

Bradykinin-induced growth inhibition of normal rat kidney (NRK) cells is paralleled by a decrease in epidermal-growth-factor receptor expression

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Normal rat kidney fibroblasts, grown to density arrest in the presence of epidermal growth factor (EGF), can be induced to undergo phenotypic transformation by treatment with transforming growth factor β or retinoic acid. Here we show that bradykinin blocks this growth-stimulus-induced loss of density-dependent growth arrest by a specific receptor-mediated mechanism. The effects of bradykinin are specific, and are not

mimicked by other phosphoinositide-mobilizing agents such as prostaglandin $F_{2\alpha}$. Northern-blot analysis and receptor-binding studies demonstrate that bradykinin also inhibits the retinoic acid-induced increase in EGF receptor levels in these cells. These studies provide additional evidence that EGF receptor levels modulate EGF-induced expression of the transformed phenotype in these cells.

INTRODUCTION

Normal rat kidney (NRK) fibroblasts have been widely used as a non-transformed indicator cell line for studying the role of polypeptide growth factors in phenotypic transformation (Van Zoelen, 1991). When cultured in the presence of epidermal growth factor (EGF) as the only growth-stimulating polypeptide, these cells have a normal phenotype and undergo density-dependent growth inhibition. When cultured in the additional presence of transforming growth factor β (TGF β), retinoic acid (RA) or platelet-derived growth factor (PDGF), however, cells acquire a transformed phenotype characterized by loss of density-dependent growth inhibition (Van Zoelen et al., 1988). These cells therefore form an attractive model system for studying the control mechanisms involved in density-dependent growth regulation, since they can first be grown to density arrest, from which they can be released by treatment with specific additional growth factors (Van Zoelen et al., 1992).

Exponentially growing NRK cells in sparse cultures have low EGF receptor levels already, but EGF binding is decreased even further at high cell densities (Rizzino et al., 1990). Neither TGF β nor RA by itself has a growth-stimulatory effect on NRK cells (Van Zoelen et al., 1988), but both are able to increase the number of EGF receptors that these cells express (Roberts et al., 1984; Assoian et al., 1984; Assoian, 1985) by transcriptional activation of the receptor gene (Thompson et al., 1988; Thompson and Rosner, 1989). These observations have led to the model whereby EGF receptor levels control density-dependent growth arrest of NRK cells cultured in the presence of EGF (Van Zoelen, 1991). When cell density increases, EGF receptor levels decrease to such a value that EGF-induced growth-stimulating signals are insufficient for induction of cell proliferation. Factors such as TGF β or RA enhance these growth-stimulating signals induced by EGF by increasing EGF receptor levels.

In this study we have investigated the effects of the nonapeptide bradykinin on phenotypic transformation of NRK cells. Bradykinin is a regulatory peptide with a variety of physiological and pharmacological activities, ranging from vasodilation and con-

trol of neurotransmission to regulation of cell proliferation (Roberts, 1989). Here we show that bradykinin inhibits the loss of density-dependent growth arrest of NRK cells induced by TGF β or RA during the first 20 h of incubation, a property which is not shared by other phosphoinositide-mobilizing agents. The observed growth inhibition induced by bradykinin is paralleled by a repression of the up-regulation of EGF receptor expression, strongly suggesting that EGF-induced proliferation of NRK cells is a direct function of EGF receptor density. These data are discussed in the light of the current hypotheses on the molecular mechanism involved in density-dependent growth control.

MATERIALS AND METHODS

Growth-stimulation assays

NRK fibroblasts (clone 49F) were plated at a density of 15000 cells/cm², and grown to confluence in serum-containing medium for 4 days, and subsequently cultured in serum-free medium for 3 days, as described by Van Zoelen et al. (1988). These cells are referred to as serum-deprived quiescent cells. These cells were then density-arrested by incubation in 5 ng/ml EGF and 5 μ g/ml insulin for an additional 48 h (Van Zoelen et al., 1988). Density-arrested cells were re-stimulated to proliferate by treatment with additional growth factors, and incorporation of [³H]thymidine (0.5 μ Ci/ml added; Amersham) was measured either cumulatively between 4 and 20 h of growth-factor addition, or as a pulse during consecutive 2 h periods (Van Zoelen et al., 1992).

EGF-binding studies

Density-arrested NRK cells (10 cm² wells) were incubated for 24 h in fresh serum-free medium without insulin and EGF. Subsequently, factors to be tested were added during the indicated time period, after which the medium was changed for 0.5 ml of binding buffer (Dulbecco's modified Eagle's medium, containing 0.1% BSA and 50 mM Bes, pH 6.8). Subsequently 1–4 ng/ml ¹²⁵I-EGF (10⁵ c.p.m./ng) and various concentrations of un-

Abbreviations used: NRK cells, normal rat kidney cells; EGF, epidermal growth factor; TGF β , transforming growth factor β ; RA, retinoic acid; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate.

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labelled EGF were added. After incubation for 1 h at room temperature, cells were washed with PBS containing 0.1% BSA, and extracted with 1% Triton X-100. Receptor-ligand binding data were analysed by the linear subtraction method (Van Zoelen, 1989).

Lipid-degradation studies

Confluent NRK cultures (10 cm² wells) were incubated for 24 h with 4 μ Ci of [³H]inositol (Amersham) in serum-containing medium. After subsequent incubation for 24 h in serum-free medium, cells were grown to density arrest by incubation for 48 h in the presence of 5 μ g/ml insulin and 5 ng/ml EGF. Subsequently agents to be tested were added together with 10 mM LiCl, and after 30 min the medium was changed for 1 ml of 10% trichloroacetic acid. Inositol phosphates were determined by elution from an AG 1X8 anion-exchange column (Bio-Rad) with 0.8 M ammonium formate/0.1 M formic acid (pH 4.5), as described in detail by Tilly et al. (1987).

Northern-blot analysis

Poly(A)⁺ RNA was isolated from density-arrested and re-stimulated NRK cells, as described previously (Van Zoelen et al., 1992). Samples (20 μ g) were denatured, electrophoresed on agarose gels and transferred to nitrocellulose blots as described by Van den Eijnden-Van Raaij et al. (1989), and hybridized with a mixture of a 0.77 kb and a 1.84 kb *Eco*RI cDNA fragment of the human EGF receptor.

Materials

¹²⁵I-EGF was generously given by Dr. T. Benraad (Department of Endocrinology, University of Nijmegen). TGF β ₁ was isolated from human platelets as described by Van den Eijnden-Van Raaij et al. (1988). RA, phorbol 12-myristate 13-acetate (PMA) and prostaglandin F_{2a} were from Sigma, bradykinin was from Boehringer, and bradykinin-related compounds were from Peninsula Laboratories.

RESULTS

Effect of bradykinin on loss of density-dependent growth inhibition

When confluent serum-deprived cultures of NRK cells are treated with EGF and insulin, cells are stimulated to undergo one additional cell cycle, after which they become quiescent in the G₁/G₀-phase as a result of density-dependent growth inhibition (Van Zoelen et al., 1988). In the continuous presence of EGF and insulin, these density-arrested cells can be re-stimulated to proliferate in a synchronous manner by treatment with additional growth factors, such as TGF β , RA, PDGF or basic fibroblast growth factor (Van Zoelen et al., 1992). In contrast with the last two factors, TGF β and RA as such have no growth-stimulating activity on NRK cells, and can only exert their transforming activity in the additional presence of EGF (Van Zoelen et al., 1988).

Figure 1(a) shows the effect of bradykinin on the loss of density inhibition of NRK cells induced by TGF β , RA and the tumour promoter PMA. Cells were density-arrested in the presence of EGF and insulin, and cumulative thymidine incorporation was subsequently measured during the first 20 h after addition of the modulating factors indicated. It is shown that, although bradykinin by itself has no significant effect on the proliferation of these density-arrested cells, it almost completely

abolishes the growth-stimulating effect of TGF β and RA. As a comparison it is shown that PMA is only a poor inducer of phenotypic transformation of NRK cells under these conditions, but that the effect induced is also sensitive to the inhibitory action of bradykinin.

Figure 1(b) shows in a time-course experiment that the growth-inhibitory effect of bradykinin depends on the time of incubation. Previous studies have indicated that 10 h after addition of TGF β or RA to density-arrested NRK cells, the first cells from these synchronized populations enter the S-phase (Van Zoelen et al., 1992). By studying 2 h pulses of [³H]thymidine incorporation, the present data show that with TGF β and RA a maximum rate of thymidine incorporation is reached within 20 h of growth-factor addition, in agreement with previous data (Van Zoelen et al., 1992). In the additional presence of bradykinin, however, these curves are shifted in time to reach a maximum around 24 h of incubation. Within the first 20 h of growth-factor treatment, bradykinin therefore appears to have a strong inhibitory effect on loss of density-dependent growth arrest induced by TGF β or RA. At later time points, however, bradykinin is shown to have growth-stimulatory effects by itself, and to enhance proliferation induced by TGF β and RA.

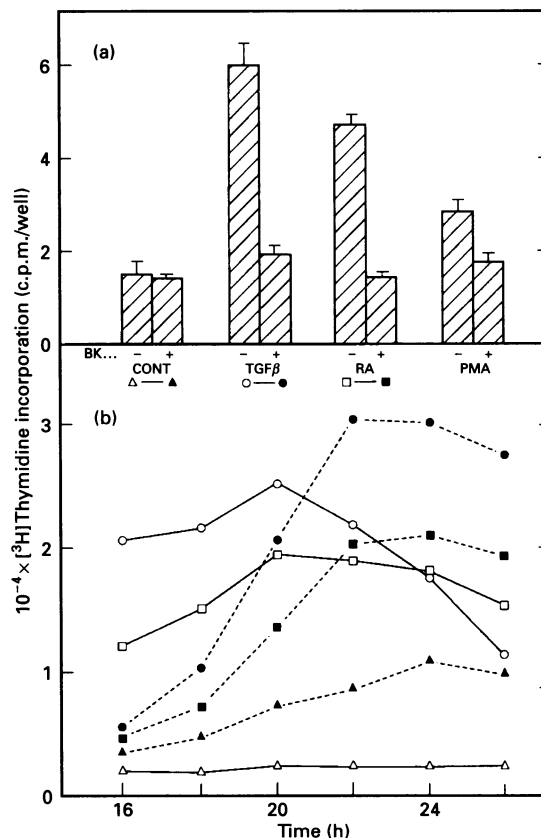


Figure 1 Effect of bradykinin on growth-factor-induced loss of density inhibition of NRK cells

(a) Density-arrested cells were incubated with 2 ng/ml TGF β , 50 ng/ml RA (retinoic acid), 50 ng/ml PMA or without stimulus (CONT), in the additional presence (+) or absence (-) of 1 μ M bradykinin (BK). Incorporation of [³H]thymidine was determined between 4 and 20 h after growth-factor addition. Indicated S.E.M. values are based on at least triplicate experiments. (b) Time course of [³H]thymidine incorporation (2 h pulses) into density-arrested NRK cells after the following treatments: Δ , no addition; \blacktriangle , 1 μ M bradykinin; \circ , 2 ng/ml TGF β ; \bullet , 2 ng/ml TGF β + 1 μ M bradykinin; \square , 50 ng/ml RA; \blacksquare , 50 ng/ml RA + 1 μ M bradykinin.

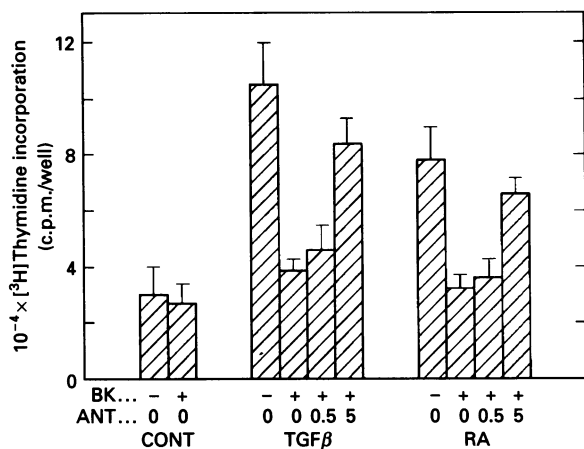


Figure 2 Reversal of bradykinin-induced growth inhibition of NRK cells by bradykinin-receptor antagonist

[³H]Thymidine incorporation into density-arrested NRK cells was measured after incubation with 2 ng/ml TGFβ, 50 ng/ml RA or no addition (CONT), in the additional presence (+) or absence (−) of 0.1 μM bradykinin (BK), and the indicated concentrations (μM) of the antagonist [D-Arg,Hyp³,Thi^{5,8},D-Phe⁷]bradykinin (ANT). Indicated S.E.M. values are based on at least triplicate experiments.

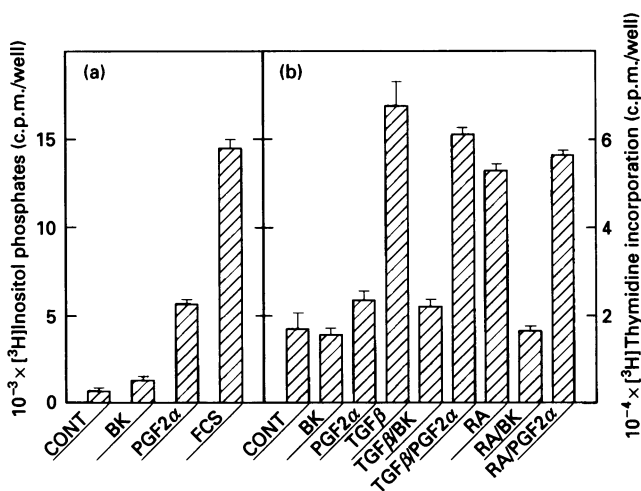


Figure 3 Comparison of bradykinin (BK) and prostaglandin F_{2α} (PGF2α) in inducing degradation of inositol-containing phospholipids (a) and growth inhibition (b) of NRK cells

Indicated stimuli were added to density-arrested NRK cells at the following concentrations: CONT, no addition; BK, 10 μM (a) or 1 μM (b); PGF2α, 1 μM; FCS, 9% fetal-calf serum; TGFβ, 2 ng/ml; RA, 50 ng/ml. Incorporation of [³H]thymidine was determined between 4 and 20 h after addition of the growth stimuli. Indicated S.E.M. values are based on at least triplicate experiments.

Bradykinin effect on cell proliferation is receptor-mediated

Bradykinin is known to exert its action by activation of a set of G-protein-coupled receptors, designated the B₁ and B₂ receptor types (Roberts, 1989). Dose-response curves showed that half-maximum inhibition of RA-induced loss of density inhibition of NRK cells was obtained at a concentration of 30 nM bradykinin (results not shown), which is indicative of a process mediated by a high-affinity receptor. Figure 2 shows that the inhibition of TGFβ- and RA-induced loss of density inhibition by 0.1 μM

bradykinin is counteracted by the B₂-receptor antagonist [D-Arg,Hyp³,Thi^{5,8},D-Phe⁷]bradykinin (Hyp, hydroxyproline; Thi, β-(2-thienyl)alanine) in a dose-dependent fashion. The bradykinin effect was also antagonized, although to a lesser extent, by [des-Arg⁹,Leu⁸]bradykinin (results not shown), which is known to have B₁-receptor-antagonist effects (Patel and Schrey, 1992). Although the present data do not give direct information on the type of bradykinin receptor involved, they show that the observed biological effects of bradykinin are receptor-mediated.

Bradykinin effect is not related to degradation of inositol-containing phospholipids

Upon receptor activation, bradykinin is known to induce degradation of inositol-containing phospholipids through activation of a phospholipase C, and to induce release of fatty acids, including arachidonic acid, by activation of a phospholipase A₂ (Roberts, 1989). It has been shown previously that bradykinin is able to mobilize Ca²⁺ from intracellular sources in NRK cells (Marks et al., 1988). Figure 3(a) shows that, under the conditions tested, bradykinin is a relatively poor inducer of inositol polyphosphate formation, compared with prostaglandin F_{2α} and fetal-calf serum. As shown in Figure 3(b), however, prostaglandin F_{2α} is unable to mimic the growth-inhibitory effect of bradykinin on NRK cells, and generally by itself induces slight mitogenic effects. Also, with respect to release of [³H]arachidonic acid, prostaglandin F_{2α} was found to be far more potent than bradykinin in NRK cells (G. B. Afink, D. C. J. G. van Alewijk and E. J. J. van Zoelen, unpublished work). Also, other agents such as the protein kinase C activator PMA and the protein kinase C inhibitor sphingosine did not mimic the growth-inhibitory effect of bradykinin on NRK cells (results not shown). Taken together, these data show that the inhibitory effect of bradykinin on loss of density-dependent growth inhibition is specific, and is not a general property of phosphoinositide-mobilizing agents.

Effects of bradykinin on EGF receptor density

It has been established in a number of cell lines, including NRK cells, that treatment with RA results in an immediate increase in the number of EGF receptors per cell (Jetten, 1982; Roberts et al., 1984). In the case of TGFβ treatment, EGF-receptor density in NRK cells first decreases, and then increases after more than 4 h of incubation (Assoian, 1985). Figure 4 shows that bradykinin blocks the RA-induced increase in EGF-binding sites during the first 4 h of incubation. Subsequently, bradykinin synergizes with RA in increasing EGF receptor levels, resulting in enhanced EGF binding after incubation for more than 6 h. During the time period investigated, bradykinin itself does not affect EGF receptor densities in NRK cells, which contrasts with observations by Earp et al. (1988) in WB cells, that hormones stimulating phosphoinositide hydrolysis may enhance levels of EGF binding.

The insert of Figure 4 shows Scatchard plots for binding of EGF to density-arrested NRK cells, before and after treatment with RA. Both plots have been fitted to a single receptor-affinity model, although the curves show a slight tendency towards curvilinearity, in agreement with data of Assoian (1985). The present Scatchard plots show that before RA treatment density-arrested NRK cells contain approx. 1100 EGF receptors with a dissociation constant of 0.5 nM, whereas after 20 h of incubation with RA cells contain approx. 9500 EGF receptors with a dissociation constant of 0.6 nM. These data underline the low number of EGF receptors in density-arrested NRK cells and the significant effect of RA on these EGF receptor levels.

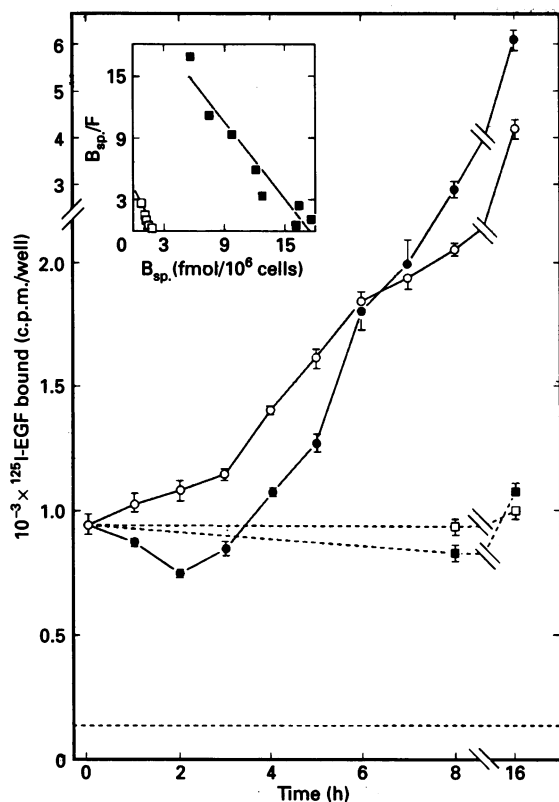


Figure 4 Effect of bradykinin on RA-induced EGF receptor levels in density-arrested NRK cells

Time course of ^{125}I -EGF (4 ng/ml) binding after addition of 50 ng/ml RA (○), 50 ng/ml RA + 1 μM bradykinin (●), 1 μM bradykinin (■) or no addition (□). The broken horizontal line represents the level of non-specific binding obtained by additional treatment with a 100-fold excess of unlabelled EGF. The insert shows a Scatchard plot for specific binding (B_{sp}) of 1 ng/ml ^{125}I -EGF and variable concentrations of unlabelled EGF, without (□) or with (■) pretreatment for 20 h with 50 ng/ml RA. Free ligand concentrations (F) are expressed in nM. Indicated S.E.M. values are based on at least triplicate experiments.

Figure 5 shows the effect of bradykinin on EGF receptor mRNA levels in a Northern-blot analysis. Rat EGF receptor mRNA chromatographs at a size of 9.6 kb, with an occasional additional transcript at 5 kb (Ober and Carpenter, 1991). The data presented here show that the steady-state mRNA levels for the EGF receptor rapidly increase with time upon RA treatment, but that within the period of 4 h tested this effect is completely abrogated by bradykinin. In TGF β -treated cells, an increase in EGF receptor mRNA levels was only visible after 4 h of incubation, whereas PDGF, which in combination with EGF also induces phenotypic transformation of NRK cells, had no effect on EGF receptor mRNA levels (results not shown). The data of Figure 5 also show that, in NRK cells density-arrested in the presence of EGF, little expression of the EGF receptor gene is detectable under the present experimental conditions. This observation agrees with the EGF-binding data in Figure 4, and is in line with the concept that EGF receptor levels decrease with increasing cell density (Rizzino et al., 1990). Interestingly, however, it has been shown in a variety of studies that EGF is able to enhance expression of its own receptor gene by a combination of both transcriptional and post-transcriptional controls (Clark et al., 1985; Earp et al., 1988; Thompson and Rosner, 1989; Fernandez-Pol et al., 1989; Hudson et al., 1989).

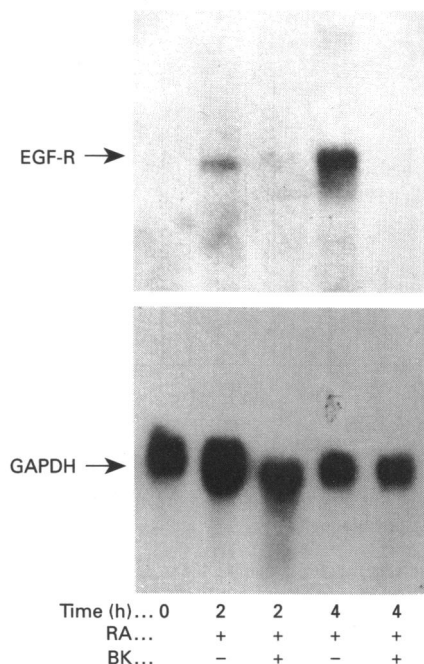


Figure 5 Northern blot of EGF receptor (EGF-R) mRNA levels in density-arrested NRK cells after incubation for various times (0, 2, 4 h) with 50 ng/ml RA in the absence (-) or the additional presence (+) of 1 μM bradykinin (BK)

Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a loading control; mRNA EGF-R 9.6 kb, GAPDH 1.4 kb.

The present data show that in NRK cells which have undergone density-dependent growth inhibition EGF is unable to induce EGF receptor expression to a detectable level, which may be an important aspect of the molecular mechanisms involved in density-dependent growth control. In conclusion, the present data show that, in parallel with a 4 h shift in RA- and TGF β -induced release from density arrest (see Figure 1b), bradykinin also blocks the RA-induced increase in EGF receptor levels during the same period of time.

DISCUSSION

In the present study we have shown that bradykinin selectively inhibits the expression of a transformed phenotype in NRK cells. We have used this observation to demonstrate that a close correlation exists between EGF receptor levels in these cells and the ability of growth-modulating agents such as TGF β and RA to release these cells from density-dependent growth arrest. Earlier studies have shown that EGF receptor levels decrease with increasing cell density (Holley et al., 1977; Rizzino et al., 1988, 1990), and that agents such as TGF β and RA enhance the number of EGF receptors in NRK cells (Roberts et al., 1984; Assoian et al., 1984; Assoian, 1985; Thompson et al., 1988; Thompson and Rosner, 1989). Taken together with the present results on the effect of bradykinin on EGF receptor expression and density-dependent growth control, these data strongly suggest that EGF receptor density is one of the major controlling parameters in density-dependent growth inhibition and phenotypic transformation of NRK cells. It is well established that EGF receptor expression can be controlled at both the transcriptional and post-transcriptional level (Hadcock and Malbon,

1991). Further experiments are required to investigate whether under the present experimental conditions bradykinin inhibits RA-induced transcriptional activation of the EGF receptor gene, or decreases EGF receptor mRNA stability.

Bradykinin has been associated with both growth stimulation (Owen and Villereal, 1983; Roberts and Gullick, 1989; Roberts, 1989, and references therein) and growth inhibition (Newman et al., 1989; Patel and Schrey, 1992) on a variety of cells. On still other cell lines, no direct growth effect of bradykinin has been observed, in spite of the presence of specific receptors and bradykinin-mediated second messengers (Van Corven et al., 1989; Ruggiero et al., 1989). Here we have shown that bradykinin induces both short-term growth inhibition and long-term growth stimulation of NRK cells. The short-term growth-inhibitory effects are paralleled by repression of TGF β - and RA-induced enhancement of EGF receptor levels. These inhibitory effects are specific for density-arrested NRK cells, since no such effects were observed on growth-stimulated NRK cells which were first made quiescent by serum deprivation. With prostaglandin F_{2a}, only long-term growth-stimulatory effects were observed, similar to those of bradykinin, but no growth-inhibitory effects.

Bradykinin exerts its action through a set of high- and low-affinity receptors, which results in degradation of choline- and inositol-containing phospholipids and release of arachidonic acid (Van Blitterswijk et al., 1991a,b; Kast et al., 1991), as well as in enhancement of tyrosine phosphorylation (Leeb-Lundberg and Song, 1991). Degradation of inositol-containing phospholipids and release of arachidonic acid are also induced in NRK cells by prostaglandin F_{2a}, endothelin (Kusuhara et al., 1992) and lysophosphatidic acid (results not shown; see also Van Corven et al., 1989), to even a larger extent than that observed for bradykinin. However, only bradykinin is a growth-inhibitory factor for NRK cells, also when added in combination with one of the other above factors. These observations suggest that bradykinin must induce a so-far unknown second messenger relevant for growth inhibition, which is not induced by other phosphoinositide-mobilizing agents. Bradykinin-induced effects on cell proliferation generally result from the induction of prostaglandin derivatives (Patel and Schrey, 1992). Preliminary results indeed show that the observed growth-inhibitory effects of bradykinin can be largely blocked by inhibitors of cyclo-oxygenase activity, such as indomethacin (G. B. Afink, D. C. G. J. van Alewijk and E. J. J. van Zoelen, unpublished work). It is at present unclear, however, which growth-inhibitory prostaglandin is specifically induced by bradykinin in NRK cells. The observed inhibitory effects could not be mimicked by addition of well-characterized prostaglandins such as F_{2a}, E₂, A₂ or D₂ to NRK cells (results not shown).

NRK cells form an attractive model for studying the molecular mechanisms involved in density-dependent growth control, since, depending on the growth factors added, cells display either a normal or a transformed phenotype (Van Zoelen, 1991). When cells are cultured in the presence of insulin and EGF they become arrested at a saturating density, from which they can be released by treatment with additional growth factors, including TGF β and RA (Van Zoelen, 1991). Still relatively little is known about the molecular mechanisms involved in density-dependent growth inhibition, and the loss of this density control upon tumorigenic transformation. The following processes, however, have been proposed as playing a possible role. It has been shown that normal cells can become quiescent at high densities because of production of autocrine growth-inhibitory factors (Holley et al., 1978; Harel et al., 1985). With NRK cells, however, no evidence for production of such factors by density-arrested cells was found (Van Zoelen, 1991). For the epithelial NRK cell line 52E

an abrupt increase in the membrane potential has been observed as the cells reach a saturating density (Binggeli and Weinstein, 1985). In line with this observation, we found that NRK-49F fibroblasts released from density arrest by TGF β rapidly depolarize, underlining the importance of the membrane potential in controlling density-dependent growth inhibition (A. P. R. Theuvenet, P. H. J. Peters and E. J. J. van Zoelen, unpublished work). In addition, gap-junction-mediated intercellular communication has been considered to control growth arrest by direct intercellular contacts (Loewenstein, 1979), but in NRK cells no significant intercellular communication was detected in density-arrested cells (Van Zoelen and Tertoolen, 1991). Wieser et al. (1990) have published evidence that a membrane glycoprotein known as contactinhibin is involved in contact-dependent growth inhibition, but as yet no effects of this factor have been described in NRK cells.

The present and previous studies (Holley et al., 1977; Rizzino et al., 1988, 1990) have shown that modulation of growth-factor receptor densities may provide an additional mechanism for controlling cell proliferation. This may be paralleled by a density-dependent modulation of protein-tyrosine phosphatase activity; together with a modulation of growth-factor receptor levels, this may control growth-factor-induced phosphotyrosine levels in the cell (Pallen and Tong, 1991). In support of this idea, we have recently observed that the phosphatase inhibitor vanadate mimics TGF β in preventing EGF-stimulated NRK cells from becoming density-arrested (Rijksen et al., 1993a,b). It is likely that all of the above mechanisms in fact play a distinct role in the regulation of density-dependent growth control. On the basis of the observation by Rizzino et al. (1990) that in NRK cells EGF receptor levels decrease with increasing cell density, and the present observations on the effect of bradykinin on EGF receptor expression, it seems that for NRK cells modulation of receptor densities is the most appropriate mechanism for describing density-dependent growth regulation. This is also underlined by the observation that mutant NRK-49F cells with only a 3-fold decrease in EGF receptor levels are fully unresponsive to the growth-stimulating activity of EGF (Hamanaka et al., 1990). It is well realized, however, that the other mechanisms mentioned above may play a more dominant role in other cell types. For example, in human glial cells, it has been shown that density-dependent growth inhibition is associated with even an increase in EGF receptor levels (Westermarck, 1977).

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