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Neuropeptide Y inhibits depolarization-stimulated catecholamine synthesis in rat pheochromocytoma cells

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Abstract

In PC12 rat pheochromocytoma cells differentiated with nerve growth factor (NGF), neuropeptide Y inhibited depolarization-stimulated catecholamine synthesis as determined by in situ measurement of 3,4-dihydroxyphenylalanine (DOPA) production in the presence of the decarboxylase inhibitor *m*-hydroxybenzylhydrazine (NSD-1015). The inhibition by neuropeptide Y was concentration-dependent and was prevented by pretreatment with pertussis toxin, suggesting the involvement of a GTP-binding protein of the G_i or G_o subtype. The neuropeptide Y analog [Leu^{31},Pro^{34}]neuropeptide Y also caused inhibition of DOPA production, but was less potent than neuropeptide Y itself, while peptide YY and neuropeptide Y-(13–36) had no significant effect. This pattern is most consistent with the involvement of the neuropeptide Y Y_3 receptor subtype. In PC12 cells differentiated with dexamethasone, neuropeptide Y also caused a concentration-dependent inhibition of DOPA production, while peptide YY was again without effect. Neuropeptide Y had no effect on DOPA production in undifferentiated PC12 cells. These results indicate that neuropeptide Y can modulate catecholamine synthesis in addition to its modulatory effects on catecholamine release.

Keywords: Neuropeptide Y; Tyrosine hydroxylase; PC12 cell; Nerve growth factor; Dexamethasone; Pertussis toxin

1. Introduction

Neuropeptide Y is a 36-amino acid peptide belonging to the family of peptides which includes peptide YY and pancreatic polypeptide (Tatemoto et al., 1982). Neuropeptide Y is widely distributed in both the central and peripheral nervous systems, where it is often co-localized with catecholamine neurotransmitters (Wahlestedt and Reis, 1993). Three subtypes of neuropeptide Y receptors have thus far been identified on the basis of the rank order of potency of neuropeptide Y and its analogs at these receptors (Grundemar and Håkanson, 1994). Neuropeptide Y Y_1 receptors are distinguished by their high affinity for [Leu^{31},Pro^{34}] neuropeptide Y, neuropeptide Y and peptide YY. Neuropeptide Y Y_2 receptors exhibit high affinity for neuropeptide Y-(13–36), neuropeptide Y, and peptide YY, while Y_3 receptors have high affinity for neuropeptide Y, intermediate affinities for [Leu^{31},Pro^{34}]neuropeptide Y and neuropeptide Y-(13–36), and very low affinity for peptide YY. All three receptor subtypes have been shown to be G-protein linked and have the ability to inhibit cAMP production (Wahlestedt and Reis, 1993). Stimulation of Y_1 receptors has also been shown to increase intracellular Ca^{2+} (Wahlestedt et al., 1992), while Y_2 receptor stimulation can decrease Ca^{2+} influx (Chen and Westfall, 1994). Y_3 receptor stimulation has been shown to increase Ca^{2+} influx in bovine chromaffin cells (Wahlestedt et al., 1992) and to decrease Ca^{2+} influx in cultured superior cervical ganglion cells (Foucart et al., 1993).

It is well established that neuropeptide Y or its analogs have the ability to inhibit the release of catecholamines from sympathetic neurons (Westfall et al., 1990), cultured superior cervical ganglion cells (Oellerich et al., 1994), and PC12 cells differentiated with

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nerve growth factor (NGF) (Chen and Westfall, 1994; DiMaggio et al., 1994). The inhibition of catecholamine release from these cells is apparently mediated by Y_2 receptors (Chen and Westfall, 1994; Westfall et al., 1990) by inhibition of Ca^{2+} influx through voltage-gated Ca^{2+} channels (Chen and Westfall, 1994; Oellerich et al., 1994).

Based on the actions of neuropeptide Y on modulation of catecholamine release, it seemed reasonable to investigate the effects of the peptide on the rate of tyrosine hydroxylation, the rate-limiting step in the biosynthesis of the catecholamines. Rat pheochromocytoma PC12 cells were chosen for this study for several reasons. These cells contain tyrosine hydroxylase and synthesize and store catecholamines, mainly dopamine (Greene and Tischler, 1976). These cells also synthesize and store neuropeptide Y (Allen et al., 1984) and can be induced to express different neuropeptide Y receptor subtypes by the application of various differentiating agents. Undifferentiated PC12 cells resemble immature adrenal chromaffin cells and express receptors of the Y_1 subtype which are not coupled to adenylate cyclase (DiMaggio et al., 1994). Differentiation of PC12 cells with NGF induces neurite outgrowth and expression of a sympathetic neuronal phenotype (Greene and Tischler, 1976). NGF-differentiated cells respond to neuropeptide Y, [Leu^{31},Pro^{34}]neuropeptide Y and neuropeptide Y-(13–36) by inhibition of forskolin-stimulated cAMP formation (DiMaggio et al., 1994). NGF-differentiated PC12 cells also respond to neuropeptide Y and neuropeptide Y-(13–36) by stimulation and inhibition, respectively, of the depolarization-induced increase in $[Ca^{2+}]_i$ (Chen and Westfall, 1994). These studies have implicated the presence of Y_1, Y_2, and Y_3 receptor subtypes in NGF-differentiated PC12 cells. Alternatively, differentiation of PC12 cells with dexamethasone induces the expression of a mature chromaffin cell phenotype (Garber et al., 1989). In dexamethasone-differentiated cells, [Leu^{31},Pro^{34}] neuropeptide Y causes inhibition of forskolin-stimulated cAMP formation, while neuropeptide Y-(13–36) is without effect, indicating that Y_2 receptors are not present on these cells (DiMaggio et al., 1994).

The purpose of the present study was to investigate the effects of neuropeptide Y on basal and depolarization-stimulated catecholamine synthesis in PC12 cells of different phenotypes and to determine the subtype of receptor mediating these effects.

2. Materials and methods

2.1. Cell culture

Stock cultures of PC12 rat pheochromocytoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 1 mM pyruvate, 5% fetal calf serum, 10% heat-inactivated horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone at 37°C in a humidified atmosphere containing 5% CO_2 in air. Cells were passaged once per week with media changes every 2–3 days. For 3,4-dihydroxyphenylalanine (DOPA) accumulation studies, cells were plated onto six-well plates (treatment C, Mattek Corp., Ashland, MA) at a density of $2–2.5 \times 10^5$ cells per well and differentiated with NGF (50 ng/ml) or dexamethasone (1 μM) for 5 days. Undifferentiated cells were plated similarly and allowed to grow for 4–5 days before use. Medium was changed and NGF or dexamethasone additions were performed every 2–3 days.

2.2. In situ assay of tyrosine hydroxylation

Tyrosine hydroxylation was assayed by measuring the accumulation of DOPA in the presence of the decarboxylase inhibitor *m*-hydroxybenzylhydrazine (NSD-1015). The medium was aspirated and the cells were washed 1 time with filter-sterilized Krebs-Ringer Hepes buffer containing 125 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.6 mM glucose, 25 mM Hepes, and 100 μM EDTA adjusted to pH 7.4 (Tischler et al., 1983). The buffer was heated to 37°C and oxygenated with 95% O_2-5% CO_2 before use. Cells were then incubated at 37°C in a 5% CO_2 atmosphere in 1.8 ml of buffer supplemented with 400 μM NSD-1015 and 100 μM L-tyrosine in the absence or presence of the indicated concentrations of neuropeptide Y or analogs. K^+-stimulated DOPA synthesis was assayed in buffer containing 55 mM KCl with a corresponding reduction in NaCl. Aliquots (200 μl) were removed from the incubation medium at 30, 60, 90, and 120 min after the beginning of the incubation and added to 50 μl of chilled 0.5 N perchloric acid. After the removal of the 120 min aliquot, 250 μl of chilled 0.5 N perchloric acid was added to the remaining incubation medium (1 ml) and the cells were scraped from the bottom of the culture well and transferred to 1.5 ml centrifuge tubes, chilled, and disrupted by sonication. The tubes were then centrifuged for 5 min at $15\,000 \times g$ and the supernatant removed. The resulting protein pellet was resuspended in 1 N NaOH (1 ml) and the amount of protein quantified by the method of Lowry using bovine serum albumin as the standard (Lowry et al., 1951). Under these conditions, at least 90% of the DOPA produced by the cells is released into the incubation medium, an effect which has also been reported by others (Dahmer et al., 1991; Vaccaro et al., 1980). The amount of DOPA in each aliquot was quantified by high-performance liquid chromatography (HPLC) with electrochemical detection as described (Chen and Westfall, 1994). This value

was then used to determine the total amount of DOPA present in the incubation medium at each time point by the formula: total DOPA = [(DOPA in aliquot) × (incubation volume/0.2 ml)] + (DOPA removed in previous aliquots). For each culture well, the amount of DOPA per mg protein was plotted against time and the slope of the resulting line was determined by linear regression to give the rate of DOPA synthesis as ng DOPA/mg protein per min. The increase in the amount of DOPA accumulated with time using this method is quite linear, with an average R-squared value of > 0.96. Incubation of cells with 1 mM 3-iodotyrosine, an inhibitor of tyrosine hydroxylase, completely blocked DOPA accumulation (data not shown).

2.3. Statistics

All data are expressed as the means ± S.E.M. Statistical analysis was performed using two-tailed unpaired Student's t test or one-way analysis of variance followed by Student-Newman-Keuls multiple comparisons test as appropriate. A P value of 0.05 or smaller was considered significant.

2.4. Materials

Porcine neuropeptide Y, neuropeptide Y-(13–36), [Leu^{31},Pro^{34}]neuropeptide Y, and peptide YY were purchased from Peninsula Laboratories (Belmont, CA) or Sigma (St. Louis, MO). Dexamethasone, NSD-1015, 3-iodotyrosine, and pertussis toxin were purchased from Sigma. NGF was purchased from Collaborator Biomedical Products (Bedford, MA). DMEM, fetal bovine serum, horse serum, and penicillin-streptomycin-fungizone were purchased from JRH Biosciences (Lenexa, KS).

3. Results

3.1. Effects of neuropeptide Y and analogs on DOPA production in NGF-differentiated PC12 cells

Basal DOPA production in NGF-differentiated PC12 cells was determined to be 12.4 ± 1.1 ng DOPA/mg protein per min ($n = 34$). Neuropeptide Y in doses from 10^{-8} M to 10^{-6} M had no effect on basal DOPA production (data not shown). Depolarization with 55 mM KCl increased DOPA production to 27.4 ± 1.7 ng DOPA/mg protein per min ($n = 34$; $P <$ 0.001 vs. basal). Neuropeptide Y caused a concentration-dependent decrease in depolarization-stimulated DOPA production, with 1 μM neuropeptide Y producing a 50% inhibition (Fig. 1). The effects of 55 mM KCl and neuropeptide Y were stable over the time period analyzed, with no desensitization occurring during the 120 min incubation (Fig. 2). The inhibitory effect of 1 μM neuropeptide Y was completely blocked by 20 h pretreatment of cells with 50 ng/ml pertussis toxin (Fig. 3).

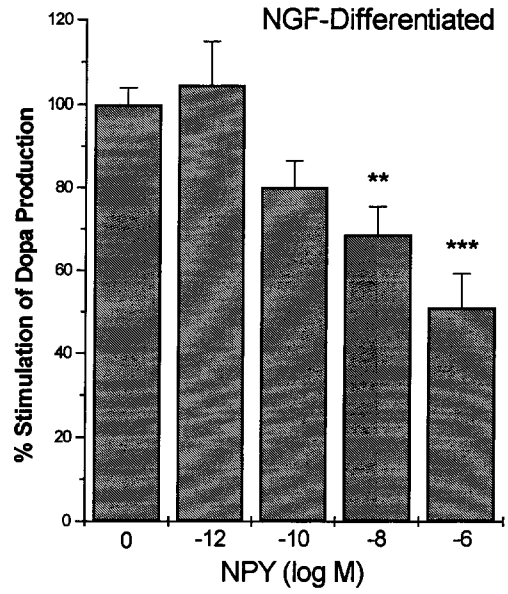

Fig. 1. Concentration-dependent effect of neuropeptide Y (NPY) on 55 mM KCl-stimulated DOPA production in NGF-differentiated PC12 cells. Results are expressed as mean percentage of the stimulation of DOPA production over basal by 55 mM KCl ± S.E.M. (n = 15–18 wells from five to six separate experiments). The mean basal DOPA production was 13.3 ± 1.3 ng DOPA/mg protein per min in these experiments, and 55 mM KCl-stimulated DOPA production was 28.9 ± 2.5 ng DOPA/mg protein per min. Significantly different from that obtained in the absence of neuropeptide Y, ** $P < 0.01$; *** $P < 0.001$.

In order to determine the subtype of receptor mediating the effects of neuropeptide Y on DOPA produc-

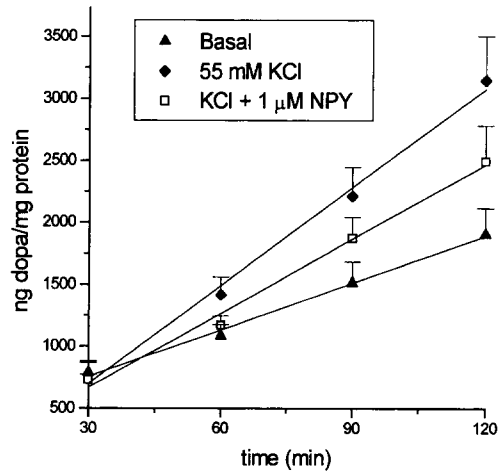

Fig. 2. Time course of effects of 55 mM KCl and 55 mM KCl + 1 μM neuropeptide Y (NPY) on DOPA production in NGF-differentiated PC12 cells. Results are expressed as mean ng DOPA/mg protein for each time point ± S.E.M. (n = 14–15 wells from five separate experiments).

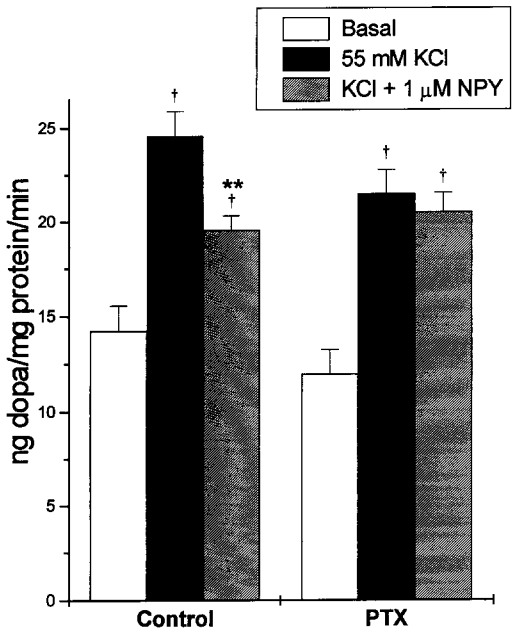

Fig. 3. Effect of pertussis toxin (PTX; 50 ng/ml) pretreatment on neuropeptide Y (NPY; 1 μM) induced inhibition of 55 mM KCl-stimulated DOPA production in NGF-differentiated PC12 cells. Results are expressed as mean DOPA production (ng DOPA/mg protein per min) $\pm$ S.E.M. ($n = 8$–9 wells from three separate experiments). Significantly different from corresponding basal, $^{\dagger} P < 0.001$. Significantly different from that obtained in the absence of neuropeptide Y, $^{**} P < 0.01$.

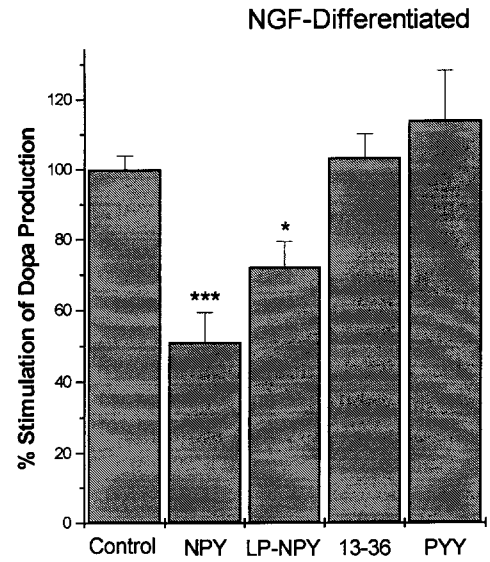

Fig. 4. Effects of 1 μM neuropeptide Y (NPY), [Leu^{31},Pro^{34}]neuropeptide Y (LP-NPY), neuropeptide Y-(13–36) (13–36), and peptide YY (PYY) on 55 mM KCl-stimulated DOPA production in NGF-differentiated PC12 cells. Results are expressed as mean percentages of the stimulation of DOPA production over basal by 55 mM KCl $\pm$ S.E.M. ($n = 9$–15 wells from three to five separate experiments for each peptide). The mean basal DOPA production was 14.0 $\pm$ 0.71 ng DOPA/mg protein per min in these experiments, and 55 mM KCl-stimulated DOPA production was 25.4 $\pm$ 1.4 ng DOPA/mg protein per min. Significantly different from control, $^{*} P < 0.05$; $^{***} P < 0.001$.

tion, the effects of several analogs were tested. [Leu^{31},Pro^{34}]Neuropeptide Y also caused an inhibition of depolarization-stimulated DOPA production, but was less effective than neuropeptide Y, producing only a 30% inhibition at 1 μM (Fig. 4). Neuropeptide Y-(13–36) and peptide YY had no significant effect on DOPA production at 1 μM (Fig. 4) or at concentrations between 10^{-9} M–10^{-7} M (data not shown).

3.2. Effects of neuropeptide Y on DOPA production in dexamethasone-differentiated PC12 cells

Basal DOPA production in dexamethasone-differentiated PC12 cells was determined to be 23.8 $\pm$ 1.8 ng DOPA/mg protein per min ($n = 21$). When incubated with 55 mM KCl, DOPA production increased to 42.9 $\pm$ 3.4 ng DOPA/mg protein per min ($n = 20$; $P < 0.001$ vs. basal). Neuropeptide Y inhibited depolarization-stimulated DOPA production in dexamethasone-differentiated cells in a concentration-dependent manner with a 50% inhibition at 1 μM neuropeptide Y (Fig. 5). Peptide YY had no effect on DOPA production in dexamethasone-differentiated cells over the concentration range tested (10^{-9} M–10^{-6} M; data not shown).

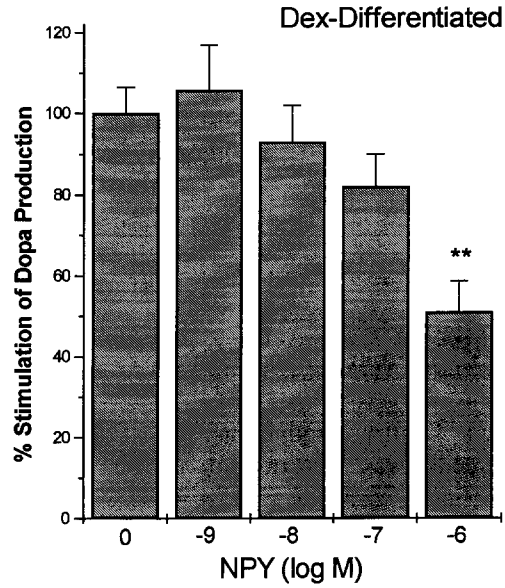

Fig. 5. Effect of neuropeptide Y (NPY) on 55 mM KCl-stimulated DOPA production in dexamethasone-differentiated PC12 cells. Results are expressed as mean percentages of the stimulation of DOPA production over basal by 55 mM KCl $\pm$ S.E.M. ($n = 12$ wells from four separate experiments). The mean basal DOPA production was 21.1 $\pm$ 2.7 ng DOPA/mg protein per min in these experiments, and 55 mM KCl-stimulated DOPA production was 41.7 $\pm$ 5.8 ng DOPA/mg protein per min. Significantly different from that obtained in the absence of neuropeptide Y, $^{**} P < 0.01$.

3.3. Effects of neuropeptide Y on DOPA production in undifferentiated PC12 cells

Basal DOPA production in undifferentiated PC12 cells was determined to be 11.3 ± 2.0 ng DOPA/mg protein per min ($n = 9$). Stimulation of DOPA production with 55 mM KCl increased this value to 18.8 ± 1.9 ng DOPA/mg protein per min ($n = 9$; $P < 0.02$ vs. basal). Neuropeptide Y in doses from 10^{-9} M to 10^{-6} M had no effect on depolarization-stimulated DOPA production in undifferentiated PC12 cells (data not shown).

4. Discussion

The present study demonstrates that neuropeptide Y inhibits depolarization-stimulated DOPA production in PC12 cells differentiated to a sympathetic neuronal phenotype with NGF or differentiated to a mature chromaffin phenotype with dexamethasone. This study also demonstrates that neuropeptide Y has no effect on basal DOPA production or on depolarization-stimulated DOPA production in undifferentiated PC12 cells. The rank order of potency of neuropeptide Y and analogs on the inhibition of depolarization-stimulated DOPA production was determined to be neuropeptide Y > [Leu^{31},Pro^{34}]neuropeptide Y > neuropeptide Y-(13–36) = peptide YY, which is most consistent with the involvement of a receptor of the Y_3 subtype (Grundemar and Håkanson, 1994). Neuropeptide Y Y_3 receptors have been identified in a number of biological systems, including rat superior cervical ganglion sympathetic neurons (Foucart et al., 1993), bovine adrenal chromaffin cells (Wahlestedt et al., 1992), rat nucleus tractus solitarii (Grundemar et al., 1991), and rat cardiac ventricular membranes (Balasubramaniam et al., 1990). The inhibitory action of neuropeptide Y on DOPA production was prevented by pretreatment with pertussis toxin, suggesting that the effect is mediated through a GTP-binding protein of the G_i or G_o type. Numerous other studies have also shown that responses to stimulation of Y_1, Y_2, or Y_3 receptors are pertussis-toxin sensitive (Chen and Westfall, 1994; DiMaggio et al., 1994; Foucart et al., 1993; Wahlestedt and Reis, 1993).

Previous studies performed in this laboratory have demonstrated that ^{125}I-neuropeptide Y binding in NGF-differentiated PC12 cells can be displaced by neuropeptide Y, [Leu^{31},Pro^{34}]neuropeptide Y, and neuropeptide Y-(13–36) (DiMaggio et al., 1994). These studies were interpreted to indicate that Y_1 and Y_2 receptors are present on these cells. However, since all of these agonists also have affinity for the Y_3 receptor, the presence of this receptor subtype using these binding studies is indeterminate. Functional studies of the effects of neuropeptide Y in NGF-differentiated PC12 cells revealed that neuropeptide Y can potentiate depolarization-induced increases in intracellular Ca^{2+} levels, probably via Ca^{2+} release from intracellular stores (Chen and Westfall, 1994). This effect of neuropeptide Y was attributed to Y_3 receptor activation, since peptide YY did not reproduce this response. These results, in combination with the results obtained in this study, indicate that Y_3 receptors are present in NGF-differentiated PC12 cells.

In dexamethasone-differentiated PC12 cells, neuropeptide Y also caused a dose-dependent inhibition of depolarization-stimulated DOPA production, while peptide YY was ineffective. The maximal observed effect of neuropeptide Y ($\sim 50\%$ inhibition) was equal in dexamethasone and NGF-differentiated cells. However, neuropeptide Y was less potent in dexamethasone-differentiated cells, with no significant effect observed until 1 μM of the peptide was used. This could indicate that fewer Y_3 receptors are expressed on dexamethasone-differentiated cells, that the receptors are less efficiently coupled to their second messengers, or that the Y_2 receptor may have some contribution to the response, as NGF- but not dexamethasone-differentiated cells express receptors of the Y_2 subtype (DiMaggio et al., 1994). Neuropeptide Y had no effect on depolarization-stimulated DOPA production in undifferentiated PC12 cells, which is consistent with the observation that neuropeptide Y receptors in undifferentiated cells are unresponsive to application of neuropeptide Y, perhaps indicating a defect in second messenger coupling (DiMaggio et al., 1994).

Several receptors which cause inhibition of catecholamine release have also been found to inhibit catecholamine synthesis, including dopamine D_2 (El Mestikawy and Hamon, 1986; Harada et al., 1990; Hetey et al., 1985), α_2-adrenergic (Pi and García-Sevilla, 1992; Yanagihara et al., 1987), γ-aminobutyric acid ($GABA_B$) (Arias-Montaño et al., 1991), serotonin (Hetey et al., 1985), and adenosine A_1 (Olianas and Onali, 1990). The mechanisms by which these receptors produce this inhibition have not been thoroughly defined, although inhibition of adenylate cyclase (El Mestikawy and Hamon, 1986; Harada et al., 1990; Olianas and Onali, 1990), blockade of Ca^{2+} entry (Arias-Montaño et al., 1991; Yanagihara et al., 1987), and inhibition of phosphoinositide hydrolysis (Olianas and Onali, 1990) have been suggested as possibilities. Each of these mechanisms is also possible in the inhibition of catecholamine synthesis by neuropeptide Y, since neuropeptide Y receptors have been shown to couple to all of these second messenger systems (Gehlert, 1994). In particular, Y_3 receptors have been shown to decrease cAMP accumulation (Balasubramaniam and Sheriff, 1990; Härfstrand et al., 1987; Wahlestedt and Reis, 1993) and to decrease Ca^{2+}

influx through voltage-gated Ca^{2+} channels (Foucart et al., 1993). Further investigation is necessary to isolate the precise mechanisms by which neuropeptide Y causes inhibition of DOPA production.

Interestingly, neuropeptide Y has been found to enhance the inhibitory effect of α_2-adrenoceptor activation on [^{3}H]noradrenaline release from preparations of several rat brain regions (Martire et al., 1986,1989). This may represent a receptor-receptor interaction by which neuropeptide Y enhances presynaptic α_2-adrenergic autoreceptor function. Furthermore, α_2-adrenoceptor activation has been shown to inhibit catecholamine synthesis in the rat brain (Pi and García-Sevilla, 1992) and in bovine adrenal medullary cells (Yanagihara et al., 1987). These results raise the possibility that α_2-adrenoceptor-mediated inhibition of catecholamine synthesis in these regions could be potentiated by neuropeptide Y in addition to its potentiation of α_2-adrenoceptor-mediated inhibition of catecholamine release.

In summary, the results of the present study demonstrate that neuropeptide Y causes inhibition of depolarization-stimulated catecholamine synthesis in NGF- and dexamethasone-differentiated PC12 cells. This inhibition seems to be mediated through the Y_3 subtype of neuropeptide Y receptor coupled to an inhibitory G protein. These results indicate that neuropeptide Y, which is co-stored and co-released with catecholamines in the central and peripheral nervous systems, can modulate catecholamine synthesis by inhibiting tyrosine hydroxylation in addition to its modulatory effects on catecholamine release.

Acknowledgements

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