephadex G-50 column. Arrows indicate migration patterns of void volume, synthetic RPP standard and column volume, respectively. RPP was eluted with 0.05 M PO_4 plus 1% BSA.

TABLE III

Percent cross-reactivity of rat pancreatic polypeptide antibody to various peptide hormones

Test hormone	Concentration tested	% Cross-reactivity
Rat-PP	5–500 PM	100%
Mouse-PP	mouse-serum	100% by linear dilution curve
Amylin	100 ng/ml	no displacement
Glucagon	33 nM	no displacement
Human-pp	1.7 nM	no displacement
Human insulin	32 mM	no displacement
Human proinsulin	170 nM	no displacement
Neuropeptide Y	430 ng/ml	no displacement
Peptide YY	430 mg/ml	no displacement
Rat C-peptide	32 nM	no displacement
Rat insulin	32 mM	no displacement

Percent cross-reactivity of rat-PP antibody was determined for various peptide hormones. The concentration of test hormone required to displace 50% of labeled rat-PP from rat-PP antibody (ED_{50}) was used to determine cross-reactivity as described under Materials and Methods. Cross-reactivities were consistently less than 0.01%.

serum PP was identical to that of synthetic rat PP, validating the serum measurement as authentic rat PP.

Rat-PP level in typical glycemic study

To validate the applicability of this assay, rat-PP levels were measured following fasting, post-feeding and during insulin induced hypoglycemia. Table IV shows fasting plasma glucose, post-feeding glycemic levels and the corresponding plasma PP levels. Plasma PP level was increased 2-fold from 40 ± 2 to 80 ± 10 pg/ml by 2 h after the animals were allowed free access to food. The increase was correspondent to the elevation in plasma glucose during same period (109 ± 2 vs. 146 ± 5 mg/dl). Glucose and PP levels in plasma fell slightly 4 h later, apparently reflecting decreased feeding drive since the experiments were by design conducted during the day (see Materials and Methods, ii) when feeding activity is known to be decreased in rats. Rat-PP release

TABLE IV

Fasting and post-feeding plasma concentrations of rat pancreatic polypeptide

Condition	Glucose (mg/dl)	PP (pg/ml)
Fasting	109 ± 2	40 ± 2
2 h post-feeding	146 ± 5	80 ± 10
4 h post-feeding	135 ± 4	44 ± 9

Fasting and post-feeding plasma concentrations of rat PP. Plasma glucose level and PP concentrations represent mean ± S.E. of means for each condition, respectively. Plasma glucose level was determined in a Beckman Glucose Analyzer 2 at 20 °C. Rat PP was measured as described under Materials and Methods.

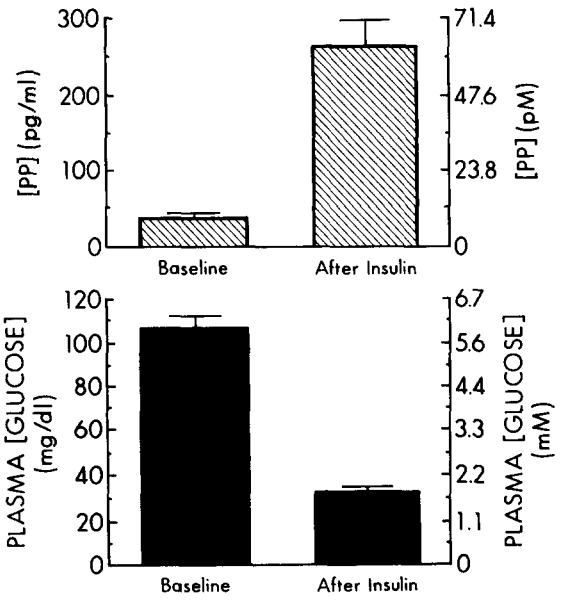

Fig. 3. Rat-PP release during insulin induced hypoglycemia in normal conscious rats. Graphs show mean ± S.E.M. of plasma RPP (upper graph) and plasma glucose level (lower graph) before and 30 min after intravenous (bolus) insulin administration (2 units/kg body weight, $n = 15$).

during insulin induced hypoglycemia in normal rats is shown in Fig. 3. Glucose levels fell significantly from 107 ± 5 to 33 ± 2 mg/dl by 30 min after a bolus insulin infusion. PP levels rose from 38 ± 5 pg/ml to 261 ± 34 pg/ml ($P < 0.0005$) at 30 min.

Discussion

Rats and mice are widely used animal models in the study of pathophysiology of human diabetes mellitus, obesity and autonomic neuropathy, etc. These experiments often require an evaluation of endogenous pancreatic polypeptide (PP) secretion. Previously, the specific measurement of pM levels of PP in rat and murine plasma was not possible. This study validates a specific and sensitive radioimmunoassay (RIA) for rat-PP in serum or plasma. The assay is simple, and is easily applicable in glycemic studies using rat and mouse models.

Previously reported RIA for rat-PP in tissues [16], pancreas perfusate [17] and serum [18] often made use of either a rabbit anti-rat PP antibody originally reported by Kimmel et al. [19] in a 48 h equilibrium incubation with a dextran/charcoal technique of separation of bound ^{125}I from free [18], or carboxyl-terminal hexapeptide antibodies in a 24 h disequilibrium incubation and a 24 h second antibody precipitation [20]. In the reports cited, with two exceptions (Refs. 16 and 18), bovine-PP tracer was used. While these assay procedure measured levels of PP, assay specificity and sensitivity appeared highly compromised. For example, carboxyl-terminal hexapeptide antiserum is also capable of cross-reacting with neuro-peptide Y and peptide YY [21,22]. Addi-

tionally, rat PP from pancreatic extracts have been shown to cross-react poorly and non-specifically with bovine-PP antibody and tracer [3]. The only available report of RIA for rat-PP in serum (Miyasaka et al. [18]) in which both the anti-rat PP antibody and rat-PP tracer were used in the assay achieved sensitivity of 80 pg/ml with a inter and intra assay %CV of 10%. Detailed characterization of the assay was not presented. PP assay sensitivity of 80 pg/ml is incapable of detecting low rat-PP levels under various experimental conditions such as fasting and feeding ad libitum without concentration of serum prior to assay. Studies of rodent PP levels are therefore frequently plagued by inconsistent values. We have developed a highly reproducible RIA for rat-PP in serum that achieves sensitivity of 19 pg/ml (4.5 pM) PP at ± 3 S.D. The assay produces no cross-reactivity with a variety of hormones and peptides likely to co-circulate in plasma, see Table III. The percent intra- and between-assay coefficient of variation are 6.4% and 5.9%, respectively (Table Ia,b). The antibody also cross-reacts well with mouse-PP as determined by linear serum dilution curves (data not presented), thus making it useful in the measurement of murine-PP.

The remarkable sensitivity of this assay is due to the antibody's high affinity constant and the result of step-wise sequence addition of antibody and labeled ligand, a procedure termed 'sequential saturation' [23]. The sensitivity of 19 pg/ml at ± 3 S.D. is particularly desirable when samples volumes available for assay are small, as in the case of multiple sampling protocols from rats or mice, and makes possible accurate assessment of the low fasting concentrations which ranged from 29 pg/ml (6.9 pM) to 45 pg/ml (10.7 pM) in normal rats.

In conclusion, we have developed and characterized a rat-PP assay which is sensitive, simple and specific for rat-PP. It is applicable in laboratory studies of normal physiology and disease processes to ascertain PP levels in serum in rodent models and also provides a new tool for reliable measurements of rat- and mouse-PP levels as indeces of parasympathetic nervous input to the pancreatic islets [24].

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