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# DNA SYNTHESIS INDUCED BY THE NEUROPEPTIDE SUBSTANCE K CORRELATES TO THE LEVEL OF MYC-GENE TRANSCRIPTS

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SUMMARY: Neuropeptides have recently been implicated in regulation of normal and neoplastic cell growth. Substance K is a neurotransmittor candidate that has been identified as a mitogen for smooth muscle cells and fibroblasts. However, the ability to respond to stimulation with substance K declines rapidly in cells serum-starved for more than 24 h and in parallel with a decrease in the intracellular level of myc-gene transcripts. Contrarily, myc-transformed cells, that inspite of a decrease demonstrated a high level of myc mRNA after 48 h in serum-free medium, maintained their ability to initiate DNA synthesis when stimulated with substance K. The results suggest that the intracellular signal of substance K-induced DNA synthesis interacts with the myc protein. 

The identification of the viral oncogenes and their cellular homologs, the protooncogenes, have increased our understanding not only of the mechanism behind neoplastic tranformation but also of the regulation of normal cell proliferation and differentiation. Mitogenic stimulation of quiescent fibroblasts induces a rapid increase in c-myc mRNA concentration. The exact function of the c-myc protein in the cell remains to be elucidated but several lines of evidence indicate that it is a prerequisite for entrance into a replicating state (1,2). Amplification of the myc gene has also been observed in several types of tumor cells (3). Agonist-induced breakdown of phosphatidylinositol 4,5-bisphosphate into inositol trisphosphate and diacylglycerol represents another early cellular response to mitogenic stimulation (4,5). Inositol trisphosphate and diacylglycerol are believed

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ABBREVIATIONS: SMC, smooth muscle cells; NQ, normal quil fibroblasts; PDGF, platelet-derived growth factor.

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to function as intracellular second messengers through their ability to regulate mobilization of calcium from intracellular stores and to activate protein kinase C (4,5). Hydrolysis of phosphatidylinositol 4,5-bisphosphate have also been found to occur in response to tachykinins (6-8). We recently reported that the tachykinins substance K and substance P stimulate initiation of DNA synthesis in smooth muscle cells and fibroblasts (9). Substance P and substance K are neuropeptide transmitter candidates derived from a common precursor molecule (10). Substance P has a widespread distribution in the nervous system (11) including a population of primary sensory neurons which also contain substance K (12). We describe here that the ability of smooth muscle cells to respond to stimulation with substance K under serum-free conditions is restricted to a limited period of time after the removal of serum. To elucidate if this diminished responsiveness was due to a decrease in the intracellular myc level, experiments using myc-transformed quail embryo fibroblasts were performed.

## MATERIALS AND METHODS

Smooth muscle cells (SMC) were enzymatically isolated from the aortic media of Sprague-Dawley rats (13). Normal quail fibroblasts (NQ; 14) and quail fibroblats transformed with v-myc containing OK 10 virus (9C; 15) were kindly supplied by Dr K. Moelling and Dr K. Alitalo, respectively. Subconfluent cultures grown on glass cover slips were serum-starved in medium MCDB 104 (Gibco) for the indicated time periods and transferred to medium MCDB104/0.1% bovine serum albumin containing 1.0 µCi/ml of $^{3}$H-thymidine (Amersham) with or without addition of $10^{-7}$M substance K (Peninsula) or 25 ng/ml of PDGF (kindly provided by Dr C.-H. Heldin) for 24h. The cells were then fixed in 3% buffered glutaraldehyde and processed for autoradiography as described elsewhere (13). The fraction of labeled nuclei was determined by counting at least 250 randomly selected cells in each specimen. The abundance of v-myc and c-myc transcripts in 9C and NQ cells were determined by northern blot analysis. 40 µg of total oRNA or 5 µg of poly(A)$^{+}$-selected RNA isolated from exponentially proliferating or serum-starved cultures was separated by formaldehyde agarose electrophoresis (1.5%), transferred to nitrocellulose and hybridized with c-myc probe (pM c-myc 54; 16) as described elsewhere (17). Sizes of transcripts were determined relative to mouse 18 S and 28 S RNA markers. Subsequent wash and rehybridization with an actin probe confirmed that approximately equal amounts of RNA was analyzed.

## RESULTS AND DISCUSSION

Arterial smooth mucsle cells (SMC) serum-starved for 48h readily start to synthesize new DNA in response to serum or platelet-derived growth factor

(PDGF). 71.8% (s.d. 3.8) of the cells given 10% serum and 47.6% (s.d. 1.0) given 25 ng/ml of PDGF were labelled with $^{3}$H-thymidine, as determined by autoradiography, after incubation for 24h. In cells given serum-free medium (MCDB 104) without addition only 12.5 % (s.d. 2.3) of the cells were labelled. However, the ability of SMC given medium MCDB 104 to initiate DNA synthesis is dependent on the length of the previous serum starvation period (Fig. 1). The finding that a limited fraction of the cells continue to synthesize DNA after 48h in serum-free medium is probably due to a small endogenous production of PDGF like molecules in the SMC (18). The extent of previous serum starvation also determines the ability of SMC to respond to the tachykinin substance K. If SMC are directly transferred to medium MCDB 104 without prior serum-starvation the rate of DNA synthesis is continuously high and no further stimulation is obtained by addition of substance K. An increased ability of substance K to stimulate DNA synthe-

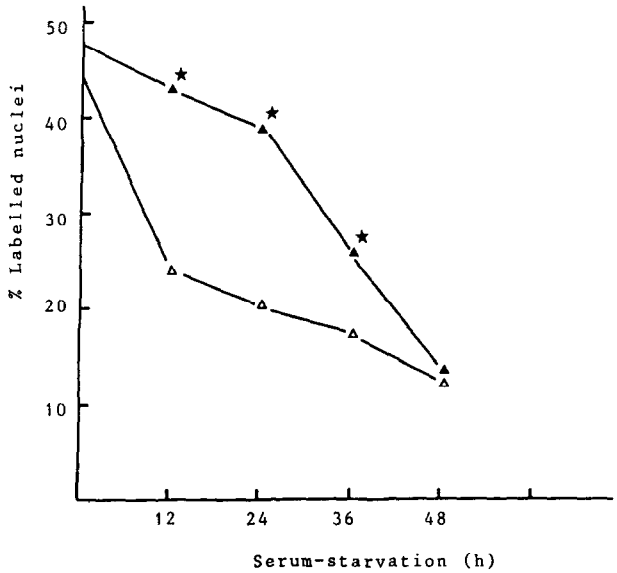


Fig. 1 Effect of the length of the serum-starvation period on initiation of DNA synthesis in unstimulated or substance K-stimulated cultures of SMC. Subconfluent cultures of SMC were serum-starved in medium MCDB 104 (Gibco) for the indicated time periods and transferred to medium MCDB 104/0.1% bovine serum albumin containing 1.0 µCi/ml of $^{3}$H-thymidine (Amersham) with (closed triangles) or without (open triangles) addition of $10^{-7}$M substance K (Peninsula) for 24h. The cells were fixed and processed for autoradiography as described in materials and methods. Values are given as means of triplicate cultures (s.d.<20%). Asterisk indicates values which are statistically different ($p<0.05$) from the respective control value.

sis, as compared to untreated controls, is observed in cells serum-starved for 12-36h (Fig.1). However, in SMC serum-starved for 48h, substance K lacked effect on the initiation of DNA synthesis (Fig.1). These findings raise the possibility that the ability of substance K to stimulate DNA synthesis is dependent on an intracellular mediator. This putative substance seems to be present in continuously growing serum-treated cultures but to decrease below a critical level in serum-starved cultures. Similar kinetics have recently been described to be characteristic for the c-myc protein (19,20). To investigate a possible interaction between tachykinin receptor activation and myc expression, experiments were performed using quail fibroblasts transformed with v-myc containing OK 10 virus. Proliferating, serum-treated cultures of this cell line (9C) have considerably increased levels of myc mRNA (> 100-fold as judged by densitrometric scanning) as compared to normal quail fibroblasts (NQ; Fig. 2A). The levels of v-myc mRNA in serum-deprived 9C cells is decreased by 83% after 24 h and by 93% after 48 h as judged by densitometric scanning, but considerable

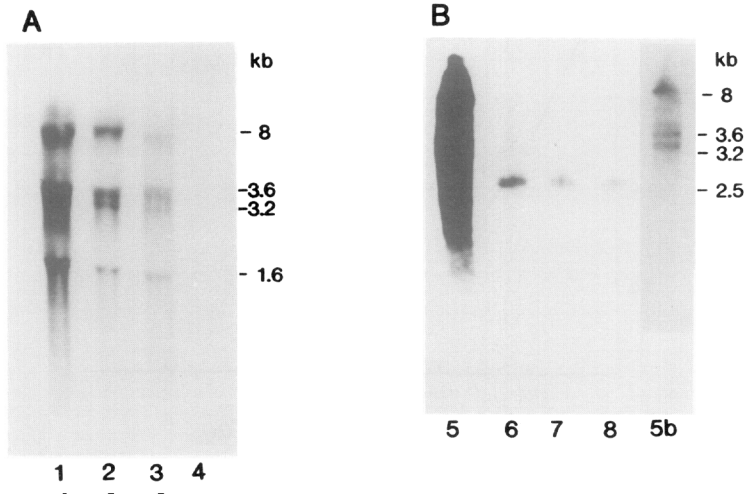


Fig. 2 Northern blot analysis of v-myc and c-myc transcripts in proliferating and serum-starved 9C and NQ cells. 40 µg of total RNA (A) or 5 µg of poly(A)$^+$-selected RNA (B) were isolated from exponentially proliferating or serum-starved cultures and hybridized with c-myc probe as described in materials and methods. The RNA analyzed was from proliferating 9C (lane 1), 9C cells serum-starved for 24 h (lane 2) or for 48 h (lanes 3, 5 and 5b), proliferating NQ cells (lanes 4 and 6), and NQ cells serum-starved for 24 h (lane 7) or 48 h (lane 8). Lane 5b represents a shorter exposure of the material represented in lane 5.

amounts of v-myc transcripts are still present in the cells (Fig. 2B). Furthermore, the v-myc level in 9C cells serum-starved for 48 h is still considerably higher than the level of c-myc in proliferating NQ cells (Fig. 2B). When NQ cells were deprived of serum the abundance of myc transcripts decreased by 55% after 24 h and by 75% after 48 h (Fig. 2B). In accordance with earlier findings on rat smooth muscle cells and human skin fibroblasts (9) substance K stimulates DNA synthesis in NQ cells, growth-arrested by transfer to serum-free medium for 24h, in a dose-dependent manner, whereas substance K lacks stimulatory effect on cells serum-starved for 48h (Fig.3). The rate of DNA synthesis in unstimulated, serum-starved 9C cells is higher than in NQ cells. In contrast to normal fibroblasts, myc-transformed cells respond with increased DNA synthesis when exposed to substance K also after 48h of serum-starvation, although a tendency to a decreased responsiveness was noted at the lowest concentration of SK (Fig.3). To investigate if the mitogenic effect of substance K could be blocked by specific interaction with tachykinin-receptors NQ and 9C cells were exposed to substance K with or without the SP-receptor antagonist spantide (21; Bachem). Spantide was found to completely remove the stimulatory effect of substance K on both NQ and 9C cells (Table 1). Spantide does not interfere with the mitogenic effect of PDGF on smooth muscle cells or human fibroblasts (9), indicating that the observed decrease in DNA synthesis is not due to an unspecific toxicity. Our results suggest that the intracellular signal of tachykinin-induced DNA synthesis interacts with the myc protein.

The nature of the intracellular signal that mediates tachykinin-induced DNA synthesis remains to be elucidated. In other cells tachykinins have been shown to induce breakdown of phosphoinositodes (6,7). Hence, it is possible that growth-stimulatory tachykinins operate via an increase in cytosolic calcium and/or an increase in calcium-calmodulin dependent phosphorylation. In this concept, it is interesting to note that PDGF-induced DNA synthesis is preceded both by a rapid breakdown of phosphainositides (22) and a 10-40 fold increase in c-myc mRNA concentration (1). On the

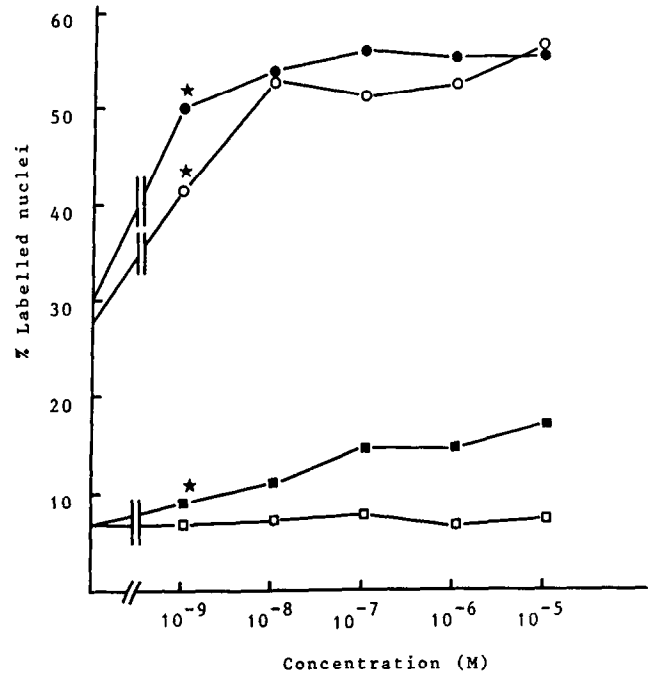


Fig. 3 Effect of substance K on initiation of DNA synthesis in normal quail fibroblasts (NQ) and myc-transfected quail fibroblasts (9C). Subconfluent cultures of NQ cells (cubes) and 9C cells (circles) were serum-starved for 24h (closed symbols) or 48h (open symbols) and transferred to medium MCDB 104/0.1% bovine serum albumin containing 1.0 μCi/ml of $^{3}$H-thymidine and different concentrations of substance K for 24h. The percent labeled nuclei were determined as described in Fig. 1. Each value represents the mean of nine determinations from three different experiments (s.d.<20%). Asterisk indicates the first value in each group which is significantly different ($p<0.01$) from the control.

Table 1: Effect of the substance P antagonist spantide on DNA synthesis induced by substance K (SK) in normal quail fibroblasts (NQ) and myc-transfected quail fibroblasts (9C)

| | Period of serum-starvation | | | |
|---|---|---|---|---|
| | 24h | | 48h | |
| | % labeled nuclei | s.d. | % labeled nuclei | s.d. |
| NQ cells | | | | |
| MCDB 104 | 5.4 | 2.0 | 6.9 | 2.0 |
| $10^{-7}$M SK | 14.9 | 2.9 | 8.3 | 3.6 |
| $10^{-7}$M SK/3x$10^{-5}$M spantide | 9.3 | 2.6 | 6.0 | 2.0 |
| 9C cells | | | | |
| MCDB 104 | 30.1 | 4.2 | 27.7 | 4.0 |
| $10^{-7}$M SK | 56.2 | 3.6 | 51.6 | 6.4 |
| $10^{-7}$ SK/3x$10^{-5}$M spantide | 26.8 | 3.7 | 24.6 | 1.6 |

The fraction of labeled nuclei was determined as described in Fig. 1 legend. Each value represents the mean of triplicate cultures.

other hand, 3T3 cells trancfected with myc remain quiescent in spite of a 40-fold increase of c-myc mRNA and Armelin et al. have reported that growth of normal 3T3 cells require both competence factors as PDGF and progression factors such as epidermal growth factor, whereas myc-transfected 3T3 cells synthesize DNA in response to epidermal growth factor alone (2). In accordance, platelet-poor plasma induce DNA synthesis in 3T3 cells into which the c-myc protein has been microinjected but not in controls (23). Since a higher expression of c-myc is characteristic for undifferentiated cells (17,19), a similar mechanism could be of importance for growth regulation during fetal development. It is tempting to speculate that tachykinins, such as substance P and substance K, released from pheripheral neurons (14) stimulate growth of undifferentiated non-neural cells during this period. Interestingly, it have been reported that the last days of fetal development in rodents are characterized by an enhanced c-myc expression (25) and a rapid increase in substance P immunoreactivity in sensory neurons (26). A similar mechanism may also be responsible for the nerve-dependent regeneration of amphibian leg in which the limb regenerates only when peripheral nerve fibers are present in connection with the blastema (27). The amplification of oncogenes is one of several changes that have been implicated with induction of cell growth and neoplastic transformation. In neuroblastomas, N-myc expression appears in stage III and IV (28,29) and several lines of evidence indicate that the amplification of N-myc is related to the development of the tumor (30). In this context it is interesting that neuroblastomas are of neuroectodermal origin and that some of these tumor cell lines may produce the tachykinin substance P (31). It is not known whether tachykinin production is of importance for the tumor cell growth, as recently reported for gastrin releasing peptide and small cell carcinoma of the lung (32).

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