Bradykinin augments fibroblast-mediated contraction of released collagen gels

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Bradykinin augments fibroblast-mediated contraction of released collagen gels. Am J Physiol Lung Cell Mol Physiol 281: L164–L171, 2001.—Bradykinin is a multifunctional mediator of inflammation believed to have a role in asthma, a disorder associated with remodeling of extracellular connective tissue. Using contraction of collagen gels as an in vitro model of wound contraction, we assessed the effects of bradykinin tissue on remodeling. Human fetal lung fibroblasts were embedded in type I collagen gels and cultured for 5 days. After release, the floating gels were cultured in the presence of bradykinin. Bradykinin significantly stimulated contraction in a concentration- and time-dependent manner. Coincubation with phosphoramidon augmented the effect of 10⁻⁹ and 10⁻⁸ M bradykinin. A B₂ receptor antagonist attenuated the effect of bradykinin, whereas a B₁ receptor antagonist had no effect, suggesting that the effect is mediated by the B₂ receptor. An inhibitor of intracellular Ca²⁺ mobilization abolished the response; addition of EGTA to the culture medium attenuated the contraction of control gels but did not modulate the response to bradykinin. In contrast, the phospholipase C inhibitor U-73122 and the protein kinase C inhibitors staurosporine and GF-109203X attenuated the responses. These data suggest that by augmenting the contractility of fibroblasts, bradykinin may have an important role in remodeling of extracellular connective tissue.

In the airways, peribronchial fibrosis is a feature of both asthma and chronic bronchitis. This lesion may lead to the narrowing of small airways and could contribute to the fixed airflow limitation that compromises respiratory function. Fibroblasts are known to generate a traction force and to participate in tissue rearrangement (15, 39). This suggests that modulation of fibroblast contraction by inflammatory mediators like bradykinin might lead to altered tissue structure.

An in vitro model for extracellular matrix rearrangement is the three-dimensional system of fibroblasts cultured in a native collagen gel (3, 11, 40). When fibroblasts are cultured in such a collagen gel, the gels are contracted by the traction force that fibroblasts generate. Platelet-derived growth factor, transforming growth factor-β, and fetal calf serum (FCS) have been known to augment the contraction (3, 21, 34), whereas β-adrenergic agonists and PGE₂ inhibit contraction (10, 31). In the present study, the effect of bradykinin on fibroblast contractility was investigated with this model. The results indicate that bradykinin can augment fibroblast-mediated gel contraction. Bradykinin therefore may affect the fibrotic process by augmenting fibroblast contractility and thus could contribute to the formation of abnormal tissue architecture as well as to acute inflammation.

METHODS

Materials. Type I collagen (rat tail tendon collagen, RTTC) was extracted from rat tail tendons by a previously published method (11). Briefly, tendons were excised from rat tails, and the tendon sheath and other connective tissues were removed carefully. After repeated washing with phosphate-buffered saline (GIBCO BRL) and 95% ethanol, type I collagen was extracted in 4 mM acetic acid. Protein concentration was determined by weighing a lyophilized aliquot from each lot of collagen solution. SDS-PAGE routinely demonstrated no detectable proteins other than type I collagen.

Bradykinin, d-Arg-[Hyp⁵,Thi⁸,9,D-Phe⁷]-bradykinin, Nα-adamantanacetyl-d-Arg-[Hyp⁵,Thi⁸,9,D-Phe⁷]-bradykinin, des-Arg⁸,[Leu⁹]-bradykinin, phosphoramidon, indomethacin, nordihydroguaiaretic acid (NDGA), pertussis toxin (PTX), cholera toxin, staurosporine, phorbol 12-myristate 13-acetate...
that the final mixture resulted in 0.75 mg/ml of collagen, 3.3 as described previously (32). Briefly, RTTC, distilled water, 105 cells/ml, a physiological ionic strength, and 1 SE of three separate determinations determined from conditions can affect the gel contraction, all data shown in number of cell passages, the batch of RTTC, and culture triplicate gels within each experiment. Inasmuch as the area measured immediately after release.

The results are expressed as the percentage of each gel compared with the area measured immediately after release.

Statistical evaluation. The results are expressed as means ± SE of three separate determinations determined from triplicate gels within each experiment. Inasmuch as the number of cell passages, the batch of RTTC, and culture conditions can affect the gel contraction, all data shown in each figure were taken from a single experiment. Results, however, were always confirmed by repeating each experiment on separate occasions at least three times. The data were analyzed using an unpaired two-tailed Student’s t-test and two-way ANOVA with repeated measures. Comparisons were considered statistically significant at P < 0.05.

RESULTS

Time- and bradykinin concentration-dependent augmentation of fibroblast-mediated contraction of collagen gel. Bradykinin significantly augmented fibroblast-mediated collagen gel contraction in a time- and concentration-dependent manner (Figs. 1 and 2). There was no detectable contraction of the control gels in 30 min. After 60 min, control gels had contracted 10.0 ± 1.4%, and they continued to contract throughout the 360-min observation period. In contrast, with 10−9 or 10−7 M bradykinin, there was detectable contraction after 30 min of incubation. Contraction at 30 min, as a percent decrease in area, was 13.1 ± 1.7% with 10−9 M bradykinin and 26.0 ± 1.6% with 10−7 M. After 60-min incubation, the contraction was 21.3 ± 0.3% with 10−9 M bradykinin and 32.2 ± 1.6% with 10−7 M (Fig. 1). Augmented contraction could be observed with 10−7 M bradykinin throughout the 360-min incubation. The difference between 10−9 M bradykinin and control, observable after 30 min, was diminished by 360 min. Bradykinin-augmented contraction was concentration dependent over the entire range of 10−10 to 10−6 M tested (Fig. 2). Because a “plateau” was not observed, it was not possible to calculate an EC50 (the concentration of bradykinin at which a 50% of maximal effect of bradykinin is induced). To determine whether adult

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Fig. 1. Time-dependent contraction of collagen gels by fibroblasts augmented by bradykinin. Fibroblasts were embedded in collagen gels and cultured for 5 days. The gels were then released and cultured with and without 10−9 or 10−7 M bradykinin. The area of each gel was then measured using an image analyzer after various incubation periods.
airway fibroblasts could respond to bradykinin, HBF were similarly tested. Bradykinin (10^{-7} M) resulted in augmented contraction (83.6 \pm 0.8\% of original size) compared with control (95.4 \pm 1.1\% of original size, \( P < 0.01 \)).

**Effect of protease inhibitors on bradykinin augmented fibroblast gel contraction.** Peptidase degradation of small peptides might be an important regulatory process in vivo (27, 42). Because fibroblasts are known to have two membrane-bound peptidases, neutral endopeptidase (NEP) (24, 28) and angiotensin-converting enzyme (ACE) (37, 45), which are capable of cleaving bradykinin, the effects of the NEP inhibitors phosphoramidon and thiorphan and the ACE inhibitor captopril (9) were evaluated. The effect of 10^{-2} and 10^{-8} M bradykinin on fibroblast gel contraction was significantly increased in the presence of 1 mM phosphoramidon (Fig. 3, \( P < 0.01 \)). In contrast, captopril showed no effect, and the addition of captopril to phosphoramidon did not further augment contraction. Thiorphan (10 \mu M) also augmented the effect of bradykinin similarly to the effect of phosphoramidon (data not shown).

**Effect of B1 and B2 antagonists.** The effect of bradykinin is mediated by specific receptors, which belong to two major categories, B1 and B2 (7, 35). To evaluate which receptor mediates the effect of bradykinin on fibroblast gel contraction, the effects of a B1 receptor competitive antagonist des-Arg^{9-}[Leu^{8}]-bradykinin and B2 receptor competitive antagonists d-Arg-[Hyp^{3},Thi^{5,8},d-Phe^{7}]-bradykinin and Na-adamantaneacetyl-d-Arg-[Hyp^{3},Thi^{5,8},d-Phe^{7}]-bradykinin (26) were evaluated. As shown in Fig. 4, the B2 antagonist Na-adamantaneacetyl-d-Arg-[Hyp^{3},Thi^{5,8},d-Phe^{7}]-bradykinin attenuated the effect of bradykinin (\( P < 0.01 \) by ANOVA), whereas the B1 antagonist des-Arg^{9}-[Leu^{8}]-bradykinin had no effect. The B2 antagonist d-Arg-[Hyp^{3},Thi^{5,8},d-Phe^{7}]-bradykinin also inhibited the effect of bradykinin (data not shown). Thus the B2 receptor appears to mediate the enhancing effect of bradykinin on fibroblast contraction of collagen gels.

**Signal transduction pathways mediating the effects of bradykinin.** Because the effects of bradykinin in some cells are known to be mediated by a PTX-sensitive G protein (6, 44), the effect of PTX on bradykinin-augmented fibroblast-mediated collagen gel contraction was investigated. PTX treatment did not affect the fibroblast-mediated collagen gel contraction in either the absence or the presence of bradykinin (Fig. 5).

Because G protein-coupled receptors, including B2, signal predominantly through the \( \beta \)-isoforms of phospholipase C (PLC) (44), the effect of the PLC inhibitor U-73122 and its inactive analog U-73343 were evaluated (37). There was no significant difference between U-73343, the inactive analog of U-73122, and vehicle alone. In contrast, U-73122 at a concentration of 10 \mu M significantly attenuated the contraction of control gels and completely abolished the augmentation of fibroblast-mediated gel contraction induced by bradykinin (\( P < 0.01 \), Fig. 6).

PLC activation is commonly followed by calcium mobilization (30). Therefore, the effect of calcium mobilization inhibitors was investigated to determine whether the response to bradykinin involved \( \text{Ca}^{2+} \) mobilization. Addition of 3,4,5-trimethoxybenzoic acid...
8-(diethylamino)-octyl ester (TMB-8), an inhibitor of intracellular Ca\textsuperscript{2+} mobilization, abolished the response to bradykinin (P < 0.01, Fig. 7A). However, EGTA, a Ca\textsuperscript{2+} chelator, attenuated the contraction of the control gel (P < 0.01) but did not affect the response to bradykinin (Fig. 7B).

The involvement of protein kinase C (PKC) in the response to bradykinin was evaluated using the PKC inhibitors staurosporine and GF-109203X (36, 41) and by pretreating with PMA to downregulate PKC. By incubating fibroblasts embedded in collagen gels with 1 μM PMA for 12 h, the augmentation of fibroblast gel contraction by bradykinin was completely abolished (Fig. 8A). Both of the PKC inhibitors tested, staurosporine and GF-109203X, also attenuated the response to bradykinin (Fig. 8, B and C).

The effects of inhibitors did not appear to be due to cytotoxicity inasmuch as there was no significant difference between the viability of the cells incubated with U-73122 and U-73343, the cells incubated with TMB-8 and control cells, or PMA-treated cells and control cells, as assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay (data not shown).

**DISCUSSION**

The current study demonstrates that bradykinin augments fibroblast-mediated collagen gel contraction. Addition of a NEP inhibitor further augmented the
effect of bradykinin. The B₂ receptor appeared to mediate the effect because a B₂ receptor competitive antagonist attenuated the response, whereas a B₁-receptor competitive antagonist did not. The PKC inhibitors staurosporine and GF-109203X and the PLC inhibitor U-73122 inhibited control and bradykinin-induced contraction. The Ca²⁺ antagonist TMB-8 abolished the bradykinin-stimulated response. PTX treatment and the addition of EGTA to the culture medium failed to modulate the response to bradykinin. Thus the effect of bradykinin seemed to depend on PLC and PKC activation and intracellular Ca²⁺ mobilization.

Rearrangement of extracellular matrix is a crucial process in both wound healing and fibrosis. One aspect of the rearrangement of extracellular connective tissue matrix is contraction. The contraction of wounded tissue can minimize the area to be covered by epithelium and may promote healing. The contraction of tissue and matrix in chronic diseases, however, may lead to disruption of tissue architecture and cause tissue dysfunction (12, 15, 17). Whereas the mechanisms involved in the contraction of fibrotic tissues during repair processes are not fully described, fibroblasts can generate a traction force and can participate in this process (22, 39). When fibroblasts are cultured in a three-dimensional native type I collagen gel, they contract the collagen gel. This phenomenon has been considered to be a model of tissue rearrangement (17). In the current study, this model was used to investigate the contractility of fibroblasts.

Bradykinin is a nonapeptide that has been implicated in the response to trauma and injury (38, 42). Bradykinin is known to increase vascular permeability and secretion of mucus, to cause vasodilation, and to contract smooth muscle both in vivo and in vitro (46). Increased levels of bradykinin have been reported in bronchoalveolar lavage fluids from asthmatic patients (2). Bradykinin also provokes bronchoconstriction in asthmatic subjects (14). Here we reported that bradykinin (10⁻¹⁰ to 10⁻⁶ M) augmented the contraction of type I collagen gels by HFL1 or HBF cells. The concentrations of bradykinin used in this study are close to the bradykinin amount found in human sputum (23). Thus bradykinin may have an important role in asthma and other inflammatory diseases.

Peptidases that are responsible for the breakdown of bradykinin may have important roles in modulating bradykinin-induced effects (27, 42). Although many proteases are able to hydrolyze kinins, two membrane-bound enzymes, NEP and ACE, seem to have important roles (9). NEP, which is present in a variety of airway cells, can cleave many peptides including bradykinin, substance P, neurokinin A, and vasoactive intestinal peptide (24, 28). ACE also is present in a variety of cells, including fibroblasts, and can degrade bradykinin (33, 37, 45). In the current study, the NEP inhibitors phosphoramidon and thiorphan augmented the effect of bradykinin, whereas the ACE inhibitor captopril had no effect. These results suggest that the effect of bradykinin can be modulated by peptidase activity and that under the culture conditions used, NEP has a more important role than ACE.

The receptors mediating responses to bradykinin have typically been divided into two major subtypes, B₁ and B₂ (35). The B₁ receptor is not expressed at significant levels in normal tissues, but its synthesis can be induced after tissue injury or inflammation. The B₂ receptor is constitutively expressed in various kinds of cells including smooth muscle cells, certain neurons, fibroblasts, and epithelial cells and is responsible for many of bradykinin's diverse biological effects. The current study suggests that the effects of bradykinin on fibroblast-mediated collagen gel contraction are mediated by the B₂ receptor. Consistent with this observation, lung fibroblasts have been demonstrated to express the B₂ receptor (1, 25).

Bradykinin activates subcellular responses not only directly but also indirectly through the release of other mediators, including arachidonic acid metabolites, nitric oxide, platelet-activating factor, tumor necrosis factor (TNF)-α, interleukin (IL)-1, norepinephrine and neuropeptides, depending on the cell types and responses (7, 35, 42). The effects of the cyclooxygenase inhibitor indomethacin, the lipoxygenase inhibitors...
NDGA and Wy-50295M (16), the nitric oxide synthesis inhibitor \( \text{NG}^2\)-monomethyl-L-arginine citrate, and the nitric oxide generator nitroprusside were evaluated. These reagents tested all failed to modulate the augmentation of the fibroblast-mediated collagen gel contraction induced by bradykinin (data not shown). Thus the effects of bradykinin appear not to be caused indirectly by eicosanoids or nitric oxide-mediated mechanisms. Bradykinin can lead to indirect effects through other mechanisms as well. In this regard, IL-1, TNF-\( \alpha \), platelet-activating factor, and substance P were all found to be without enhancing effect on fibroblast-mediated gel contraction in our system (data not shown), suggesting that the bradykinin effect is not mediated indirectly through these agonists either.

The signaling pathway(s) by which bradykinin enhances fibroblast-mediated gel contraction was investigated. Bradykinin receptors are members of the G protein-coupled receptor superfamily, and bradykinin can induce cellular effects by activation of either PTX-sensitive or -insensitive G proteins (7, 44). PTX failed to inhibit the effects of bradykinin, suggesting involvement of a PTX-insensitive G protein pathway.

One of the PTX-insensitive signaling pathways that can be activated by bradykinin in other cells is activation of the phosphoinositide-specific PLC enzyme, leading to hydrolysis of phosphatidylinositol 4,5-bisphosphate and formation of inositol 1,4,5-trisphosphate and diacylglycerol (13, 29, 30). Inositol trisphosphate leads to mobilization of intracellular calcium and diacylglycerol leads to activation of PKC. It has been reported that the stimulatory effects on fibroblast-mediated gel contraction of serum, endothelin-1, and platelet-derived growth factor are dependent on activation of PKC (8, 18–20). Our results with bradykinin are consistent with these data, although the contraction assay used by Guidry and associates (18–20) was slightly different from that used in the current study. Importantly, inhibition of PLC or PKC in the present study inhibited control gel contraction as well as bradykinin-augmented contraction. Thus it is possible that PKC and PLC are needed for gel contraction, and their role in bradykinin signal transduction in this regard is not yet established.

In conclusion, the current study demonstrates that fibroblast-mediated collagen gel contraction can be augmented by bradykinin. The ability of bradykinin to modulate this process suggests that bradykinin may have an important role in fibrotic processes. The possibility of modulating this process by targeting bradykinin receptors or the subsequent signaling pathway

![Figure 8](http://ajplung.physiology.org/)

**Fig. 8.** Effects of phorbol 12-myristate 13-acetate (PMA), staurosporine, and GF-109203X on the augmentation of fibroblast-mediated gel contraction induced by bradykinin. The gels were incubated with 1 \( \mu \)M PMA for 12 h (A), with 100 nM staurosporine for 30 min (B), with 10 \( \mu \)M GF-109203X for 1 h (C), or with appropriate vehicle. The gels were then released and transferred to dishes with medium containing bradykinin with and without PMA, staurosporine, or GF-109203X. The area of gels was measured immediately and after 1-h incubation.
involved might offer a novel therapeutic opportunity in a variety of destructive and fibrotic diseases.

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