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Lysophosphatidic Acid and Bradykinin Have Opposite Effects on Phenotypic Transformation of Normal Rat Kidney Cells

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Abstract The bioactive lipid lysophosphatidic acid is besides a strong mitogen for quiescent fibroblasts, a potent inducer of phenotypic transformation of normal rat kidney cells. The lysophosphatidic acid induced loss of density-arrest is strongly inhibited by bradykinin. Although their effects on normal rat kidney cell proliferation are opposite, bradykinin mimics many of the intracellular effects induced upon lysophosphatidic acid receptor activation, including phosphoinositide turnover, Ca^{2+}-mobilization and arachidonic acid release. Bradykinin does not counteract the lysophosphatidic acid induced reduction of cAMP levels in normal rat kidney cells. However, bradykinin inhibits the lysophosphatidic acid and other growth factor induced phenotypic transformation through the induction of a so far uncharacterized prostaglandin G/H synthase product. The growth inhibitory effect of bradykinin is limited to density-arrested cells, while upon prolonged treatment bradykinin itself is capable to induce the loss of density-dependent growth control. It is concluded that bradykinin is a bifunctional regulator of normal rat kidney cell proliferation and that its inhibitory effects are mediated via the induction of a prostaglandin derivative.

Key words: contact-inhibition, prostaglandins, cAMP, phosphatidyl inositol, cyclooxygenase, arachidonic acid, PDGF, retinoic acid, TGFβ, LPA

Density-dependent growth inhibition or contact-inhibition is an important mechanism by which normal non-transformed cells become limited in their growth. However, upon tumorigenic transformation this density-dependent growth control is lost and cells become able to proliferate without restrictions. Density-dependent growth control is one of the most prominent differences between the growth characteristics of transformed and non-transformed cells. The precise molecular mechanisms that underly this growth inhibition at increasing cell densities are not completely understood [reviewed in Van Zoelen, 1991]. Several polypeptide growth factors are capable to induce the loss of density-dependent growth inhibition and thus phenotypically transform cells. The cell line NRK-49F, derived from normal rat kidney fibroblasts, provides an excellent model system to study the role of growth factors in this transformation process. NRK cells become density-arrested when cultured in the sole presence of epidermal growth factor (EGF). However, the cells are reversibly transformed and lose their density-dependent growth inhibition if additional transforming growth factor β (TGFβ), retinoic acid (RA) or platelet-derived growth factor (PDGF) is present [Van Zoelen et al., 1988]. At the molecular level this TGFβ and RA induced loss of density-dependent growth inhibition in NRK cells can be explained by the regulation of EGF receptor numbers. EGF receptor densities on the surface of NRK cells decrease with increasing cell densities [Rizzino et al., 1988, 1990]. At the stage of density-dependent growth arrest these EGF receptor numbers have been decreased to a level at which EGF is no longer able to provide a mitogenic signal to the cells. However, addition of TGFβ or RA, which by themselves are not mitogenic for these cells, results in an increase of EGF receptor numbers in NRK cells [Roberts et al., 1984; Assoian et al., 1984; Assoian, 1985; Van Zoelen et al., 1994]. As a result the cells become respondent to EGF again and resume growing with a transformed phenotype.

The lipid lysophosphatidic acid (LPA) is a potent mitogen for a number of different fibro-

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blasts [Van Corven et al., 1989]. A putative LPA receptor has been described, which is most likely G-protein coupled [Van der Bend et al., 1992; Van Corven et al., 1993; Howe and Marshall, 1993]. The intracellular second messenger systems affected upon LPA activation include phosphatidyl inositol (PI)-hydrolysis, Ca^{2+}-mobilization, PLA_2-activation, and a decrease in cAMP levels [Van Corven et al., 1989; Jalink et al., 1990]. Recently Van Corven et al. [1993] have also reported that the protooncogene $p21^{RAS}$ is rapidly activated upon LPA stimulation. In these studies the nonapeptide bradykinin has been shown to mimic a large variety of the intracellular messengers induced by LPA. However, in contrast to LPA, bradykinin has no growth stimulatory effect on the investigated fibroblasts. Moreover we have recently shown that bradykinin is a strong inhibitor of the RA and TGFβ induced increase in EGF receptor number and the concomitant loss of density-dependent growth inhibition of NRK cells [Van Zoelen et al., 1994].

To gain more insight in the working mechanisms of LPA and bradykinin we studied the effects of these growth regulatory agents on the phenotypic transformation of NRK cells. LPA showed to be both a strong mitogen and a very potent inducer of phenotypic transformation in these cells. Bradykinin on the contrary inhibits not only the TGFβ and RA induced phenotypic transformation but also the induction of this process by LPA. This inhibitory effect of bradykinin is specific for density-arrested cells. Quiescent, serum-starved NRK cells are not affected by bradykinin. The specific LPA induced reduction in cAMP levels is not the target for the inhibitory effect of bradykinin, because bradykinin is not able to prevent this decrease in cAMP. However, the inhibition of growth factor induced phenotypic transformation by bradykinin is mediated through an arachidonic acid (AA) metabolite and more specifically via the induction of a prostaglandin G/H (PGH) synthase product.

METHODS

Phenotypic Transformation Assay

NRK-49F cells were grown to confluency in 24-well tissue culture dishes in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY), supplemented with 10% newborn calf serum (Hyclone, Logan, UT). Confluent cells were made quiescent by a subsequent three days incubation in serum-free medium, as described previously [Van Zoelen et al., 1988]. These cells were grown to density-arrest by an additional 48 hours incubation in the presence of 5 ng/ml EGF (Collaborative Biomedical Products, Bedford, USA) and 5 μg/ml insulin (Sigma, St. Louis, MO). After treatment with additional growth factors, the capacity of these growth factors to induce phenotypic transformation was determined by the measurement of [^{3}H]-thymidine (TdR) incorporation (0.5 μCi/ml, Amersham International, England), either in a cumulative manner between 4–19 h after agonist addition, or in consecutive 2 h pulse intervals.

Inositol Phosphate Determination

Confluent NRK cultures in 6-well tissue culture dishes were labeled for 24 h with 4 μCi/ml [^{3}H]-inositol (Amersham International, England) in serum-containing medium. After a subsequent 24 h incubation in serum-free medium, cells were grown to density-arrest by a 48 h treatment with 5 ng/ml EGF and 5 μg/ml insulin. The cells were stimulated with agonists for 30 min in the additional presence of 10 mM LiCl. The reactions were terminated by changing the medium for 10% trichloro-acetic acid (TCA). Inositol phosphates were determined by elution from a AG 1X8 anion exchange column (BioRad, Richmond, CA) with 0.8 M ammonium formate/0.1 M formic acid (pH 4.5), as described in detail by Tilly et al. [1987].

cAMP Measurements

Density-arrested NRK cells in 6-well tissue culture dishes were preincubated for 10 min with 0.5 mM 3-isobutyl-1-methylxanthine (Sigma, St. Louis, MO), followed by the addition of agonists. After the appropriate incubation times the cells were rinsed with ice-cold phosphate-buffered saline and the reactions were stopped by treatment with 1 ml 10% ice-cold TCA. The TCA was removed by a fivefold extraction with diethylether and the samples were lyophilysed. cAMP content was determined using the Amersham [^{3}H]-cAMP kit, according to the instructions of the manufacturer.

[^{3}H]-Arachidonic Acid Release

Density-arrested NRK cells in 6-well tissue culture dishes were labeled with 1 μCi/ml [^{3}H]-arachidonic acid (180–240 Ci/mmol, NEN) for 15 h. After the labeling the medium was re-

placed by 1.5 ml Hepes buffered Dulbecco's modified Eagle medium with additional 0.1% fatty acid free bovine serum albumin (Sigma, St. Louis, MO). This medium was changed several times to remove unincorporated label. Agonists were added and after 1 h 0.5 ml of the culture medium was withdrawn and total radioactivity released into the medium was determined by liquid scintillation analysis.

Measurements of Intracellular Ca^{2+}

Cells were grown on gelatin-coated glass coverslips instead of tissue culture plastic as described above. Density-arrested NRK cells were loaded for 90 min with 5 μg/ml Fura-2AM (Molecular Probes, Eugene, OR) and 0.01% pluronic acid in Hepes/bicarbonate-buffered serum-free medium at room temperature. Subsequently the cells were washed and incubated in Hepes/bicarbonate-buffered saline (in mM: 25 NaCl, 5 KCl, 2 $CaCl_2$, 10 glucose, 15 Hepes, and 17.6 $NaHCO_3$) at 25°C. Intracellular calcium measurements were performed using a spectrofluorometer (SPF-500 Aminco), at an excitation wavelength of 340 nm (4 nm bandwidth) and an emission wavelength of 480 nm (8 nm bandwidth). Data were sampled and stored at a rate of 0.1 Hz using a data processor. Growth factors were added directly to the incubation medium. At the end of the experiment, ionomycin (5 μM) and Mn^{2+} (1 mM) were added to obtain an estimate of maximum and background calcium values.

Materials

TGFβ was purified from human platelets according to the protocol of Van den Eijnden-Van Raaij et al. [1988]. Recombinant PDGF-BB was a generous gift from Dr. C-H Heldin (Uppsala, Sweden). LPA was purchased from both Sigma (St. Louis, MO) and Serdary (London, Ontario, Canada). RA, indomethacin, nordihydroguaiaretic acid, 2-methyl-1,2-3-pyridyl-1-propanone, and prostaglandin $F_{2\alpha}$ were from Sigma, bradykinin was from Boehringer (Mannheim, Germany).

RESULTS

Effect of LPA and Bradykinin on the Loss of Density-Dependent Growth Arrest

We have previously shown that bradykinin acts as a potent inhibitor of RA and TGFβ induced phenotypic transformation of NRK cells [Van Zoelen et al., 1994]. The inhibitory action

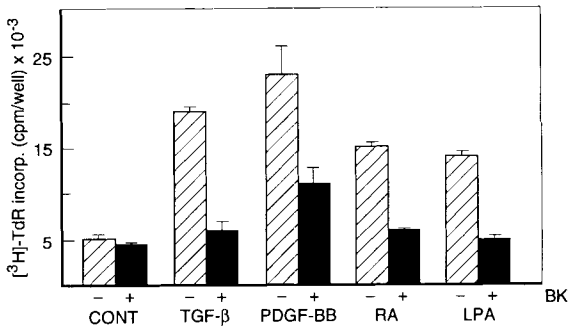

Fig. 1. Effect of bradykinin on growth factor induced loss of density-arrest. Density-arrested NRK cells were incubated with 2 ng/ml TGFβ, 5 ng/ml PDGF-BB, 50 ng/ml RA, 100 μM LPA, or without stimulus (CONT) in the absence (−) or presence (+) of an additional 1 μM bradykinin. [3H]-TdR incorporation was determined between 4 and 19 h after the additions. Values represent the mean ± standard deviation of triplicate determinations.

of bradykinin has been correlated with a specific inhibition of EGF receptor mRNA formation. LPA has been described as a very potent mitogen for serum-starved quiescent fibroblasts [Van Corven et al., 1989, 1992]. Figure 1 shows the effect of LPA on the [3H]-TdR incorporation in density-arrested NRK cells, compared with the transforming effects of TGFβ, RA, and PDGF-BB. LPA induces the loss of density-dependent growth arrest to the same extent as RA. The transforming capacities of LPA were confirmed by induction of anchorage-independent growth (data not shown). Also shown in this figure is the inhibitory effect of bradykinin on this growth factor induced loss of density-arrest. Bradykinin does not only inhibit the RA and TGFβ induced loss of density-arrest, but it abolishes also the transforming capacities of LPA and to a lesser extent those of PDGF-BB. These results might suggest that bradykinin is a general inhibitor of NRK cell growth, but this is not the case. The inhibitory action of bradykinin is specific for density-arrested cells as shown in Figure 2. This figure compares the effects of LPA and bradykinin on the [3H]-TdR incorporation in both serum-starved, quiescent cells (Fig. 2A) and density-arrested cells (Fig. 2B). LPA displays besides its transforming effect on density-arrested cells also a strong mitogenic response on quiescent NRK cells. More importantly however is the observation that bradykinin does not inhibit this mitogenic action of LPA on quiescent cells, while it completely inhibits the transforming effect of LPA on density-arrested cells.

It is concluded that bradykinin is a potent inhibitor of growth factor induced phenotypic

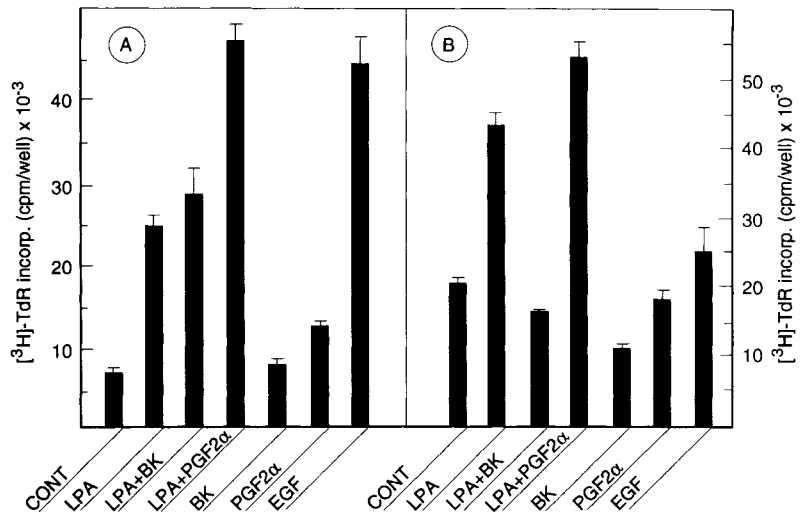

Fig. 2. The inhibitory effect of bradykinin on NRK cells is specific for density-arrested cells. Serum starved, quiescent (**A**) or density-arrested (**B**) NRK cells were incubated with the indicated substances. Final concentrations: 100 μM LPA, 1 μM bradykinin, 1 μM $PGF_{2\alpha}$, and 5 ng/ml EGF, or no addition (CONT). [^{3}H]-TdR incorporation was determined between 3 and 22 h (A) or between 2 and 16 h (B) after addition of the growth factors. Values represent the mean ± standard deviation of triplicate determinations.

transformation, but this inhibition is specifically limited to the conditions of density-arrest. Furthermore LPA shows to be both a potent mitogen and a transforming growth factor for NRK cells.

The Opposite Effects of Bradykinin and LPA Are Not Correlated to the Induction of Inositol Phosphates, Arachidonic Acid, Ca^{2+}-Release or Changes in cAMP Levels

In Rat-1 and HF cells bradykinin mimics several intracellular responses evoked by LPA, including PI-hydrolysis, Ca^{2+}-mobilization, and AA-release [Van Corven et al., 1989; Jalink et al., 1990]. However the biological effects of LPA and bradykinin on density-arrested NRK cells are completely opposite. This observation can be used to characterize the intracellular mechanisms involved in the process of phenotypic transformation. To investigate the role of these second messenger systems in the cell proliferation of density-arrested NRK cells, the effect of $PGF_{2\alpha}$ on NRK cell growth was studied, which is also shown in Figure 2. $PGF_{2\alpha}$ is like LPA and bradykinin a G-protein coupled agonist. $PGF_{2\alpha}$ however does not display a remarkable effect on either quiescent or density-arrested cell proliferation. It also does not inhibit the LPA induced cell growth, but it rather synergises with LPA to stimulate NRK cell growth.

The stimulation of PI-hydrolysis, Ca^{2+}-mobilization, and AA-release by LPA, bradykinin, and $PGF_{2\alpha}$ does not correlate with their effects on cell growth as shown in Figure 3. Figure 3A displays the data of the cumulative PI-hydrolysis in density-arrested NRK cells after treatment with these agonists. LPA and bradykinin generate a similar, rather weak PI-response when compared to $PGF_{2\alpha}$. Although $PGF_{2\alpha}$ elicits a much stronger PI response than LPA and bradykinin, the resulting Ca^{2+} transients are the same as shown in Figure 3B. Typical is the very strong PI-response when LPA and $PGF_{2\alpha}$ are added together. This synergistic action was also observed in the [^{3}H]-TdR incorporation assays (Fig. 2). Figure 3C gives a comparable picture for the LPA, bradykinin, and $PGF_{2\alpha}$ stimulated AA-release. $PGF_{2\alpha}$ is also the most potent inducer of AA-release compared to LPA and bradykinin. $PGF_{2\alpha}$ generates the same amount of AA-release as the phospholipase A_2 activator mellitin. Again, bradykinin and LPA give a equal response in AA-release. The same experiments on serum-starved, quiescent NRK cells show similar results (data not shown). Although $PGF_{2\alpha}$ generates the strongest PI and AA response, it does not show a pronounced effect on NRK cell proliferation compared to LPA and bradykinin. LPA and bradykinin however display a similar response in both AA-release and PI-hydrolysis,

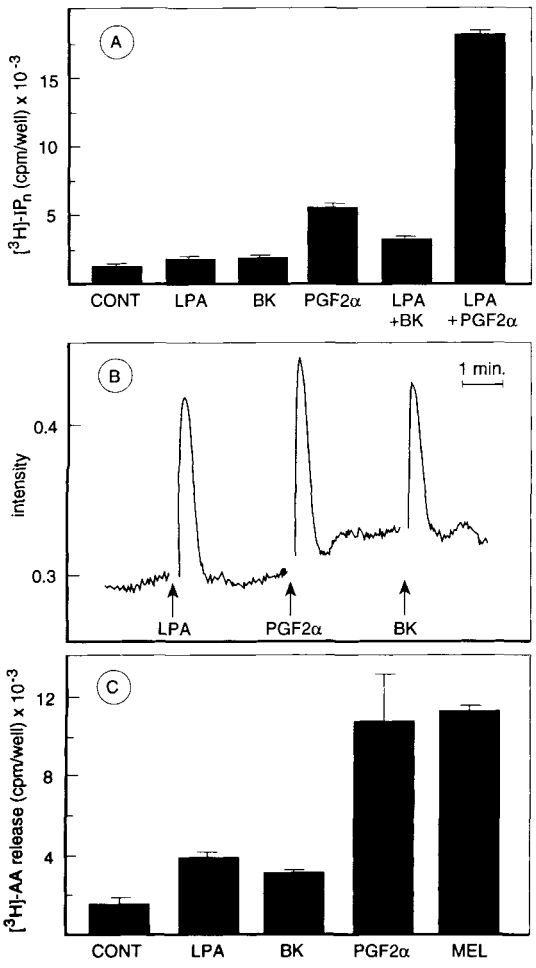

Fig. 3. Induction of inositol phosphates, arachidonic acid, or Ca^{2+}-mobilization by LPA, bradykinin and $PGF_{2\alpha}$ in NRK cells. **A:** Density-arrested, [^{3}H]-inositol labeled NRK cells were incubated for 30 min with the indicated agonists and the cumulative total inositol phosphate mobilization was determined. Final concentrations: LPA 100 μM, bradykinin (BK) 10 μM, $PGF_{2\alpha}$ 10 μM, or no addition (CONT). Values represent the mean ± standard deviation of duplicate determinations from a representative experiment. **B:** Typical Ca^{2+}-responses to sequential addition of LPA (10 μM), $PGF_{2\alpha}$ (1 μM), and bradykinin (BK, 1 μM) to density-arrested NRK cells. Ca^{2+} levels are represented as Fura2 fluorescence intensities. **C:** [^{3}H]-arachidonic acid labeled, density-arrested NRK cells were incubated with the indicated agonists. The [^{3}H]-arachidonic acid release into the medium was measured after 1 h incubation at 37°C. Final concentrations are the same as under A, with the addition of 1 μg/ml. mellitin (MEL). Values represent the mean ± standard deviation of duplicate determinations from a representative experiment.

but they have completely opposite effects on NRK cell growth. Taken together these data strongly suggest that there is no correlation between the potency of bradykinin and LPA to generate PI-hydrolysis, Ca^{2+}-mobilization, or AA-release and their effect on density-arrested NRK cell growth.

Another second messenger pathway that might be involved in the growth regulatory actions of LPA and bradykinin is cAMP. Increases in intracellular cAMP levels can inhibit fibroblast proliferation [Hollenberg and Cuatrecasas, 1973; Heldin et al., 1989; Burgering et al., 1989]. As shown in Figure 4A, proliferation of NRK cells is also inhibited when intracellular cAMP levels are elevated. The adenylate cyclase agonist forskolin inhibits the growth factor induced loss of density-arrest of NRK cells in a concentration dependent manner. Decreases in cAMP levels have been reported as an important early signaling event for LPA [Van Corven et al., 1989; Kumagai et al., 1993]. The data in Figure 4B clearly demonstrate that also in NRK cells LPA dramatically decreases forskolin stimulated cAMP levels. However bradykinin does not show any effect on either basal, elevated, or decreased cAMP levels. Thus it seems unlikely that cAMP plays a role in the inhibitory effect of bradykinin on NRK cells. The rapid decrease in elevated cAMP levels is specific for LPA, but not required for phenotypic transformation. The other investigated transforming growth factors RA, TGFβ, and PDGF-BB do not affect cAMP levels in NRK cells (data not shown).

The combined data on intracellular messengers suggest that PI-hydrolysis, Ca^{2+}-mobilization, AA-release, and changes in cAMP levels per se do not mediate the inhibitory effect of bradykinin. PI-hydrolysis, Ca^{2+}-mobilization, and AA-release are also not sufficient for the transforming effect of LPA. The only specific LPA effect is the decrease in forskolin stimulated cAMP levels, but this effect is not the target for the inhibitory action of bradykinin.

The Inhibitory Effect of Bradykinin Is Mediated Through a PGH Synthase Product

In previous reports on bradykinin mediated inhibition of cell growth, this effect could always be abolished by indomethacin, an inhibitor of PGH synthase [Straus and Pang, 1984; Patel and Schrey, 1992; McAllister et al., 1993a]. It is known that several products of the metabolic conversion of AA by PGH synthase can be potent inhibitors of cell growth [reviewed in Fukushima, 1992]. In our system indomethacin also completely abolished the inhibitory effect of bradykinin on growth factor induced loss of density-arrest (Fig. 5), as did other PGH synthase inhibi-

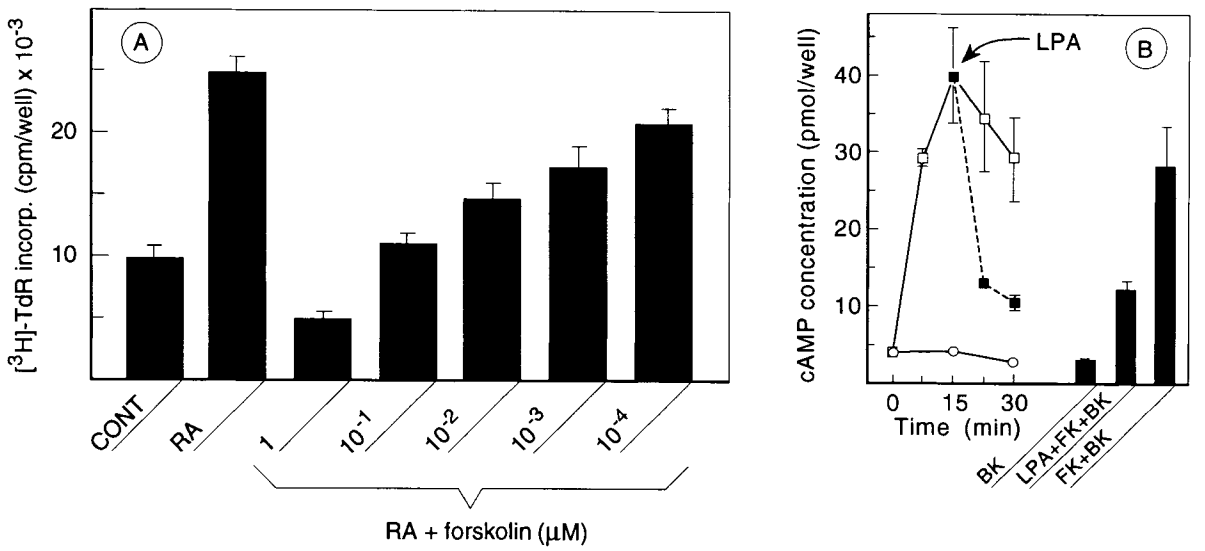

Fig. 4. **A:** Effect of forskolin on loss of density-arrest. Density-arrested NRK cells were treated with 50 ng/ml RA in the absence or additional presence of indicated concentrations of forskolin. CONT represents untreated cells. [^{3}H]-TdR incorporation was determined between 4 and 19 h after the additions. Values represent the mean ± standard deviation of triplicate determinations from a representative experiment. **B:** Effect of LPA and bradykinin on cAMP levels in NRK cells. Time course of cAMP levels in density-arrested NRK cells after treatment with 0.1 μM forskolin (□—□), 15 min. 0.1 μM forskolin followed by the addition of 100 μM LPA (arrow, ■---■) or no addition (○—○). The bar graphs on the right show the cAMP levels after a 30 min treatment with 1 μM bradykinin (BK) or a 15 min treatment with 0.1 μM forskolin followed by a subsequent 15 min incubation in the additional presence of 1 μM bradykinin (FK + BK) or 100 μM LPA and 1 μM bradykinin (LPA + FK + BK). All data are from the same experiment. Values represent the mean ± standard deviation of duplicate determinations from a representative experiment.

tors such as flurbiprofen and ibuprofen (data not shown). It is also shown in Figure 5 that inhibitors of lipoxygenase (nordihydroguaiaretic acid) or epoxygenase (2-methyl-1,2-di-3-pyridyl-1-propanone), two other AA converting enzymes, had no effect on the bradykinin inhibition of phenotypic transformation. These results give a strong indication that one or more PGH synthase products mediate the inhibitory effect of bradykinin.

To test whether the observed effect was mediated by one of the well characterized PGH synthase products several AA metabolites were added to RA induced density-inhibited NRK cells, including the prostaglandins A_2, D_2, E_1, E_2, $F_{2\alpha}$, I_2, and the thromboxane A_2 agonist U46619. However, no inhibition of phenotypic transformation by any of these compounds was observed (data not shown). Preliminary data with conditioned medium from bradykinin stimulated, density-arrested NRK cells however indicate that a growth inhibitory compound is released from the cells (DHTP Lahaye and EJJ Van Zoelen, unpublished observation). These observations suggest that bradykinin induces the formation of a so far uncharacterized PGH synthase product which is responsible for the inhibition of phenotypic transformation.

Bradykinin Is a Potential Transforming Growth Factor for NRK Cells

An important observation in the phenotypic transformation assays was that the inhibitory effect of bradykinin was fading after prolonged incubation times. To investigate this phenomenon in more detail, time courses of [^{3}H]-TdR incorporation into NRK cells were made under different growth conditions. In Figure 6A the time course of [^{3}H]-TdR incorporation after the addition of LPA to density-arrested cells with or without additional bradykinin is shown. LPA displays a typical stimulation of [^{3}H]-TdR incorporation with a maximum incorporation rate between 19–21 h after the addition. These kinetics are similar to those reported for TGFβ but are delayed when compared to the PDGF stimulated [^{3}H]-TdR incorporation in density-arrested NRK cells [Van Zoelen et al., 1992]. These delayed kinetics may reflect that the LPA induced loss of density-arrest is a indirect mechanism, like that of TGFβ. However, LPA does not

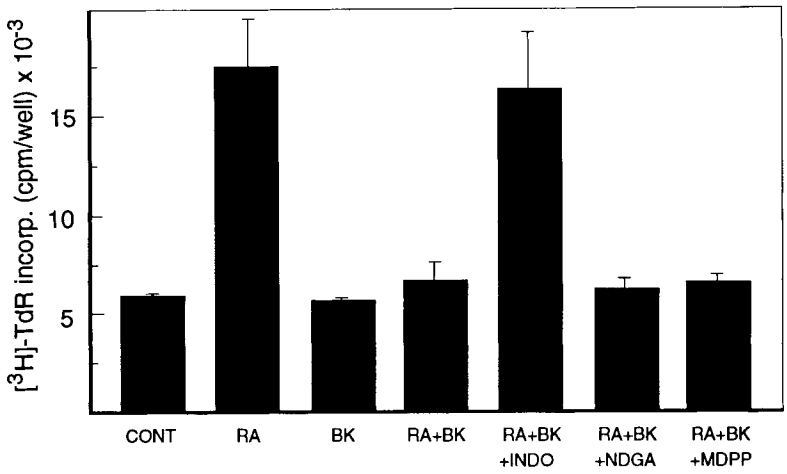

Fig. 5. Inhibitory effect of bradykinin is abolished by indomethacin. Density-arrested NRK cells were incubated with the indicated growth factors at the following concentrations: 50 ng/ml RA, 1 μM bradykinin (BK), or no addition (CONT). In the indicated lanes the cells were incubated with 1 μM indomethacin (INDO), 1 μM nordihydroguaiaretic acid (NDGA), or 1 μM 2-methyl-1,2-di-3-pyridyl-1-propanone (MDPP) before the addition of RA and bradykinin. [^{3}H]-TdR incorporation was determined between 4 and 19 h after the addition of the growth factors. Values represent the mean ± standard deviation of triplicate determinations.

require EGF for its transforming activity (data not shown). Van Corven et al. [1992] have also shown that the kinetics for LPA induced mitogenesis in quiescent Rat-1 fibroblasts are slower than for EGF induced mitogenesis. Thus it might be possible that the relative slow kinetics of LPA rather reflect a slow working mechanism than a indirect mechanism.

In the additional presence of bradykinin the LPA induced loss of density-arrest is inhibited, but not permanently. Bradykinin causes a delay of the LPA stimulated growth curve of approximately 8 h. Importantly, bradykinin alone displays a growth stimulatory effect with the same kinetics as LPA plus bradykinin, although the maximum incorporation rate is somewhat lower. This slow growth stimulatory effect of bradykinin explains the fading of the inhibitory effect in the [^{3}H]-TdR incorporation assays. When RA, TGFβ, LPA, or PDGF-BB stimulated density-arrested cells in these cumulative assays are incubated for more than 19 h in the presence of bradykinin, the growth stimulatory effect of bradykinin becomes visible and compensates its inhibitory effect.

The fact that bradykinin is able to induce the loss of density-dependent growth inhibition makes it a potential transforming growth factor. The question remains whether this bradykinin-induced loss of density-arrest is EGF dependent, similar to the transforming activities of RA and TGFβ [Van Zoelen et al., 1988]. To address this problem the kinetics of bradykinin stimulated [^{3}H]-TdR incorporation in serum-starved, quiescent NRK cells were investigated in the presence and absence of EGF (Fig. 6B). Addition of EGF to quiescent NRK cells induces a single round of DNA synthesis, after which the cells become density-arrested. However in the additional presence of bradykinin the cells continue for another round of DNA synthesis, which is indicative for the loss of density-dependent growth inhibition [Van Zoelen, 1991]. It is clear from the data in Figure 6B that bradykinin needs the presence of EGF for its transforming effect. When added alone, bradykinin is not able to induce [^{3}H]-TdR incorporation. These data are very similar to those obtained with TGFβ and RA as described in detail previously [Van Zoelen et al., 1988].

In conclusion, bradykinin displays a biphasic effect on density-arrested NRK cell growth. Initially bradykinin transiently inhibits the growth factor induced loss of density-arrest. However, in the additional presence of EGF, extended incubation periods with bradykinin cause the phenotypic transformation of these cells.

DISCUSSION

The bioactive lipid LPA has obtained much attention in recent years for its role in fibroblast proliferation [Van Corven et al., 1989, 1992] and regulation of cytoskeletal elements [Ridley and Hall, 1992; Edwards et al., 1993; Jalink et al., 1993]. However, the intracellular signals that

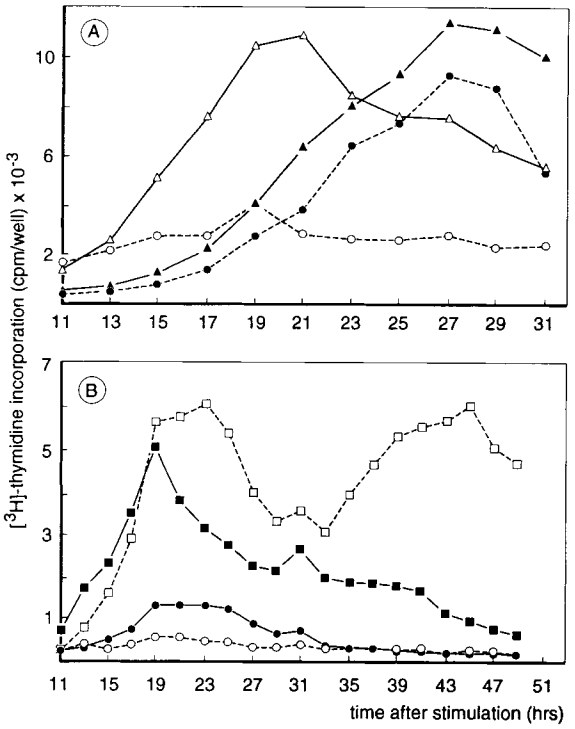

Fig. 6. Kinetics of the growth stimulatory and inhibitory effect of bradykinin. **A:** Time course of the [^{3}H]-TdR incorporation (2 h pulses) into density-arrested NRK cells after the addition of 100 μM LPA (△—△), 100 μM LPA, and 1 μM bradykinin (▲—▲), 1 μM bradykinin (●---●), or without stimulus (○---○). **B:** Time course of the [^{3}H]-TdR incorporation (2 h pulses) into serum-starved, quiescent NRK cells after the addition of 5 ng/ml EGF (■—■) 5 ng/ml EGF, and 1 μM bradykinin (□---□), 1 μM bradykinin (●—●), or no stimulus (○---○). Values represent the mean of triplicate determinations (standard deviation within 15% of mean value).

convey the LPA actions still remain obscure. In the present study the intracellular effects of LPA were compared with those of bradykinin. Bradykinin is a small peptide with a wide range of reported biological effects including neurotransmission, pain perception, smooth muscle contraction, and cell proliferation [Roberts, 1989]. Bradykinin mimics many of the LPA induced intracellular responses, including PI-hydrolysis, Ca^{2+}-mobilization, and AA-release as described in this paper and previously by others [Van Corven et al., 1989; Jalink et al., 1990]. However their effect on density-arrested NRK cell proliferation is completely opposite. LPA induces the loss of density-arrest in NRK cells, while this process is strongly inhibited by bradykinin. The differential growth effects of LPA and bradykinin could not be correlated to differences in PI-hydrolysis, Ca^{2+}-mobilization, or AA-release. The specific effect of LPA on forskolin stimulated cAMP levels was not counteracted by bradykinin, indicating that the growth inhibitory effect of bradykinin is not mediated by cAMP. Although the inhibitory action of bradykinin is not correlated with the level of AA per se, it appears to be mediated through the formation of a specific AA metabolite by PGH synthase. Other studies on the inhibitory effect of bradykinin on fibroblast proliferation have also indicated the involvement of a PGH synthase product in this process [Straus and Pang, 1984; Patel and Schrey, 1992; McAllister et al., 1993a]. The exact nature of the PGH synthase product has not been characterized yet, but externally added prostaglandins tested so far did not mimic the bradykinin inhibition of NRK cells. This is in contrast to the observations of Patel and Schrey [1992], and McAllister et al. [1993a]. In both studies bradykinin could be replaced by externally added prostaglandin E_2 to elicit an inhibitory effect on fibroblast cell growth. This indicates that in NRK cells another prostaglandin is involved in the inhibitory action of bradykinin. Current studies are underway to identify this prostaglandin.

An intriguing observation is the specificity of the bradykinin inhibition for density-arrested cells. McAllister et al. [1993b] have also reported a cell density-dependent inhibitory effect of bradykinin on human gingival fibroblasts, but not as strict as in NRK cells. These authors correlate the bradykinin responsiveness to the increase in bradykinin receptor numbers at increasing cell densities. It remains to be investigated whether the bradykinin receptor number increases upon density-arrest in NRK cells, but bradykinin induced PI-hydrolysis and AA-release are similar in both quiescent and density-arrested cells. Another option is an induction of a different bradykinin receptor subtype when NRK cells become density-arrested. In general, bradykinin receptors are defined in two subtypes. The type 2 subtype is widely expressed in many cell types and seems to be responsible for the bradykinin induced PI-responses and stimulation of cell growth [Roberts, 1989; Roberts and Gullick, 1989]. The type 1 subtype is in general not expressed, but its involvement in bradykinin mediated inhibition of human breast fibroblasts has been reported [Patel and Schrey, 1992]. The exact contribution of either subtype in the inhibition of NRK cells has not been established yet [Van Zoelen et

al., 1994]. Regulation of the phosphorylation state of the bradykinin receptor [Jong et al., 1993] or regulation of processes downstream of the receptor upon density-arrest can also be responsible for the specificity of the bradykinin inhibition. However at present not much is known about these processes and possible modes of regulation.

Upon prolonged incubation bradykinin induces the loss of density-dependent growth arrest of NRK cells. The mechanism by which bradykinin phenotypically transforms NRK cells is similar to that of RA and TGFβ, although the kinetics are much slower [Van Zoelen et al., 1988]. The EGF dependence of this transforming effect of bradykinin suggests that bradykinin, like RA and TGFβ, upregulates EGF receptor numbers to release the cells from density-arrest. ^{125}I-EGF binding is indeed increased upon bradykinin treatment of density-arrested NRK cells (unpublished observation). Stimulation of PI-turnover might be responsible for this effect on EGF receptor levels and the concomitant loss of density-arrest. The correlation between agents linked to the phosphoinositide signaling system and upregulation of EGF receptors has been reported previously by Earp et al. [1988]. The observation that $PGF_{2\alpha}$ is also able to induce loss of density-arrest, in a EGF dependent manner, with the same kinetics as bradykinin (data not shown) further support this hypothesis. LPA also increases ^{125}I-EGF binding in density-arrested cells (unpublished observation). However, the phenotypic transformation of NRK cells is not dependent on EGF. This suggests that the transforming effect of LPA is mediated through another, as-yet-unidentified pathway.

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