Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 65 (2003) 1507–1513
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Role of nerve growth factor in the regulation of parotid cell differentiation induced by rat serum

Takashi Takeuchi[1], John M. Aletta, Suzanne G. Laychock, Yingrao Tian, Ronald P. Rubin[*]

Department of Pharmacology & Toxicology, School of Medicine & Biomedical Sciences, State University of New York at Buffalo, 102 Farber Hall, Buffalo, NY 14214, USA

Received 9 April 2002; accepted 30 September 2002

Abstract

The present study was undertaken to examine the factors that regulate rat serum (RS)- and nerve growth factor (NGF)-induced differentiation in a rat parotid acinar cell line. RS elicited extracellular signal-regulated kinase (ERK1/ERK2) activation within 5 min, while cyclic AMP (cAMP) levels transiently rose after 6 hr. RS also elicited a rise in amylase mRNA levels within 30 min, which preceded the rise in amylase protein levels. A possible role for NGF was suggested by the findings that parotid cells express both TrkA and p75 receptors. The immunoreactivity of these NGF receptors was reduced during exposure to RS. Following prolonged incubation in RS when ERK activity subsided to near basal levels, NGF restored ERK1/ERK2 activity to the elevated level initially observed in RS. NGF was ineffective when cells were incubated in fetal bovine serum. NGF, when incubated in combination with the cAMP-generating neuropeptides, calcitonin gene-related peptide and vasoactive intestinal peptide, markedly enhanced the cellular amylase content produced by RS. We conclude that parotid cell differentiation arises from an activation of cell surface receptors by humoral factors in combination with NGF and cAMP-generating neuropeptides.

Keywords: Nerve growth factor; Parotid acinar cells; Trk receptors; Calcitonin gene-related peptide; Vasoactive intestinal peptide; cAMP; MAP kinase

1. Introduction

Differentiation is a complex, integrated biological process involving a number of different mediators. The parasympathetic and sympathetic nerves of the autonomic nervous system are generally considered to be key regulatory factors in salivary gland development (see Ref. [1]). The parotid gland of the rat also receives nerve fibers containing the neuropeptides CGRP and VIP [2]. These cAMP-generating neuropeptides have been implicated in salivary secretion [3–5] and in non-adrenergic, non-cholinergic salivary secretion in response to parasympathetic nerve stimulation [6]. Furthermore, VIP exerts general trophic effects in rat parotid glands [7], and, like other neuropeptides, induces neuronal differentiation in cell lines [8,9].

To create an experimental model for long-term studies on differentiation, proliferation, and functional maturation, we developed a clonal rat parotid acinar cell line (3–9 cells) [10]. Using biochemical, immunological, and morphological markers, we found that parotid cells cultured in medium containing 10% RS undergo morphological and biochemical changes resulting in differentiation to a parotid acinar cell-like phenotype. In response to RS, parotid cell cultures divide more slowly, acquire secretory organelles, and express parotid-specific proteins in greater abundance, particularly secretory proteins. Moreover, exposure to RS unmasked the ability of receptor agonists to elicit a rise in cytoplasmic Ca^{2+} and promote amylase secretion [10]. The ability of RS to promote a more differentiated phenotype in transformed parotid cells suggests that certain components of RS regulate the expression of specific gene products that are involved in differentiation.

[*] Corresponding author. Tel.: +1-716-829-3048; fax: +1-716-829-2801.
E-mail address: rprubin@acsu.buffalo.edu (R.P. Rubin).
[1] Present address: Department of Veterinary Physiology, Faculty of Agriculture, Tottori University, Tottori 680-0945, Japan.

Abbreviations: cAMP, cyclic AMP; CGRP, calcitonin gene-related peptide; ERK, extracellular signal-regulated protein kinase; FBS, fetal bovine serum; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; PVDF, polyvinylidene fluoride; RS, rat serum; and VIP, vasoactive intestinal peptide.

0006-2952/03/$ – see front matter © 2003 Elsevier Science Inc. All rights reserved.
doi:10.1016/S0006-2952(03)00116-3

Murine salivary glands also synthesize large quantities of NGF [11,12], which is a member of a family of growth factors (neurotrophins) important for the differentiation and survival of neuronal and non-neuronal cells [11,13]. NGF binds to two receptors, a high-affinity site with tyrosine kinase activity ($p140^{trk}$), also known as TrkA, and $p75^{NGFR}$ (p75), which possesses a lower affinity for NGF [14]. Activation of TrkA by NGF triggers a signaling cascade that includes the stimulation of MEK (MAPK or ERK) kinase [15,16]. Although differentiative responses are generally attributed to TrkA receptors, p75 receptors are likely to interact, either physically and/or functionally, to transduce widely divergent NGF signals [17].

NGF has potentially important effects on the rat parotid gland. For example, the administration of NGF *in vivo* induces a proliferative effect on the rat parotid gland that is equivalent to that elicited by the β-adrenergic agonist isoproterenol and is mediated mainly through the autonomic nervous system [18–20]. Still, there has been a lack of specific information concerning the presence of NGF receptors on parotid acinar cells and the nature of their role in cell differentiation. In addition, since cAMP enhances NGF-induced neurite formation and can convert EGF from a proliferative factor to a differentiating one [21–23], cAMP may be implicated in the action of NGF on parotid cells.

Differentiation of parotid acinar cells has been studied only sporadically owing to the lack of convenient models. In the present investigation, we used RS and NGF to promote the differentiation of parotid (3–9) cells as assessed by the expression of the phenotypic marker amylase at the mRNA and protein levels. We demonstrated that RS-induced activation of MAPK is an early step in the integrative processes associated with parotid cell differentiation, while MAPK is a later target for NGF action. The experiments also provide evidence of temporally disparate actions of NGF- and cAMP-dependent signaling pathways on parotid cell differentiation.

2. Materials and methods

2.1. Preparation of the parotid cell line

Acinar cells were prepared from male Sprague–Dawley rat parotid glands by a previously described procedure using trypsin and collagenase digestion [24]. A parotid acinar cell line was then prepared as described previously in detail [10]. In brief, primary cultures of parotid cells were grown for 3 days at 35° before transfection using pSV-neo plasmid vectors containing SV40 promoter and large T antigen. Two neomycin-resistant genes were used to immortalize the cells by DNA transfection using lipofectamine. Although three clones were isolated, the 3–9 clone became the focus of our studies because of its more differentiated phenotype. The optimum temperature for the cell culture, which was generally maintained in FBS, was 33°. Substances including NGF (prepared from salivary glands as previously described [25]), VIP (Calbiochem), and CGRP (Sigma) were added directly to the culture medium as 100–1000× stock solutions in physiological salt solution or DMSO. The DMSO concentration never exceeded 0.1% (v/v) in the culture medium and was ineffective by itself in altering the parameters under study. To avoid cell density-dependent responses to RS, NGF, or neuropeptides, all experiments were initiated by plating cells at a density of 750,000/100-mm culture dishes. All other cell culture conditions were similar to those described by Zhu *et al*. [10], which were consistent with the induction of a differentiated phenotype.

2.2. Amylase assay

For the measurement of amylase, an aliquot (450 μL) derived from approximately 10^5 parotid cells was treated with 0.2% Triton X-100 and analyzed for total cell content of amylase. For measurement of amylase release, cells were centrifuged (12,000 *g* for 10 min at room temperature) through Nyosil oil in a microfuge, and the supernatant was analyzed for α-amylase activity [10]. Amylase activity was defined as micrograms of maltose formed/milligram protein/unit time. Results for amylase release were converted to relative stimulation (% total tissue content) after subtraction of basal activity.

2.3. Western blot analysis for Trk proteins

For Western blotting of NGF receptors (anti-TrkA and anti-$p75^{NGFR}$) in 3–9 cells, 75 μg protein of total cell extract was loaded on 7.5 and 10% gels for the high- and low-affinity receptors, respectively. The proteins were transferred to PVDF membranes and blocked for 60 min with 5% dry milk protein in PBS. The blots were incubated overnight with anti-TrkA (Santa Cruz) (1:1000) or anti-p75 (Chemicon) (1:1000), which is a well characterized mouse monoclonal antibody [26]. After incubation with a secondary antibody (anti-rabbit IgG), immunoreactivity was detected by chemiluminescence (ECL, Pierce) and quantitated by densitometric scanning using Molecular Analyst software.

2.4. Measurement of ERK1 and -2 activity

Extracts were obtained from 3–9 cells that were grown in either 10% FBS for 3 days or 10% RS for various time periods. Equal amounts of protein (100 μg) were subjected to western blot analysis using polyclonal antibodies for active, phosphorylated ERK (1:2500) (Promega) and total ERK enzyme (1:2000) (Santa Cruz). For these studies, a ^{125}I-anti-rabbit detection system based on X-ray film autoradiography was used to measure activated, phosphorylated ERK and total ERK expression.

The quantitative scale of this detection system was calibrated with serially diluted radiolabeled protein and was linear over an 18-fold range.

2.5. Measurement of amylase gene expression

Amylase gene expression was measured by the RNase protection assay. pcXP101, a full-length amylase cDNA clone, was prepared by fusing pcXP38 and pcXP101 (provided by Dr. Raymond MacDonald of Southwestern Medical Center). A 381-bp fragment of the amylase cDNA was obtained by *Eco*RI digestion of the clone. The fragment was subcloned into a pBSK (+/−) plasmid (Stratagene) for making the cRNA probe. *In vitro* transcription using T3 RNA polymerase (Ambion) yielded an antisense cRNA probe that protects a 381-bp amylase fragment. Total RNA was prepared using TRIzol reagent (GIBCO-BRL) according to the instructions of the manufacturer. Total RNA (10 μg) was subjected to overnight hybridization with [^{32}P]UTP antisense probes. Following RNase A and T_1 treatment to digest the unhybridized RNA and cRNA probe, the protected double-stranded RNA fragments were heat-denatured at 95° and resolved on a gel composed of 5% acrylamide/8 M urea in a mixture of 100 mM Tris, 100 mM borate, and 2 mM EDTA. After separation of the protected bands, they were visualized by autoradiography and quantified using a Phosphoimage Scanner. β-Actin mRNA was used as an internal control for normalizing the amount of sample loading. For this purpose, a rat β-actin riboprobe (giving one protected band of 126 bases) was obtained from Ambion and labeled in a similar fashion to the amylase riboprobe. Total RNAs from each sample were hybridized overnight at 45° with 2 fmol of labeled amylase antisense RNAs and 5 fmol of labeled β-actin antisense RNAs.

2.6. Measurement of cAMP levels

Cells were cultured for 1 hr to 4 days in the presence of RS. One hour before the experiment was terminated, 3-isobutyl-1-methylxanthine (IBMX) (0.1 mM) was added. The reaction was stopped by the addition of 100 μL HCl (1 N) to 1 mL of culture medium, and the samples were frozen at −70°. The samples were subsequently thawed and microcentrifuged (1600 *g* for 30 min at 4°), and 50-μL aliquots were neutralized with 25 μL sodium acetate (250 mM) and diluted to 250 μL with deionized H_2O. Samples and cAMP standards were acetylated and then assayed by radioimmunoassay as described previously [27].

3. Results

3.1. Identification of high- and low-affinity Trk receptors

To investigate the effects of NGF on acinar cells, we first determined whether neurotrophin receptors are expressed by our parotid cell model. Indeed, Fig. 1 shows that parotid cells express both TrkA (high affinity) and p75 (low affinity) receptors. The mobility patterns of the high and low molecular weight forms of the proteins that cross-reacted with anti-TrkA and -p75 antibodies were comparable to those detected in PC12 cells, a neuronal model of NGF action [14,28] (Fig. 1). The immunoreactivity for p75 receptors in cells incubated in FBS was much more intense than that for TrkA receptors (Fig. 1).

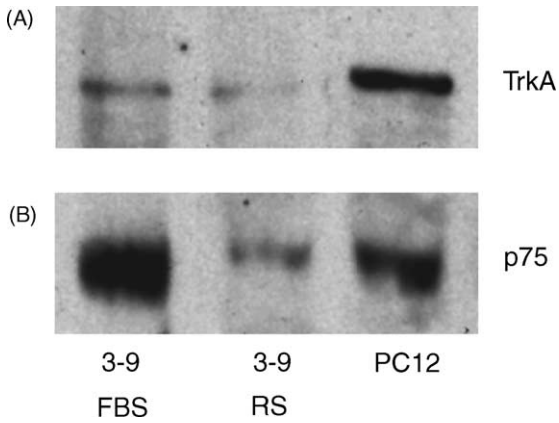

Fig. 1. Western blot analysis of TrkA and p75 receptor expression in parotid cells. Extracts obtained from parotid (3–9) cell cultures grown in either 10% FBS or 10% RS and PC12 (neuronal) cells grown in RPMI-1640 were analyzed by SDS–PAGE and immunoblotting. In both experiments, rabbit polyclonal antibodies (1:1000) against (A) the TrkA receptor (140 kDa) or (B) the p75 receptor (75 kDa) were incubated at 4° overnight, and a secondary antibody (anti-rabbit IgG, 1:1000) was incubated for 1 hr at room temperature. In (A), the PVDF membranes were blocked with 14% milk, and a 150-μg aliquot of protein was applied to a 7.5% SDS–polyacrylamide gel in lane designated 3-9 FBS and lane designated 3-9 RS and 75 μg protein was loaded in lane designated PC12. In (B), the PVDF membranes were blocked with 8% milk, and cell extracts were analyzed using a 10% gel by loading equal amounts of protein in each lane (25 μg/lane). The protein bands representing both NGF receptors expressed in parotid cells migrated with mobilities similar to those expressed in PC12 cells. Data are representative of three separate experiments.

Prolonged exposure of cells to RS produced a decrease in TrkA and p75 immunoreactivity, with immunoreactive p75 displaying a greater loss of intensity than TrkA (Fig. 1).

3.2. Functional activity of NGF receptors

In certain cells, sustained activation of MAPK is not only required but is sufficient to induce differentiation [13,15]. We conducted further experiments to assess a possible role for the MAPK pathway in 3–9 cell differentiation. ERK1/ERK2 activation was monitored by western blot analysis using a phospho-specific ERK antibody that only recognizes activated, phosphorylated ERK. As shown in Fig. 2A, cells incubated in RS elicited a time-dependent ERK activation. The activation was relatively rapid and transient, peaking at 30–60 min (Fig. 2A, upper panel), and decreasing by 3 days to low levels that were comparable to those detected in FBS-treated cells (Fig. 2B, upper panel). Blotting for total ERK protein

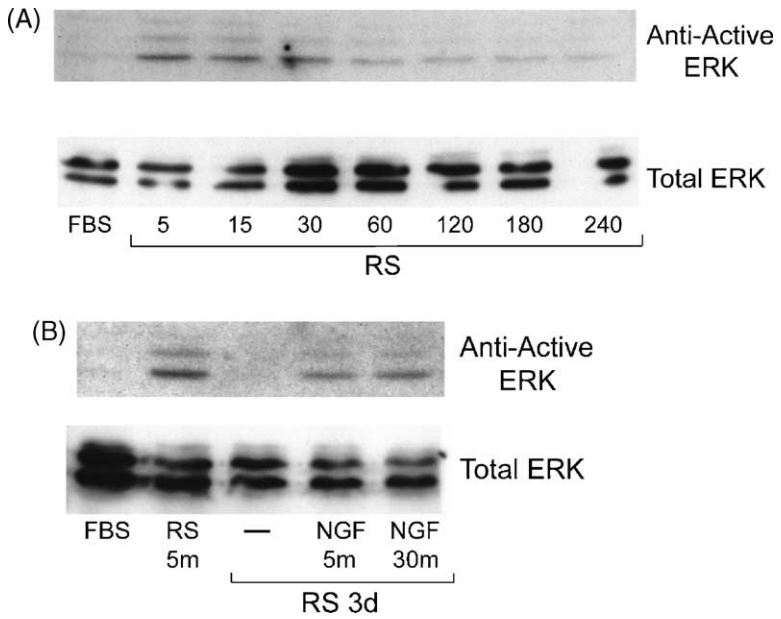

Fig. 2. Activation of ERK1 and -2 by rat serum and NGF. In (A), extracts obtained from parotid cells were grown in either 10% FBS for 3 days or 10% RS for 5 min to 4 hr at 37°. Equal amounts of protein (100 μg) were subjected to SDS–PAGE and blotted for active, phosphorylated ERK (upper panel). In (B), extracts were incubated in FBS and then transferred to RS for 5 min to 3 days. NGF (10 ng/mL) was added after 3 days in RS. Equal amounts of protein were subjected to SDS–PAGE and blotted for phosphorylated ERK (upper panel). Western blotting with an antibody against total ERK protein is shown in the lower panels of (A) and (B) to document equivalent loading. Data depicted in (A) and (B) are representative of three or more separate experiments.

showed similar sample loading for each time point (Fig. 2A and B, lower panel).

Binding of neurotrophins to TrkA receptors leads to the recruitment of adaptor proteins that couple the receptors to intracellular signaling cascades, including the Ras/ERK pathway. This pathway is essential for differentiation of PC12 cells, neurons, and diverse cell types [16,29]. Hence, the ability of NGF to modify this signaling pathway was investigated in parotid cells. After prolonged RS treatment (3 days), when ERK activity had fallen to levels that were comparably low to those observed in FBS (cf. Fig. 2A), the subsequent addition of NGF for 5–30 min restored ERK activation to a level that was observed during the initial period of RS incubation (Fig. 2B, upper panel). Blotting for total ERK protein showed equivalent sample loading for each treatment (Fig. 2B, lower panel). Prior to RS-induced differentiation, NGF was incapable of activating ERKs (data not shown).

To provide additional evidence that the NGF receptors on differentiated parotid cells are functional, the effect of NGF on amylase release (measured enzymatically) was investigated. After basal secretion was subtracted, a 30-min exposure to 10 ng/mL of NGF stimulated amylase output relative to non-stimulated cells incubated in RS by an average of 48% in two separate experiments (54 and 42%). By comparison, the potent secretagogue isoproterenol, at a maximal stimulatory concentration (10 μM), augmented secretion in two experiments by an average of 124% (138 and 109%). Parotid cells cultured in FBS failed to display NGF-stimulated amylase release (data not shown).

3.3. Measurement of mRNA levels in differentiating parotid cells

To establish that changes in amylase protein levels during exposure to RS were a response to events at the transcriptional or translational level, amylase gene expression was monitored in 3–9 cells incubated in FBS or RS using the RNase protection assay (see "Section 2"). Figure 3 reveals that cells incubated in FBS exhibited a low level of amylase mRNA that increased within 30 min following

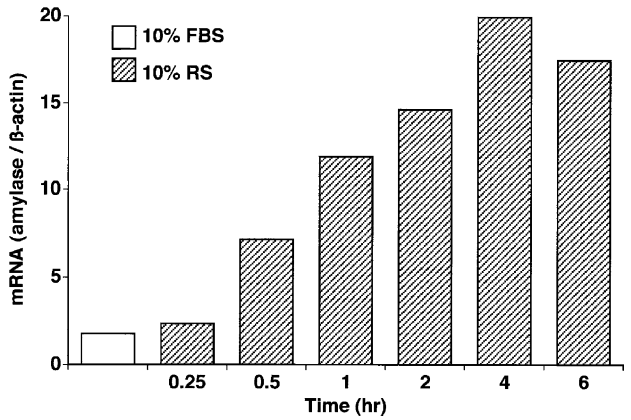

Fig. 3. Time dependence of the effect of RS on amylase gene expression during RS-induced differentiation. Totals RNAs (10 μg/lane) from parotid cells were subjected to the RNase protection assay by being hybridized to the ^{32}P-labeled amylase probe or the β-actin cRNA probe as described in "Section 2." The protected amylase fragment was quantitated by densitometry and normalized to β-actin message. Although they are not shown, actin levels were invariant. The figure shows the results taken from one of three separate experiments.

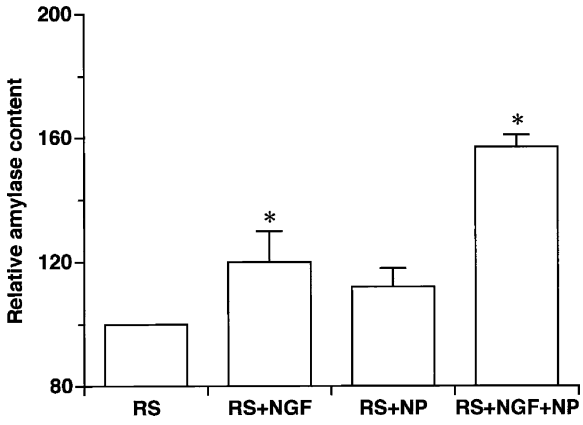

Fig. 4. Stimulatory effect of the neuropeptides CGRP and VIP on cell amylase content. Amylase activity was measured in parotid cells grown for 4 days in 10% RS alone or together with the following additions: 10 ng/mL of NGF; 100 nM CGRP plus 10 nM VIP (NP); or NGF plus NP. Amylase activities are expressed as a percentage of the activity measured in cells incubated in RS alone. Results shown are means ± SEM of 4–5 independent experiments. Key: (*) $P < 0.05$ as compared to control values in RS alone (Student's unpaired *t*-test).

exposure of the cells to RS. Peak amylase mRNA levels were attained within 4 hr. These findings taken together with our previous work [10] demonstrate that RS induces an early rise in mRNA levels that precedes the elevation in protein levels.

3.4. Promotion of cellular amylase levels by neuropeptides

The fact that the rat parotid gland receives nerve fibers containing the cAMP-generating neuropeptides CGRP and VIP led us to investigate the contribution of neuropeptide signaling to parotid cell differentiation. CGRP and VIP elicited small and variable increases in cellular amylase levels (~10%). NGF alone induced a modest but significant (~20%) increase in cellular amylase content (Fig. 4). However, when CGRP and VIP were added together to cells incubated in RS plus NGF, a 57% increase in cellular amylase content relative to cells incubated in RS alone was observed (Fig. 4). These findings are reminiscent of those obtained in PC12 cells in which neuropeptides acting as cAMP agonists in combination with NGF induce a level of neurite outgrowth (differentiation) that greatly exceeds the effect of NGF or neuropeptide alone [23]. By contrast, cells incubated in RS plus a combination of CGRP/VIP in the absence of NGF elicited a smaller and more variable increase in cellular amylase levels (11%) (Fig. 4). The selectivity of CGRP/VIP action was demonstrated by the inability of substance P, in combination with either CGRP or VIP, to mimic the synergistic effect with NGF (data not shown). Thus, the combined presence of NGF and cAMP appears to regulate the developmental expression of parotid acinar cells.

To determine whether cAMP plays a role in RS-induced differentiation, we measured cAMP levels following exposure of parotid cells to RS. Following incubation in FBS, cells treated with RS exhibited a 25-fold rise in cAMP levels (from 8 to 200 fmol/μg protein) after 6 hr, which subsequently returned to basal levels by 24 hr (mean from two independent experiments). These low levels were maintained during the remainder of the 4-day incubation period.

4. Discussion

The present work extends our previous analysis of RS-induced differentiation of parotid cells to include more rapid cellular responses than those documented previously [10]. Because of its relative abundance and prominent functional role in salivary acinar cells, cell amylase levels serve as a specific marker of differentiation [30]. Thus, we have established that changes in amylase protein levels during exposure to RS are a response to changes in parotid amylase gene expression by demonstrating a rapid rise in amylase RNA levels (within 30 min). RS also produced a rapid stimulation of ERK activation (within 5 min), suggesting that this signaling pathway represents a key element in regulating parotid cell development (see Fig. 2). The fact that the rapid rise in ERK activity is followed by a time-dependent increase in amylase message provides support for the idea that the MAPK pathway is involved in parotid cell differentiation.

Although NGF is a key factor in neuronal cell differentiation, targets of NGF include cells of non-neuronal origin, such as pancreatic β cells, ovary cells, and pituitary cells [31,32]. Since the physiological significance of NGF in salivary glands is not well established, one of the goals of this study was to gain knowledge of the functional role of NGF on parotid cells. While there is abundant evidence that mouse submandibular and submaxillary salivary glands store and excrete large quantities of NGF [11,12], to our knowledge there is no documented evidence for the localization of TrkA or p75 receptors in rat parotid gland. Potential insight into this problem was established by our findings that undifferentiated parotid acinar cells (incubated in FBS) express both TrkA and p75 protein. Since TrkA and p75 receptors are key elements required for the transmission of NGF signals, our findings strongly implicate these receptors as effectors of NGF actions in parotid cells. The relatively high levels of p75 protein compared to TrkA protein detected in non-differentiated cells and the differential decrease in p75 expression observed during differentiation prompt speculation that during exposure to RS the loss of p75 activity facilitates the generation of a differentiation signal through the TrkA receptor pathway. In contrast to p75, RS-induced parotid cell differentiation is generally associated with an increase in the levels of specific proteins, including amylase and proline-rich protein (PRP) [10].

The cooperative interaction of TrkA and p75 receptors has also been proposed in other cell types to modulate

certain neurotrophin responses, including cell differentiation and survival [33–35].

Our finding that NGF is capable of activating ERK in parotid cells coincides with other work demonstrating that NGF triggers a signaling cascade resulting in the activation of the ERK pathway in neuronal cells [15,16,29]. Whether the action of NGF to activate the ERK pathway leads to parotid cell development will require further study. In addition to demonstrating that components in RS enable NGF to express its stimulatory action on ERK, the results of the present study also show that NGF is able to synergize with neuropeptides in promoting amylase content. Furthermore, the fact that NGF was able to modestly stimulate amylase release from parotid cells treated with RS, but not with FBS, provides further support for the notion that parotid acinar cells are NGF sensitive.

The diverse effects of NGF on parotid cells treated with RS to promote the development of a more differentiated phenotype and enhance amylase secretion as reported herein are in accord with the ability of transient versus prolonged stimulation by NGF to produce diverse effects on a given cell [29]. However, it should be noted that since NGF is unable to augment amylase content in parotid cells incubated with FBS (data not shown), it is not responsible for RS-induced parotid cell differentiation. This supposition is supported by the following pieces of evidence: (a) the concentration of NGF in 10% RS (<0.5 ng/mL), relative to the concentrations used in our studies (10 ng/mL), is much too low to be biologically active [36]; and (b) RS is unable to duplicate the effects of NGF on the differentiation of sympathetic neurons [37].

The additional finding of an early rise in cAMP levels (within 6 hr) during RS-induced cell differentiation also suggests a role for cAMP in parotid cell development. Additionally, the fact that NGF enhanced the responses of the cAMP-generating neuropeptides VIP and CGRP in augmenting cellular amylase content reveals that the combined presence of NGF and cAMP regulates the developmental expression of parotid acinar cells. The original concept that NGFs and neurotransmitters function in concert with neuropeptides was first proposed more than a decade ago [38]. This concept was predicated upon the knowledge that autonomic and sensory nerves innervating the parotid gland contain several neuropeptides that could serve complementary roles in acinar cell development and functional maturation.

The interaction between NGF and the cAMP-generating neuropeptides as demonstrated in this study is reminiscent of the synergism exhibited in PC12 cells by a combination of NGF and agents that elevate cAMP levels to promote neuronal differentiation [8,9,21]. However, while NGF can induce a rise in cAMP levels, the cyclic nucleotide does not serve as a second messenger for NGF-induced differentiation in neuronal cells [23]. One target of signaling pathways mediated by NGF is the cAMP response element-binding protein CREB [39], which may be one possible site of the convergence of the NGF- and cAMP-mediated pathways in PC12 cells and perhaps in parotid cells as well.

Differentiation is likely expressed through integrated responses involving ERK and cAMP. However, results from different laboratories have shown that depending upon the cell type, cAMP can either activate or inhibit the ERK pathway [40,41]. Our findings that RS induced ERK1/ERK2 activation within 5 min, while a rise in cAMP was observed only after 6 hr, indicate that activation of the MAPK pathway is not dependent upon the cAMP-protein kinase A signaling pathway. However, the cAMP pathway may play an inhibitory role in a later phase of the differentiation process, with MAPK serving as a downstream target of the cAMP signaling pathway.

In conclusion, the present study has developed a new paradigm of cell differentiation by demonstrating that the NGF receptor system is expressed in parotid cells and its differentiative effects are enhanced by the physiologically relevant co-transmitters CGRP and VIP through receptor-specific signal transduction mechanisms. The fact that the effects of NGF and the neuropeptides are observed only in cells differentiating in response to RS and are not detectable when the cells are incubated in FBS indicates that components present in RS are obligatory for cell differentiation induced by neurotrophins and neuropeptides. While the parotid cell line utilized in this study may not duplicate all of the cellular events that take place *in vivo*, further experimentation utilizing this cell line will facilitate a greater understanding of the processes that control the development and functional maturation of parotid acinar cells.

References

[1] Virta E, Tornwall J, Uusitalo H. Substance P and neurokinin A immunoreactive nerve fibres in the developing salivary glands of the rat. Histochemistry 1992;98:317–25.
[2] Ekstrom J, Ekman R, Hakanson R, Luts A, Sundler F. Developmental studies on vasoactive intestinal peptide, substance P, and calcitonin gene-related peptide in salivary glands of postnatal rats. Acta Physiol Scand 1994;151:107–15.
[3] Scott J, Baum BJ. Involvement of cyclic AMP and calcium in exocrine protein secretion induced by vasoactive intestinal polypeptide in rat parotid cells. Biochim Biophys Acta 1985;847:255–62.
[4] Ekstrom J. Neuropeptides and secretion. J Dent Res 1987;86:524–30.
[5] Salo A, Virta E, Uusitalo H. Calcitonin gene-related peptide (CGRP) and its effects on protein release *in vitro* in the developing submandibular gland of the rat. Regul Pept 1995;55:155–65.
[6] Ekstrom J, Ekstrom PF. Acinar degranulation in the rat parotid gland induced by neuropeptides. Exp Physiol 2001;86:733–8.
[7] Mansson B, Nilson B-O, Ekstrom J. Effects of repeated infusions of substance P and vasoactive intestinal peptide on the weights of salivary glands subjected to atrophying influences in rats. Br J Pharmacol 1991;101:853–8.
[8] Kozlowski MR, Rosser MP, Hall E, Longden A. Effects of bradykinin on PC-12 cell differentiation. Peptides 1989;10:1121–6.
[9] Okumura N, Miyatake Y, Takao T, Tamaru T, Nagai K, Okada M, Nakagawa H. Vasoactive intestinal peptide induces differentiation and MAP kinase activation in PC12h cells. J Biochem (Tokyo) 1994;115:304–8.

[10] Zhu Y, Aletta JM, Wen J, Zhang X, Higgins D, Rubin RP. Rat serum induces a differentiated phenotype in a rat parotid acinar cell line. Am J Physiol 1998;275:G259–68.
[11] Levi-Montalcini R. The nerve growth factor: thirty-five years later. EMBO J 1987;6:1145–54.
[12] Shooter EM. Early days of the nerve growth factor proteins. Annu Rev Neurosci 2001;24:601–29.
[13] Davies AM. The neurotrophic hypothesis: where does it stand? Philos Trans R Soc Lond B Biol Sci 1996;351:389–94.
[14] Bothwell M. Functional interactions of neurotrophins and neurotrophin receptors. Annu Rev Neurosci 1995;18:223–53.
[15] Segal RA, Greenberg ME. Intracellular signaling pathways activated by neurotrophin factors. Annu Rev Neurosci 1996;19:463–89.
[16] Kaplan DR, Miller FD. Signal transduction by the neurotrophin receptor. Curr Opin Cell Biol 1997;9:213–21.
[17] Bibel M, Hoppe E, Barde Y-A. Biochemical and functional interactions between the neurotrophin receptors *trk* and $p75^{NTR}$. EMBO J 1999;18:616–22.
[18] Schneyer CA, Humphreys-Beher MG. Nerve growth factor-induced increase in [^{3}H]thymidine incorporation into parotid and submandibular glands of young rats and its partial blockade by propranolol or partial sialoadenectomy. Biochem Pharmacol 1990;39:1679–86.
[19] Schneyer CA, Hall DH. Autonomic regulation of the immature and adult rat parotid gland. In: Schneyer CA, Schneyer LH, editors. Secretory mechanisms of salivary glands. New York: Academic Press; 1967. p. 155–77.
[20] Schneyer CA, Humphreys-Beher M, Hall HD. Nerve growth factor effects on inhibition by submandibular gland ablation or autonomic denervation of activity-induced parotid growth. J Auton Nerv Syst 1992;37:137–44.
[21] Frodin M, Peraldi P, Van Obberghen E. Cyclic AMP activates the mitogen-activated protein kinase cascade in PC12 cells. J Biol Chem 1994;269:6207–14.
[22] Yao H, Labudda K, Rim K, Capodieci P, Loda M, Stork PJS. Cyclic adenosine monophosphate can convert epidermal growth factor into a differentiating factor in neuronal cells. J Biol Chem 1995;270: 20748–53.
[23] Gunning PW, Landreth GE, Bothwell MA, Shooter EM. Differential and synergistic actions of nerve growth factor and cyclic AMP in PC-12 cells. J Cell Biol 1981;89:240–5.
[24] Rubin RP, Adolf MA. Cyclic AMP regulation of calcium mobilization and amylase release from isolated permeabilized rat parotid cells. J Pharmacol Exp Ther 1984;268:600–6.
[25] Mobley WC, Schenker A, Shooter EM. Characterization and isolation of proteolytically modified nerve growth factor. Biochemistry 1976;15: 5543–51.
[26] Chandler CE, Parsons LM, Hosang M, Shooter EM. A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells. J Biol Chem 1984;259:6882–9.
[27] Laychock SG. Impaired cyclic AMP response to stimuli in glucose-desensitized rat pancreatic islets. Mol Cell Endocrinol 1995;113: 19–28.
[28] Kaplan DR, Stephens RM. Neutrophin signal transduction by the Trk receptor. J Neurobiol 1994;25:1404–17.
[29] Huang EJ, Reichardt LF. Neurotrophins: roles in neuronal development and function. Annu Rev Neurosci 2001;24:677–736.
[30] MacDonald RJ, Crerar MM, Swain WF, Pictet RL, Thomas G, Rutter WJ. Structure of a family of rat amylase genes. Nature 1980; 287:117–22.
[31] Kanaka-Gantenbein C, Tazi A, Czernichow P, Scharfman R. *In vitro* presence of the high affinity nerve growth factor receptor Trk-A in the rat pancreas: differential localization during pancreatic development. Endocrinology 1995;136:761–9.
[32] Rosenbaum T, Vidaltamayo R, Sanchez-Soto MC, Zentella A, Hiriart M. Pancreatic β cells synthesize and secrete nerve growth factor. Proc Natl Acad Sci USA 1998;95:7784–8.
[33] Maliartchouk S, Saragovi HU. Optimal nerve growth factor trophic signals mediated by synergy of Trk A and p75 receptor-specific ligands. J Neurosci 1997;17:6031–7.
[34] Yoon SO, Casaccia-Bonnefil P, Carter B, Chao MV. Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival. J Neurosci 1998;18:3273–81.
[35] Powers JF, Shahsavari M, Tsokas P, Tischler AS. Nerve growth factor receptor signaling in proliferation of normal adult rat chromaffin cells. Cell Tissue Res 1999;295:21–32.
[36] Korsching S, Thoenen H. Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: correlation with density of sympathetic innervation. Proc Natl Acad Sci USA 1983;80:3513–6.
[37] Chun LLY, Patterson PH. Role of nerve growth factor in the development of rat sympathetic neurons in vitro. II. Developmental studies. J Cell Biol 1977;75:705–11.
[38] Hokfelt T. Neuropeptides in perspective: the last ten years. Neuron 1991;7:867–9.
[39] Ginty DD, Bonni A, Greenberg ME. Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-*fos* transcription via phosphorylation of CREB. Cell 1994;77:713–25.
[40] Houslay MD, Kolch W. Cell-type specific integration of cross-talk between extracellular signal-regulated kinase and cAMP signaling. Mol Pharmacol 2000;58:659–68.
[41] Qiu W, Zhuang S, von Lintig FC, Boss GR, Pilz RB. Cell type-specific regulation of B-Raf kinase by cAMP and 14-3-3 proteins. J Biol Chem 2000;275:31921–9.