Neuropeptide Y in neuroblastoma × glioma hybrid cells

Response to dexamethasone and nerve growth factor

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High concentrations of a newly-identified biologically potent peptide, neuropeptide Y, have been demonstrated in 3 related mouse neuroblastoma-derived clonal cell lines, N18TG2 0.35 pmol/mg protein, NG108-15 0.44 pmol/mg protein and NCB-20 0.39 pmol/mg protein. The NG108-15 cell line was chosen for further evaluation. Dexamethasone (10 μM) and nerve growth factor (10 ng/ml) resulted in a 2-fold increase in cellular neuropeptide Y concentrations. The response to dexamethasone was demonstrated to be dose-dependent. Exposure to both agents in combination resulted in a more than additive effect, indicating synergism.

Neuropeptide Y *Neuroblastoma cell line* *Dexamethasone* *Nerve growth factor*

1. INTRODUCTION

Culture of NG108-15 neuroblastoma × glioma somatic hybrid cells has allowed the investigation of differentiated neuronal function at a molecular level. The NG108-15 cell line possesses many neuronal properties including electrical excitability [1,2] mediated by activation of specific sodium channels [3] and the ability to form synapses in vitro [1,2,4]. These cells synthesize the neurotransmitter acetylcholine which is released in response to stimulation [5]. The cells were derived by hybridization of the N18TG2 mouse neuroblastoma with the C6BU-1 rat glioma clone.

Recently it has become apparent that some or all neurones may not only possess a 'classical' neurotransmitter, but also a neuropeptide which may also fulfill a neurotransmitter role. This has led to the concept of 'co-localisation' of neurotransmitters [6]. Although the control of acetylcholine synthesis and release has been investigated extensively, little attention has been directed towards the possible peptidergic nature of cultured neuroblastoma cells, although they have been reported to contain enkephalins [7]. We have examined a number of neuroblastoma and neuroblastoma hybrid cell lines (including NG108-15) and have investigated the presence of a novel peptide in these cells. Neuropeptide Y (NPY) is a 36 amino acid peptide characterized by N-terminal tyrosine and C-terminal tyrosine amide residues [8,9]. Since its isolation from porcine brain, it has been demonstrated in abundance within neurones both in the central [10] and peripheral [11] nervous systems. It has been identified within the adrenal medulla [12] and in ganglioneuroblastomas [13]. The possibility that NPY might be synthesized in vitro by one or more established cell lines would allow experimental investigation under controlled conditions to examine the regulation of its synthesis and release.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

Cells were cultured in flasks (75 cm^2, Falcon Plastics) in Dulbecco's modified Eagle medium (DMEM, Gibco Bio-Cult), containing 10% foetal calf serum (Gibco Bio-Cult), 100 μM hypoxanthine, 1 μM aminopterin, 16 μM thymidine and 50 μg/ml gentamicin (Sigma). Cultures were maintained at 37°C in a humidified atmosphere of 93% air and 7% CO_2.

The cells were harvested as follows. The medium was removed from each dish and replaced with Dulbecco's phosphate-buffered saline (no Ca^{2+} or Mg^{2+} ions). Cells were tapped free from the surface of the dishes and the resultant suspension centrifuged at 150 × *g* for 5 min. The supernatant was removed and the cells were subsequently extracted in aprotinin (Trasylol)-supplemented Tris–phosphate buffer (5 mM) at pH 7.4 (final aprotinin concentration of 1000 KIU/ml) using ground glass homogenisers to ensure cell rupture. The extracts were acidified with glacial acetic acid (30 μl/ml) to give a final molarity of 0.5 M, and then boiled for 10 min. A sample of each extract was removed for protein determination [14] using bovine serum albumin as standard. NPY-like immunoreactivity (NPY-LI) was subsequently determined in extracts of 3 cell lines N18TG2 (mouse neuroblastoma), NG108-15 (mouse neuroblastoma × rat glioma) and NCB-20 (mouse neuroblastoma × hamster brain) [4].

Experiments employing the NG108-15 cell line alone were designed to show the effects of dexamethasone and nerve growth factor (NGF) on the cell content of NPY. Cells were plated onto multiwell (35-mm diameter) dishes (Falcon Plastics), and when the cells were adherent to the flasks, the medium was replaced by that containing 10 μM cytosine arabinoside (Sigma). After 24 h, the medium was changed and replicate cultures were maintained in medium without cytosine arabinoside supplemented with either of the following: (i) 2.5 S mouse salivary gland NGF (10 ng/ml, Sigma); (ii) dexamethasone disodium phosphate (10 μM, Sigma); (iii) both NGF (10 ng/ml) and dexamethasone (10 μM).

The cells were then cultured for selected times up to 10 days with medium changes every 48 h. Triplicate cultures were harvested in each group on the days indicated, and measurements were made of NPY-LI and cell protein. The results were expressed as mean ± standard deviation of replicate cultures.

2.2. *Radioimmunoassay*

Concentrations of NPY-LI in the cell extracts were determined using a recently developed specific radioimmunoassay. The antibody was raised in a rabbit to NPY conjugated with carbodiimide to bovine serum albumin. Natural porcine NPY was iodinated using chloramine T and purified on a G-50 Sephadex superfine (Pharmacia) column prior to use as a tracer in the assay. Natural porcine NPY was used as the standard. The final dilution of the antibody was 1:10000 in an assay volume of 600 μl containing 60 mM sodium phosphate buffer (pH 7.2), 50 mM sodium EDTA and 1% (w/v) bovine serum albumin. The assay detection limit is 2 fmol/assay tube with 95% confidence, and shows 30% cross-reaction with the structurally related peptide YY. There is no cross-reaction with other members of the pancreatic polypeptide (PP) family of peptides (including avian PP and human PP) at concentrations up to 1000 fmol/assay tube.

2.3. *High-performance liquid chromatography analysis*

Cell extracts (NG108-15) were separated chromatographically for analysis of NPY-LI using high performance liquid chromatography (HPLC) with a μ-Bondapak C-18 reverse phase column, dimensions 3.9 × 300 mm. A linear gradient elution system was used from 35% to 45% acetonitrile in 0.2% aqueous trifluoroacetic acid, with a flow rate of 2 ml/min. Fractions of 1 ml were collected for subsequent radioimmunoassay. Natural porcine NPY was used as a standard marker.

3. RESULTS

Measurable quantities of NPY-like immunoreactivity (NPY-LI) were present in extracts of all 3 cell lines examined (N18TG2 0.35 pmol/mg protein; NG108-15 0.44 pmol/mg protein; NCB-20 0.39 pmol/mg protein). The synthesis of NPY-LI was increased after incubation of

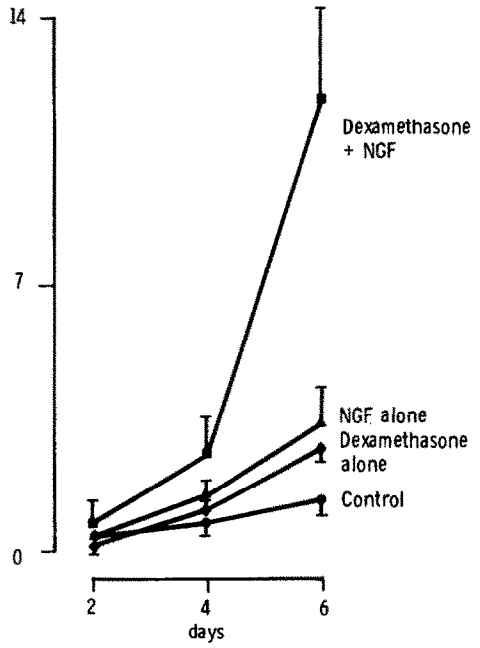

Fig.1. Results show the changes in intracellular NPY-LI concentration of cultured NG108-15 cells in the absence or presence of dexamethasone (10 μM), nerve growth factor (10 ng/ml) or both. The cells were harvested at the times shown from 3 replicate dishes. Data points show means ± SD.

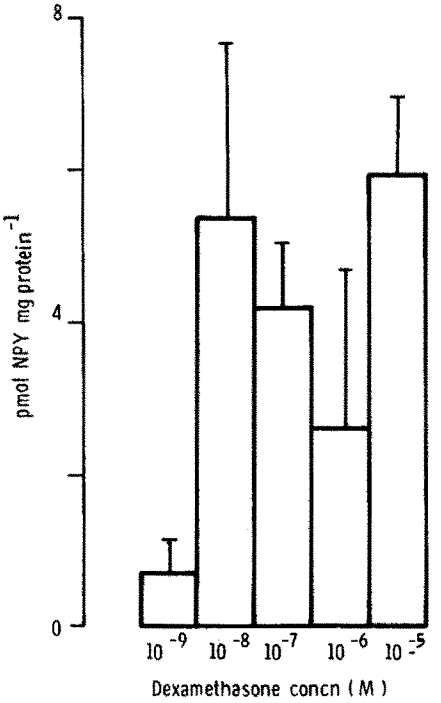

Fig.2. Results show the increase in cellular NPY-LI concentration that accompanies culture of NG108-15 cells for 6 days in the absence or presence of dexamethasone at concentrations between 1 nM and 10 μM. Error bars show SD of triplicate results.

the cells with dexamethasone (10 μM) or NGF (10 ng/ml) alone. The mean concentration of NPY-LI in cells incubated in medium containing both NGF and dexamethasone for a period of 6 days was 11.9 ± 2.35 pmol/mg protein. In cells cultured without these additions, the concentration was 1.5 ± 0.7 pmol/mg protein. Cells incubated in dexamethasone alone contained a maximum NPY level of 2.75 ± 0.53 pmol/mg protein and those incubated with NGF a level of 3.39 ± 0.96 pmol/mg protein (fig.1). Culture in the presence of either agent alone was accompanied by a 2-fold increase in cell content of NPY-LI, whereas the response seen in cells exposed to both agents represents an 8-fold increase in NPY-LI cell content.

In all cases the maximum response was seen after 6 days incubation with a subsequent decline in the concentration of NPY-LI (not shown).

NG108-15 cells were also incubated with selected concentrations of dexamethasone between 1 nM and 10 μM for a period of 6 days (fig.2). Results

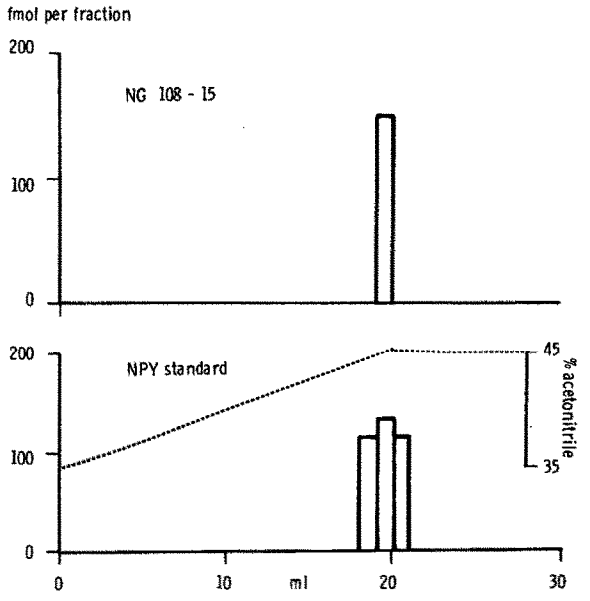

Fig.3. HPLC profiles of porcine NPY standard, and extracts of NG108-15 cells. Details of procedure are given in section 2.

of this indicate a maximum response between 10 and 100 nM which is sustained at higher concentrations.

HPLC chromatographic analysis of cell extracts showed that the NPY-LI co-eluted in the position of the porcine NPY standard at 45% acetonitrile (fig.3). Recovery from the columns exceeded 85% in all cases.

4. DISCUSSION

Cells of the neuroblastoma or neuronal somatic hybrid clones have been shown to contain high concentrations of NPY-LI. Although only recently discovered, this mammalian neuropeptide has been identified in numerous anatomical sites, but is confined to cells derived from the neural crest. Its role within the central nervous system remains obscure, but peripherally NPY possesses potent vasoconstrictor properties [15,16]. NPY is located in noradrenergic neurones [17,18], and electrical field stimulation of mouse vas deferens has suggested that NPY, in common with the enkephalins, may modulate noradrenaline release [19]. It is of interest therefore to have identified high concentrations of NPY in a cholinergic cell line (NG108-15). This is the first report of NPY localization in cells expressing features of differentiated cholinergic nerves.

The cellular concentrations of NPY were found to be increased on exposure of the NG108-15 cells to dexamethasone or nerve growth factor. In these experiments cell division had been arrested by culture for 24 h in cytosine arabinoside. In many neuroblastoma and hybrid cell lines, cellular differentiation may be initiated by NGF, elevation of cyclic-AMP or other stimuli. The expression of more highly differentiated neuronal functions in the cultured cells, e.g., synapse formation with addition of dibutyryl cyclic AMP to NG108-15 cells [2], is accompanied by a significant reduction in the rate of cell division. To eliminate from these experiments the non-specific effects of arrested cell division on the synthesis of NPY, all manipulations were performed on a population of non-dividing cells. Over 6 days, both agents resulted in a doubling of NPY concentration. Both agents had profound morphological effects upon these cells; in particular nerve growth factor accelerated the formation of neurite outgrowths. Continued exposure of the cells to dexamethasone and nerve growth factor resulted in an increase in NPY concentrations that was more than additive indicating synergism between these two agents.

The changes in cellular concentration of NPY appear to accompany the increased neuronal differentiation in these cells. Dexamethasone alone has been shown to increase concentrations of the two opiate peptides; methionine- and leucine-enkephalin [20]. In contrast to this study the response was rapid, occurring within the first 24 h of exposure to dexamethasone. However, in both studies the question has not been resolved whether the increase in concentration reflects more rapid synthesis (transcriptional or post transcriptional) or decreased degradation. In the case of the enkephalins, however, the ratio of Met to Leu enkephalin was preserved suggesting induction of the common precursor.

In conclusion, the presence of a biologically active peptide (NPY) within several neuronal cell clones has been demonstrated. There is good reason to suppose that these cells will provide a useful system for the further examination of the synthesis of NPY and related peptides from their precursor molecules.

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