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Inhibition of glucose stimulated insulin secretion by neuropeptide Y is mediated via the Y1 receptor and inhibition of adenylyl cyclase in RIN 5AH rat insulinoma cells

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Summary Neuropeptide Y (NPY) has been shown to inhibit insulin secretion from the islets of Langerhans. We show that insulin secretion in the insulinoma cell line RIN 5AH is inhibited by NPY. ^{125}I-Peptide YY (PYY) saturation and competition-binding studies using NPY fragments and analogues on membranes prepared from this cell line show the presence of a single class of NPY receptor with a Y1 receptor subtype-like profile. Inhibition of insulin secretion in this cell line by NPY fragments and analogues also shows a Y1 receptor-like profile. Both receptor binding and inhibition of insulin secretion showed the same orders of potency with NPY > [Pro^{34}]-NPY > NPY 3–36 > > NPY 13–36. The Y1 receptor antagonist, BIBP 3226, blocks NPY inhibition of insulin secretion from, and inhibits ^{125}I-PYY binding to, RIN 5AH cells. Northern blot analysis using a Y1-receptor specific probe shows that NPY Y1 receptors are expressed by RIN 5AH cells. Y5 receptors are not expressed in this cell line. Neuropeptide Y inhibition of insulin secretion is blocked by incubation with pertussis toxin, implying that the effect is via a G-protein (G_i or G_o) coupled receptor. Neuropeptide Y inhibits the activation of adenylyl cyclase by isoprenaline in RIN 5AH cell lysates, and the stimulation of cAMP by glucagon-like peptide-1 (7–36) amide (GLP-1). It also blocks insulin secretion stimulated by GLP-1, but not by dibutyryl cyclic AMP. Hence, we suggest that NPY inhibits insulin secretion from RIN 5AH cells via a Y1 receptor linked through G_i to the inhibition of adenylyl cyclase. [Diabetologia (1998) 41: 1482–1491]

Keywords Neuropeptide Y, Y1 receptor, insulin secretion, insulinoma cells.

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Abbreviations: NPY, Neuropeptide Y; PYY, peptide YY; PP, pancreatic polypeptide; [Pro^{34}]-NPY, NPY analogue with proline substituted for isoleucine at position 34; NPY 3–36 and NPY 13–36, NPY fragments with the first two and twelve N-terminal amino acids removed respectively; Y1-Y6, NPY/PYY/PP receptor subtypes; GTP-γ-S, guanosine 5′-O-(3-thiotriphosphate); ATP-γ-S, adenosine 5′-O-(3-thiotriphosphate); FCS, fetal calf serum; GLP-1, glucagon like peptide 1 (7–36) NH_2; HEPES, N-[2-hydoxyethyl]piperazine-N′-[2-ethanesulphonic acid]; DMEM, Dulbecco's modified Eagle's medium.

Insulin secretion from the beta cells of the pancreas is under many regulatory influences, including neuropeptide Y (NPY). This 36 amino acid peptide is one of the most widespread peptide messengers in the body [1]. Among its many actions, an inhibition of insulin secretion by NPY has been found in mouse [2], pig [3] rat [4–6] and human [7]. In the islets, most NPY can be found in sympathetic nerves [8] and a role in the sympathetic regulation of insulin secretion has therefore been suggested. Recently, we have shown the presence of messenger RNA for NPY in islets from rats treated with 6-hydroxydopamine to remove sympathetic inervation [9]. This is supported by reports from others [10, 11] and by work showing synthesis of NPY by clonal insulin-secreting cell lines [9, 12]. These results suggest local production of NPY in the islet, and a paracrine or autocrine regulation of

insulin secretion. In normal rat islets, NPY secretion decreases in a similar manner to glucagon following an increase in the glucose concentration [6]. In insulin resistant dexamethasone treated rats, however, islet NPY secretion followed a pattern more similar to insulin secretion, with a sixfold rise seen with increased glucose [6]. These results suggest a complicated system of NPY production in the islets and this is possibly mirrored by complexity in the response to NPY.

Neuropeptide Y, peptide YY (PYY) and pancreatic polypeptide (PP) form a family of peptides with similar structures. There are currently six recognised receptor subtypes for these peptides (Y1-Y6) [13–18]. The different subtypes of receptor for this family have been identified and characterised by their ability to bind NPY, PYY and PP fragments and analogues. The Y1 receptor binds with high affinity only full length NPY or full length analogues such as the NPY analogue [Pro^{34}]NPY with proline substituted for isoleucine at position 34, and has reduced affinity for C-terminal fragments such as NPY fragments with the first twelve N-terminal amino acids removed (NPY 13–36). The Y2 receptor, in contrast to Y1, has a much higher affinity for C-terminal fragments than for substituted analogues such as [Pro^{34}]-NPY.

A group of receptors sharing low affinity for PYY have been labelled y3 [19]. Peptide YY is reported to have similar effects to NPY on insulin secretion [20] and this receptor therefore seems unlikely to be involved in the effect of NPY on the beta cell. The recently cloned Y4 receptor [17, 21] is characterised by its high affinity for PP and the Y5 [14] and Y6 [15] receptors are both receptors with Y1-like profiles found in the hypothalamus. Although the Y6 receptor has been cloned from mouse genomic DNA, a physiological correlate has yet to be described and in a number of species the receptor has undergone a frame-shift mutation.

The mechanism of the inhibitory action of NPY on insulin secretion is not known. In many other systems NPY acts via the inhibitory GTP-binding protein (G-protein), G_i, and inhibition of adenylyl cyclase [22–24]. In pancreatic perfusion studies, however, NPY inhibited secretion stimulated by dibutyryl cyclic AMP [25], suggesting that this effect might be distal to cyclic AMP generation. Alternative modes of action might include interaction with K^+ channels, to block membrane depolarisation (as seen with diazoxide, and with the neuropeptide galanin [26]), or interaction with Ca^{2+} channels [27].

To investigate the receptor and second messenger systems involved in the inhibition of insulin secretion by NPY we have used the clonal beta-cell line RIN 5AH [28]. Clonal cells were chosen for initial characterisation of this system because experiments with isolated islets are both difficult to do, and difficult to interpret, because of the confounding influence of multiple cell types. These intra-islet paracrine influences are important for the functioning of the normal islet, and therefore experiments with clonal cell lines, which are a model of individual cell types, do not tell the whole story. To understand the intra-islet interactions it is, however, first necessary to understand the actions and responses of the individual cell types involved. For this, clonal cell lines provide an invaluable tool. Clonal cell lines also have the disadvantage of being transformed. This often leads to phenotypes altered from the cells of which they are supposed to be models, and this should be born in mind when interpreting results obtained using them. RIN 5AH was chosen in preference to some other insulin secreting cell lines because of its glucose responsiveness (cf. RIN m5F, which is not glucose responsive but responds to other secretagogues such as glyceraldehyde). We have examined the NPY receptors present on this cell line and the inhibition of insulin secretion by NPY fragments and analogues.

Materials and methods

Peptides and iodination. The porcine sequence was used for all NPY fragments and analogues unless otherwise specified. NPY, NPY 3–36, NPY 13–36 and [Pro^{34}]-NPY were synthesised by P. Byfield (Haemostasis unit, Clinical Science Centre, Hammersmith Hospital, UK). Peptide YY was synthesised by IAF (Quebec, Canada). BIBP 3226 was kindly donated by Dr Karl Thomae GmBH (Biberach an der Riss, Germany). All cell culture reagents were supplied by Gibco (Gibco BRL, Life Technologies Ltd., Paisley, UK). All other materials were supplied by Sigma or Merck (both Poole, Dorset, UK) unless otherwise stated.

Porcine PYY was iodinated using the iodogen method as described previously [29]. Peptide (5 nmol) was dissolved in 10 µl 0.2 mol/l phosphate buffer (pH 7.2) and added to a polypropylene tube containing 10 µg 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (iodogen, Pierce, Rockford, Ill., USA), plus 37 MBq $Na^{125}I$ (Amersham International, Amersham, Bucks., UK). This was incubated on ice for 5 min and products separated by reversed phase C_{18} HPLC (Waters Novapak column, Millipore, Milford, Mass., USA), developed with a 15–45 % acetonitrile (v/v) water 0.05 % trifluoroacetic acid (v/v) gradient. Fractions (1.5 ml) were collected and radioactive peaks assayed for receptor binding activity. Active fractions were aliquoted, freeze-dried and stored at –20 °C. The specific activity of the radioligand was 27 Bq/fmol.

Cell culture. RIN 5AH cells from passage number 14 to 20 were maintained routinely in RPMI 1640 medium containing 5.5 mmol/l glucose and 5.5 mmol/l glutamine and supplemented with 10 % fetal calf serum (FCS) (v/v) and antibiotics (penicillin 100 IU/ml and streptomycin 100 µg/ml) [30]. Medium was changed every 48 h and cells passaged when they reached 70 % confluence (approximately 7 days). In the same way SK-N-MC cells (Dr. S. Legon, Dept. Metabolic Medicine, Hammersmith Hospital, ICSM), which are the de facto standard for investigation of Y1 receptors [31, 32], were grown routinely in 50 % Dulbecco's modified Eagle's medium (DMEM)/50 % HAMS F12 medium, supplemented with 10 % FCS and antibi-

otics [33]. HEK 293 cells (Dr. S. Legon) were stably transfected with the NPY Y5 receptor cDNA according to the methods of Gerald et al. [14], and grown in DMEM with 10 % FCS and antibiotics.

Insulin secretion experiments. RIN 5AH cells were grown as above in 24-well plates for 96 h. Medium was replaced for 2 h before the start of the experiment with glucose free, FCS free medium. Cells were then incubated for 2 h in FCS free medium with 1.2 mmol/l glucose and test substances. The medium was then removed and stored at –20 °C for subsequent RIA. Dose response curves were constructed for NPY 1–36, NPY 3–36, NPY 13–36, [Pro^{34}]-NPY and BIBP 3226. The effect of NPY (100 nmol/l) on insulin secretion in the presence of glucagon like peptide (GLP)-1 (10 nmol/l), and dibutyryl cyclic AMP (1 mmol/l) (both in the presence of 1.2 mmol/l glucose) was tested. All secretion experiments were carried out in triplicate, and were repeated between 6 and 12 times.

To test the ability of pertussis toxin to inhibit the action of NPY on RIN 5AH cells, cells were preincubated for 2 h in glucose free, serum free RPMI 1640 medium containing 200 ng/ml pertussis toxin (Sigma Pode, UK). They were then exposed to medium containing combinations of glucose (1.2 mmol/l), pertussis toxin (200 ng/ml), NPY (1 μmol/l), and somatostatin (1 μmol/l). Medium was removed after 3 h and insulin was measured by radioimmunoassay (RIA).

Insulin radioimmunoassay. Culture medium was assayed in duplicate for insulin by an established RIA using porcine insulin standard and Glu5 antibody at a final dilution of 1:150,000 [6]. The assay had a detection limit of 2 fmol/tube at 95 % confidence limits. Both inter-assay and intra-assay variations were below 10 %.

Membrane preparation. Cell membranes were prepared by osmotic lysis and differential centrifugation as described previously [33]. Briefly, cells were lysed in 100 mls of 1 mmol/l N-[2-hydoxyethyl]piperazine-N′-[2-ethanesulphonic acid] (HEPES) buffer (pH 7.4) containing protease inhibitors (benzamidine (100 μg/ml), bacitracin (100 μg/ml), aprotinin (30 μg/ml), soya bean trypsin inhibitor (10 μg/ml), pepstatin (0.5 μg/ml), leupeptin (0.5 μg/ml), and antipain (0.5 μg/ml)) at 4 °C. The lysate was centrifuged at 4 °C for 15 min at 3 000*g*. The supernatant was then discarded and the pellet disrupted in 50 mmol/l HEPES (pH 7.4) with protease inhibitors and centrifuged again at 4 °C for 15 min at 3 000*g*. The supernatant was then centifuged at 4 °C for 60 min at 48 000*g*, and the resulting pellet resuspended in the same buffer to a final concentration of 2–4 mg/ml, as determined by biuret protein assay [34]. Aliquots were stored at –80 °C until use.

Receptor binding. Receptor binding was carried out as described previously [33]. Membranes (100 μg protein) were incubated with 1000 Bq (75 pmol/l) [^{125}I]-PYY in the presence or absence of unlabelled peptides as indicated. Binding was carried out in a final volume of 500 μl assay buffer (20 mmol/l HEPES pH 7.4, 5 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 1 % (w/v) bovine serum albumin) for 90 min at 30 °C. Bound and free label were then separated at 4 °C by centrifugation for 2 min at 15 000*g*. The pellet was resuspended in 1 ml of ice-cold assay buffer and re-centrifuged. Bound ^{125}I-PYY was then quantified in a γ-counter. Total specific binding was defined as the difference in counts between assays in the presence (non-specific) and absence (total) of 200 nmol/l NPY. Analysis of ^{125}I-PYY binding experiments was carried out using ReceptorFit programs (Lundon Software Inc., Cleveland, Ohio, USA). Two-site and one-site curves were compared by *F*-test. Two-site curves were considered to be of a significantly better fit than one-site when p was less than 0.05.

Adenylyl cyclase activity. Cell lysates were made as described previously [33]. Briefly, confluent cultures of RIN 5AH cells grown in 75 cm^2 flasks were washed with ice-cold phosphate-buffered saline and then with ice-cold 1 mmol/l Tris/HCl (pH 7.4) containing 2 mmol/l EDTA, pepstatin (0.5 μg/ml), leupeptin (0.5 μg/ml), and antipain (0.5 μg/ml). The cells were then allowed to lyse in 3 ml of the same buffer for 15 min at 4 °C and then disrupted in a glass teflon homogeniser. Aliquots (20 μl) of the lysate were incubated for 10 min at 25 °C in a total volume of 100 μl reaction mixture containing 25 mmol/l Tris/HCl (pH 7.4), 2 mmol/l $MgCl_2$, 1 mmol/l EDTA, 100 μmol/l GTP, 100 μmol/l ATP, 1 mmol/l cyclic AMP, 20 mmol/l creatine phosphate, 2 mmol/l isobutyl methylxanthine, 20 μg creatine kinase, 20 μg myokinase, 1 μCi [α-^{32}P] ATP and 33 nCi ^{3}H cyclic AMP (Amersham International) and, where indicated, various experimental agents. The reaction was stopped by the addition of 10 μl 62.5 % (v/v) trichloroacetic acid, and insoluble matter separated by centrifugation (15 000*g*, 3 min). The supernatant was assayed for ^{32}P-labelled cyclic AMP as described previously [35]. Assays were carried out with a replicate number of 6, and were repeated at least three times.

Cyclic AMP measurement. RIN 5AH cells were grown on 24-well plates as described for insulin secretion experiments. On the day of the experiment, medium was replaced with glucose-free and serum-free RPMI 1640. After 2 h preincubation, this was replaced with serum-free RPMI 1640 containing 1 mmol/l isobutylmethylxanthine, either no glucose or 1.2 mmol/l glucose and experimental agents. After 15 min this medium was removed, and 1 ml acid ethanol (70 % ethanol in 0.05 N HCl) added to the cells. This was left overnight at –20 °C and then dried down by centrifugal evaporation in a Savant Speed-vac Plus (Life Sciences International, Basingstoke, Hants., UK). The residue was taken up in 300 μl assay buffer (50 mmol/l CH_3COONa, 0.1 % NaN_3, 8 mmol/l EDTA, pH 6.2) and assayed using the NEN Flash Plate cAMP assay kit (NEN Life Sciences, Hounslow, UK).

Northern blot analysis. Total RNA was extracted using the guanidinium thiocyanate/phenol/chloroform method [36]. Poly $(A)^+$ RNA was prepared from total RNA by oligo($dT_{12–18}$) cellulose (Amersham Pharmacia Biotech, St. Albans, Herts., UK) chromatography [37]. Northern blot analysis was done as described previously [36, 37]. Briefly, 50 μg of poly (A) + RNA was size separated on a denaturing 3-(n-morpholino) propane-sulphonic acid (MOPS)/formaldehyde 1 % agarose gel and transferred to a Hybond-N membrane (Amersham International). The RNA was fixed by baking at 80 °C for 2 h. Analysis of Y1 receptor mRNA was carried out using an oligonucleotide probe corresponding to nucleotides 465–489 of the rat Y1 receptor sequence [38]. The probe was labelled using [α-^{32}P]-dATP (Amersham International) and terminal transferase (Promega, Southampton, UK) and the blot was treated as described previously [36]. Briefly, the blot was hybridised with 2 ng/ml oligonucleotide probe in 5 × SSC (1 × SSC = 0.15 mmol/l NaCl, 0.015 mmol/l Sodium Citrate, pH 7.0), 10 × Denhardts solution (1 × Denhardts solution = 0.02 % ficoll, 0.02 % polyvinylpyrrolidone, 0.02 % BSA fraction V), 20 mmol/l Na_3PO_4, 100 μg/ml herring sperm DNA, 7 % sodium dodecyl sulphate and 10 % dextran sulphate for 18 h at 55 °C. The blot was then washed in 1 × SSC, 1 % sodium dodecyl sulphate for 30 min at 60 °C. Y5 receptor mRNA was analysed using a cDNA probe corresponding to the entire coding region of the rat Y5 receptor sequence [14], labelled

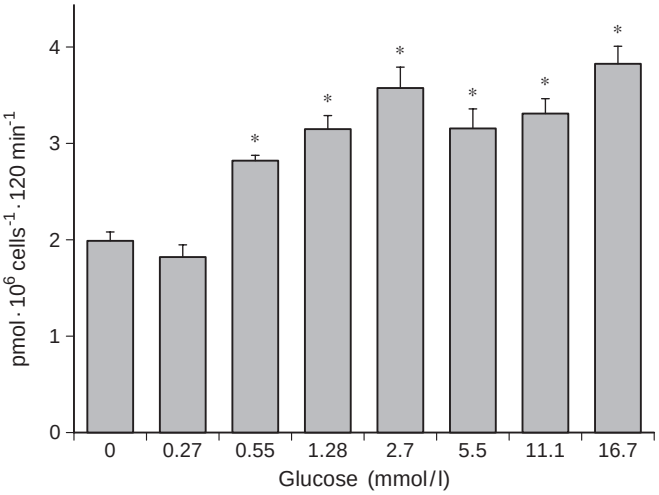

Fig. 1. Insulin secretion from RIN 5AH cells in response to glucose. $*p < 0.05$ vs zero glucose. Results are the means ± SEM of 6 experiments, each carried out in triplicate

by random primer extension using [α-^{32}P]-dCTP and the Klenow fragment as described previously [37]. Briefly, hybridisation included 1 ng/ml probe in 50% formamide, 5 × SSC, 5 × Denhardts solution, 100 μg/ml herring sperm DNA and 10% dextran sulphate for 18 h at 42 °C. The blot was washed in 0.1 × SSC, 0.1% sodium dodecyl sulphate for 20 min at 60 °C.

Results

Insulin secretion. RIN 5AH cells secreted insulin in response to glucose in a dose dependent manner, with 1.2 mmol/l glucose giving maximum stimulation (Fig. 1). Basal insulin secretion was 2.0 ± 0.1 fmol · 10^6cells^{-1} · min^{-1} and this was raised to 3.8 ± 0.2 fmol · 10^6cells^{-1} · min^{-1} by maximum glucose stimulation (means ± SEM of six individual experiments, each in triplicate). Neuropeptide Y is able to inhibit insulin secretion by 50.4 ± 7.5%, with an approximate EC_{50} of 0.1 nmol/l for this effect and [Pro^{34}]-NPY is almost as potent an inhibitor of insulin secretion in RIN 5AH as NPY itself (approximate EC_{50} = 0.3 nmol/l) (Fig. 2), suggesting that the peptide could be acting through a Y1-like receptor. Neuropeptide Y 13–36, was unable to inhibit insulin secretion at concentrations up to 1 μmol/l (Fig. 2 d), further supporting the involvement of a Y1-like receptor. Neuropeptide Y 3–36 inhibits insulin secretion with a potency about 18-times lower than NPY 1–36 (Fig. 2 c, approximate EC_{50} = 1.8 nmol/l), again fitting the expected profile for the Y1 receptor. The Y1 receptor antagonist BIBP 3226 was able to block the inhibition of glucose stimulated insulin secretion by 10 nmol/l NPY (Fig. 2 f, approximate EC_{50} = 3.2 μmol/l) but had no effect itself on glucose stimulated insulin secretion (Fig. 2 e). Pertussis toxin (200 nmol/l) is able to completely block the inhibition of insulin secretion by NPY (1 μmol/l) and the positive control, somatostatin (1 μmol/l) (Fig. 3).

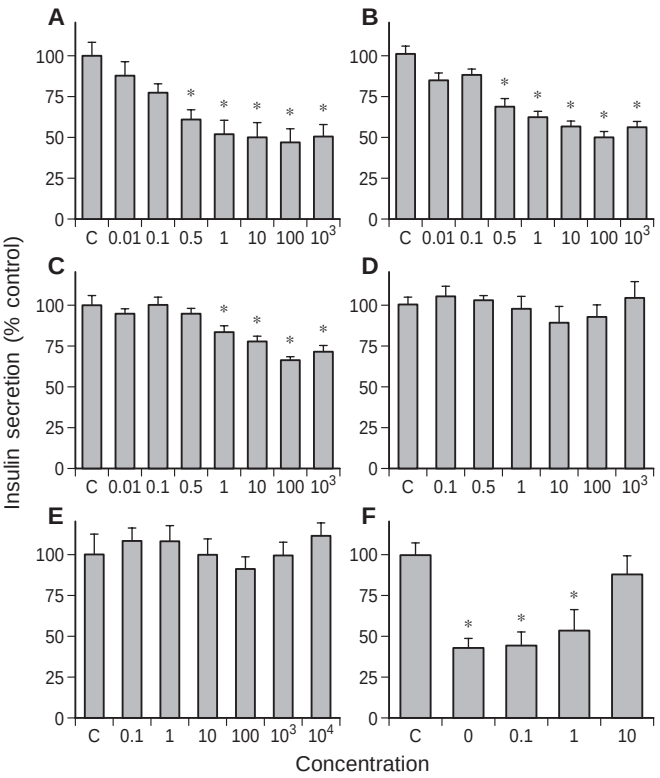

Fig. 2A–F. Insulin secretion from RIN 5AH cells, as a percentage of maximum glucose response. The dose response curves for the inhibition of glucose stimulated insulin secretion by NPY 1–36(**A**), [Pro^{34}]-NPY (**B**), NPY 3–36 (**C**), and NPY 13–36 (**D**). **E** shows the lack of effect of BIBP 3226 on stimulated insulin secretion. **F** is the dose response curve for antagonism of the effect of 10 nmol/l NPY by BIBP 3226. Concentrations of treatment are in nanomolar for **A**,**B**,**C**, **D** and **E**, and in μmolar for **F**. $*p < 0.05$ vs glucose stimulated secretion. Results are the means ± SEM of 6–12 experiments, each carried out in triplicate

Glucagon like peptide-1 (100 nmol/l) potentiates glucose-stimulated insulin secretion from RIN 5AH cells (6.4 ± 0.4 fmol · 10^6cells^{-1} · min^{-1} in 1.2 mmol/l glucose control, 26.4 ± 0.9 fmol · 10^6cells^{-1} · min^{-1} in the presence of 100 nmol/l GLP-1, 410% increase, $p < 0.01$ vs glucose control, $n = 5$ each carried out in triplicate) (Fig. 4). Neuropeptide Y (100 nmol/l) is able to completely block this effect (GLP-1 + NPY = 92 ± 5% of 1.2 mmol/l glucose control, $p < 0.01$ vs GLP alone, $n = 5$ experiments each carried out in triplicate) as well as insulin secretion in the absence of GLP-1 (NPY alone = 31 ± 3% of 1.2 mmol/l glucose control, $p < 0.05$ vs glucose control). Dibutyryl cyclic AMP causes a dose dependent increase in insulin secretion, with 1 mmol/l being the lowest concentration to cause an increase (4.0 ± 0.2 fmol · 10^6cells^{-1} · min^{-1} in 1.2 mmol/l glucose control, 5.8 ± 0.3 fmol · 10^6cells^{-1} · min^{-1} in the presence of 1 mmol/l dibutyryl cyclic AMP, 140% increase,

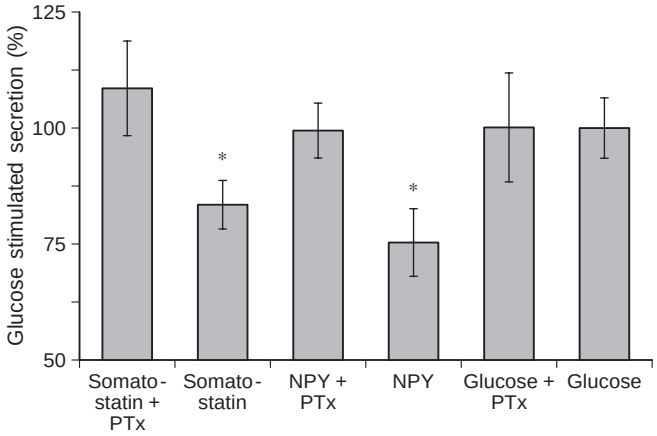

Fig. 3. The effect of pertussis toxin (PTx, 200 ng/ml) on NPY and somatostatin inhibition of insulin secretion from RIN 5AH. Cells were preincubated with pertussis toxin for 2 h and then incubated with pertussis toxin and peptide for a further 2 h. Somatostatin is included as a positive control. Results are expressed as percentage of insulin secreted in response to 1.2 mmol/l glucose (i.e. difference between zero glucose and 5.5 mmol/l glucose). The results shown are the means ± SEM of 3 experiments, each carried out with 6 replicates

$p < 0.01$ vs control) (Fig. 4). A concentration of 10 mmol/l caused a further increase (6.8 ± 0.3 fmol $\cdot$ 10^6cells^{-1} $\cdot$ min^{-1}, 170% increase, $p < 0.01$ vs control). The ability of NPY to inhibit dibutyryl cyclic AMP (1 mmol/l) stimulated insulin secretion was investigated (Fig. 4c). In this experiment, dibutyryl cyclic AMP was again found to potentiate glucose stimulated insulin secretion from RIN 5AH cells (5.6 ± 0.2 fmol $\cdot$ 10^6cells^{-1} $\cdot$ min^{-1} in 1.2 mmol/l glucose control, 12.7 ± 0.5 fmol $\cdot$ 10^6cells^{-1} $\cdot$ min^{-1} in the presence of 1 mmol/l dibutyryl cyclic AMP, 230% increase, $p < 0.01$ vs glucose control, $n = 5$). Neuropeptide Y (100 nmol/l) did not inhibit this action (200 ± 10% of 1.2 mmol/l glucose control, $p > 0.05$, $n = 5$).

Binding of ^{125}I-PYY to RIN 5AH cell membranes. Binding experiments with RIN 5AH membranes consistently gave greater than 50% specific binding (66.7 ± 3.4%, $n = 10$). Binding of increasing concentrations of ^{125}I-PYY to RIN 5AH membranes showed a single, saturable binding site with a $K_D = 395 \pm 72$ pmol/l and $B_{max} = 286 \pm 47$ fmol/mg protein ($n = 3$) (Fig. 5). Inset in this figure is a scatchard analysis of binding data to verify the single site model, but is not used for calculation of B_{max} and K_D, since it is less accurate than the non-linear method used [39]. Binding of ^{125}I-PYY was inhibited in a dose-dependent manner by the non-hydrolysable GTP analogue, guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S, approximate $IC_{50} \sim 3$ μmol/l, maximum inhibition = 95.5 ± 4%), but not by adenosine 5′-O-(3-thiotriphosphate) (ATP-γ-S, $IC_{50} > 100$ μmol/l), suggesting a requirement for guanine nucleotides, and hence G-protein linkage of the receptor (Fig. 6). The

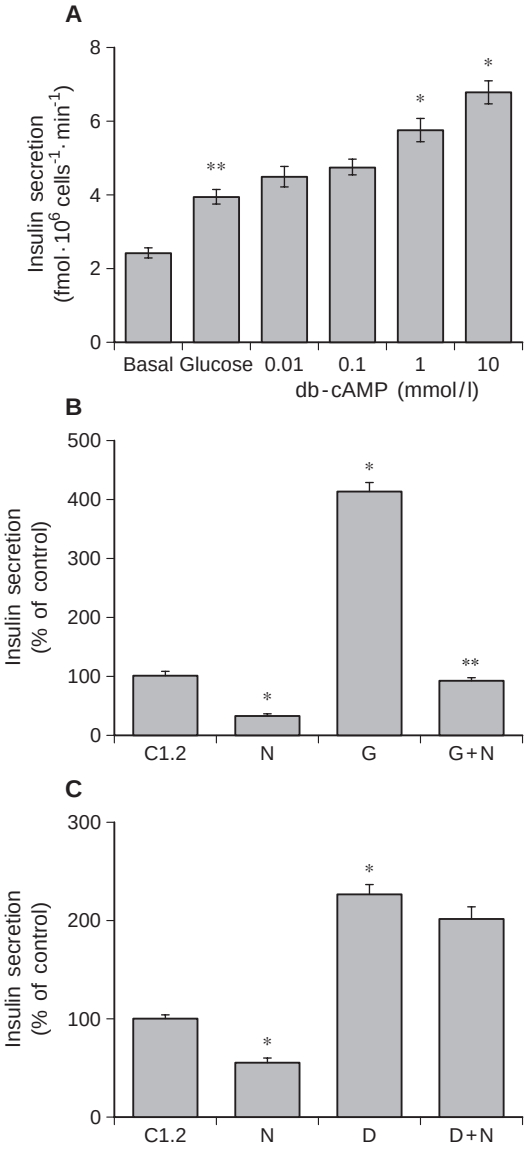

Fig. 4. A The effect of dibutyryl-cyclic AMP on insulin secretion from RIN 5AH cells. The lowest effective dose was 1 mmol/l, and this was used for subsequent experiments. The results shown are the means ± SEM of 3 experiments each carried out in quadruplicate. $^{**}p < 0.005$ vs basal secretion; $^{*}p < 0.01$ vs glucose stimulated insulin secretion. The effect of NPY (100 nmol/l) on **B** GLP-1 (100 nmol/l) and **C** dibutyryl-cyclic AMP (1 mmol/l) stimulated insulin secretion from RIN 5AH cells. $^{*}p < 0.01$ vs 1.2 mmol/l glucose control, $^{**}p < 0.01$ vs GLP-1). C1.2 = control (1.2 mmol/l glucose alone). N = 100 nmol/l NPY, G = 100 nmol/l GLP-1, G + N = 100 nmol/l GLP-1 + 100 nmol/l NPY, D = 1 mmol/l dibutyryl cyclic AMP, D + N = 1 mmol/l dibutyryl cyclic AMP + 100 nmol/l NPY. The results shown are the means ± SEM of 6 experiments, each carried out in triplicate

ability of NPY fragments and analogues to compete for ^{125}I-PYY binding sites was examined in equilibrium competition binding experiments. Competition curves were constructed using NPY ($IC_{50} = 0.14 \pm 0.025$ nmol/l), PYY ($IC_{50} = 0.45 \pm 0.15$ nmol/l), [Pro^{34}]-NPY ($IC_{50} = 0.34 \pm 0.17$ nmol/l), NPY 3–36

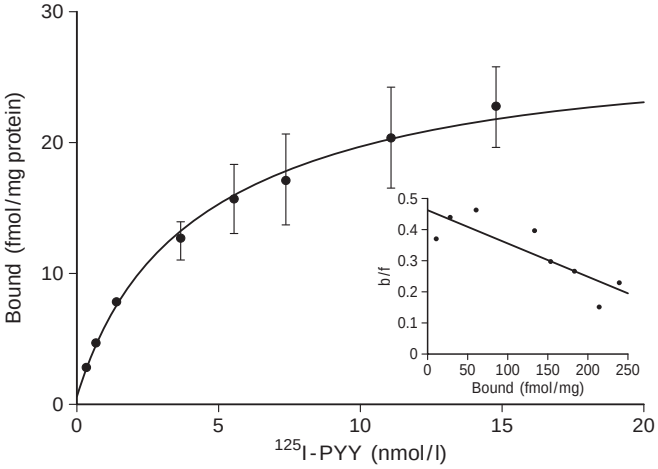

Fig. 5. Saturation binding of ^{125}I-PYY to RIN 5AH cell membranes. Cells were incubated with increasing concentrations of ^{125}I-PYY in the presence (non-specific binding) or absence (total binding) of 200 nmol/l NPY. Specific binding is shown and was calculated as the difference between total and non-specific binding. K_D and B_{max} were calculated by non-linear regression analysis. Inset is Scatchard analysis of the same data [45], used to visually check one-site binding. Mean and SEM are calculated from 3 experiments

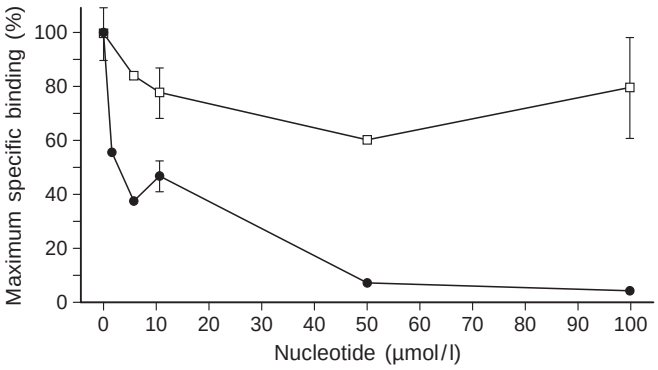

Fig. 6. The effect of GTP-γ-S (●) and ATP-γ-S (□) on ^{125}I-PYY binding to RIN 5AH cell membranes. Membranes were incubated in the presence of increasing nucleotide concentration, in the presence (non-specific binding) or absence (total binding) of 200 nmol/l NPY. Specific binding was calculated as the difference between total and non-specific binding. Only specific binding is shown for the sake of clarity. Total and non-specific binding were carried out in triplicate, and the result of a representative experiment is shown here

(IC_{50} = 0.98 ± 0.16 nmol/l), and NPY 13–36 (IC_{50} = 17 ± 4 nmol/l). These experiments show a receptor with a Y1-like profile, with ligands showing the order of potency NPY > [Pro^{34}]-NPY = PYY > NPY 3–36 > > NPY 13–36. The specific Y1 antagonist, BIBP 3226 bound to RIN 5AH cell membranes with an IC_{50} of 37 nmol/l (Fig. 7).

Adenylyl cyclase activity. To measure the inhibition of adenylyl cyclase, RIN 5AH cell lysates were incubated with isoprenaline to stimulate adenylyl cyclase activity. Isoprenaline (10 μmol/l) gave a robust, but not maximum, stimulation (242 ± 8% vs control, $p < 0.05$, results not shown), and hence this concentration was used for further experiments. Neuropeptide Y (1 μmol/l, 10 μmol/l) was able to completely inhibit isoprenaline stimulated adenylyl cyclase activity (113 ± 4% of control and 93 ± 9% of control respectively, $p < 0.05$ vs isoprenaline) (Fig. 8). It (10 μmol/l) produced a small but significant reduction in basal adenylyl cyclase activity (60 ± 3% of control, $p < 0.05$ vs control). Somatostatin (10 μmol/l), used as a positive control, reduced isoprenaline induced adenylyl cyclase activity by 70% (159 ± 8% of control, $p < 0.05$ vs isoprenaline) but had no effect on basal activity (97 ± 14% control, $p > 0.05$).

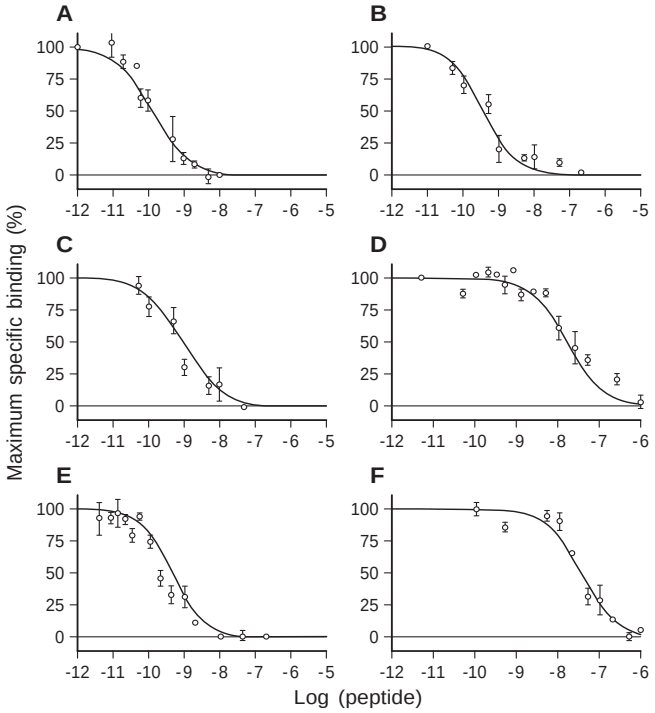

Fig. 7A–F. Competition for ^{125}I-PYY binding to RIN 5AH membranes by NPY 1–36 (**A**), [Pro34]-NPY (**B**), NPY 3–36 (**C**), NPY 13–36 (**D**), PYY (**E**) and BIBP 3226 (**F**). IC_{50} values were calculated by non-linear regression analysis. Each curve is the average of three curves carried out in triplicate. The means ± SEM of 3 experiments is shown here

Cyclic AMP measurement in whole cells. Glucagon like peptide at a concentration of 100 nmol/l was found to increase cAMP production in RIN 5AH cells (0.42 ± 0.04 pmol · 10^6cells^{-1} · min^{-1} in basal control, 0.84 ± 0.09 pmol · 10^6cells^{-1} · min^{-1} in GLP-1 stimulated, $p < 0.01$). Neuropeptide Y was able to inhibit this stimulation, with 100 nmol/l being the lowest effective dose (0.7 ± 0.07 pmol · 10^6cells^{-1} · min^{-1}, $p < 0.01$ vs GLP-1 alone), and 1 μmol/l completely removing the effect of GLP-1 (0.44 ± 0.09 pmol · 10^6cells^{-1} · min^{-1}, not significantly different from basal) (Fig. 9).

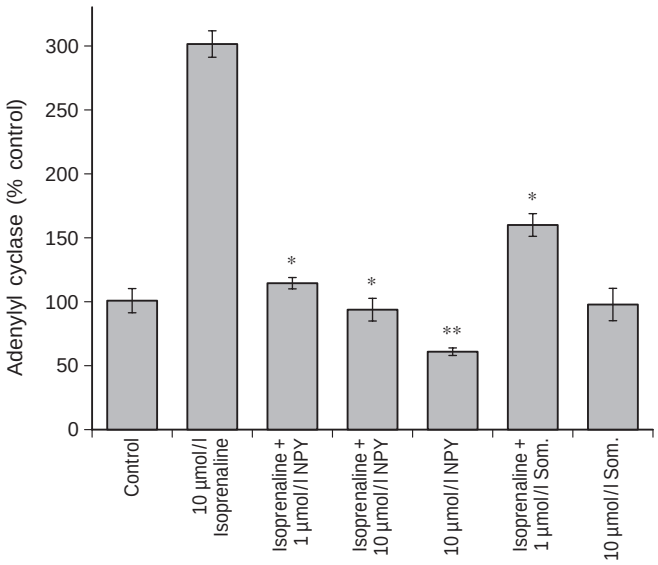

Fig. 8. Adenylyl cyclase activity in RIN 5AH cell-lysates. Isoprenaline stimulates adenylyl cyclase activity and this is inhibited by NPY (1 µmol/l, 10 µmol/l) and somatostatin (Som, 10 µmol/l). Somatostatin is included as a positive control. Somatostatin had no effect on basal adenylyl cyclase activity. *$p < 0.05$ vs isoprenaline control; ** $p < 0.05$ vs saline control. Results shown are the means ± SEM of 3 experiments carried out with 9 replicates

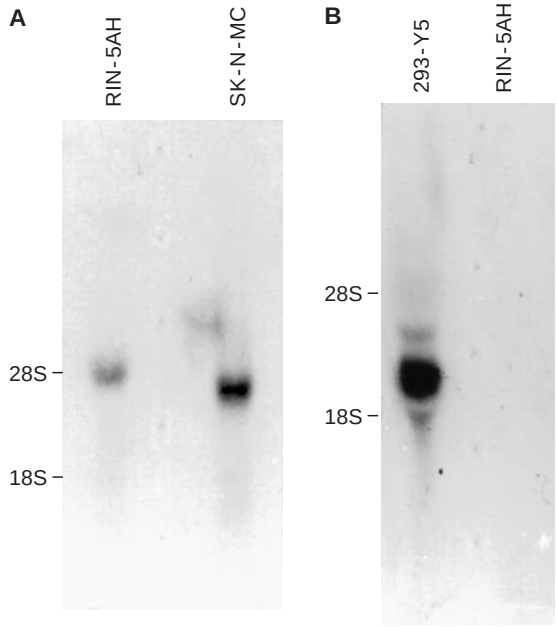

Fig. 10A, B. Northern blot analysis of poly-A$^+$ RNA from RIN 5AH in comparison **A** with SK-N-MC, probed with a Y1 specific probe and **B** with HEK 293 stably expressing Y5 receptors, and probed with a Y5 specific probe. 18 S and 28 S ribosomal RNA bands are used as size markers

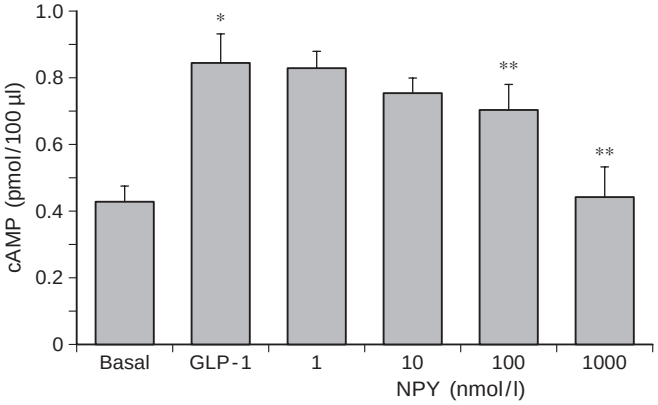

Fig. 9. Cyclic AMP measurement in whole cells. The effect of NPY on GLP-1 (7–36) NH_2–stimulated accumulation of cAMP in RIN 5AH cells was measured by RIA. NPY is shown to dose-dependently inhibit the effect of GLP-1 (7–36) NH_2. *$p < 0.001$ vs basal cAMP; **$p < 0.001$ vs GLP-1 alone. Results shown are means ± SEM of 3 experiments each carried out in quadruplicate

Northern blot analysis. Northern blot analysis of RIN 5AH and SK-N-MC cell poly (A)$^+$ RNA showed single bands of comparable size in both lanes when probed with an oligo probe specific for the Y1 receptor (Fig. 10 a). The bands are not of identical size but ran at a position in keeping with the reported size of the cloned Y1 receptor of 3.5 kilobases (for the human receptor) [13] and 4 kilobases (for the rat) [38]. When poly (A)$^+$ RNA from RIN 5AH was probed with a specific probe for the NPY Y5 receptor, no band was visible. A clear band was seen when poly (A)$^+$ RNA from HEK 293 cells stably expressing rat NPY Y5 receptors was similarly probed (Fig. 10).

Discussion

Neuropeptide Y has been shown to inhibit glucose-stimulated insulin secretion in vivo [9, 10] in isolated rat islets [6] and in clonal insulinoma cell lines [9, 12]. The receptor with which it interacts, and the second messenger system by which it affects insulin secretion have not, however, been identified. In this paper we show that in the insulinoma cell line, RIN 5AH, NPY interacts with the NPY Y1 receptor and inhibits insulin secretion via a reduction in adenylyl cyclase activity.

Receptor binding indicated that a single population of NPY binding sites was present on RIN 5AH cell membranes. Non-linear regression analysis was carried out on the data derived from saturation experiments incubating RIN 5AH membranes with different concentrations of ^{125}I-PYY. Statistical comparison of the goodness of fit of one-site and two-site models suggested that the one-site fit was better, and therefore, that either a single class of receptor was present, or that a number of classes were present all with similar affinities for ^{125}I-PYY. The ligand profile of this receptor was investigated using competition curves. Non-linear regression analysis, again suggest-

ed a single population of binding site for each of the peptides tested.

Competition curves generated using ^{125}I-PYY binding to RIN 5AH membranes showed a Y1-like profile, with [Pro^{34}]-NPY having a high affinity (IC_{50} ~ 0.3 nmol/l), and NPY 13–36 binding only in the mid-nanomolar range (IC_{50} ~ 17 nmol/l). The rank order of binding seen is NPY > [Pro^{34}]-NPY = PYY > NPY 3–36 > NPY 13–36. Insulin secretion experiments on RIN 5AH cells show that NPY (approximate EC_{50} = 0.1 nmol/l), [Pro^{34}]-NPY (approximate EC_{50} = 0.3 nmol/l), and NPY 3–36 (approximate EC_{50} = 1.8 nmol/l), all inhibited glucose-stimulated insulin secretion. The rank order of potency matches that of receptor affinity, with NPY > [Pro^{34}]-NPY > NPY 3–36 > > NPY 13–36. The potency of NPY 3–36 seems slightly higher than might be expected at the Y1 receptor, and might suggest an activation profile more similar to the Y5 receptor. We have reported previously that in SK-N-MC cells, which express the Y1 receptor, NPY 3–36 has an affinity about 35 times lower than NPY 1–36 [33], whereas, at the Y5 receptor, NPY 1–36 and NPY 3–36 are reported to be virtually equipotent [14]. Although, in RIN 5AH cells, the affinity of NPY 3–36 is only 7 times lower than that of NPY 1–36 and its potency in inhibiting insulin secretion about 18 times lower than NPY 1–36, the profile is still more similar to the Y1 receptor than to the Y5 receptor. The difference between the binding affinity of NPY 13–36 to RIN 5AH NPY receptors and its potency on inhibition of insulin secretion suggests that it may bind to the receptor but with low efficacy.

Although these findings suggest that the Y1 receptor mediates the effect of NPY at the RIN 5AH cell, it remains a possibility that the Y5 receptor, which has a Y1-like binding profile, rather than the authentic Y1 is present. To test this, the ability of BIBP 3226 to inhibit the actions of NPY was investigated. BIBP 3226 is an antagonist at the Y1 receptor, and is reported not to bind to any other NPY receptor so far described including the Y5 receptor [14]. This antagonist blocks the inhibition by NPY of insulin secretion from RIN 5AH cells but has no effect on basal insulin secretion and binds to the NPY receptor on RIN 5AH cell membranes. The lack of effect of the antagonist on basal secretion suggests that there is no tonic activation of NPY receptors on these cells. In keeping with this, we were not able to observe any NPY-like immunoreactivity in medium in which RIN 5AH cells have been grown using a specific radioimmunoassay (results not shown). This is further evidence that the receptor mediating the inhibition by NPY of insulin secretion from RIN 5AH cells is the Y1 receptor and suggests that the Y5 receptor is not involved. Northern blot analysis shows two similarly sized bands in RIN 5AH and SK-N-MC cell polyA^+ RNA when probed with an oligo-probe for the Y1 receptor. That the two bands are not identical in size is not surprising since RIN 5AH is a rat cell line, whereas SK-N-MC is a human cell line, and since the rat and human Y1 mRNA s have been shown to be of different sizes (rat = 4 kb [38], human = 3.5 kb [13]). Northern blot analysis was not able to show expression of the Y5 receptor by RIN 5AH cells.

The ability of GTP-γ-S, but not ATP-γ-S, to completely block the specific binding of ^{125}I-PYY to RIN 5AH membranes suggests that the receptor is G-protein linked, and the ability of pertussis toxin to block NPY's inhibition of insulin secretion further supports this. This latter effect suggests that NPY receptors in RIN 5AH cells are most likely coupled to G_i, or G_o. NPY has been shown to be coupled to these GTP-binding proteins (G-proteins) in a number of systems [22, 40]. Evidence from other NPY systems would suggest that the most likely effector system to be activated by the receptor-G-protein complex is inhibition of adenylyl cyclase activity.

We have shown that NPY receptors in RIN 5AH cells are indeed linked to inhibition of this system, by directly measuring adenylyl cyclase activity and showing inhibition of GLP-1 (7–36) NH_2 stimulated cAMP production in whole cells. Other experiments provide direct evidence that NPY inhibits insulin secretion by inhibiting adenylyl cyclase. Glucagon like peptide-1 increases insulin secretion by increasing adenylyl cyclase activity and cyclic AMP [13]. Neuropeptide Y is able to completely block this effect, presumably by preventing stimulation of adenylyl cyclase. Dibutyryl cyclic AMP, on the other hand, stimulates insulin secretion through the same mechanisms as cyclic AMP but bypasses the requirement for adenylyl cyclase. Neuropeptide Y is not able to reduce insulin secretion stimulated by this agent, even though the dose of dibutyryl cAMP used produced a less than maximum stimulation of insulin secretion. This could suggest that NPY has no important effects distal to increased cyclic AMP, at least in RIN 5AH cells.

Taken together these experiments represent strong evidence that, in RIN 5AH cells, NPY inhibits insulin secretion via inhibition of adenylyl cyclase activation. There is, however, evidence from the literature suggesting that inhibition of adenylyl cyclase activity is possibly not the sole means by which NPY inhibits insulin secretion. For instance, in the perfused rat pancreas NPY is reported to inhibit insulin secretion in the presence of dibutyryl cyclic AMP [25]. PYY is reported to have similar effects in perifused mouse islets [41]. In this study PYY is reported to have no effect on protein kinase C-stimulated insulin secretion, or on intracellular calcium or potassium efflux. In agreement with our findings, this paper reported a reduction in glucose stimulated cyclic AMP concentrations by PYY. The whole pancreas or islet are both far more complex systems than that which

we are examining here, and it is possible that in these system, other factors and other effects of NPY cloud the results.

Additionally, it is not thought that raised adenylyl cyclase activity is a major factor in the stimulation of insulin secretion by glucose [42]. Our results strongly suggest that NPY does inhibit adenylyl cyclase activity and, that by doing so, it inhibits the increase in insulin secretion seen following GLP-1 treatment. It has also been reported that, in the dog, PYY is able to inhibit insulin secretion stimulated by gastric inhibitory polypeptide [43], another peptide known to stimulate insulin secretion by raising cyclic AMP concentrations [44]. It clearly remains possible that NPY inhibits insulin secretion stimulated by glucose by a means other than inhibition of adenylyl cyclase activity. At least in the RIN 5AH cell, inhibition of adenylyl cyclase activity appears an important mechanism by which NPY inhibits glucose-stimulated insulin secretion. It remains to be further investigated whether this system is equally important in the islet itself.

In summary, RIN 5AH cells express a single group of NPY receptors, with a Y1-like ligand-binding profile. This profile is similar to that seen for the inhibition of insulin secretion. The specific Y1 antagonist, BIBP 3226 blocks the inhibition of insulin secretion by NPY. The RIN 5AH cells express Y1 receptor mRNA, but not Y5 receptor mRNA. This receptor appears to inhibit insulin secretion by G_i-mediated inhibition of adenylyl cyclase activity.

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