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Stimulation of neuropeptide Y release in rat pheochromocytoma cells by nitric oxide

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Abstract

Neuropeptide Y and nitric oxide (NO) synthase are colocalized in nervous tissues. We tested the hypothesis whether or not NO might be involved in the release of neuropeptide Y. Neuropeptide Y concentration in the supernatant of PC12 rat pheochromocytoma cells, shown to express NO synthase I by immunohistochemistry, rose threefold in a time- and dose-dependent manner following sodiumnitroprusside and 3-morpholinosydnonimine (SIN-1) incubation. Neuropeptide Y mRNA expression was induced by NO-donors as a function of incubation-time. Neuropeptide Y production rose fivefold with zaprinast, an inhibitor of the phosphodiesterase V and threefold with nerve growth factor (NGF). Combined application of zaprinast and NGF did not further increase neuropeptide Y production while combination of zaprinast and sodiumnitroprusside potentiated the NO effect on neuropeptide Y release. The data suggest that NO regulates neuropeptide Y secretion of PC12 pheochromocytoma cells on the mRNA level. © 1997 Elsevier Science B.V.

Keywords: Nitric oxide (NO) pharmacology; Neuropeptide Y; PC12 cells

1. Introduction

Neuropeptide Y, a 36-amino-acid peptide first isolated from porcine brain (Tatemoto et al., 1982), is found in many central and perivascular neurons. The release of neuropeptide Y is regulated by various substances, including nerve growth factor (NGF) and dexamethasone as could be demonstrated in PC12 pheochromocytoma cells (Allen et al., 1984, 1987; Minth-Worby, 1994). Like neuropeptide Y, nitric oxide (NO) is known to be a neurotransmitter, involved in glutamate-induced neurotransmission (Moncada et al., 1989; Garthwaite, 1991), the formation of memory (Bohme et al., 1991; Shibuki and Okada, 1991), vasorelaxation, gut motility, and sphincter relaxation (Moncada and Higgs, 1993). Recent morphological studies revealed a colocalisation of neuropeptide Y and NO in various peripheral nervous tissues, including postganglionic sympathetic neurons (Nichols et al., 1994; Smet et al., 1994; Hohler et al., 1995). We therefore hypothesized that NO might be involved in the release of neuropeptide Y. Since many studies with regard to the regulation of neuropeptide Y release were performed on PC12 pheochromocytoma cells we tested the hypothesis in these cells grown in monolayers.

2. Material and methods

2.1. Cell culture

PC12 rat pheochromocytoma cells were cultured in RPMI 1640 cell culture medium (Gibco, Eggenstein, Germany) supplemented with 5% fetal calf serum, 10% horse serum and 2% penicillin/streptomycin at 37°C in air containing 5% CO_2. 5×10^5 PC12 cells were plated in 25 cm^2 cell culture flasks (Falcon, Becton&Dickinson, Summit, NJ, USA) and grown for three days before being used for the experiments. Monolayers were incubated with the NO-donors sodiumnitroprusside (100 μM) and 3-morpholinosydnonimine (100 μM SIN-1), with nerve growth factor as a positive control (50 ng/ml NGF) and the inhibitor of cGMP-phosphodiesterase zaprinast (100 μM; all chemicals from Sigma, Munich, Germany). For every incubation

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appropriate controls were used. After incubation the supernatant was stored at −20°C until used for measurement. Monolayer cells were trypsinized, an aliquot was used for determination of the number of cells while most cells were immediately frozen in liquid nitrogen and stored at −80°C until used for RNA extraction. The number of cells was counted with an electronical cell counting system (Casy, Schärfe, Reutlingen, Germany). DNA synthesis as a marker of proliferation was assessed using a commercial 5-bromo-2′-deoxyuridine assay (Boehringer-Mannheim, Mannheim, Germany) and an ELISA-reader (KUCO-21, Zinsser Analytic, Frankfurt, Germany).

2.2. Immunohistochemistry

Immunohistochemistry was performed as described elsewhere (Hohler et al., 1995). Monolayers of PC12 cells were preincubated for 1 h with phosphate-buffered saline (PBS) containing 10% normal porcine serum, 0.1% bovine serum albumin and 0.5% Tween 20. Following overnight incubation with the polyclonal NO synthase I antiserum (1:1500, Klatt et al., 1992) the cells were washed in PBS and reacted for 1 h with biotinylated anti-rabbit immunoglobulin G (IgG) antiserum (1:200; Amersham Buchler, Braunschweig, Germany). Subsequently, the cells were washed again and incubated for 1 h with streptavidin–Texas Red (1:200; Amersham Buchler) to label the biotinylated secondary antibody. The specimen were then viewed by epifluorescence microscopy. Specificity controls for the primary antisera included replacement by non-immune serum and preabsorption (20 μg antigen/ml antiserum diluted to working concentration) with NO synthase isolated from porcine cerebellum.

2.3. Radioimmunoassay

Supernatant of PC12 cells was diluted with 0.5 ml/ml 1% trifluoro acid solution and extracted by using octadecasilyl silica cartridges (Sep-Pak; Milipore, Eschborn, Germany). The columns were prewashed with 10 ml 100% methanol and 10 ml triethylamine buffer (20 mM; pH 4) solution (TBS). Supernatant was placed on the column and washed with 10 ml TBS. The eluate (4 ml of 80% methanol in TBS) was concentrated to dryness in a vacuum centrifuge (Hetovac; Nunc, Wiesbaden, Germany), redissolved in 0.1 phosphate-buffered solution and assayed.

Neuropeptide Y in extracts was measured by a radioimmunoassay as recently described (Rascher et al., 1993).

2.4. RNA extraction and Northern blot analysis

RNA extraction and Northern blot were performed as described elsewhere (Hänze et al., 1991). RNA was extracted from the cells using guanidine–thiocyanate acid phenol and after glyoxylation run on a phosphate buffered 1.2% agarose gel and transferred onto nylon membranes (Nytran N, Schleicher & Schuell, Dassel, Germany) by capillary blot procedure. After pre-hybridization, hybridization was carried out overnight at 68°C by using 50 ng digoxigenin-labeled RNA probe (Boehringer-Mannheim) per ml hybridization solution (see below). To detect the digoxigenin-labeled probes, the membrane was incubated for 30 min at room temperature with a 2% solution of blocking agent (Boehringer-Mannheim) and then for 30 min at room temperature in the same solution containing 1:10 000 dilution of a polyclonal anti-digoxigenin sheep antibody fab fragment conjugated to alkaline phosphatase (Boehringer-Mannheim). After the membrane was washed with 100 mM Tris–HCl (pH 7.4) and 150 mM NaCl, alkaline phosphatase activity was determined by addition of the chemiluminescence substrate solution (CSPD) and exposure of the membrane to an X-ray film. The intensity of specific mRNA bands was quantified by densitometry (Sharp JX-330 Colour Scanner, Sharp, Japan; One-Dscan, Scanalytics, Billerica, MA, USA). All specific mRNA signals were normalized to actin expression. A specific cDNA probe for rat neuropeptide Y (Hänze et al., 1991) and human β-actin (Moos and Gallwitz, 1983) cloned in pGEM4, respectively, was used.

2.5. Statistic evaluation

All results are given as mean ± standard deviation. Differences in neuropeptide Y secretion and mRNA expression over time and as a function of NO donor concentration were compared using ANOVA. A P-value of < 0.05 was considered significant.

3. Results

As assessed by immunohistochemistry PC12 cells expressed considerable amounts of NO synthase I (Fig. 1). Cell proliferation was reduced by NO donors and zaprinast in a dose dependent manner. Sodiumnitroprusside de-

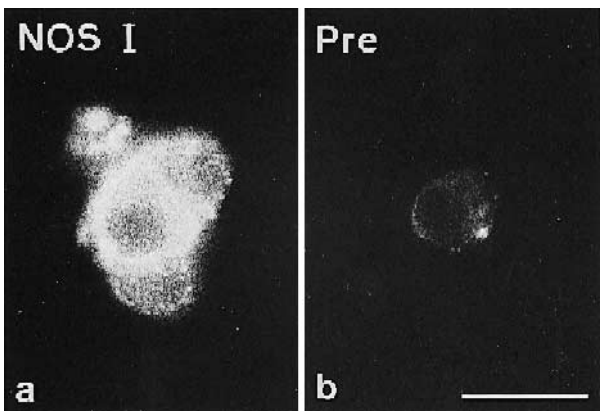

Fig. 1. PC12 cells expressing NO synthase I immunoreactivity (a) which was absent after preabsorption with the antigen (b). Bar = 10 μm.

creased 5-bromo-2′-deoxyuridine labeling from an absorbance ($A_{370\ nm} - A_{492\ nm}$) of 1.6 ± 0.1 to 1.5 ± 0.2 (10 μM), 1.4 ± 0.2 (30 μM) and 0.9 ± 0.4 (100 μM) ($P < 0.01$). Similar results were obtained for the incubation with SIN-1, zaprinast (0.8 ± 0.3, $P < 0.01$) and the combination of zaprinast and sodiumnitroprusside (0.7 ± 0.2, $P < 0.01$). Consequently, neuropeptide Y concentrations in the supernatant of PC12 cells were corrected for the total number of cells and are given in pM/24 h per cell. Incubation of PC12 cells with the NO donors sodiumnitroprusside and SIN-1 increased neuropeptide Y concentration in the supernatant in a time- and dose dependent-manner (Fig. 2a and b), whereas neuropeptide Y release remained constant in untreated control cells at 6, 24 and 48 h. Northern blot analysis shows an incubation time-dependent increase of the ratio between neuropeptide Y mRNA expression and actin mRNA expression in these cells (Fig. 3A). Neuropeptide Y concentration in supernatant rose fivefold following administration of the phosphodiesterase inhibitor zaprinast, whereas nerve growth factor (NGF, 50 ng/ml) increased neuropeptide Y production threefold (Fig. 3B). The effect of zaprinast was potentiated by simultaneous administration of zaprinast/sodiumnitroprusside. However, combined treatment of zaprinast and nerve growth factor (NGF) did not further increase neuropeptide Y release (Fig. 3B).

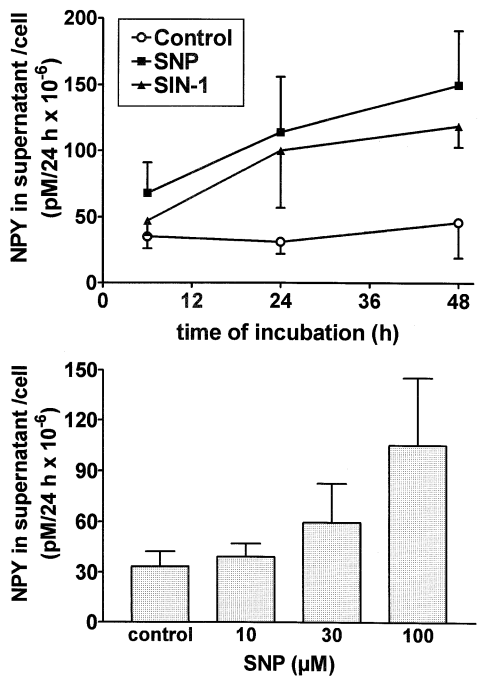

Fig. 2. Neuropeptide Y in supernatant of PC12 cells incubated with NO-donors. Neuropeptide Y concentrations were measured by radioimmunoassay. Mean and standard deviation are shown. (a) Neuropeptide Y concentrations following treatment with sodiumnitroprusside (100 μM, squares) and 3-morholinosydnonimine (SIN-1 100 μM, triangles) as a function of incubation time. Untreated controls are represented by circles. Neuropeptide Y secretion in treated cells was significantly higher than in controls ($P < 0.02$ for SIN-1 and $P < 0.01$ for sodiumnitroprusside incubation). (b) Neuropeptide Y concentration as a function of sodiumnitroprusside concentration after 24 h of incubation. Neuropeptide Y secretion was significantly higher in cells treated with 30 and 100 μM sodiumnitroprusside than in controls ($P < 0.05$ and $P < 0.01$, respectively).

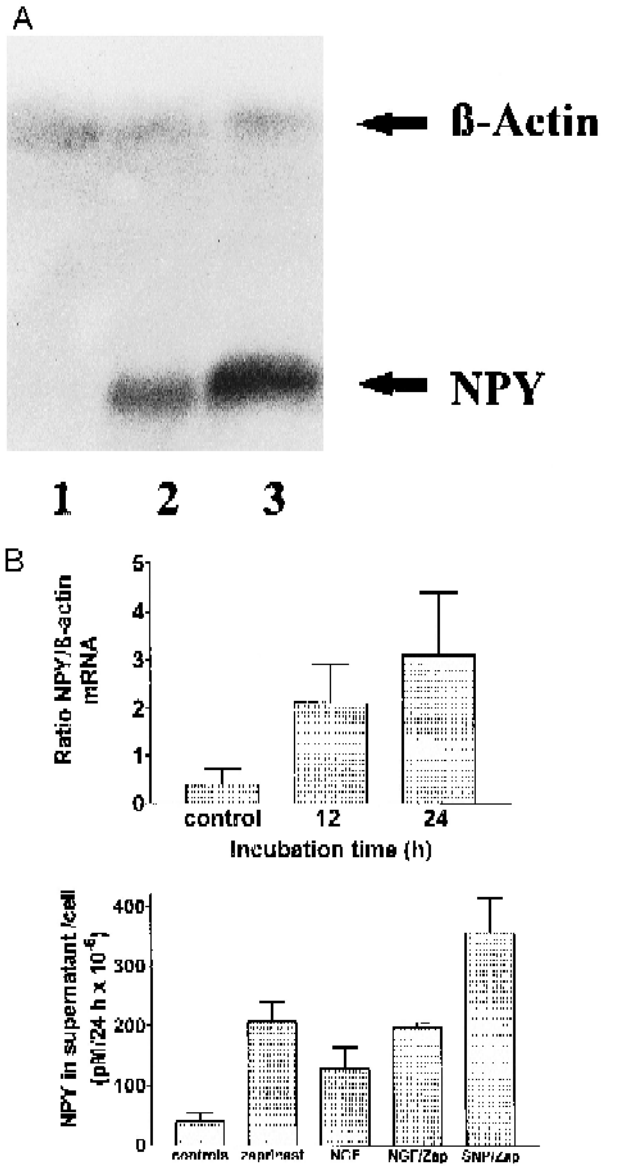

Fig. 3. (A) Northern blot for the detection of neuropeptide Y mRNA expression in PC12 cells with and without sodiumnitroprusside incubation using a specific cRNA probe. Neuropeptide Y (NPY) mRNA expression was quantified using a cRNA probe for β-actin. (a) Increase in neuropeptide Y mRNA expression as a function of sodiumnitroprusside incubation time (controls = lane 1; 12 h 100 μM sodiumnitroprusside = lane 2; 24 h 100 μM sodiumnitroprusside = lane 3). (b) Neuropeptide Y mRNA/ β-actin ratio as evaluated by optic densitometry rose significantly after 12 and 24 h of sodiumnitroprusside incubation ($P < 0.01$ and $P < 0.001$, respectively). (B) Neuropeptide Y concentration in supernatant of PC12 cells. Controls: supernatant of untreated PC12 cells. NGF (50 ng/ml) and zaprinast (Zap) treated cells: significantly higher secretion than in control cells ($P < 0.005$ and $P < 0.001$, respectively). The effect of zaprinast was not influenced by simultaneous treatment with NGF and zaprinast but potentiated by zaprinast/sodiumnitroprusside treatment ($P < 0.01$).

4. Discussion

The data demonstrate that NO released by NO-donors induces neuropeptide Y secretion of PC12 pheochromocytoma cells in a time- and dose-dependent manner. This effect appears to be regulated on a transcriptional level as evidenced by increased neuropeptide Y mRNA expression in PC12 cells, although posttranscriptional mechanisms regulating mRNA stability can not be excluded entirely. Zaprinast induced inhibition of the phosphodiesterase V, that catalyzes the inactivation of the second messenger cGMP, increases neuropeptide Y production. The latter effect is potentiated by the combination of zaprinast with NO-donors.

These findings indicate that NO enhances the neuropeptide Y production in PC12 cells. In addition, stimulation of the cGMP second messenger system, among others used by nitric oxide, led to an increase in neuropeptide Y release from PC12 cells. Since PC12 cells express NO synthase I as evidenced by immunohistochemistry, it may be speculated that the release of neuropeptide Y may be triggered by endogenous NO formation in these cells. Morphological studies have shown colocalization of neuronal nitric oxide synthase and neuropeptide Y in enteric neurons and in postganglionic sympathetic neurons (Hohler et al., 1995; Nichols et al., 1994; Smet et al., 1994) and our data may add a functional role to the anatomical findings in that NO stimulates the neuropeptide Y release.

The effects of nitric oxide on nervous cells have been studied with respect to catecholamines. NO has been shown to inhibit the action of norepinephrine from adrenergic nerves in canine and guinea-pig pulmonary vessels (Cederqvist et al., 1991; Greenberg et al., 1989; Yamamoto et al., 1994). More recently it could be demonstrated that the inhibition of dopamine and norepinephrine appears to be mediated through oxidation of catecholamines by NO leading to an inactivation of these compounds (Macarthur et al., 1995). Similar observations were made with regards to the modulation of endothelin-1 and its action through the endothelin-A-receptor by NO (Goligorsky et al., 1994). In that study it was hypothesized that NO may interfere with the postreceptor pathway for Ca^{2+} and displace bound endothelin-1 from its receptor. In addition to this modulators effect on the endothelin signaling a recent communication demonstrated attenuation of endothelin-1 release in adrenal glands independently of cGMP (Hinson et al., 1996).

In conclusion, NO is involved in the secretion of neuropeptide Y by PC12 cells. This finding may contribute to the understanding of the role of NO in the modulation of neurotransmitters and vasoactive peptides.

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