Crosstalk: phosphorylation of α_{1b}-adrenoceptors induced through activation of bradykinin B2 receptors

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Abstract The action of bradykinin was studied in rat-1 fibroblasts stably expressing α_{1b}-adrenoceptors. It was observed that bradykinin and kallidin markedly increase cytosol calcium concentration, but that the B1 agonist, des-Arg^9-bradykinin, only mimicked this effect to a minimal extent. Antagonists, selective for the B2 subtype, such as Hoe 140, blocked this effect of bradykinin and kallidin. Similarly, bradykinin and kallidin stimulated the production of inositol phosphates and B2 antagonists blocked their actions.

The possibility that bradykinin could modulate α_{1b}-adrenoceptors was studied. It was observed that bradykinin and kallidin increased α_{1b}-adrenoceptor phosphorylation and that such effect was also blocked by Hoe 140. Interestingly, the ability of norepinephrine to increase intracellular calcium concentration was not altered by pretreatment of the cells with bradykinin, i.e. bradykinin induced α_{1b}-adrenoceptor phosphorylation but this did not lead to receptor desensitization.

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Key words: α_{1b}-Adrenoceptor; Receptor phosphorylation; Bradykinin receptor; Rat-1 fibroblast; Desensitization

1. Introduction

Protein phosphorylation is a well-known cardinal event in the regulation of cellular functioning. The properties (i.e. activity, intracellular location, etc.) of many proteins, such as those involved in the control of metabolism or in signal transduction are critically influenced by phosphorylation. Among those proteins involved in signal transduction, G protein-coupled receptors are important targets of protein kinases [1,2].

α_{1b}-Adrenoceptors are members of the seven transmembrane domains G protein-coupled families of receptors that modulate phosphoinositide turnover and calcium signalling [3–5]. The function of these receptors seems to be regulated by phosphorylation/dephosphorylation reactions. Agonist stimulation induces homologous desensitization of these receptors, which is associated to receptor phosphorylation [6–11]; there is evidence that kinases specific for the agonist-occupied G protein-coupled receptors, such as GRK2 and GRK3 participate in this desensitization [10]. In addition, activation of non-adrenergic receptors can also lead to α_{1b}-adrenoceptor (heterologous) desensitization [7,11–13]. We have previously shown that endothelin through ET_A receptors induces the phosphorylation and desensitization of α_{1b}-adrenoceptors in rat-1 fibroblasts; protein kinase C and putative protein tyrosine kinase(s) seem to participate in this type of desensitization. However, the information that exists about this process is very limited.

Bradykinin is a nonapeptide local mediator, prototypic member of the kinins. This peptide mediates important physiological functions such as smooth muscle contraction, cell growth or ion transport in a large variety of cells [14,15]. It also participates in pathophysiological processes such as pain sensation, hypotension and edema formation [14]. The actions of bradykinin are mediated through two types of receptors, B1 and B2; most of its physiological actions involve the B2 subtype [14,15]. In the present paper we show that activation of bradykinin B2 receptors induces phosphorylation of α_{1b}-adrenoceptors.

2. Materials and methods

Bradykinin, kallidin (Lys-bradykinin), des-Arg^9-bradykinin, *N*α-adamantaneacetyl-D-Arg-[Hyp^3,Thi^{5,8},D-Phe]-bradykinin, des-Arg^9-[Leu^8]-bradykinin, (−)-norepinephrine, endothelin, protease inhibitors and all other reagents, unless stated otherwise, were obtained from Sigma Chemical Co. Hoe 140 and des-Arg^{10}-Hoe 140 were from Research Biochemicals International. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, trypsin, antibiotics and other reagents used for cell culture were from Gibco BRL. Myo-[2-^{3}H]inositol (22.9 Ci/mmol) and [^{32}P]Pi (8500–9120 Ci/mmol) were from New England Nuclear. Sepharose-coupled protein A was from Upstate Biotechnology and Fura-2/AM was from Molecular Probes.

Rat-1 fibroblasts transfected with the hamster α_{1b}-adrenoceptor [16], were generously provided to us by Drs. R.J. Lefkowitz, M.G. Caron and L. Allen (Duke University). The cells were cultured in glutamine-containing high glucose DMEM supplemented with 10% fetal bovine serum, 300 μg/ml of the neomycin analog, G-418 sulfate, 100 μg/ml streptomycin, 100 U/ml penicillin and 0.25 μg/ml amphotericin B at 37°C under a 95% air/5% CO_2 atmosphere, as described previously [11,17]. In the present experiments, cells at confluence were serum-deprived in unsupplemented DMEM for 24 h.

Receptor phosphorylation studies were performed as described in detail [11]. In brief, cells were maintained in phosphate-free DMEM during 1 h and then incubated in 3 ml of the same medium containing [^{32}P]Pi (0.05 mCi/ml) for 3 h at 37°C. Labeled cells were stimulated as indicated, then they were washed twice with ice-cold phosphate buffered saline and solubilized with 1 ml of ice-cold solubilization buffer [11]. The extracts were centrifuged and the supernatants transferred to tubes containing anti-α_{1b}-adrenoceptor antiserum and sepharose-coupled protein A and immunoprecipitated as described [11]. The amount of phosphorylated receptor was determined by PhosphorImager analysis. At least three independent experiments were performed for each treatment. Data were analyzed and plotted using commercial software (Prism 2.01, GraphPad Software).

Intracellular calcium concentration ($[Ca^{2+}]_i$) was quantified as described [17]. In brief, fibroblasts were incubated overnight in DMEM without serum and antibiotics. Cells were loaded with Fura-2/AM in Krebs-Ringer-HEPES containing 0.05% bovine serum albumin, pH 7.4 for 1 h at 37°C. Fluorescence measurements were performed

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with excitation monochromators set at 340 and 380 nm, with a chopper interval of 0.5 s, and the emission monochromator set at 510 nm. The $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. [18] using the software provided by AMINCO-Bowman; traces were directly exported to the graphs. K_i values were calculated according to Cheng and Prusoff [19].

Production of [^{3}H]inositol phosphates was performed essentially as described [17]. Briefly, cells were labeled with [^{3}H]inositol (6 μCi/ml) for 18–24 h in 1 ml of inositol-free DMEM containing 1% fetal bovine serum. Cells were washed twice with Krebs-Ringer-HEPES buffer containing 1.3 mM $CaCl_2$ and preincubated for 20 min in buffer containing 10 mM LiCl. Incubations with the agents to be tested were for 15 min and were terminated by the addition of perchloric acid. [^{3}H]Inositol phosphates were separated by Dowex AG1-X8 chromatography [20]. Total [^{3}H]inositol phosphate production is presented.

3. Results and discussion

Previous studies have shown that there is clonal variation in the presence of endogenous bradykinin receptors in rat-1 fibroblasts. In some clones, a small number of receptors have been found [21] whereas in others no binding sites or functional responses have been detected [22]. In addition, it has been reported that the expression of some genes, such as the *ras* oncogene, markedly increases the expression of bradykinin receptors in these cells [21]. Therefore, we investigated the presence of endogenous bradykinin receptors in rat-1 fibroblasts stably expressing α_{1b}-adrenoceptors, by studying two functional responses, i.e. the ability of bradykinin to increase $[Ca^{2+}]_i$ and to induce the production of [^{3}H]inositol phosphates.

It can be observed in Fig. 1 that bradykinin and kallidin increased in concentration-dependent fashion $[Ca^{2+}]_i$ in these cells; both natural agonists had similar potency ($EC_{50} \sim 50$ nM) and equal efficacy. In contrast, the B1-selective agonist, des-Arg^9-bradykinin only induced a marginal increase in $[Ca^{2+}]_i$ (Fig. 1, upper panel). The effect of 100 nM bradykinin on this parameter was inhibited in concentration-dependent fashion by the B2 antagonists, Hoe 140 and *N*α-adamantaneacetyl-D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin; the former was consistently more potent than the latter (K_i values 3.5 nM and 18 nM, respectively) (Fig. 1, middle panel). Des-Arg^{10}-Hoe 140, a B1-selective antagonist, only partially inhibited the effect of bradykinin at the highest concentration tested whereas des-Arg^9-[Leu^8]-bradykinin, another B1-selective antagonist, was completely ineffective in the range of concentrations tested (Fig. 1, middle panel). The effect of kallidin on $[Ca^{2+}]_i$ was also completely blocked by Hoe 140 (Fig. 1, lower panel). The antagonists by themselves did not modify basal $[Ca^{2+}]_i$. The data clearly indicated that bradyki-

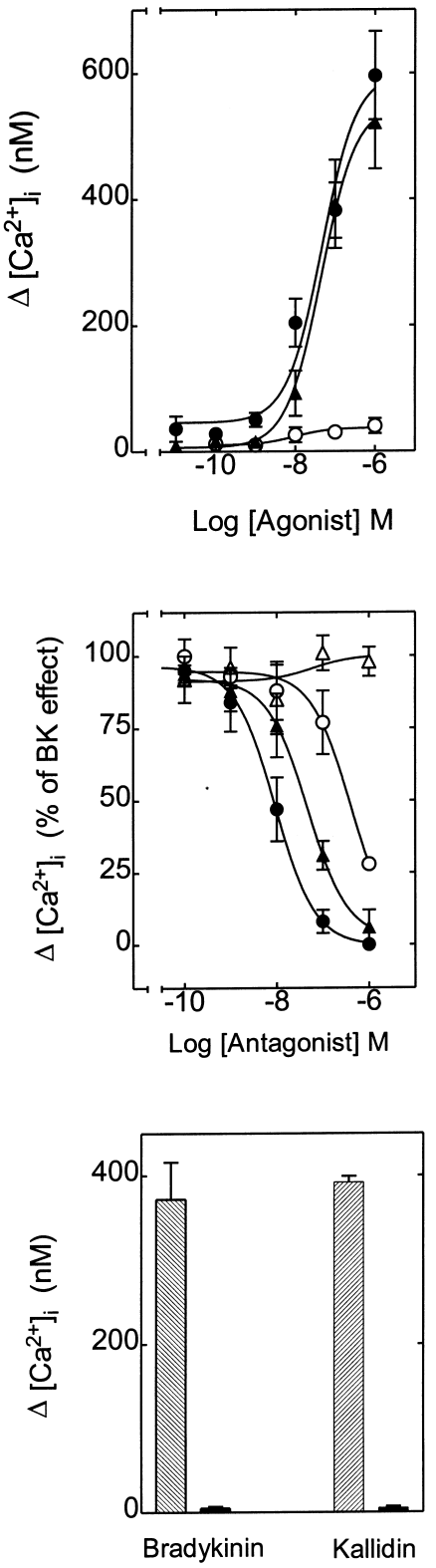

Fig. 1. Effect of bradykinin agonists and antagonists on intracellular free calcium concentration. Upper panel: cells were incubated in the presence of different concentrations of bradykinin (closed circles), kallidin (closed triangles) or des-Arg^9-bradykinin (open circles). Data are the changes in intracellular calcium in response to the agents; basal $[Ca^{2+}]_i$ was 128 ± 19 nM. Plotted are the means ± S.E.M. of 3–7 experiments using different cell cultures. Middle panel: cells were incubated for 1 min in the presence of different concentrations of the antagonists: Hoe 140 (solid circles), *N*α-adamantaneacetyl-D-Arg-[Hyp^3,Thi^{5,8},D-Phe]-bradykinin (solid triangles), des-Arg^{10}-Hoe 140 (open circles) or des-Arg^9-[Leu^8]-bradykinin (open triangles); after this preincubation 100 nM bradykinin (BK) was added. Data are the changes in intracellular calcium in response to bradykinin as compared to the effect of that of bradykinin alone (100%). Plotted are the means ± S.E.M. of 3–4 experiments using different cell cultures. Lower panel: cells were preincubated for 1 min in the absence of antagonist (dashed columns) or presence of 1 μM Hoe 140 and challenged with 100 nM bradykinin (left group) or 100 nM kallidin (right group). Data are the changes in intracellular calcium in response to the agents; basal $[Ca^{2+}]_i$ was 101 ± 15 nM. Plotted are the means ± S.E.M. of 3 experiments using different cells cultures.

nin receptors of the B2 subtype were present in rat-1 fibroblasts stably expressing α_{1b}-adrenoceptors.

The ability of bradykinin to increase $[Ca^{2+}]_i$ was observed in buffer containing 2.5 mM EGTA (data not shown) which indicated that calcium mobilization from intracellular stores was involved. Therefore, the ability of these agents to stimulate the production of [^{3}H]inositol phosphates was tested. Bradykinin and kallidin stimulated the production of [^{3}H]inositol phosphates and their effects were completely blocked by Hoe 140 (Fig. 2). The data confirmed that these cells endogenously express B2 bradykinin receptors and further indicated that these receptors are coupled to phosphoinositide turnover and calcium mobilization, as observed in many other models [14,15,22]. It should be mentioned that the effect of bradykinin on this parameter is relatively small (40–50% over basal), since in the same experiments 10 nM endothelin was able to increase [^{3}H]inositol phosphate production up to 2.5-fold (data not shown).

We next examined the possibility that activation of B2 bradykinin receptors may lead to α_{1b}-adrenoceptor phosphorylation. As shown in Fig. 3, bradykinin induced phosphorylation of the adrenergic receptor in a concentration-dependent fashion. The maximum increase was ~40–50% over basal and it was observed at concentrations between 1 and 3 μM, with an EC_{50} of ~500 nM. Kallidin was also able to induce α_{1b}-adrenoceptor phosphorylation and the effect of both natural agonists, bradykinin and kallidin, was completely blocked by the B2 antagonist, Hoe 140 (Fig. 4). The antagonist by itself was without effect on the basal receptor labeling. These data clearly indicate that the same B2 receptors mediate both actions of bradykinin, increase in $[Ca^{2+}]_i$ and induction of adrenoceptor phosphorylation. The 10-fold difference in EC_{50} values observed for these effects of the peptide is not surprising. This difference suggests that bigger receptor occupation than calcium mobilization is required to induce receptor phosphorylation, or expressed in other words, that a small generation of IP_3 could be sufficient to increase $[Ca^{2+}]_i$ but that other factors are important to induce receptor phosphorylation.

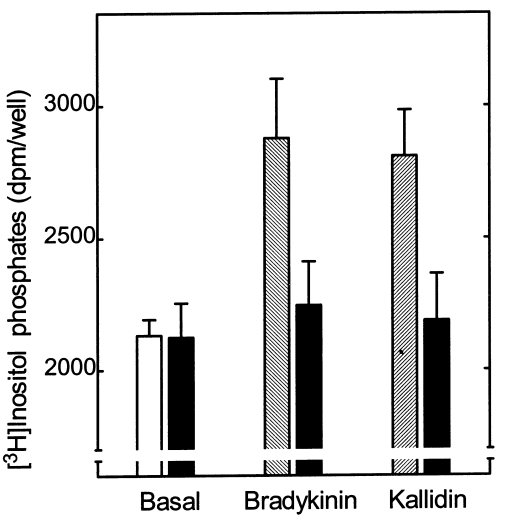

Fig. 2. Effect of bradykinin agonists and antagonists on [^{3}H]inositol phosphate production. Cells were incubated in the absence or presence (solid columns) of 1 μM Hoe 140 with no other agent (left group), 100 nM bradykinin (middle group) or 100 nM kallidin (right group). Plotted are the means ± S.E.M. of 5–7 experiments using different cell cultures.

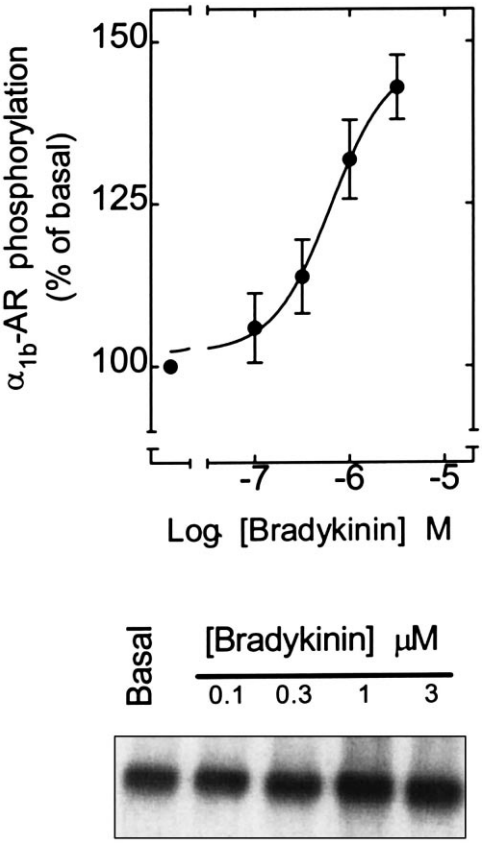

Fig. 3. Effect of bradykinin on α_{1b}-adrenoceptor phosphorylation. Cells were incubated in the absence (basal) or presence of different concentrations of bradykinin. Plotted are the means ± S.E.M. of 4–5 experiments using different cell cultures. In the lower panel a representative autoradiograph is presented.

We next studied the functional consequences of bradykinin-induced adrenoceptor phosphorylation by examining the effect of norepinephrine on $[Ca^{2+}]_i$. As shown in Fig. 5, 10 μM norepinephrine induced a marked increase in $[Ca^{2+}]_i$. When cells were pretreated with 1 μM bradykinin the effect of norepinephrine was not affected. Under the same conditions, 10 nM endothelin markedly decreased the effect of norepinephrine on this parameter (Fig. 5). It should be mentioned that even lower concentrations of endothelin that induce a much smaller effect on $[Ca^{2+}]_i$ were able to diminish clearly the effect of norepinephrine (data not shown, please see [11]). In addition, we studied if the actions of smaller concentrations of norepinephrine were affected by the action of the peptide without observing any clear effect (data not shown). The possibility that a longer exposure to bradykinin (up to 15 min) could be required to affect the action of the catecholamine was tested without success (data not shown). It was clear, therefore, that in spite of inducing receptor phosphorylation, bradykinin did not lead to α_{1b}-adrenoceptor desensitization in rat-1 fibroblasts.

Our results are consistent with data previously published in DDT_1 MF-2 cells in which it was observed that bradykinin induced a small effect on phospholipid labeling and also phosphorylation of α_1-adrenoceptors [7]. However, some marginal

desensitization of the α_{1b}-adrenergic action was detected in those experiments [7]. It is possible, therefore, that the strength of the signal generated by bradykinin could be insufficient to desensitize α_{1b}-adrenergic action in rat-1 fibroblasts but that in other cells where α_{1b}-adrenoceptors and B2 bradykinin receptors are coexpressed, such interaction may have functional consequences. Nevertheless, it is clear that receptor phosphorylation does not necessarily indicate receptor desensitization. In fact, it has been observed that overexpression of GRK5 and GRK6 markedly increases α_{1b}-adrenoceptor phosphorylation but does not affect the receptor-mediated responses [10]. Additionally, our data clearly indicate that agonist-induced calcium mobilization is not sufficient to induce desensitization of α_{1b}-adrenoceptors. Activation of protein kinase C and putative protein tyrosine kinase(s) seem to mediate α_{1b}-adrenoceptor phosphorylation induced by heterologous stimuli [11]. Interestingly, bradykinin is able to activate protein kinase C [23] and there is also evidence that the kinin induces protein tyrosine phosphorylation [24].

In summary, our data indicate that bradykinin B2 receptors are endogenously expressed in rat-1 fibroblasts and that activation of such receptors induce increases in $[Ca^{2+}]_i$, phosphoinositide turnover and leads to α_{1b}-adrenoceptor phosphorylation.

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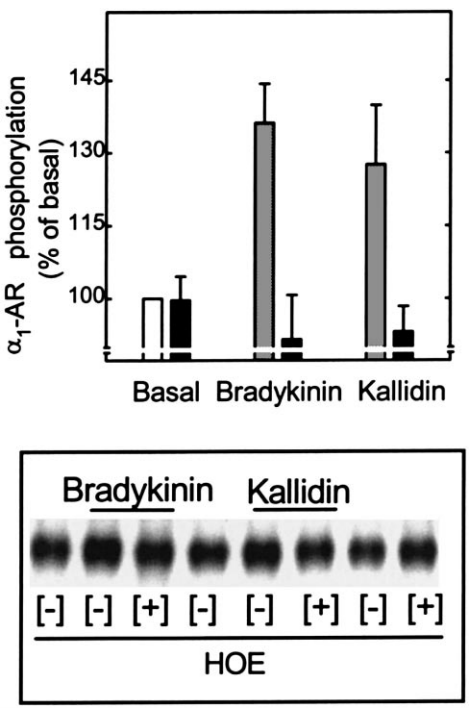

Fig. 4. Effect of bradykinin agonists and antagonists on α_{1b}-adrenoceptor phosphorylation. Cells were incubated in the absence or presence (solid columns) of 1 μM Hoe 140 with no other agent (left group, basal), 1 μM bradykinin (middle group) or 1 μM kallidin (right group). Plotted are the means ± S.E.M. of 4–5 experiments using different cell cultures. In the lower panel a representative autoradiograph is presented.

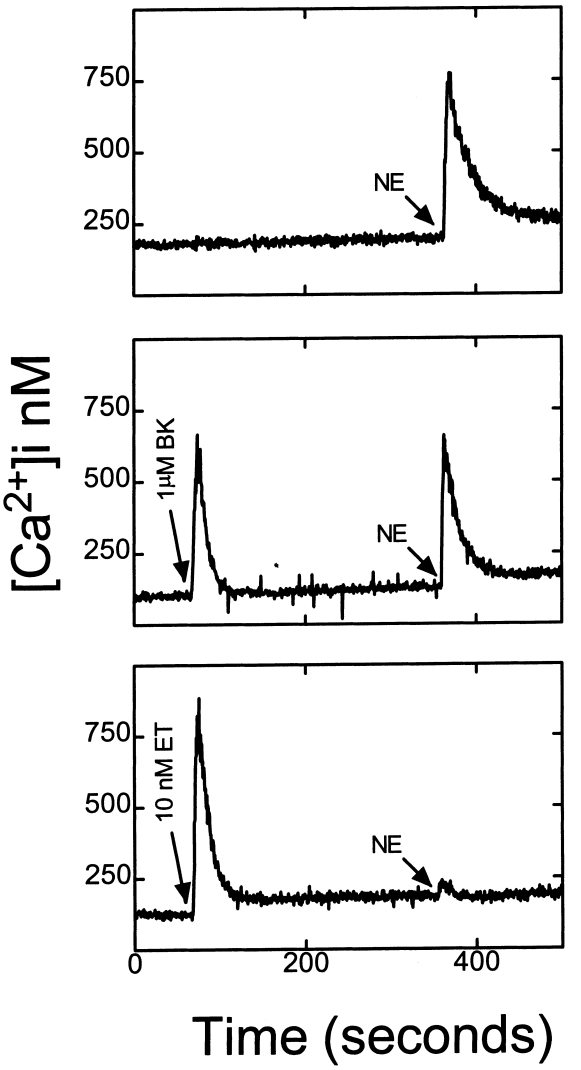

Fig. 5. Effect of bradykinin and endothelin on the increase in intracellular calcium induced by norepinephrine. Cells were challenged with 10 μM norepinephrine (NE), 1 μM bradykinin (BK) or 10 nM endothelin (ET). Data were directly imported from the fluorometer computer and are representative of 3 experiments using different cell cultures.

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