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NPY Regulates Catecholamine Secretion from Human Adrenal Chromaffin Cells

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The aim of the present work was to find out whether NPY synthesized in human adrenal chromaffin cells controls in an autocrine/paracrine fashion the release of catecholamines by these cells. Accordingly, the constitutive and regulated release of both NPY and catecholamines was measured simultaneously in cultured human chromaffin cells. In addition, by using both RT-PCR and a combination of specific agonists and antagonists, we characterized the expression of NPY receptors on these cells as well as their pharmacology. Our results were as follows. 1) Human chromaffin cells constitutively secrete NPY. 2) Nicotine elicits a rapid increase in the release of both catecholamines and NPY; this release of NPY is more sustained than that of catecholamines. 3) RT-PCR shows expression of Y1, Y2, Y4, and Y5 receptor mRNA by chromaffin cells; these receptors are functional, as various receptor specific agonists elicit an increase in intracellular calcium. 4) Peptide YY, in contrast to NPY, is not able to stimulate the release of catecholamines. This finding was corroborated by the observation that no receptor-specific antagonists were able to reduce constitutive catecholamine release, whereas an NPY-immunoneutralizing antibody markedly attenuated the secretion. Taken together, these data suggest that NPY originating from the adrenal medulla locally enhances the secretion of catecholamines, presumably by acting via the putative y3 receptor. (*J Clin Endocrinol Metab* 86: 5956–5963, 2001)

NPY is a 36-amino acid peptide present in the brain (1) and peripheral nervous system in nerve endings, from which it is coreleased with norepinephrine (NE) during stress (2). Several observations suggest that NPY is an important neurotransmitter involved in the central and peripheral control of blood pressure (3, 4). NPY stimulates at least six types of G protein-coupled receptors, called Y1, Y2, y3, Y4, Y5, and Y6 (5–10). Activation of the Y1 receptor by NPY results in vasoconstriction and postsynaptic potentiation of the catecholamine effect on blood pressure (11). NPY also presynaptically inhibits the release of catecholamines through the Y2 receptor (12). No functions in the cardiovascular system have been associated with the Y4 and Y5 receptors. The Y6 receptor is not functional in man. The y3 receptor has not yet been cloned and is pharmacologically characterized by its inability to be activated by peptide YY (PYY) (13). NPY is also produced by the adrenal medulla of various species including man (14–19) and by human pheochromocytomas (20).

The reported effects of NPY on catecholamine release by the adrenal medulla in rats (21, 22) and from bovine cells (23, 24) are conflicting depending on the experimental set-up and the animal species. As NPY potentiates the effects of catecholamines released from sympathetic nerves, we hypothesized that NPY originating from the adrenal medulla might also participate in this effect by locally regulating the secretion of catecholamines by chromaffin cells. Thus, the goal of this investigation was to examine whether *in vitro* NPY affects the catecholamine secretory activity of isolated adrenal chromaffin cells, and if so, which receptor(s) is responsible for this effect.

Abbreviations: $[Ca^{2+}]_i$, Intracellular free calcium concentration; EP, epinephrine; hPP, human pancreatic polypeptide; KB, Krebs buffer; NE, norepinephrine; PYY, peptide YY; TBST, 5 mM Tris and 0.1% Triton X-100, pH 7.6; TH, tyrosine hydroxylase.

Materials and Methods

Peptides and antagonists

NPY-(1–36), NPY-(3–36), [Leu^{31},Pro^{34}]NPY, [Pro^{34}]NPY, and PYY were purchased from Novabiochem (Laufelfingen, Switzerland), human pancreatic polypeptide (hPP) was obtained from Bachem (Bubendorf, Switzerland); hNPY-(13–36), BIBP3226 (25), and BIBP3242 were gifts from Dr. K. Hofbauer (Novartis, Basel); T_4[NPY-(33–36)]$_4$ (26) was synthesized in our laboratory; the Y5 antagonist 2-[4-(2-oxo-2,3-dihydrobenzoimidazol-1-yl)-piperidin-1-yl]-*N*-(9-oxo-9H-fluoren-3-yl)-acetamide hydrochloride was synthesized according to patent WO9835957 (27).

Adrenal glands and cell culture

The study was approved by the hospital transplantation review board and the medical direction. Adrenal glands were obtained from kidney transplant donors (seven women and eight men; 36 ± 17 yr; age range,10–62 yr). All the donors were brain-dead patients whose relatives had accepted multiorgan procurement. The abdominal organs of the donors, including the adrenal glands, were perfused *in situ* via aortic cannula with University of Wisconsin solution at 4 C. The adrenals were then procured *en bloc* with the kidneys and preserved in an ice bath at 4 C. Consequently, the preparation of the glands could be made immediately after harvesting when surgery was performed in our own hospital. When it was performed at a distant site this was delayed by a maximum of 4 h. The glands were cleaned, and a cannula was inserted into the suprarenal vein (28). The glands were then perfused with 5 ml HBSS (136 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.44 mM KH_2PO_4, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, and

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5 mM D-glucose) until clear perfusate was seen dripping from the gland. Five to 7 ml HBSS containing 0.2% collagenase, type I (Sigma, St. Louis, MO), were then injected into the gland and shaken at 37 C for 30 min. Thereafter, the gland was opened, and the medulla was separated from the cortex using a scalpel by scraping off the brown interdigited islets of chromaffin cells. The medullary tissue was chopped, collected in 10 ml 0.2% collagenase solution, and incubated for 10 min. The remaining tissue was treated twice with 10 ml collagenase solution to fully digest the medullary tissue. The digested tissue was further washed twice with HBSS to remove collagenase, and cell pellets were resuspended in 15 ml culture medium [DMEM/Ham's F-12 (Life Technologies, Inc., Grand Island, NY), 1:1, with 15 mM HEPES, 14 mM $NaHCO_3$, L-glutamine (Life Technologies, Inc.), pyridoxine (Life Technologies, Inc.), 10% inactivated FCS (Seromed, Munich, Germany), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.)]. Fibroblasts were removed from the chromaffin cell preparation by plating the cells consecutively three times for 60 min each time on different flasks, and cells were then cultured for 2–6 d at 37 C with 5% CO_2. Bovine adrenal glands were obtained from a local slaughterhouse, and chromaffin cells were isolated by the procedure described above.

Immunohistochemistry

Cells were cultured on glass coverslips and washed three times with PBS, pH 7.4, before fixation with 1:1 acetone/methanol at −20 C for 15 min. Immunohistochemistry was performed as follows. After washing twice with PBS and once with TBST (5 mM Tris and 0.1% Triton X-100 (Sigma) at pH 7.6), coverslips were incubated for 54 h at 4 C in a solution containing antityrosine hydroxylase (anti-TH) antibody (Sigma) diluted 1:10,000, or NPY02, an anti-NPY monoclonal antibody (29), diluted 1:12,000 in TBST with 0.1% NaN_3. After three washes with TBST, cells were incubated with goat antimouse Ig (Nordic Immunological Laboratories, Tilburg, The Netherlands; 1:30) and rinsed in TBST before the addition of mouse peroxidase-antiperoxidase complex (Sternberger Monoclonals, Inc., Baltimore, MD; 1:200) in TBST. The cells were then washed in TBST, incubated with the peroxidase substrate/3,3′-diaminobenzidine tetrahydrochloride solution (Polyscience, Inc., Niles, IL), and counterstained with toluidine blue (1%). The coverslips were destained, dehydrated in successive baths of ethanol, and mounted with a second coverslip.

RT-PCR

Total RNA was isolated from chromaffin cells using the RNeasy kit (QIAGEN, Chatsworth, CA). Genomic DNA was removed by digestion with ribonuclease-free deoxyribonuclease I in the presence of RNasin ribonuclease inhibitor. RNA was then purified by phenol/chloroform extraction (30). cDNA was synthesized by incubating total RNA with oligo(deoxythymidine)$_{15}$ primer, RNasin, and Moloney monkey leukemia virus reverse transcriptase (Promega Corp., Madison, WI) for 1 h at 37 C. PCR was performed using specific primer pairs for Y1, Y2, Y4, and Y5 receptors and NPY (Life Technologies, Inc.). Y1 primers were: forward, 5′-TAT GTA GGT ATT GCT GTG ATT TG; and reverse, 5′-CTG GAA GTT TTT GTT CAG GAA; product size was 519–616 bp; annealing temperature was 58 C (31). Y2 primers were: forward, 5′-AAA TGG GTC CTG TCC TGT GCC; and reverse, 5′-TGC CTT CGC TGA TGG TAG TGG; product size was 442 bp; annealing temperature was 66 C (32). Y4 primers were: forward, 5′-CGC GTG TTT CAC AAG GGC ACC TA; and reverse, 5′-TGC CAC TTA GCC TCA GGG ACC C; product size was 376 bp; annealing temperature was 67 C (33). Y5 primers were: forward, 5′-GGG TCC CCA CTT GCT TTG AGA TA; and reverse, 5′-GTT CTT TCC TTG GTA AAC AGT GAG; product size was 350 bp; annealing temperature was 61 C (34). NPY primers were: forward, 5′-TGC TAG GTA ACA AGC GAC TG; and reverse, 5′-CTG CAT GCA TTG GTA GGA TG; product size was 386 bp; annealing temperature was 60 C (35). Positive controls used were SK-N-MC cells for Y1 (36), LN 319 cells for Y2 (26), human nasal mucosa for Y4 (33), and human placenta for Y5 (34) and NPY (37). All amplifications were performed in buffer containing 1.5 mM $MgCl_2$, 200 μM dNTP (Amersham Pharmacia Biotech, Arlington Heights, IL), 0.5 pM of each primer, and 25 U/ml hot start *Taq* DNA polymerase (Perkin-Elmer Corp., Foster City, CA). Reactions were performed as follows: 15 min at 95 C, 40 cycles at 95 C for 1 min, annealing temperature for 1 min, 72 C for 1 min, and final extension at 72 C for 10 min. PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide. All experiments were repeated with chromaffin cells from three different adrenal glands.

Assay of intracellular free calcium concentration ($[Ca^{2+}]_i$)

Chromaffin cells were cultured on glass coverslips coated with poly-D-lysine (Sigma; 2.5 $\mu g/cm^2$), and $[Ca^{2+}]_i$ was determined using the fluorescent probe fluo-3/AM (Molecular Probes, Inc., Eugene, OR) as previously described (26). The changes in $[Ca^{2+}]_i$ were evaluated in single cells on whole images containing 8–15 cells using the NIH image analyzer program.

Assay of cAMP

As NPY receptors are usually coupled to G_i proteins (13), cAMP concentrations were determined in chromaffin cells as previously described (26). The results are expressed as femtomoles of cAMP per μg protein.

Release experiments

Perifusion. Cells were plated on glass coverslips placed in a perifusion chamber warmed at 37 C and stabilized for 90 min at a flow rate of 0.75 ml/min with Krebs buffer (KB). Samples were then collected alternately every 30 sec for NPY and catecholamine assays. For blockade experiments, fractions were collected for catecholamine determinations every 3 min, with a flow rate of 0.35 ml/min. For catecholamine assays, samples were acidified with 0.4 M $HClO_4$ and quick frozen. Catecholamines were extracted on alumina and determined by HPLC with electrochemical detection (Waters Corp., Milford, MA) (28). NPY concentrations were measured by enzyme immunoassay (28).

Static release

Chromaffin cells were plated in 24-well plates (100,000 cells/well). Culture medium was aspirated, and the wells were washed twice with KB warmed to 37 C. After incubation for 10 min at 37 C with KB with or without drugs, samples were collected and acidified with 0.4 M $HClO_4$ and frozen. Catecholamine cell contents were extracted with KB containing 0.4 M $HClO_4$ and 10 mM EDTA. Catecholamine release was expressed as the percentage of total intracellular content, and the effects of the drugs in each experiment were expressed as the percent change over the control value. Immunoneutralization of endogenous NPY has been performed with NPYO5, a monoclonal antibody that binds to the C-terminal part of NPY required for bioactivity at the NPY Y1 receptor (29), and also at the Y2 and Y5 receptors (data not shown).

Statistical methods

All values are expressed as the mean ± SEM. The data were compared by one-way ANOVA with *post-hoc* analysis using unpaired *t* test according to Bonferroni's method. $P < 0.05$ was considered significant.

Results

Human chromaffin cells in culture contain catecholamines and NPY

Digestion of 15 glands provided 1.5–5 million chromaffin cells/gland. Cell viability, as determined by trypan blue dye exclusion, was generally 95%. After a few hours in culture, chromaffin cells formed clusters of 5–20 cells, reminiscent of the *in vivo* disposition of chromaffin cells in the intact adrenal gland. Sixty percent of chromaffin cells in culture stained positively for TH (Fig. 1A), and 30% stained positively for NPY-like immunoreactivity (Fig. 1B). After 5 d of culture, many cells extended neurite-like processes strongly immunoreactive for TH.

The catecholamine and NPY contents in chromaffin cells were in line with the immunohistochemical observations.

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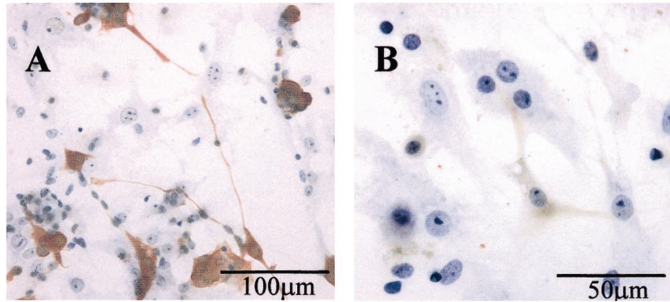

FIG. 1. Staining of human chromaffin cells with antibodies against TH (A) and NPY (B).

Intracellular contents were 314 ± 128 fmol NPY/10^5 cells (mean ± SD of six different cell cultures), 166 ± 148 pmol NE/10^5 cells (mean ± SD; n = 13; range = 5–87) and 251 ± 65 pmol epinephrine (EP)/10^5 cells (mean ± SD; n = 13; range, 7–137). The ratio of NE and EP in each culture was relatively constant (38 ± 6% for NE and 62 ± 6% for EP). Dissection of adrenal gland followed by plating and 4 d of cell culture did not change the proportion of NE relative to EP in chromaffin cells. On a molar basis, cells contain 1250-fold more catecholamines than NPY.

Differential response of catecholamine and NPY release to nicotine

In perifused chromaffin cells, catecholamines and NPY were constitutively released (Fig. 2A). NE, EP, and NPY were secreted at rates of 0.022 ± 0.002%, 0.018 ± 0.008%, and 0.04 ± 0.01% of intracellular content/0.5 min, respectively. Nicotine (100 μM) infusion for 10 min increased the release of both NPY and catecholamines by about 7- and 4-fold, respectively (Fig. 2, B and C). The pattern of secretion was divided into two phases; a few seconds after exposure to nicotine, NE, EP, and NPY secretion was enhanced, with a peak at 2 min. However, NE and EP secretion returned to baseline within 5 min, whereas 25 min after removal of nicotine, the NPY secretion rate was still increased (Fig. 2A).

Human chromaffin cells express NPY and NPY receptors

Next, we investigated which NPY receptor subtypes were expressed by chromaffin cells in culture. We analyzed RNA isolated from cells obtained from three different subjects for the presence of NPY, Y1, Y2, Y4, and Y5 mRNAs by RT-PCR. We detected mRNA for NPY and Y1, Y2, Y4, and Y5 receptors in all samples (Fig. 3).

Signal transduction induced by NPY receptors

We then examined the functional pharmacology of NPY receptors expressed on individual human chromaffin cells by measuring $[Ca^{2+}]_i$ increases evoked by NPY. NPY induced a rapid increase in $[Ca^{2+}]_i$, followed by a relatively long-lasting decrease, possibly due to calcium entry (Fig. 4A). The basal $[Ca^{2+}]_i$ level was 158 ± 12 nM (n = 312). NPY (100 nM) caused an increase in $[Ca^{2+}]_i$ by 245 ± 65 nM (Table 1). NPY stimulated 21% of the cells at 10 nM and 31% at 100 nM. PYY, $[Leu^{31},Pro^{34}]$NPY a Y1/Y5 agonist, and NPY13–36 or TASPV, selective Y2 agonists (38), increased $[Ca^{2+}]_i$ in chromaffin cells, all at 100 nM (Table 1). In contrast, hPP (100 nM; Y4 agonist) weakly increased cytosolic calcium release. These data are in agreement with RT-PCR experiments indicating that Y1/Y5, Y2, and Y4 receptors are expressed on chromaffin cells.

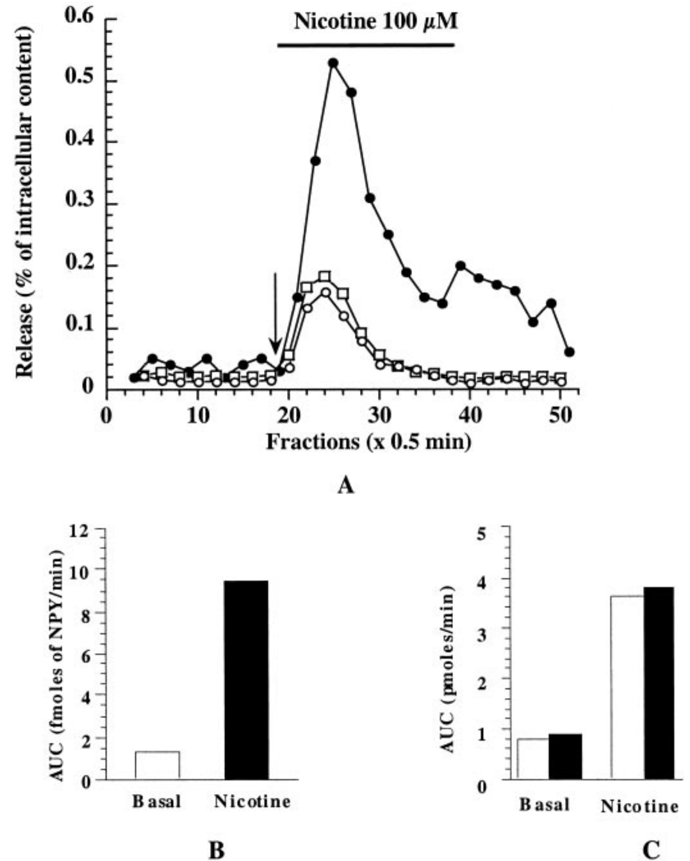

FIG. 2. Constitutive and regulated release of NPY, NE, and EP from human chromaffin cells. The cells were plated onto two glass coverslips and introduced in a perifusion chamber. Every 30 sec, perifusion samples were collected alternatively for NPY and catecholamine assays. A, Release of NPY(●), NE (□), and EP (○) is expressed as a percentage of the intracellular content. The amounts of NPY (B), NE (□; C), and EP (■; C) released per min (constitutive release) or with nicotine (100 μM; regulated release) are indicated. One representative experiment of three is shown.

We investigated the effect of NPY on the response to agents that stimulate adenylate cyclase. NPY inhibited forskolin-stimulated cAMP accumulation in two cultures obtained from two different glands (Fig. 4B), with maximal inhibition (42%) at 5 nM NPY. These results suggest functional coupling of NPY receptors and adenylate cyclase activity in chromaffin cells.

NPY stimulates catecholamine secretion

To evaluate the effect of NPY on catecholamine secretion, we incubated chromaffin cells for 10 min with 1–100 nM NPY, and observed a 30–60% increase in catecholamine release over the control value (Fig. 5).

NPY analogs, but not PYY, evoke catecholamine release

To characterize the receptor(s) involved in catecholamine release, different NPY selective agonists were studied in

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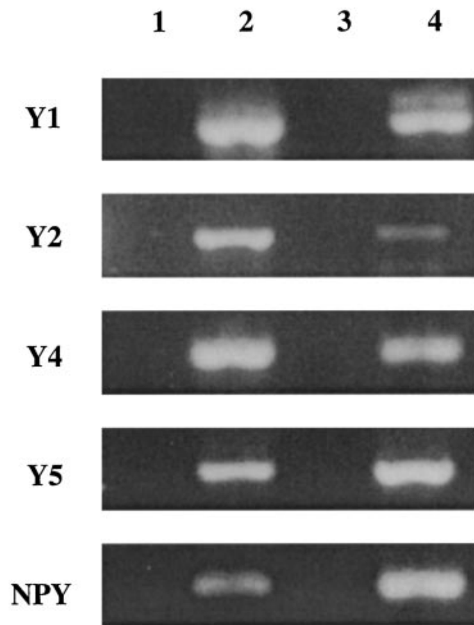

FIG. 3. Expression of NPY Y1, Y2, Y4, and Y5 receptors. NPY receptor and NPY mRNAs were detected in primary cultured human adrenal chromaffin cells and control tissues. These data are representative of three or four different cell preparations obtained from different donors. cDNAs synthesized from total RNA of human tissues were used as templates for PCR reactions using specific primers. Lane 1, Negative PCR control (no template); lane 2, positive control; lane 3, RNA of chromaffin cells without reverse transcriptase; lane 4, RNA of chromaffin cells with reverse transcriptase.

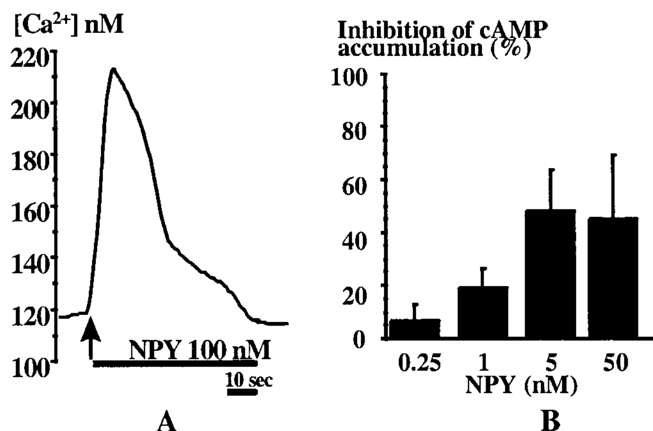

FIG. 4. Signal transduction pathways activated by NPY receptors on human chromaffin cells. A, NPY increased $[Ca^{2+}]_i$ release from human chromaffin cells. NPY caused a transient free calcium rise that reached a peak in 10 sec and returned to basal levels within 50 sec. This trace is representative of 20 different recordings. B, NPY inhibited forskolin-stimulated cAMP accumulation in chromaffin cells. Cells (100′000/well) were incubated with 10 μM papaverine, 1 μM forskolin, and different concentrations of NPY, and cAMP was measured. Data are the mean of two separate experiments performed in triplicate.

static release experiments. hPP (Y4 agonist), NPY-(13–36) (Y2 agonist), and NPY-(3–36) (Y2/Y5 agonist) evoked catecholamine secretion at 100 nM. Surprisingly, exposure to 100 nM [Leu^{31},Pro^{34}]NPY, [Pro^{34}]NPY (Y1/Y5 agonists), or PYY did not elevate catecholamine release (Fig. 5).

The rank order of potency of NPY and analogs in terms of stimulation of catecholamine secretion was: NPY>NPY-(13–36), NPY-(3–36), PP>>>^{34}ProNPY = [Leu^{31},Pro^{34}]NPY = PYY, which is most consistent with the involvement of a receptor of the putative y3 receptor subtype (36, 39–41).

TABLE 1. Effect of NPY and analogs on $[Ca^{2+}]_i$ of chromaffin cells

	n	$[Ca^{2+}]_i$ (nM) in the total cell population	$\Delta[Ca^{2+}]_i$ (nM) in responding cells	% of responding cells
Basal	358	158 ± 12	0	0
NPY (100 nM)	154	183 ± 29	245 ± 65	31
NPY (10 nM)	34	202 ± 38	188 ± 79	21
PYY (100 nM)	33	188 ± 34	214 ± 86	27
[Leu^{31},Pro^{34}]NPY (100 nM)	39	238 ± 47	191 ± 86	15
NPY(13–36) (100 nM)	44	150 ± 24	183 ± 88	14
PP (100 nM)	68	160 ± 39	17 ± 6	9
TASP-V (100 nM)	37	284 ± 103	226 ± 155	40

Data were obtained from five different glands. n, The total number of cells undergoing individual calcium measurement. Responding cells represent the proportion of cells that increase their free cytosolic calcium by at least 2 SD over the mean of basal fluctuation of $[Ca^{2+}]_i$ (10 nM). Data are expressed as the mean ± SEM increase in $[Ca^{2+}]_i$ in responding cells.

NPY has a tonic role on catecholamine release by chromaffin cells

We then studied the effects of different NPY receptor antagonists to assess the involvement of endogenous NPY on catecholamine secretion by chromaffin cells. Cells were preincubated with 1 μM BIBP 3226 (Y1 antagonist) or its inactive isomer BIBP 3242 (25), T4 [NPY-(33–36)]4 (Y2 antagonist) (26), or 100 nM of a Y5 antagonist (27). None of these significantly altered the constitutive release of catecholamines in either static or perifusion systems (Fig. 6A). These results are consistent with our previous postulate that a y3 receptor may be responsible for catecholamine release. To determine whether NPY stimulates catecholamine secretion through a non-Y1, -Y2, and -Y5 receptor via an autocrine/paracrine loop, we used an anti-NPY monoclonal antibody, NPY05, for immunoneutralization experiments. NPY05 caused significant reductions of the basal release of NE and EP by 35% and 28%, respectively (Fig. 6, B and C). The reduction of catecholamine secretion induced by NPY05 was progressive and lasted even after removing the antibody from the perifusion medium (Fig. 6B); NPY05 did not affect all stimuli to catecholamine secretion, as KCl depolarization still evoked large releases of NE and EP. To rule out the possibility of a nonspecific effect of NPYO5, we used an unrelated IgG, directed against the C-flanking peptide of NPY (CPON01), as a negative control (42) at the same concentration as NPYO5. CPON01 was devoid of any effect on catecholamine secretion (Fig. 6A).

Discussion

The present data demonstrate that human adrenal chromaffin cells constitutively secrete catecholamines and NPY, and that NPY stimulates the release of catecholamines. The data obtained with various selective agonists and antagonists

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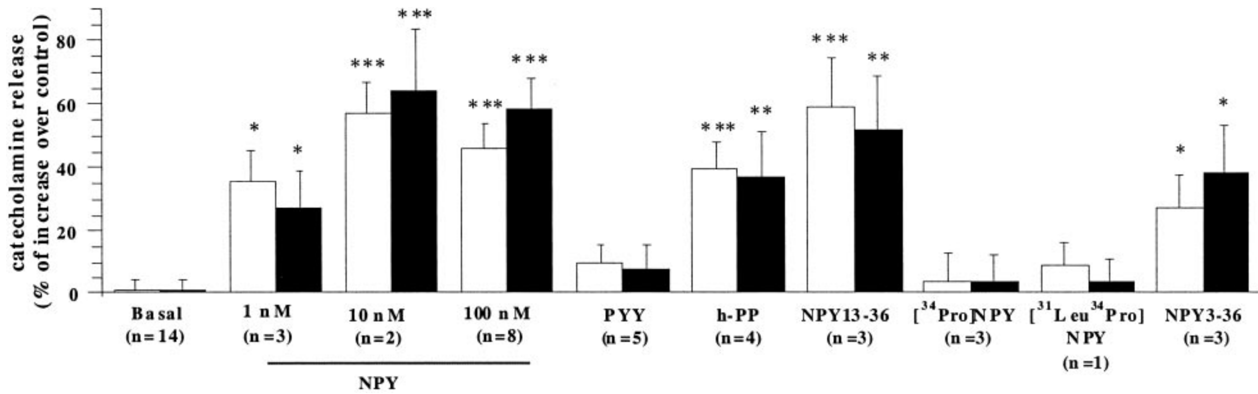
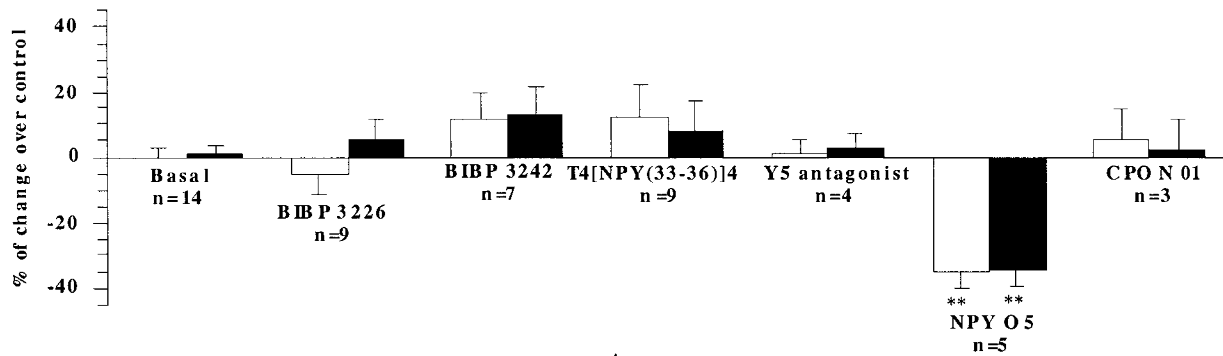
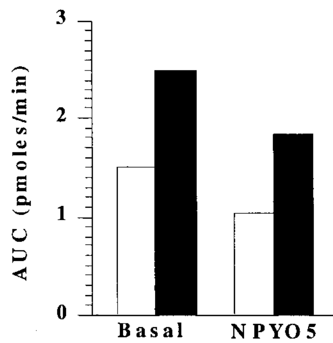


FIG. 5. Effects of exogenous NPY and analogs (at 100 nM) on NE (□) and EP (■) release from chromaffin cells. Cells were incubated for 10 min at 37 C in KB (basal) or in the presence of different NPY analogs, and catecholamines were measured by HPLC. Data are expressed as the percent increase in release compared with basal (mean ± SEM of experiments with one to eight different adrenal glands performed in triplicate). *, $P < 0.05$ compared with basal (by unpaired t test according to Bonferroni's method).





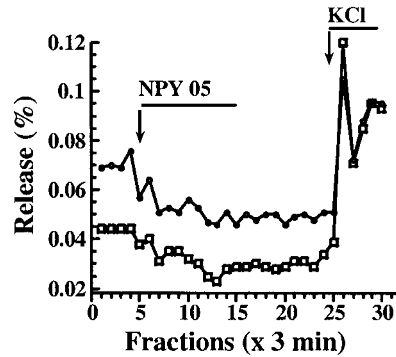


FIG. 6. Endogenous NPY is involved in tonic catecholamine release from chromaffin cells. A, Effects of different NPY antagonists on basal release of NE (□) and EP (■) by chromaffin cells. Cells were preincubated for 30 min with 1 μM Y1 and Y2 antagonists, 100 nM Y5 antagonist, and 6 μg/ml NPY05 or CPON01 antibodies, and release of NE and EP was measured for 10 min. Data are expressed as the percent change in catecholamine release compared with basal release (mean ± SEM of experiments with three to eight different adrenal glands performed in triplicate). *, $P < 0.05$ compared with basal (by unpaired t test according to Bonferroni's method). B, Immunoneutralization of endogenous NPY by NPY05 decreases the catecholamine secretion rate. Cells were perifused with KB, and fractions were collected every 3 min for catecholamine assay. The antibody NPYO5 (6 μg/ml) was added at fraction 5 (15 min), and 56 mM KCl was added at fraction 25 (75 min). Release of NE (□) and EP (●) is expressed as a percentage of the intracellular content. C, Amounts of NE (□) and EP (■) released per min. The area under the curve (AUC) of catecholamine release from cells was divided by the duration of perifusion (60 min) with KB (basal) or NPYO5 (6 μg/ml). The mean ± SEM of perifusion performed using three different adrenal glands are shown. *, $P < 0.05$ compared with basal (by t test).

suggest that NPY elicits catecholamine release by stimulating the putative y3 receptor, in that NPY05, an anti-NPY monoclonal antibody used to neutralize endogenous peptide released from cells, but not Y1, Y2, or Y5 antagonists, reduced the constitutive release of catecholamines from chromaffin cells. These data taken together suggest that NPY released from chromaffin cells may act in an autocrine/paracrine manner to modulate catecholamine secretion from chromaffin cells.

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We first established our primary cell culture of adrenal chromaffin cells and demonstrated that the ratio of NE to EP found in our cell culture was constant over the time of cell culture and similar to that we found in the intact adrenal gland. However, this ratio (62%) is relatively high compared with that previously reported in the intact human adrenal gland, where NE was found to be only 10% of total catecholamines (43). A possible explanation is that the adrenals used in our studies were preserved in an organ conservation solution, whereas in previous studies chromaffin cell homogenates were prepared from adrenal medulla dissected from patients at autopsy, with no indication of the time lag between death and tissue preparation (43).

We then found that NPY induced the secretion of catecholamines by human chromaffin cells. The present data contrast with other studies showing a weak inhibitory effect of NPY on NE and EP release from bovine chromaffin cells evoked by a cholinergic agonist (23). Conflicting results, however, have also been seen in perfused bovine adrenal glands, where NPY was shown to stimulate the secretion of catecholamines in the presence of cholinergic agents (24). We performed similar studies to ensure that our experimental system was not responsible for this discrepancy and found that NPY inhibits the cholinergic-induced secretion of catecholamines from bovine chromaffin cells by 20% (data not shown).

The effect of NPY in the adrenal medulla of other species is not clearly established; for instance, although NPY evokes the release of catecholamines from intact rat adrenal capsular tissue (21), it has also been reported that NPY immunoneutralization enhances the nicotine-mediated release of catecholamines from cultured rat adrenal chromaffin cells (22). It therefore, appears that the effect of NPY on the adrenal medulla depends on the experimental conditions used and the animal species studied.

RT-PCR experiments on mRNA extracted from cultured chromaffin cells indicated that NPY, Y1, Y2, Y4, and Y5 receptors are expressed. However, these data must be interpreted cautiously. Although differential plating allows preferential selection of chromaffin cells over fibroblasts, we cannot exclude the possibility that some cortical cells may contaminate our preparations. Therefore, the crucial step in determining the NPY receptor involved in catecholamine secretion was to use selective NPY agonists in the functional tests of catecholamine secretion. The rank order of potency of NPY and analogs in terms of stimulation of catecholamine secretion was: NPY (Y1/Y2/y3/Y4/Y5)>NPY-(13–36) (Y2), NPY-(3–36) (Y2/Y5), PP (Y4)>>>[Leu^{31},Pro^{34}]NPY = [Pro^{34}]NPY (Y1/Y4/Y5) = PYY (Y1/Y2/Y4/Y5), suggesting the involvement of the y3 receptor subtype (36, 39–41). A common feature of y3 receptors is their inability to be activated by PYY. However, our data contrast with those reported with bovine chromaffin cells suggesting the involvement of y3 and Y_1 receptors in catecholamine secretion (23, 24, 36, 39).

Relatively few tissues that express y3 receptors have been identified: bovine adrenal chromaffin cells (23, 36, 39), rat superior cervical ganglia sympathetic neurons (44), rat nucleus tractus solitarii (45), rat cardiac ventricular membranes (46), rat distal colon (41), and PC12 cells differentiated with nerve growth factor (47). Also, the potency of NPY analogs on y3 receptors depends on the species and tissues studied; for instance, [Pro^{34}]NPY is effective in bovine chromaffin cells and rat nucleus tractus solitarius, but not in human chromaffin cells; NPY-(13–36) is effective in human and bovine chromaffin cells, but not in PC-12 cells or rat nucleus tractus solitarius; [Leu^{31},Pro^{34}]NPY can stimulate the y3 receptor of PC-12 cells and rat distal colon, but not human chromaffin cells (23, 26, 40, 41, 47). Therefore, Y1, Y2, Y4, and Y5 receptors, despite their presence on chromaffin cells, do not appear to be implicated in NPY-evoked catecholamine release from human chromaffin cells.

The fact that NPY stimulates catecholamine release from human chromaffin cells raises the question of whether endogenous NPY plays a tonic role in catecholamine secretion. To address this question, we used Y1, Y2, and Y5 receptor antagonists and established that these compounds had no effect on basal catecholamine release. In the absence of specific y3 antagonists, we neutralized endogenous NPY with NPYO5, an anti-NPY monoclonal antibody that significantly reduced the basal release of catecholamines (29), thus suggesting that endogenous NPY modulates catecholamine secretion in chromaffin cells. Unfortunately, the absence of y3 antagonists precludes clinical experiments to assess this mechanism in humans.

With regard to cell signaling, NPY significantly decreased forskolin-stimulated cAMP accumulation, in contrast with reports on bovine chromaffin cells that NPY had no effect on adenylate cyclase activity (36). An intriguing question remains concerning the effect of NPY agonists on calcium mobilization. In our studies, PYY, [Leu^{31},Pro^{34}]NPY, and [Pro^{34}]NPY increased $[Ca^{2+}]_i$, presumably via Y1, Y2, Y4, or Y5 receptors. As the major signal for catecholamine exocytosis in chromaffin cells is an increase in $[Ca^{2+}]_i$, via calcium entry rather than mobilization of intracellular Ca^{2+} stores, we suggest that the y3 receptor is probably the only receptor to be efficiently coupled to the calcium increase-exocytosis mechanism via the influx of Ca^{2+} (48).

The finding that nicotine, during the first phase of stimulation, evokes a simultaneous release of NPY, EP, and NE from chromaffin cells might reflect a common storage of NPY within NE and/or EP granules. Consequently, our data do not support two conflicting immunohistochemical reports that NPY is selectively colocalized with EP (46) or NE (49). We postulate that during the initial phase of release, NPY acts on NPY y3 receptors to amplify catecholamine release. Moreover, although NPY and catecholamines might in some instances be located in common vesicles, the prolonged secretion of NPY (even beyond the end of nicotine infusion) favors a distinct pool of vesicles containing mainly NPY available for exocytosis during the phase when catecholamine secretion returned to constitutive levels.

At the periphery, NPY is colocalized with NE and released from sympathetic nerve terminals upon sympathetic stimulation (2–4). NPY potentiates the vasoconstrictor effect of NE in rats (11) as well as humans (50) through the Y1 receptor. In addition to the sympathetic nervous system, we previously found that the rat adrenal was a significant source of NPY, as circulating NPY levels in rats are 35% lower in the absence of adrenal medulla (51), but similar data in man are

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lacking. Therefore, NPY released from the adrenal medulla may contribute to the increase in blood pressure observed during sympathoadrenal stimulation; this is probably the case for the cold water test, as NPY Y1 antagonist administration attenuates the elevation in blood pressure seen during this test in rats (52). Therefore, we postulate a dual role of NPY as a neurotransmitter (originating from the nerve endings) and a hormone (deriving from the adrenal).

In conclusion, the present study suggests a new role for NPY as a local hormone that modulates catecholamine actions during stress, and it is postulated that this function is mediated via the y3 receptor in man. Consequently, better understanding of the y3 receptor might allow the development of specific receptor antagonists that could be useful for treating hyperadrenergic states as well as patients with pheochromocytoma and increased secretion of NPY associated with hypertensive crises.

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