Inhibition of DNA Synthesis and Growth in Human Breast Stromal Cells by Bradykinin: Evidence for Independent Roles of B₁ and B₂ Receptors in the Respective Control of Cell Growth and Phospholipid Hydrolysis¹

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ABSTRACT

The paracrine and intracellular mechanisms controlling stromal cell growth in the normal or neoplastic breast are unknown. This in vitro study uses human breast fibroblasts to investigate a potential role for the inflammatory peptide mediator bradykinin (BK) in the regulation of DNA synthesis and signal transduction in these cells. Bradykinin stimulated a dose-dependent increase in inositol lipid hydrolysis and cytosolic Ca²⁺ levels in serum-starved fibroblasts derived from both normal and breast tumor tissue. Bradykinin also caused a dose-dependent decrease in cell growth and [³²P]thymidine incorporation into DNA in breast fibroblasts. Bradykinin inhibited this mitogenic effect of EGF but not that due to insulin-like growth factor 1. The binding of [¹²⁵I]EGF to fibroblasts was also inhibited by BK. Prostaglandin E₂ also inhibited fibroblast DNA synthesis, and the cyclooxygenase inhibitor indomethacin partially reversed the inhibitory action of BK on DNA synthesis. Studies with BK receptor antagonists and agonists indicate that inositol lipid signalling and arachidonic acid mobilization in response to BK are B₂ receptor-mediated pathways, whereas the inhibition of DNA synthesis appears to be via B₁ receptors. Although these data support a role for prostaglandins and EGF receptor down-regulation in the inhibitory action of BK on DNA synthesis in breast fibroblasts, a BK receptor-mediated pathway is also implicated. This study highlights a potential pathophysiological role for BK as a negative regulator of breast stromal cell growth.

INTRODUCTION

Many human tumors, particularly those of the breast, are characterized by an intense fibrotic response which has been attributed to an increased stromal cell population (1). However, the cellular mechanisms regulating this stromal cell proliferation are unknown. Although breast stromal cells are thought to play an important paracrine role in determining the proliferation and differentiation states of neighboring epithelial cells in both normal and neoplastic breast tissue (2–5), convincing evidence also suggests that epithelial-stromal cell interactions in the breast are bidirectional (1, 3). Furthermore, a potential role exists for the action of both paracrine and autocrine growth factors on stromal cells, since breast cancer cells produce a variety of growth factors, including EGF and IGF-1, which have been implicated in mediating responses such as collagen formation and DNA synthesis in fetal lung fibroblasts (14) and more recently prostaglandin synthesis in rabbit dermis fibroblasts (17) and murine osteoblasts (18). The B₁ receptor appears to be inducible in some tissues as a result of growth factor stimulation (14), and cell division (10) are mediated via B₂ receptors. Several mammalian cell types also express B₁ receptors which have been implicated in mediating responses such as collagen formation and DNA synthesis in fetal lung fibroblasts (14) and more recently prostaglandin synthesis in rabbit dermis fibroblasts (17) and murine osteoblasts (18). The B₁ receptor appears to be inducible in some tissues as a result of an inflammatory response (19).

Although polypeptide growth factors and regulatory peptides appear to activate independent intracellular signalling systems, these multiple pathways may converge or interact, resulting in synergism of various cellular responses including proliferation (20–22). Indeed, regulatory peptides that activate the inositol signalling pathway often require an additional, synergizing growth factor to evoke a mitogenic response. The present study seeks to increase our knowledge of the regulatory mechanisms controlling breast stromal cell growth by monitoring DNA synthesis in human breast fibroblasts in response to BK and the growth factors EGF and IGF-1. We describe here a potent stimulation of BK by EGF to increase Ca²⁺ mobilization in breast fibroblasts. Surprisingly, this activation of putative mitogenic signalling by BK is accompanied by an inhibition of both basal and EGF-stimulated DNA synthesis in these cells.


[3] The abbreviations used are: EGF, epidermal growth factor; BK, bradykinin; IGF-1, insulin-like growth factor 1; TPA, 12-O-tetradecanoylphorbol-13-acetate; DMEM, Dulbecco’s modified essential medium; HEPES, (S)-2-amino-2-(hydroxymethyl)-1-propanesulfonic acid; FCS, fetal calf serum; EMEM, Eagle’s minimal essential medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HBS, Hanks’ balanced salt solution; FURA 2-AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-onyl]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetracetic acid, pentaacetoxymethyl ester; PGD₂, prostaglandin D₂; PGF₂α, prostaglandin E₂; PGE₂, prostaglandin E₂; PGF₂α, prostaglandin F₂α.
MATERIALS AND METHODS

Preparation of Human Breast Fibroblasts. Human breast fibroblasts were prepared by a modification of methods previously described (5) and grown from either surgically removed malignant breast tumors or from tissue obtained from women undergoing reductive mammoplasty. Tissue obtained at surgery was immediately transported to the laboratory in an ice-cold mixture (1:1) comprising DMEM and Ham's F-10 were prepared by a modification of methods previously described (5).

MATERIALS AND METHODS

Excess fat was removed prior to mincing. The resulting minced tissues were then treated with DMEM:Ham's F-10 mixture containing 200 units/ml of collagenase type II and 0.2% fetal calf serum (v/v). All cell lines obtained after subculture were subsequently grown to confluence, which usually occurred within 14 days. Cells were then subcultured into 6-cm dishes at a 1:2 or 1:4 split and were then subsequently grown in complete EMEM, buffered with 20 mM HEPES (pH 7.4), and supplemented with glutamine (2 mM), bicarbonate (4.5 mM), and containing 10% FCS (v/v). All cell lines obtained after subculture (HBF 1-5) exhibited typical fibroblast-like morphology at confluence, forming whorls and parallel bundles comprising spindle-shaped cells. Cells with epithelial morphology were absent. Immunohistochemical staining for marker indicated the presence of fibronectin and the absence of the epithelial tonofilament marker LE61.

Measurement of [3H]thymidine Incorporation into Fibroblast DNA. Human breast fibroblasts grown in 75-cm² flasks between passages 3 and 7 were used to seed into 3.8-cm² 12-well multiplates at a density of approximately 5 x 10⁵ cells per well, in complete EMEM medium, containing additional bicarbonate at 26 mM. Cells were maintained at 37°C and under 5% CO₂ in humidified air until they were confluent. Once confluence was reached, cells were washed once with 2 ml PBS and incubated in 2 ml of serum-free EMEM for 48 h. After this period, the medium was removed, and 1 ml of the respective treatments was added, again in serum-free EMEM for 18 h. Four h prior to the end of this incubation period, the cells were pulsed with [3H]thymidine (1 μCi/ml), added in a volume of 25 μl. Incubations were terminated by aspirating off the medium and washing the cells once with 0.5 ml of ice-cold PBS, twice with 0.5 ml of 10% (w/v) trichloroacetic acid, and once with 0.5 ml of methanol. The resulting precipitate material was solubilized over a 20-min period at 24°C with 0.5 M NaOH. After neutralization with 0.5 M HCl, aliquots were removed and counted for radioactivity.

Cell Proliferation. Near-confluent human breast fibroblasts grown in 75-cm² flasks were used to seed into 3.8-cm² 12-well multiplates at a density of approximately 5 x 10⁴ cells per well, in complete EMEM medium, containing additional bicarbonate at 26 mM. Cells were maintained at 37°C and under 5% CO₂ in humidified air until they were confluent. Once confluence was reached, cells were washed once with 2 ml PBS and incubated in 2 ml of serum-free EMEM for 48 h. After this period, the medium was removed, and 1 ml of the respective treatments was added, again in serum-free EMEM for 18 h. Four h prior to the end of this incubation period, the cells were pulsed with [3H]thymidine (1 μCi/ml), added in a volume of 25 μl. Incubations were terminated by aspirating off the medium and washing the cells once with 0.5 ml of ice-cold PBS, twice with 0.5 ml of 10% (w/v) trichloroacetic acid, and once with 0.5 ml of methanol. The resulting precipitate material was solubilized over a 20-min period at 24°C with 0.5 M NaOH. After neutralization with 0.5 M HCl, aliquots were removed and counted for radioactivity.

To a subsequent 5-min incubation with or without BK. Aliquots of the incubation medium were then counted to measure release of radiolabel from the cells.

Measurement of Cytosolic Ca²⁺, [Ca²⁺]i. Confluent fibroblast cultures were harvested by trypsinization and resuspended in Tyrodes' salt solution containing 10 mM HEPES (pH 7.4), Ca²⁺ at 1.0 mM, and 6 μM FURA 2-AM. After a loading period of 30 min at 37°C the cell suspension was centrifuged and resuspended in fresh Tyrodes' solution. Fluorescence measurements were performed in a PTI Deltascan dual-wavelength spectrofluorimeter using previously described techniques (25).

Expression of Data. All values are presented as the means ± SD from individual representative experiments. Statistical evaluation of the data when comparing control groups with treatment groups was by the Students t test. Comparisons between the individual means of different treatment groups were analyzed by analysis of variance followed by a t test.

Materials. All BK-related peptides, mouse EGF, FURA 2-AM, and TPA were obtained from Sigma Chemical Company (United Kingdom). Human recombinant IGF-1 was purchased from Bachem, Ltd. (United Kingdom). [123I]-labeled mouse EGF (96 μCi/μg), [methyl-3H]thymidine (40 to 60 Ci/mm), [1-14C]arachidonic acid (50 to 60 Ci/mm), and myo-2-14H]inositol were purchased from Amer sham International (United Kingdom). Dowex 1 × 8 (200 to 400 mesh) anion-exchange resin was obtained from Bio-Rad Laboratories (United Kingdom). All tissue culture media and cell culture products were obtained from Flow Laboratories, Ltd. (United Kingdom). All other chemicals and reagents were of analytical reagent AR grade and were obtained from Fison (United Kingdom).

RESULTS

Bradykinin Inhibits DNA Synthesis and Proliferation in Human Breast Fibroblasts. Incubation of serum-starved human breast fibroblasts with BK for 24 h consistently caused a dose-related inhibition of [3H]thymidine incorporation into trichloroacetic acid-precipitable material in all cell lines examined (Table 1). This inhibitory effect was evident in fibroblasts obtained from both normal (HBF-1, -3, -4) and malignant (HBF-2 and -5) breast tissue (Table 1). Inhibition was maximal at 100 nM with a 50% inhibitory concentration around 15 nM (Fig. 1). At lower concentrations of BK (1 nM) a small stimulation of DNA synthesis was sometimes observed (Fig. 1). Fetal calf serum consistently stimulated DNA synthesis in breast fibroblasts (Table 1). Bradykinin also inhibited breast fibroblast
Bradykinin Inhibits EGF-stimulated DNA Synthesis and 125I-EGF Binding. Both EGF and IGF-1 were mitogenic for human breast fibroblasts, as indicated by an increased [3H]thymidine incorporation into DNA (Fig. 3). Bradykinin caused a dose-dependent inhibition of EGF-stimulated DNA synthesis which was significant at 0.1 nM and maximal at 1 μM (Fig. 3). The EGF-induced increase in [3H]thymidine incorporation was reduced by 10-fold in the presence of BK (1 μM), the respective changes due to EGF in the absence and presence of BK (1 μM) being 5317 ± 758 and 506 ± 135 cpm/well (mean ± SD, n = 3). The mitogenic effect of IGF-1 was unaffected in the presence of BK (Fig. 3).

In mammalian fibroblasts the binding of 125I-EGF to its cellular receptors is inhibited by agonists which activate protein kinase C (see Ref. 26). The phorbol ester TPA, a potent activator of protein kinase C, inhibited 125I-EGF binding in breast fibroblasts by 70% (Fig. 4). Bradykinin also inhibited 125I-EGF binding in breast fibroblasts at the same concentrations as the inhibition of EGF-stimulated DNA synthesis by BK with maximal inhibition of 38% observed at 1 μM (Fig. 4).

Effect of Indomethacin and Prostaglandins on Fibroblast DNA Synthesis. Treatment of breast fibroblasts with the cyclooxygenase inhibitor indomethacin partially reversed the inhibition of [3H]thymidine incorporation caused by BK (Fig. 5), while also reducing basal DNA synthesis at high concentrations. In three separate experiments BK-induced inhibition of DNA synthesis was reversed from 43 ± 13% inhibition to 16 ± 2%.

### Table 1 Effect of bradykinin and FCS on [3H]thymidine incorporation into DNA in human breast fibroblast lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Age (days)</th>
<th>Control</th>
<th>0.01</th>
<th>1</th>
<th>10% FCS</th>
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<tbody>
<tr>
<td>HBF-2</td>
<td>24</td>
<td>5.0 ± 0.1</td>
<td>5.6 ± 0.3</td>
<td>3.3 ± 0.7(a)</td>
<td>49.8 ± 0.9(b)</td>
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<tr>
<td>HBF-3</td>
<td>45</td>
<td>3.6 ± 0.5</td>
<td>2.8 ± 0.2</td>
<td>1.8 ± 0.1(a)</td>
<td>12.3 ± 2.2(a)</td>
</tr>
<tr>
<td>HBF-4</td>
<td>61</td>
<td>3.8 ± 0.1</td>
<td>2.5 ± 0.2(b)</td>
<td>2.1 ± 0.1(a)</td>
<td>31.2 ± 1.0(b)</td>
</tr>
<tr>
<td>HBF-5</td>
<td>49</td>
<td>8.9 ± 0.8</td>
<td>7.2 ± 0.4(c)</td>
<td>3.7 ± 0.3(b)</td>
<td>20.2 ± 1.6(b)</td>
</tr>
</tbody>
</table>

* Mean ± SD of triplicate determinations from five different HBF lines.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.75 ± 0.18(a)</td>
<td>3.07 ± 0.52</td>
</tr>
<tr>
<td>Bradykinin (10^-9 m)</td>
<td>1.21 ± 0.10(a)</td>
<td>1.5 ± 0.18(b)</td>
</tr>
<tr>
<td>Bradykinin (10^-8 m)</td>
<td>0.98 ± 0.13(b)</td>
<td>1.52 ± 0.18(b)</td>
</tr>
<tr>
<td>Des-Arg^9 bradykinin (10^-8 m)</td>
<td>3.54 ± 0.33</td>
<td>2.12 ± 0.06(c)</td>
</tr>
<tr>
<td>Des-Arg^9 bradykinin (10^-7 m)</td>
<td>2.97 ± 0.17(b)</td>
<td>4.69 ± 0.58(c)</td>
</tr>
</tbody>
</table>

* Mean ± SD.

Fig. 1. Dose response of bradykinin-induced changes in inositol monophosphate production and thymidine incorporation in human breast fibroblasts, HBF-3. Inositol monophosphate production (®) was measured during a 60-min incubation and [3H]thymidine incorporation (©) was determined after 18 h (see "Materials and Methods" for details). Points, mean of triplicate determinations; bars, SD. * P < 0.05; † P < 0.01; ‡ P < 0.001 compared with values obtained in the absence of bradykinin.

proliferation during a 7-day growth period, whereas fetal calf serum stimulated cell growth (Table 2). No net cell loss was apparent during BK exposure at the end of this growth period.

Bradykinin Stimulates Inositol Lipid Hydrolysis and [Ca2+]i. Inositol monophosphate production in response to BK was monitored in [3H]inositol-labeled breast fibroblasts and exhibited a dose-dependent increase (Fig. 1). Inositol lipid hydrolysis was maximal at 100 nM with a 50% effective dose around 1 nM (Fig. 1). Bradykinin also stimulated increases in inositol trisphosphate production, the respective values (mean ± SD) for control, 0.1 nM and 1 μM BK being 1.0 ± 0.2, 1.5 ± 0.1 (P < 0.02), and 4.5 ± 0.5 (P < 0.001), 10^-9 × cpm/incubation.

Changes in [Ca2+]i were measured in breast fibroblasts in response to bradykinin by monitoring FURA-2 fluorescence. After bradykinin administration there was a rapid transient increase in free Ca2+ (Fig. 2). This was dose dependent with regard to both onset and peak Ca2+ levels attained (Fig. 2).

**INHIBITION OF DNA SYNTHESIS BY BRADYKININ**

Bradykinin inhibits DNA synthesis in human breast fibroblast lines, as described in "Materials and Methods." HBF-1, -3, and -4 were derived from normal breast tissue. HBF-2 and -5 were derived from malignant breast tissue.
INHIBITION OF DNA SYNTHESIS BY BRADYKININ

Effect of bradykinin on growth factor-stimulated DNA synthesis. HBF-3 cells were treated for 18 h with increasing concentrations of bradykinin in the absence (○) and presence of EGF, 10 ng/ml (O), or IGF-1, 10 ng/ml (●). [3H]Thymidine incorporation was measured as described in "Materials and Methods." Points, mean of triplicate determinations and expressed as a percentage of the control values (12,317 ± 2,237 cpm/well) obtained in the absence of bradykinin or growth factor; bars, SD. *P < 0.05; **P < 0.01; ***P < 0.001 compared with control; #, P < 0.05; ##, P < 0.01; ###, P < 0.001 compared with EGF alone.

Bradykinin-stimulated total inositol phosphate production was significantly reduced in the presence of d-Arg-[Hyp5,Thr5,8,D-Phe7] BK (Fig. 7A). Basal inositol lipid hydrolysis was unaffected by either the B2 receptor antagonist or the B1 receptor agonist Des-Arg9-BK (Fig. 7A). Bradykinin also stimulated arachidonic acid release from [3H]arachidonic acid-prelabeled fibroblasts, whereas the B1 agonist had no effect (Fig. 7B). Epidermal growth factor, while having no effect on basal arachidonic acid release, potentiated the response to BK (Fig. 7B).

Effect of BK-related Peptides on DNA Synthesis and Phospholipid Hydrolysis. The B2 receptor antagonist D-Arg-[Hyp5,Thr5,8,D-Phe7] BK had no effect on the ability of BK to inhibit [3H]thymidine incorporation into breast fibroblasts (Fig. 6A). This B2 receptor antagonist alone caused a small stimulation of DNA synthesis (Fig. 6A). In contrast the B1 receptor antagonist Des-Arg9-BK, like bradykinin, also inhibited [3H]thymidine incorporation (Fig. 6B). Nonetheless, the inhibitory effect of bradykinin on DNA synthesis was reversed in the presence of the B1 receptor antagonist (Fig. 6B).

The B1 receptor agonist Des-Arg9-bradykinin had no effect at 10 nM but was equipotent with bradykinin in inhibiting [3H]thymidine incorporation at 1 μM (Fig. 6C). Similarly in another fibroblast line (HBF-5) derived from malignant breast tissue, Des-Arg9-BK (1 μM) also reduced basal [3H]thymidine incorporation from 8615 ± 942 to 4417 ± 224 cpm/incubation (mean ± SD, n = 3, P < 0.01). Furthermore, cell proliferation was also inhibited by the B1 agonist at 1 μM but not at 10 nM (see Table 2).

Bradykinin-stimulated total inositol phosphate production was significantly reduced in the presence of d-Arg-[Hyp5,Thr5,8,D-Phe7] BK (Fig. 7A). Basal inositol lipid hydrolysis was unaffected by either the B2 receptor antagonist or the B1 receptor agonist Des-Arg9-BK (Fig. 7A). Bradykinin also stimulated arachidonic acid release from [3H]arachidonic acid-prelabeled fibroblasts, whereas the B1 agonist had no effect (Fig. 7B). Epidermal growth factor, while having no effect on basal arachidonic acid release, potentiated the response to BK (Fig. 7B).

Table 3 Effect of prostaglandins on [3H]thymidine incorporation into DNA in human breast fibroblasts

<table>
<thead>
<tr>
<th>[3H]Thymidine incorporation (cpm/well)</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8,944 ± 753</td>
<td>9,824 ± 1,179</td>
<td>8,615 ± 942</td>
</tr>
<tr>
<td>Bradykinin (10^(-8) M)</td>
<td>7,189 ± 364</td>
<td>4,934 ± 1,111 (a)</td>
<td>6,005 ± 929 (b)</td>
</tr>
<tr>
<td>Bradykinin (10^(-7) M)</td>
<td>3,269 ± 311 (c)</td>
<td>3,771 ± 259 (a)</td>
<td>6,776 ± 306 (b)</td>
</tr>
<tr>
<td>PGE2 (10^(-8) M)</td>
<td>7,239 ± 713</td>
<td>6,927 ± 548 (d)</td>
<td>3,064 ± 449 (c)</td>
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<tr>
<td>PGE2 (10^(-7) M)</td>
<td>10^(-8) M</td>
<td>7,239 ± 941</td>
<td>10^(-9) M</td>
</tr>
<tr>
<td>PGE2 (10^(-6) M)</td>
<td>9,253 ± 487</td>
<td>11,311 ± 971</td>
<td>5,483 ± 1,297</td>
</tr>
<tr>
<td>PGE2 (10^(-5) M)</td>
<td>7,790 ± 103</td>
<td>11,311 ± 971</td>
<td>5,724 ± 2,276</td>
</tr>
</tbody>
</table>

*Mean ± SD of triplicate determinations from three representative experiments.

Significant differences in comparison with control values are indicated as P < 0.01 (a), P < 0.05 (b), P < 0.001 (c), and P < 0.02 (d).
compared with values obtained in the presence of the B2 antagonist alone. In A, bradykinin was present at 10 nM, and the B2 antagonist Des-Arg'-bradykinin was present at 1 μM. *P < 0.05 compared with values obtained in the absence of either peptide; t, P < 0.05 compared with values obtained in the presence of the B2 antagonist alone. In B, bradykinin was present at 10 nm, and the B2 antagonist Des-Arg^[8]-bradykinin was present at 5 μM. *, P < 0.02; **, P < 0.001 compared with values obtained in the absence of either peptide. C, comparative effect of bradykinin and des-Arg^[8] bradykinin. *P < 0.001 compared with values obtained in the absence of peptide. Columns, mean; bars, SD.

DISCUSSION

In the present study with human breast fibroblasts, BK was weakly mitogenic at low concentrations (1 nm), but at 10 nm and above a dose-dependent inhibition of DNA synthesis was observed, with up to 50% reduction of basal activity seen in the presence of 1 μM BK. This inhibitory effect was seen in all breast fibroblast cell lines studied, whether derived from normal or malignant tissue. In contrast, EGF, IGF-1, and fetal calf serum, which was used as a positive control in all experiments monitoring [3H]thymidine incorporation, all consistently stimulated DNA synthesis in human breast fibroblasts. Furthermore, our studies on cell growth demonstrated a respective inhibition and stimulation of breast fibroblast proliferation by BK and fetal calf serum, confirming our observations on DNA synthesis. No cell loss was apparent during exposure to BK in the growth studies, suggesting a cytostatic rather than cytotoxic effect of BK.

The reported effects of BK on mammalian fibroblast cell growth have been varied. Bradykinin has been shown to be mitogenic in human dermal fibroblasts (27), rabbit dermal fibroblasts (17), and murine Swiss 3T3 cells (28). In contrast, BK alone was reported to have no effect on DNA synthesis in human amniotic fluid-derived fibroblasts (17), human dermal fibroblasts (12), or IMR-90 human fetal lung fibroblasts (10). Nonetheless, an inhibitory effect of BK on [3H]thymidine incorporation was clearly evident at 1 μM in the latter study (10). Although the basis of this differential action of BK in mammalian fibroblasts is unknown, it presumably reflects differences in species, tissue, and/or culture conditions (e.g., presence of serum, insulin, or other growth factors). Similar differential effects of histamine on the stimulation (29) and inhibition (30) of DNA synthesis in human skin fibroblasts have also been recently reported. Thus, BK is not unique in its ability to inhibit DNA synthesis in human fibroblasts, since histamine (via an H1 receptor) also inhibited [3H]thymidine incorporation in a human skin fibroblast cell line (30).

Signal transduction pathways which have been implicated in the action of mitogenic peptides such as BK, vasopressin, and bombesin include phospholipase C-mediated inositol lipid hydrolysis (see Ref. 31). Activation of this pathway leads to the potential respective elevation of cytosolic Ca++ levels (32) and concomitant stimulation of protein kinase C (33) by the two second messengers generated in response to phosphatidylinositol 4,5-bisphosphate hydrolysis (inositol 1,4,5-trisphosphate and diacylglycerol). Human breast fibroblasts exhibited a marked dose-dependent increase in both inositol lipid hydrolysis and [Ca++]i in response to BK. Despite this potent activation of putative mitogenic signals by BK, this response was always accompanied by an inhibition of DNA synthesis. Stimulation of DNA synthesis in human breast fibroblasts by EGF was also progressively inhibited in a dose-dependent manner by BK. However, IGF-1-stimulated [3H]thymidine incorporation was unaffected by BK. This differential sensitivity of growth factor action to inhibition by BK again suggests that this is not a general cytotoxic effect of BK on human breast fibroblasts.

One possible mechanism mediating this inhibition of EGF action could be via a decrease in EGF receptor number, since BK did indeed significantly decrease the specific binding of 125I-EGF to human breast fibroblasts. Bradykinin has also been recently reported to block the mitogenic action of EGF on human fetal lung fibroblasts. However, this effect was not accompanied by changes in EGF receptor occupancy (13).

Fig. 6. Effect of bradykinin-related peptides on DNA synthesis in breast fibroblasts. HBF-4 cells were incubated with or without bradykinin antagonists as indicated and in the absence (open bars) or presence of bradykinin (hatched bars) or Des-Arg^[8] bradykinin (dotted bars) for 18 h. [3H]Thymidine incorporation into DNA was measured during the last 4 h of treatment (see Materials and Methods for details). In A, bradykinin was present at 10 nM, and the B2 antagonist Des-Arg^[8]-bradykinin (B2 agonist) was present at 10 μM, *, P < 0.05 compared with values obtained in the absence of either peptide; t, P < 0.05 compared with values obtained in the presence of the B2 antagonist alone. In B, bradykinin was present at 10 nM, and the B2 antagonist Des-Arg^[8]-Leu^[6] bradykinin was present at 5 μM. *, P < 0.02; **, P < 0.001 compared with values obtained in the absence of either peptide. C, comparative effect of bradykinin and des-Arg^[8] bradykinin. *P < 0.001 compared with values obtained in the absence of peptide. Columns, mean; bars, SD.

Fig. 7. Effect of bradykinin-related peptides on inositol lipid hydrolysis (A) and arachidonic acid release (B) in breast fibroblasts. Fibroblasts were incubated in the absence (open bars) or presence of bradykinin (hatched bars) or des-Arg^[8]-bradykinin (dotted bars). In A, inositol monophosphate production in HBF-4 cells was measured during a 60-min incubation as described in Materials and Methods. Bradykinin, Des-Arg^[8]-bradykinin (B2 agonist), and Des-Arg^[8]-bradykinin (B2 antagonist) were present at 10 nM, 8.6 μM, and 1 μM, respectively. *, P < 0.001 compared with basal values in the absence of peptide; **, P < 0.001 compared with bradykinin alone. In B, arachidonic acid release from HBF-5 cells prelabeled with [3H]arachidonic acid was measured in response to a 5-min incubation with or without bradykinin (1 μM) or Des-Arg^[8]-bradykinin (1 μM) after pretreatment with or without EGF (10 ng/ml). *, P < 0.01 compared with control in the absence of peptides; **, P < 0.01 compared with bradykinin alone. Columns, mean; bars, SD.
Nonetheless, agonists which lead to protein kinase C activation are known to inhibit EGF binding in a variety of cell types. Indeed, TPA, a potent activator of protein kinase C, also caused a marked decrease in \(^{125}\)I-EGF binding in breast fibroblasts. Some isoforms of protein kinase C may be selectively activated by unsaturated fatty acids (34) and, in this regard, BK is known to mobilize free arachidonic acid, following phospholipase A\(_2\) activation, in various cell types (35, 36) including human breast fibroblasts in the present study.

An alternative well-established fate for arachidonic acid is, of course, conversion to various eicosanoid products. Prostaglandins have been previously implicated as negative regulators during growth stimulation of human diploid fibroblasts by BK (10, 14) or EGF (37). Indeed, BK is a potent stimulator of prostaglandin production in many cell types including human fibroblasts (14). In this regard it is significant that the cyclooxygenase inhibitor indomethacin did partially reverse the inhibitory effect of BK on DNA synthesis in the present study. Furthermore, prostaglandin E\(_2\) also mimicked the inhibitory effect of BK on breast fibroblast DNA synthesis. Our present observations are thus consistent with a role for prostaglandins as inhibitory mediators of the BK-induced inhibition of DNA synthesis in human breast fibroblasts. Indeed, BK also stimulated prostaglandin precursor release in breast fibroblasts. This effect was potentiated by EGF. Although the biochemical basis for this synergism is unknown, EGF receptor-associated tyrosine kinase (38) has been recently implicated in the regulation of phospholipase A\(_2\). Similar synergistic responses have been previously reported on arachidonic acid release from rat mesangial cells in response to vasopressin and EFG (39).

Bradykinin receptors have been classified as B\(_1\) or B\(_2\) according to the relative potencies of a series of agonists and antagonists. The selectivity of the respective putative bradykinin B\(_1\) and B\(_2\) receptor antagonists Des-Arg\(^{9}\)[Leu\(^\text{IV}\)] BK and D-Arg-[Hyp\(^\text{V}\),Thi\(^{\text{IV}}\),D-Phe\(^{\text{V}}\)] BK has been previously established in a variety of receptor-specific systems (40–42). Inositol lipid hydrolysis and arachidonic acid release in breast fibroblasts in response to BK appear to be B\(_2\) receptor-mediated events, as evidenced by the ability of B\(_2\) receptor antagonist d-Arg[Hyp\(^{\text{V}},\text{Thi}^{\text{IV}},\text{D-Phe}^{\text{V}}\)] BK to inhibit BK-stimulated inositol phosphate production and the impotence of the B\(_1\) agonist Des-Arg\(^{9}\)-BK in effecting inositol phosphate production and arachidonate mobilization. On the other hand, the B\(_2\) receptor antagonist had no effect on BK-induced inhibition of DNA synthesis, whereas the B\(_1\) antagonist Des-Arg\(^{9}\)-[Leu\(^{\text{IV}}\)] BK reversed this inhibitory effect of BK. Furthermore, the B\(_1\) agonist Des-Arg\(^{9}\)-BK also inhibited DNA synthesis and growth in breast fibroblasts. These data thus tend to dissociate the activation of inositol lipid signalling by BK from its inhibition of DNA synthesis, the latter phenomenon appearing to be a B\(_2\) receptor-coupled pathway. Whether the inhibitory effect of BK on DNA synthesis and growth is partially mediated by Des-Arg\(^{9}\)-BK following the action of any endogenous kininase I is unknown. However, the fact that 10 nM BK consistently inhibited both DNA synthesis and growth, whereas Des-Arg\(^{9}\)-BK was without effect at this concentration, does not support such a role for kininase I in BK action on breast stromal cells. It is perhaps relevant to note that the B\(_1\) antagonist, Des-Arg\(^{9}\)-[Leu\(^{\text{IV}}\)] BK albeit at 5 \(\mu\)M also inhibited DNA synthesis in the present study. Similarly, this B\(_1\) antagonist also exhibits BK agonist activity in other systems such as histamine release by mast cells and norepinephrine release in the adrenal medulla (see Ref. 43 for review). This phenomenon has raised the question as to whether such effects are mediated by receptor-independent mechanisms (42, 43). In this regard, recent reports indicate that various basic peptides including BK may stimulate histamine release by interacting directly with G-proteins (44).

The nature of the intracellular mechanisms used by the B\(_1\) receptor to inhibit DNA synthesis in human breast fibroblasts remains unknown. Since BK-stimulated arachidonic acid release does not appear to be a B\(_1\) receptor-mediated event, any proposed role for eicosanoid production during the action of B\(_1\) agonists implies regulation distal to precursor release. Although increases in both prostaglandins and precursor release in response to BK occur via B\(_2\) receptors in a variety of cell types including fibroblasts (14, 36), recent studies have reported stimulation of prostaglandin production by Des-Arg\(^{9}\)-BK in rabbit dermis fibroblasts (17) and mouse osteoblast-like cells (18). Interestingly, in the latter study the temporal onset of BK-stimulated PGE\(_2\) synthesis was rapid (within minutes), whereas the response to Des-Arg\(^{9}\)-BK occurred only after 24 h (18), suggesting the existence of different intracellular mechanisms for B\(_1\) and B\(_2\) receptor-mediated prostaglandin production. Alternatively, certain actions of BK mediated by the arachidonic acid cascade appear to operate by mechanisms independent from either B\(_1\) or B\(_2\) receptors (42). We are continuing to investigate a role for prostaglandins during BK action on breast fibroblasts.

Finally, caution must be exercised when extrapolating any \textit{in vitro} finding to the \textit{in vivo} environment. Thus, whether our present observation on the regulation of breast fibroblast DNA synthesis and cell growth represents a real physiological/pathological role for BK in the normal/neoplastic human breast must remain speculative. A role for kinins in carcinoma of the breast is unknown. Nonetheless, inflammatory reactions may play an important role in the surveillance mechanisms used by the host against neoplasia. Indeed, patients with medullary breast carcinoma associated with abundant inflammatory stroma demonstrated the most favorable prognosis following tumor removal, compared with other histologically defined groups (45). Bradykinin is a key mediator of the body's response to trauma and is generated locally during inflammatory reactions at the site of tissue injury by the proteolytic cleavage of kininogen by kallikrein (46). Bradykinin action is often associated with prostaglandin production in target cells, along with other cellular responses related to inflammation, including vasodilation, increased vascular permeability, pain perception, and the regulation of stromal cell proliferation (see Ref. 46). Interestingly, BK has been previously reported to inhibit tumor growth \textit{in vivo}. Thus, intralesional injection of BK markedly inhibited the growth of spontaneous mammary tumors (MM46) and sarcomas (Sa180) in mice (47), as well as SV-40 virus-induced fibrosarcomas in hamsters (48). Our present findings also demonstrate a potential antimitogenic role for this inflammatory peptide mediator in the regulation of human breast stromal cell growth.

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