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Immunopharmacology 33 (1996) 9–15

Immunopharmacology

Human lung fibroblasts express multiple means for enhanced activity of bradykinin receptor pathways

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Abstract

Human lung fibroblasts represent important targets for the biologic activities of bradykinin (BK). We have identified multiple mechanisms in these cells which may extend their potential for BK receptor responsiveness, particularly with regard to generation of arachidonate metabolites. These fibroblasts can constitutively express B_2 and B_1 BK receptors concurrently, both coupled to the pathway for arachidonate metabolism resulting in generation of PGE_2 and the potent vasoactive lipid mediator Thromboxane A_2. Although expression patterns for B_2 and B_1 receptors have classically been regarded as 'constitutive' and 'inducible', respectively, we demonstrate that in human lung fibroblasts both can be expressed spontaneously at equivalent biologic activity levels without selective induction by other mediators. Concurrent B_2/B_1 receptor expression extends the scope of fibroblast response potential to both BK and des-Arg^9-BK in the same time frame. We have identified additional short-term and long-term cellular events, involving both protein kinase pathways through which BK receptors act and those which act upon BK receptors, that result in enhanced BK receptor response potential. These properties of BK receptors may affect whether fibroblast behaviors maintain controlled activities of normal homeostasis or foster escalating cellular responses which may influence the progression of certain human disease states.

Keywords: B_2 Receptor; Signaling; Phosphorylation; Affinity; Mobilization

1. Introduction

Certain biochemical mediators play a dual role in both normal body homeostasis and in the response to environmental situations which perturb that homeostasis. Bradykinin (BK) represents such a versatile peptide mediator addressing both the nervous system, where it modulates sensory and sympathetic synaptic transmission, and the human inflammatory system protecting against microbial invaders and mechanical injury. This latter host defense system denotes the initial presence of injury and ultimately sets the stage for repair. BK is generated through activation of the coagulation pathway, in an ongoing fashion at a low environmental concentration that may reflect physiologic cellular regulation and/or a continuum of minor injuries. Generation of the BK nonapeptide interacting with B_2 receptors can escalate by two orders of magnitude, and this product

Abbreviations: BK, bradykinin; TxA_2, thromboxane A_2; PMA, 12-*O*-tetradecanoylphorbol 13-acetate

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PII S0162-3109(96)00075-6

further can undergo C-terminal cleavage to des-Arg^9-BK selective for B_1 BK receptors, thus broadening the scope of BK biologic activity toward target cells and tissues. (See Becherer et al., 1982; Baenziger et al., 1992; Marceau, 1995 for reviews). In responding to mediators generated over this range from modest fine-tuning of normal cellular functions to outright crisis management, the inflammatory system presents a fragile balance between host protection and destruction. At the same time it offers a paradigm by which the mechanisms causing cells to cross the line between protection and destruction may be uncovered, and in the long-term may afford a potential basis for designing interventions. We have identified behaviors and regulatory modes for BK receptors expressed by human lung fibroblasts which may tend to promote escalation of cellular responses capable of progressing to adverse consequences for the host.

2. Materials and methods

The non-immortalized human lung fibroblast line WI-38 (American Type Culture Collection) was cultured in α-MEM containing 10% fetal calf serum (Irvine Scientific) and assessed for BK receptor biologic activities and radioligand binding parameters as described previously (Becherer et al., 1982; Baenziger et al., 1992; Jong et al., 1993). Briefly, dose response curves were generated for production of arachidonate metabolites mediated by BK or B_1 agonist des-Arg^9-BK. PGE_2 and Thromboxane A_2 (TxA_2) in buffer overlays from 4–10 min incubations of the cells with peptide agonists were assayed by standard immunoassay technologies, measuring the latter arachidonate metabolite as its stable end-product TxB_2. Elevation of intracellular cAMP levels in response to BK receptor activity was assessed by radioimmunoassay. Identification of receptor subtypes governing responses was monitored by B_2 and B_1 receptor antagonist inhibition of BK-mediated arachidonate metabolism as well as the B_1-specific agonist above. Binding of [^{3}H]BK (Amersham, DuPont/NEN) was analyzed by Scatchard analysis using the EBDA/LIGAND program (Elsevier/Biosoft). Peptide agonists and antagonists were from Sigma, Peninsula Laboratories, and Bachem.

3. Results

3.1. Enhancing BK receptor response potential via receptor subtypes: concurrent expression of B_2 and B_1 receptors linked to the same arachidonate metabolic pathway

We defined fibroblast BK receptor-directed functions that result in elaboration of extracellular lipid mediators which can serve to communicate with neighboring cells. Fig. 1 demonstrates B_1 receptors constitutively expressed in WI-38 fibroblasts and participating in arachidonate metabolism at a level equivalent to that of concurrently expressed B_2 receptors. The relative expression of both subtypes varied on a spontaneous basis in 99 cultures tested, presenting 3 general profiles or cellular phenotypes. The majority displayed the profile shown in Fig. 1A, in which only a B_2 receptor prompted PGE_2 production. This B_2-predominant phenotype was observed in 57% of cultures, in which the BK receptor biologic response was completely resistant to 100 μM B_1 antagonist des-Arg^{9}Leu^8-BK. In a subset of cultures randomly chosen from the total and evaluated by additional criteria for receptor subtype assignment, 7/13 (54%) showed BK-mediated PGE_2 production that was inhibited 86–95% by the B_2 receptor antagonist [Thi^{5,8}D-Phe^7]-BK at concentrations of 50–100 μM and was unresponsive to the B_1 agonist des-Arg^9-BK at concentrations of 1–5 μM, confirming this prevalent relative representation of the B_2 predominant phenotype. A smaller proportion of WI-38 human lung fibroblast cultures (16%) expressed the phenotype shown in Fig. 1B, in which a constitutive B_1 response predominated for BK-mediated arachidonate metabolism. In 16% of cultures in the total set, BK-mediated PGE_2 production was inhibited $\geq$ 80% by des-Arg^9[Leu^8]-BK, and in the randomly chosen subset 1/13 responded equivalently to saturating concentrations of 100–500 nM des-Arg^9-BK and $\geq$ 250 nM BK.

Constitutive B_1 receptor expression occurred most frequently in conjunction with B_2 expression. Fig. 1C demonstrates this B_2/B_1 dual expression phenotype with both receptors linked to the same PGE_2 biosynthetic pathway, accounting for 27% of the total set of cultures. Maximal inhibition of B_1 receptor activity by 100 μM des-Arg^9[Leu^8]-BK de-

creased total BK-mediated PGE_2 production in these cells by 30–50% in individual experiments. Equivalence of B_1 and B_2 receptor activity linked to PGE_2 biosynthesis in Fig. 1C was confirmed via dose response curves for to the B_1 receptor agonist des-Arg^9-BK (data not shown), where the maximal response at saturating des-Arg^9-BK (100–500 nM)

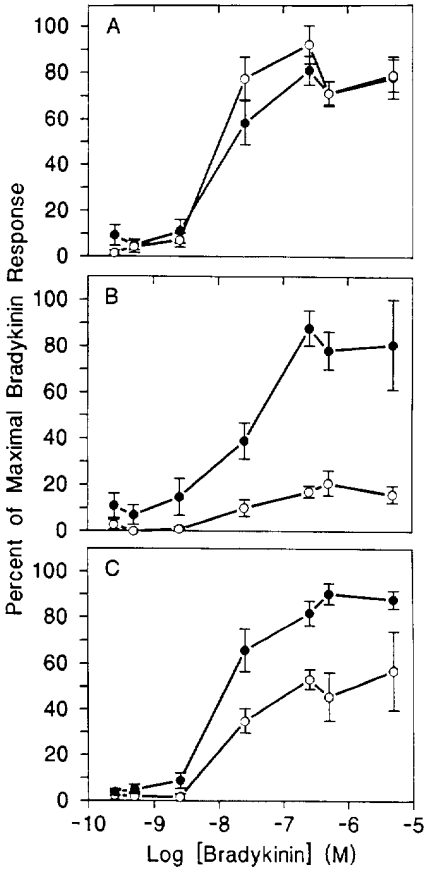

Fig. 1. Constitutive expression of both B_2 and B_1 receptors mediating PGE_2 production in human lung fibroblasts. WI-38 human lung fibroblasts cultured in α-Minimum Essential Medium with 10% fetal calf serum were treated with the indicated concentrations of BK in the absence (●) or presence (○) of the B_1 antagonist [des-Arg^9.Leu^8]BK (100 μM). PGE_2 production was measured as described (Dalemar et al., 1992). Profiles reflect mean ± SEM for each set of representative cultures evaluated. (A) B_2 predominant phenotype resistant to B_1 antagonist ($n = 9$); (B) B_1 predominant phenotype demonstrating >80% inhibition by B_1 antagonist ($n = 6$); (C) Dual B_2/B_1 phenotype with each subtype providing equal contribution toward PGE_2 production ($n = 8$). Representative profiles shown here reflect a total of 99 cultures.

was 39 ± 4.5% of the amount of PGE_2 produced at saturating BK (500 nM $^{-1}$ μM ($n = 17$). Thus both B_2 and B_1 receptor populations efficiently coupled to the G-protein-mediated pathway for PGE_2 production.

In 9 WI-38 cultures, subtype expression assessed by antagonist inhibition of [^{3}H]BK binding further confirmed the relative frequency of the 3 phenotypes as expressed spontaneously in mediating PGE_2 production. The B_2 antagonist at 50 μM completely inhibited [^{3}H]BK binding (250 nM^{-1} μM) in 6/9 cultures where radioligand binding was also totally resistant to displacement by 50 μM des-Arg^9[Leu^8]-BK. In 2/9 cultures radioligand binding was maximally inhibited by 50% in the presence of either the B_2 or the B_1 antagonist, whereas binding in 1 culture was completely resistant to the B_2 antagonist.

3.2. Enhancing BK receptor response potential via diverse arachidonate metabolites: labile / stable mediators and short-term versus sustained local effects

In WI-38 fibroblasts of the dual B_2/B_1 expression phenotype (Fig. 1C), we identified arachidonate metabolites synthesized in response to both B_1 and B_2 receptor activation. PGE_2 production was 47 ± 10 ng/10^6 cells (mean ± SE, $n = 32$) when measured at saturating BK (3.2 μM). Table 1 indicates that these cells also produced the potent labile lipid mediator TxA_2 in addition to the stable product PGE_2; output was maximal by 4 min. TxA_2 production averaged 4.2 ± 0.5 ng/10^6 cells, a level that was 9% of the PGE_2 produced at saturating BK. At 3.2 nM BK the TxA_2 produced was 1.6 ng/10^6 cells, compared with 28 ng PGE_2/10^6 cells; thus similar relative proportions of labile and stable arachidonate metabolites were generated at a lower concentration range of BK receptor activity. When arachidonate release and metabolism was stimulated by mechanical agitation of the cells, by-passing the BK receptor-mediated system, a greater amount of both PGE_2 and TxA_2 was produced, again with the same relative proportionality. Thus, stimulation of the lung fibroblast BK receptor complement by either B_2- or B_1-specific ligands prompts elaboration into the surrounding milieu of a defined proportion of short-acting mediator TxA_2 and stable PGE_2 exerting more sustained effects in this environment.

Table 1
BK receptor responses/second messengers in human lung fibroblasts

Treatment	Arachidonate metabolites(ng/10^6 cells)		cAMP levels(pmol/10^6 cells)
	Thromboxan A_2	PGE_2	
Unstimulated	0.2– 0.7	0.2– 0.7	3.5– 8.7
BK	3.3– 5.3	16.6– 60.0	33.8–75.0
Mechanical agitation	6.7–13.9	94.0–143.0	n.d.

WI-38 human lung fibroblasts in 16–35 mm culture wells were treated with buffer alone, 3.2 μM BK, or mechanical scraping and agitation for 4 min. Buffer overlays were removed for immunoassay of elaborated arachidonate metabolites; cell layers were disrupted by microwave radiation and intracellular cAMP levels determined by immunoassay (Becherer et al., 1982; Dalemar et al., 1992). Results demonstrate the range of values obtained in four experiments for arachidonate metabolites and two experiments for cAMP.
n.d., not determined.

3.3. Enhancing BK receptor response potential via short-term and sustained modulation of intracellular Ser / Thr phosphorylation pathways: roles of protein kinases A and C

We further defined short-term vs sustained effects at the level of intracellular metabolic pathways through which BK receptors act and which may in turn act on expression of BK receptors. Table 1 demonstrates that BK stimulated a 10-fold increase in intracellular cAMP levels in the same rapid 4 min time frame as BK receptor-mediated arachidonate metabolism. We have reported elsewhere that activation of protein kinase A in these cells evoked a rapid shift in B_2 receptors toward a higher affinity state (K_2 440 pM) which tends to promote saturation rather than continued propagation of the receptor-mediated PGE_2 biosynthetic response (Dalemar et al., in press). The results in Table 1 indicate that BK action itself on these fibroblasts may provide the stimulus for such a rapid shift in conjunction with enhanced cellular PGE_2 production.

In contrast, we reported (Dalemar et al., in press) that activation of protein kinase C strongly enhanced PGE_2 biosynthetic activity mediated by B_2 receptors of intermediate to lower affinity (K_d values of 5 nM and 42 nM, respectively), in a similarly rapid manner. This outcome would tend to escalate fibroblast BK receptor response potential, as the cells may continue responding to rising levels of BK in the extracellular environment by synthesizing more PGE_2 (Baenziger et al., 1992). We tested whether protein kinase C activation by phorbol-12-myristate 13-acetate (PMA) might further enhance BK receptor response potential by increasing B_1 receptor expression, as has been reported for vascular B_1 receptor expression in isolated rabbit aorta (Bouthillier et al., 1987). In WI-38 fibroblasts 30 min of treatment with 25–250 nM PMA did not significantly shift their phenotype toward increased relative expression of the B_1 receptor-mediated pathway in producing PGE_2 ($n = 8$); treatment for 24–48 h with 250 nM PMA also did not selectively enhance B_1 representation ($n = 5$). However, a more sustained impact of protein kinase C activation was clearly evident as shown in Table 2. PMA exposure, while continuing to enhance BK-mediated PGE_2 production, also caused non-BK-mediated PGE_2 production to rise dramatically, up to 10-fold. Thus the basic nature of the cellular response extended to a broader scope, mobilizing additional elements of the cellular pathway for PGE_2 production whose activities are not solely governed by BK receptors.

Table 2
Broadening of fibroblast PGE_2 biosynthetic response by prolonged PKC activation

	No PMA		With PMA	
	−BK	+BK	−BK	+BK
Experiment 1	05	22	34	172
Experiment 2	04	48	41	114

Culture wells of WI-38 fibroblasts were treated for 24 h with 250 nM PMA or solvent vehicle dilution alone, then incubated for 10 min with 500 nM BK. PGE_2 elaborated into the buffer supernatant was measured by ELISA. All values shown are ng PGE_2/10^6 cells.

4. Discussion

Human lung fibroblasts utilize activities mediated by the BK receptor system for a number of important cellular programs: communicating with neighboring cells via elaborated first messengers such as prostaglandins, and autoregulating fibroblast functions such as mitogenesis and protein synthesis via these mediators, as well as cellular second messengers (Becherer et al., 1982; Goldstein and Polgar, 1982; Goldstein and Wall, 1984; Baenziger et al., 1992). We have shown how these basic receptor activities may be subject to enhancement, expanding upon some element of the fibroblast repertoire: diverse receptors within the BK receptor family able to recognize a broader spectrum of interacting ligands, the nature of biologic activities contributed by BK receptor-associated pathways, or the time frame surrounding events and mediators linked to the receptor system. These levels of versatility in BK receptor expression are likely important for the maintenance of cellular homeostasis or the generation of an appropriate protective host response under injury conditions. However, this same versatility may serve to set up an extension of the BK receptor response beyond that which is helpful to that which is disadvantageous.

Expression of the dual B_2/B_1 BK receptor phenotype expands the range of environmental cues to which fibroblasts respond. The B_2 receptor has traditionally been regarded as 'constitutive' and the B_1 as 'inducible', with the latter property being important in progression of inflammation; induction occurs in response to certain cytokines evoked while plasma exudation fosters generation of des-Arg^9-BK due to increased BK exposure to C-terminal cleavage (Bouthillier et al., 1987; Marceau, 1995; Menke et al., 1994; Regoli et al., 1978; Regoli et al., 1986). Expression of B_1 receptors in human lung fibroblasts displays constitutive properties and specificity of G-protein-coupled pathways. In IMR90 human lung fibroblasts constitutive expression of a dual B_2/B_1 phenotype has been noted but the two subtypes mediated different functions; B_1 receptors in these cells were cytokine-inducible (Goldstein and Wall, 1984; Menke et al., 1994). We have shown that WI-38 human lung fibroblasts can bypass the requirement for the driving force of cytokines or protein kinase C activation to achieve commensurate levels of B_1 and B_2 receptor biologic activity for PGE_2 production. Forces which may regulate B_1 receptor expression in cells such as WI-38 fibroblasts will be better understood once regulatory elements at the gene level are defined as is emerging for the B_2 receptor (Ma et al., 1994). When and where human fibroblast populations in vivo may likewise acquire B_1 receptor capability, and thus an enhanced response addressing additional molecular forms of BK peptides generated in their surroundings, is an important question whose answer awaits development of molecular probes at the requisite level of sensitivity.

Expression of one subtype or of concurrent B_2/B_1 receptors linked to the same pathways gives rise to short-term as well as more sustained actions of the signaling program. Among the extracellular signals, although TxA_2 is generated in relatively low quantities its much greater potency and different spectrum of action provide an important adjunct for the biological contributions of PGE_2 to the environment surrounding fibroblasts in interstitial tissues (Feinmark and Bailey, 1982). Immediate strong vasoconstrictor and platelet release responses to TxA_2 would rapidly decline via its short half-life (30″), against a background of somewhat more sustained PGE_2 vasodilator and fibroblast mitogenic regulatory activity. TxA_2 is a pathophysiologically significant BK-evoked mediator in the lung (Rossoni et al., 1980; Schulman et al., 1982), with fibroblasts representing an important potential source for it, and hence a contributor to the potential for enhanced effects of BK receptor activity.

In the realm of intracellular signals, the significance we established for Tyr kinases *acting on* BK receptors to govern their expression, and for Tyr kinases that BK receptors *act through* in their signal transduction (Jong et al., 1993), clearly also extends to Ser/Thr kinases. The ability of BK to activate protein kinase A rapidly, as we have shown here, points toward a potential autoregulatory loop, and enhances activity of BK receptors themselves and re-directs them toward higher affinity interactions (Dalemar et al., in press). The longer-term impact of protein kinase C activation we have identified leads instead to a cellular situation in which the specificity of prostaglandin biosynthetic responses for the BK

receptor pathway is compromised. Activities elsewhere in the pathway such as phospholipase A_2 and cyclooxygenase represent possible targets contributing to such alteration in specificity (Burch et al., 1988). This capability of fibroblasts to now produce pathophysiologic quantities of mediators such as TxA_2, independent of BK receptor mechanisms, means that controlling progression of inflammation at the BK receptor level beyond the earliest events may be difficult. We have noted a similar post-protein kinase C persistent up-regulation for a fibroblast mediator management system involving histamine (Baenziger et al., 1994a,b).

The commonality of longer-term up-regulation in the inflammatory system via signaling pathways suggests that this process presents a prevalent escape mode for receptors to attain elevated and potentially detrimental activity in diseases not limited to inflammation. Specific functions known to have gone awry in the brain in Alzheimer's disease, including proteolytic processing of the amyloid precursor protein (APP) and phosphorylation of *tau* microtubule protein to yield aggregated insoluble molecular species, lie in the path of BK-mediated intracellular Ca^{2+} regulation and phosphorylation signaling cascades. We have demonstrated that neuronal BK receptors display structures, properties, and modes of regulation by intracellular signalling pathways that are comparable to those of fibroblasts (Jong, Y.-J.I., Baenziger N.L., submitted), underscoring the role of human fibroblast BK receptor behaviors as an appropriate paradigm for their human neuronal cognates (Willars and Nahorski, 1995). BK receptor pathway activity is reported increased in Alzheimer's disease fibroblasts (Ito et al., 1994; Huang et al., 1995). Insights gained from an initial inflammatory perspective in human lung fibroblasts, identifying elements that enhance overall receptor activity, can ultimately shed light on comparable mechanisms contributing to the pathophysiology of Alzheimer's disease.

Acknowledgements

The authors thank Dr. Aubrey Morrison for immunoassay of TxB_2 and Dr. Steven J. Harris for his contribution to studies of des-Arg^9-BK responses identifying the B_1 bradykinin receptor subtype. This work was supported by the Alzheimer's Association.

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