

# Neuropeptide Y inhibits vasoactive intestinal peptide- and dopamine-induced cyclic AMP formation in human Ewing's sarcoma WE-68 cells

F. van Valen, E. Keck\* and H. Jürgens

*Universitätskinderklinik, Abt. für Hämatologie und Onkologie, D-4000 Düsseldorf and \*Rheumaklinik II, D-6200 Wiesbaden, FRG*

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Neuropeptide Y (NPY) regulation of intracellular cyclic AMP accumulation was studied in human Ewing's sarcoma cell line, WE-68. NPY inhibited vasoactive intestinal peptide (VIP)- and dopamine-stimulated but not basal cyclic AMP formation. The peptide effect was rapid (<2 min), concentration-dependent with a half-maximal effective concentration ( $EC_{50}$ ) of 8 nM NPY, and maximal inhibition reaching 60–70% with 100 nM NPY. Prior exposure of WE-68 cells to pertussis toxin completely abolished the inhibitory action of NPY. It is concluded that NPY attenuates agonist-stimulated cyclic AMP formation in Ewing's sarcoma WE-68 cells, and may do so via the inhibitory guanine nucleotide regulatory protein of adenylate cyclase.

Neuropeptide Y; Pertussis toxin; cyclic AMP; (Ewing's sarcoma cell)

## 1. INTRODUCTION

Neuropeptide Y (NPY) is a 36-amino-acid polypeptide and was originally isolated from porcine brain [1]. NPY is localized in both central and peripheral neurons, and evidence is accumulating that the peptide acts as a neurotransmitter or neuromodulator controlling various physiological events [2]. Specific recognition sites for NPY have been identified in rat cerebral cortex [3] and rat pheochromocytoma cells [4]. With regard to transmembrane signalling systems, NPY has been shown to inhibit cyclic AMP production in the nucleus tractus solitarius region of rat brain [5] and human neuroblastoma cells [6].

Ewing's sarcoma is the second most frequent childhood tumor of bone and was first described in 1921 [7]. Few data are available as yet concerning the regulatory role of hormones or other factors on

second messenger systems in Ewing's sarcoma cells. WE-68 is a recently established human Ewing's sarcoma cell line which displays neural features, namely neurofilaments, S-100 protein, Leu-7 antigen and neuron-specific enolase [8]. In addition, the WE-68 cyclic AMP-generating system responds to classical neurotransmitters such as  $\beta$ -adrenergic and dopaminergic agonists [9,10] as well as the neuropeptide vasoactive intestinal peptide (VIP) [11]. Based on these findings it was opportune to study the effects of the neuropeptide NPY, both alone and in combination with VIP and dopamine, upon cyclic AMP production in the WE-68 cell line. Our data provide the first evidence for dual regulation of cyclic AMP metabolism by neuroactive ligands in Ewing's sarcoma cells.

## 2. MATERIALS AND METHODS

Synthetic human NPY and human VIP were obtained from Peninsula (St. Helens, England) and dopamine, pertussis toxin and 3-isobutyl-1-methylxanthine (IBMX) from Sigma (München).

*Correspondence address:* F. van Valen, Universitätskinderklinik, Abt. für Hämatologie und Onkologie, Moorenstrasse 5, D-4000 Düsseldorf, FRG

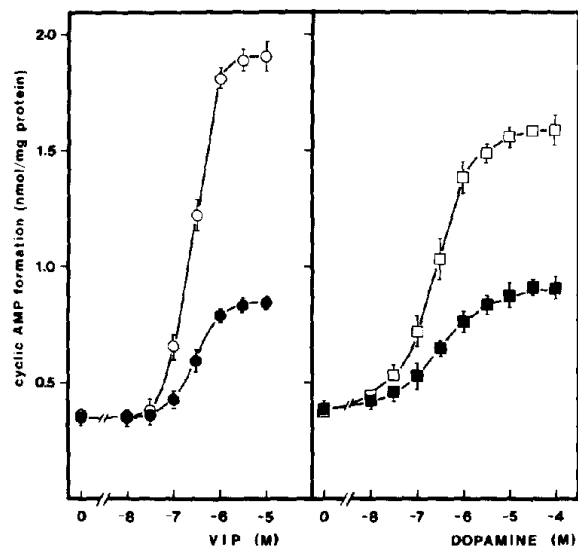


Fig.1. Cyclic AMP formation in human Ewing's sarcoma WE-68 cells incubated with increasing concentrations of VIP (○,●) or dopamine (□,■) in the absence (open symbols) and presence (closed symbols) of  $1 \mu\text{M}$  NPY. Values are means  $\pm$  SE of triplicate determinations on four separate culture preparations.

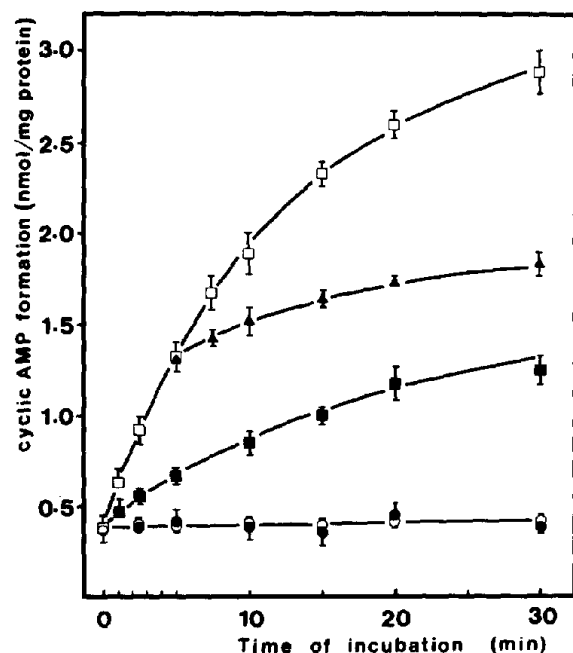


Fig.2. Time course of cyclic AMP formation in human Ewing's sarcoma WE-68 cells incubated in the absence (○,●) or presence (□,■,▲) of  $1 \mu\text{M}$  VIP without (open symbols) and with (closed symbols)  $200 \text{ nM}$  NPY added either at 0 min (●,■) or after 5 min (▲) of incubation. Values are means  $\pm$  SE of triplicate determinations on four separate culture preparations.

Ewing's sarcoma WE-68 cells were maintained in Falcon T-75 flasks in Dulbecco's modified Eagle's medium/Ham's formula-12 (1:1; DME/F12) supplemented with 5% FCS [10]. For cyclic AMP studies, cells were propagated into 24-well Costar plates. Confluent cultures were incubated with pertussis toxin or vehicle (PBS, pH 7.4) for 18 h at the indicated concentrations in DME/F12 supplemented with 0.5% FCS before testing cyclic AMP response to agonists.

The procedure for incubating the cells in order to study cyclic AMP accumulation has been described [9]. Briefly, cells were preincubated for 15 min in  $180 \mu\text{l}$  Hanks' buffered with  $20 \text{ mM}$  Hepes (HH, pH 7.4) containing  $1 \text{ mM}$  IBMX. Thereafter,  $20 \mu\text{l}$  agonist solution was added and cells incubated for an additional 15 min unless otherwise indicated. Incubations were stopped by adding  $20 \mu\text{l}$  of 55% (w/v) trichloroacetic acid to each well. After neutralization of the incubation mixture with  $80 \mu\text{l}$  of  $1 \text{ M}$  Tris, total cell and medium cyclic AMP was determined by radioimmunoassay [10]. Cell protein was measured by the Bio-Rad protein assay (Bio-Rad, München) using human serum albumin as standard.

Data shown represent means  $\pm$  SE of four experiments each performed in triplicate.

### 3. RESULTS

As demonstrated in fig.1,  $1 \mu\text{M}$  NPY attenuated the concentration-dependent stimulatory effect of

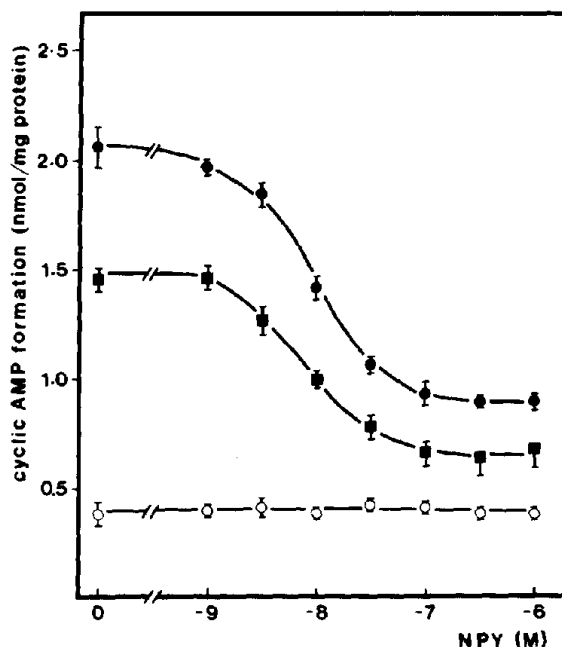


Fig.3. Cyclic AMP formation in human Ewing's sarcoma WE-68 cells incubated with increasing concentrations of NPY in the absence (○) and presence of either  $2 \mu\text{M}$  VIP (●) or  $10 \mu\text{M}$  dopamine (■). Values are means  $\pm$  SE of triplicate determinations on four separate culture preparations.

VIP on cyclic AMP generation in human Ewing's sarcoma WE-68 cells. In the presence of NPY, maximal cyclic AMP response to VIP (3  $\mu$ M) was suppressed by 70%. The  $EC_{50}$  for VIP stimulation was 0.2  $\mu$ M in both the absence and presence of the inhibitory peptide. A similar pattern was observed with the action of NPY on the cyclic AMP response to dopamine (fig.1). The  $EC_{50}$  for dopamine elevation of cyclic AMP was 0.3  $\mu$ M and remained unchanged in the presence of 1  $\mu$ M NPY whereas this concentration of the inhibitory peptide caused 60% reduction in the 10  $\mu$ M dopamine-stimulated cyclic AMP accumulation. NPY (200 nM), added either simultaneously with VIP or after 5 min of incubation with VIP, caused inhibition of cyclic AMP formation after as little as 2 min incubation (fig.2). NPY was without effect on basal cyclic AMP levels over the entire time course studied. NPY inhibited both VIP (2  $\mu$ M)- and dopamine (10  $\mu$ M)-induced cyclic AMP formation (fig.3) with an  $EC_{50}$  of 8 nM NPY and maximal inhibition for 100 nM. Basal cyclic AMP levels in WE-68 cells were not influenc-

ed by NPY in the concentration range 1 nM-1  $\mu$ M. The mechanism of NPY action was evaluated by using pertussis toxin as a probe for the inhibitory pathway of cyclic AMP formation [12]. Pertussis toxin treatment of WE-68 cells abolished the inhibitory effect of NPY on VIP- and dopamine-stimulated cyclic AMP accumulation (fig.4) with an  $EC_{50}$  of 2 ng/ml of the toxin. The stimulatory action of both VIP and dopamine was unaffected by pertussis toxin at up to 1  $\mu$ g/ml.

#### 4. DISCUSSION

Our data clearly show that NPY is a potent inhibitor of cyclic AMP accumulation in human Ewing's sarcoma WE-68 cells. NPY does not affect basal cyclic AMP levels whereas the neuropeptide attenuates the increase in cyclic AMP formation due to the stimulatory effects of VIP and dopamine. A permissive role upon the NPY inhibitory cyclic AMP response has been reported for VIP and isoproterenol [6] and the adenylate cyclase activator forskolin [5,13,14] with other intact cells and tissues. NPY decreases maximal stimulation of cyclic AMP formation by both VIP and dopamine without changing their potencies, suggesting an interaction of NPY with its own receptors in WE-68 cells. Furthermore, the demonstration of the NPY effect in the presence of the phosphodiesterase inhibitor IBMX indicates that NPY acts through direct inhibition of adenylate cyclase and not via increased cyclic AMP degradation. In this regard, NPY has been shown to inhibit adenylate cyclase activity in human cerebral cortex [15], rat hippocampus [16] and a cell line from a human neuroblastoma [6]. Our data obtained with pertussis toxin suggest that NPY receptor inhibition may be mediated by  $N_i$ , the inhibitory guanine nucleotide regulatory protein of adenylate cyclase. It is now well accepted that the stimulation and inhibition of adenylate cyclase appear to be mediated by the distinct guanine nucleotide regulatory proteins  $N_s$  and  $N_i$ , respectively [17]. A common  $\beta$ -subunit appears to be the link and, therefore, the effector of the equilibrium between the two systems [18]. Pertussis toxin selectively ADP-ribosylates and inactivates  $N_i$  [19].

In conclusion, the present results in the WE-68 human Ewing's sarcoma cell line suggest the existence of NPY receptors coupled to adenylate

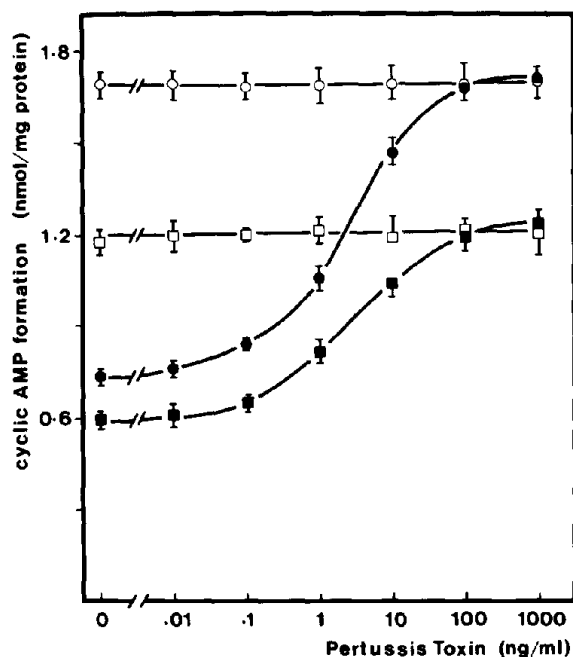


Fig.4. Cyclic AMP formation in human Ewing's sarcoma WE-68 cells pretreated for 18 h with increasing concentrations of pertussis toxin and then exposed to either 1  $\mu$ M VIP (○,●) or 0.5  $\mu$ M dopamine (□,■) without (open symbols) and with (closed symbols) 100 nM NPY. Values are means  $\pm$  SE of triplicate determinations on four separate culture preparations.

cyclase in an inhibitory manner, probably through  $N_i$ .

From a histological point of view, our data represent additional support to the concept of the expression of neural characteristics in the Ewing's bone tumor cell line, WE-68 [8,11].

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