Bradykinin-induced tyrosine phosphorylation of proteins in cultured human keratinocytes

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The stimulating effect of bradykinin on phosphorylation of proteins at tyrosine residues was visualized on human keratinocytes in primary culture. Keratinocytes were subjected either to short-time (30 s) or to long-time stimulation (4 h) with 200 nM bradykinin. Especially keratinocytes of the G1 phase showed bright immunofluorescence with monoclonal anti-phosphotyrosine antibody. Solubilized membrane proteins were fractionated by gel filtration and tested for tyrosine phosphorylation by ELISA. Short-time stimulation induced a broad peak with a shoulder at 90 kDa, the main peak at about 60 kDa and a second shoulder at 44 kDa. After long-time stimulation an additional distinct peak at 180 kDa appeared, phosphorylation at 90, 60 and 44 kDa was less pronounced.

Tyrosine phosphorylated proteins were further characterized by SDS-polyacrylamide gel electrophoresis, Western blotting and detection by monoclonal anti-phosphotyrosine antibody. After short-time stimulation with bradykinin tyrosine phosphorylation was confined to distinct bands at 82, 76, 70, 57, 54, 48, 40 and 39 kDa and a diffuse band at 62 kDa. After long-time stimulation tyrosine phosphorylation increased for the 76 kDa band and the bands at 48 and 40 kDa became more diffuse, the 39 kDa band remained and the others disappeared.

Among these proteins, MAP kinase, actin, paxillin and the EGF receptor were the most likely candidates for bradykinin-induced tyrosine phosphorylation. Therefore, these effects in keratinocytes might be associated with events related to mitosis, adhesion and variation in cell shape.

Key terms: *bradykinin, human keratinocytes, monoclonal anti-phosphotyrosine antibody, tyrosine phosphorylation*

INTRODUCTION

The nonapeptide bradykinin is known to play a role in both skin physiology and pathophysiology. Small amounts of subcutaneously applied bradykinin can induce short term effects resembling an inflammatory response such as the increase in capillary permeability, dilatation of vessels, edema and pain (Ferner *et al*, 1989; Treede *et*

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al, 1990). Among the cellular components of skin in particular keratinocytes appear to be involved in inflammatory processes by releasing specific proinflammatory mediators such as arachidonic acid and its metabolites, *e.g.*, induced by contact allergens and ultraviolet radiation (Talwar *et al*, 1990; Barker *et al*, 1991).

We have recently shown the presence of high affinity bradykinin receptors of the B2 subtype in human skin and in primary cultures of human keratinocytes by direct receptor binding studies. By autoradiographic techniques, we further localized B2 bradykinin receptors of intact skin to the mitotic active layer of the epidermis (Schremmer-Danninger *et al*, 1995). As shown in keratinocyte cell lines, B2 bradykinin receptors are coupled to pertussis toxin sensitive GTP binding protein and ligand binding is inducing the release of arachidonic acid and of other secondary mediators such as cAMP, inositolphosphates or diacylglycerol (Kast *et al*, 1991, 1993; Johnson *et al*, 1992; Rosenbach *et al*, 1993; Haase *et al*, 1997a,b). Bradykinin may also stimulate cell proliferation in particular serving as a comitogen in the presence of other growth factors such as EGF or PDGF (Olsen *et al*, 1988; Godin *et al*, 1991). Higher cellular proliferation rates induced by activation of the kallikrein-kinin system may play a significant role in the repair of tissue injury, but also in human neoplastic processes or psoriasis. For example, significantly higher values of plasma kallikrein –probably leading to enhanced liberation of bradykinin– have been proposed to be involved in the skin irritation of psoriatic patients (Thomas *et al*, 1992). Several reports also appear to indicate that malignant cells are more sensitive to the mitogenic actions of bradykinin (Roberts & Gullick, 1989; Sethi & Rozengurt, 1991; Muns *et al*, 1994). This may point to bradykinin as a cofactor in tumor growth.

In mediating the mitogenic effects of bradykinin, tyrosine phosphorylation at proteins is involved as a very basic mechanism of the proliferative response. In a variety of cultured cells (mesangial, PC12, endothelial, smooth muscle, Swiss 3T3) bradykinin-induced tyrosine phosphorylation has been reported to occur at some defined proteins such as tubulin, paxillin (between 68 and 77 kDa) and especially at the mitogen activated (MAP) protein kinase (42/44 kDa) (Jaffa *et al*, 1997; Zwick *et al*, 1997; Fleming *et al*, 1995; Casamassima & Rozengurt, 1997).

Various growth factors like EGF possess intrinsic tyrosine kinase activity involved in autophosphorylation of their receptors, as well as in phosphorylation of other proteins which are thought to play a role in cell proliferation. Interestingly, bradykinin is also reported being capable to phosphorylate the EGF receptor at tyrosine residues, thereby inducing a comitogenic effect by EGF receptor transactivation (Zwick *et al*, 1997).

In keratinocytes, however, the bradykinin-induced protein phosphorylation at tyrosine residues is only poorly characterized so far, except for a study of Coutant *et al* (1995) that reported tyrosine phosphorylation of the EGF receptor and of focal adhesion proteins after stimulation by bradykinin. Therefore, in this study we analyzed the effect of bradykinin stimulation on tyrosine phosphorylation of proteins in human keratinocytes in primary culture.

MATERIAL AND METHODS

Tissue preparation and keratinocyte cell culture.

Foreskin was obtained from healthy newborn babies undergoing elective circumcision and processed as described by Eisinger (1985), Green *et al* (1979) and Green (1989) with minor modifications. Tissue samples were transported and stored up to 4 h at 4°C in DMEM (Sigma, Deisenhofen, Germany) supplemented with vancomycin (Lilly, USA) and gentamycin (Merck, Darmstadt, Germany), 100 µg/ml each. Then skin samples were rinsed in cold saline and connective tissue was removed. Small slices were cut and stored for 5 min in 0.02% EDTA solution and

then transferred for 16 h into 0.1% trypsin in PBS at 4°C, containing 100 µl 0.1% glucose per 9 ml. After removal of the trypsin solution, saline was added and epidermis mechanically removed from dermis. To obtain a suspension of separate cells, pieces of epidermis were slowly moved in cold trypsin-EDTA solution (trypsin 0.05%, EDTA 0.02%) for 15 min. The resulting suspension was filtered onto a cushion of FCS through a fivefold layer of gaze to block trypsin activity and then centrifuged at 180 g for 10 min. For seeding, the pellet was resuspended in 1 ml FCS and 10 ml keratinocyte medium: DMEM and DMEM/Ham's 1:1, 10% FCS, 1.8 x 10^{-4} M adenine, 5 µg/ml insulin, 10^{-10} M cholera toxin, 2 mM glutamine, 5 µg/ml transferrin human, 2x10^{-9} M 3,3',5-tri-iodo-L-thyronine (Sigma, Deisenhofen, Germany) and 0.4 µg/ml hydrocortisone (Hoechst, Frankfurt, Germany).

The isolated keratinocytes were seeded onto a mitomycin (Medac, Frankfurt, Germany) –treated 3T3 fibroblast feeder–layer (Rheinwald & Green, 1975). At day 4 the keratinocyte medium used for seeding was exchanged by an equivalent medium containing 10 ng/ml EGF (Sigma, Deisenhofen, Germany); this medium was also used for subsequent passages.

Immunofluorescence with monoclonal anti-phosphotyrosine antibody.

Tyrosine phosphorylation of proteins can originally be detected with the aid of monoclonal anti-phosphotyrosine antibody (Glenney *et al*, 1988). Human keratinocytes from the 2nd or 3rd passage were seeded at approximately 600,000 cells per ml onto glass chamber slides (Nunc, Wiesbaden, Germany) and cultivated for 48 h at 37°C, 10% CO_2 and 95% humidity. After stimulation with 200 nM bradykinin, keratinocytes were washed twice with phosphate buffered saline (PBS), dried at room temperature and fixed for 10 min at 4°C in 100% methanol. Fixation was followed by incubation with 5% bovine serum albumin (BSA) in PBS buffer for 30 min at room temperature.

Incubation with 1st antibody: Anti-phosphotyrosine (Boehringer, Mannheim, Germany) was diluted with PBS/0.5% BSA 1:20 and 60 µl of the solution applied onto a marked circle on the slide. Control incubations were done in the presence of 2-phospho-DL-tyrosine (0.1 M). The same volume of a two fold concentrated anti-phosphotyrosine solution was mixed with PBS/0.5% BSA or 2-phospho-DL-tyrosine (0.2 M) in PBS/0.5% BSA and also applied in 60 µl volumes as described above. Slides were incubated for 2 h at 37°C in a humid chamber and then rinsed in PBS/0.5% BSA three times for 10 min each.

Incubation with 2nd antibody: Fluorescein isothiocyanate (FITC) conjugated anti-mouse-IgG (Boehringer, Mannheim, Germany) was diluted with PBS/0.5% BSA 1:32 and 60 µl was applied onto the marked circle on the slide. Slides were incubated for 1 h at 37°C in a humid chamber and processed as described for incubation with the 1st antibody.

To avoid bleaching, keratinocytes were covered with a solution according to Johnson and de Nogueira Aranjo (1981).

Isolation of tyrosine phosphorylated proteins.

Keratinocytes from the 3rd passage were stimulated with bradykinin in 75 cm^2 flasks. Medium was removed and the keratinocyte monolayer washed twice with 10 ml ice cold 20 mM piperazine-N,N'-bis(2-ethane sulfonic acid) (PIPES) buffer, pH 6.8. Keratinocytes were removed mechanically with a rubber policeman in a volume of 1 ml, while the flask was placed on ice. The cells were disrupted, homogenized by brief sonication and the resulting suspension was centrifuged at 50,000 g for 15 min at 2°C.

The pellet was resuspended in 2 ml 20 mM PIPES buffer, pH 6.8, 4 mM CHAPS. Membrane proteins were solubilized by stirring the solution for 5 h at 4°C (Faussner *et al*, 1991, modified). Supernatant containing the solubilized membrane proteins was obtained after centrifugation at 50,000 g for 15 min at 2°C.

ELISA of tyrosine phosphorylated proteins.

The antigen, *i.e.* solubilized membrane proteins, was bound to a poly L lysine covered micro ELISA plate (Dynatech, Denkendorf, Germany). The volume of 25 µl (protein content 50 µl/ml) was laid onto a cushion of 25 µl 1 M Tris buffer and addition of 50 µl covering buffer (0.05 M Na_2CO_3, 0.05 M $NaHCO_3$, pH 9.6) completed the reaction volume to 100 µl. Antigen binding took place at 4°C overnight. Thereafter, each cavity was washed twice with 200 µl washing buffer (10 mM Tris-HCl, 0.85% NaCl, 0.5% BSA, 0.05% Tween, pH 7.4) and incubated with another 200 µl of the same solution for 30 min at 37°C in order to block potentially free binding places.

The 1st antibody, anti-phosphotyrosine (Boehringer, Mannheim, Germany), was added in a 50 µl aliquot after being diluted 1:200 with washing buffer and incubated for 2 h at 37°C. After three washing steps, 100 µl of the 2nd antibody, peroxidase-conjugated anti mouse IgG, was added per cavity after a 1:1000 dilution and incubated for 1 h at 37°C. Three washing steps followed before peroxidase activity was detected by extinction reading at 492 nm. Controls were done with nonspecific mouse IgG.

SDS-PAGE and Western blotting.

CHAPS solubilized proteins were dialyzed against 0.05 M NH_4 Ac buffer, pH 6.5 and concentrated to a final volume of 300 µl by Amicon procedure. Bromophenolic blue solution (2 µl) was added and CHAPS solubilized proteins separated by SDS-PAGE on a 10 to 25% gradient gel. To enable specific immunodetection of the separated proteins, they were transferred to PVDF (polyvinylidendifluoride) membranes (Millipore, Eschborn, Germany).

Immunodetection of tyrosine phosphorylated proteins on PVDF membranes.

Free binding sites were blocked by slightly moving the PVDF membrane overnight at 4°C in 10 mM Tris-HCl buffer supplemented with 0.85% NaCl, 2% BSA, 0.3% gelatine and 10% goat serum, pH 7.4.

Marked strips of PVDF membrane were incubated between filter strips soaked with anti-phosphotyrosine antibody (1st antibody), diluted 1:20 with transfer buffer (39 mM glycine, 48 mM Tris-HCl, 0.0375% sodium dodecylic sulfate and 20% methanol, pH 10.4) overnight at 4°C between glass plates in a humid chamber. After incubation, membrane strips were washed in transfer buffer three times for 10 min each.

For detection with the immunogold system, strips were slightly moved for 1 h at 37°C in goat anti mouse IgG Aurion GP-bioassay (Biotrend, Aurion, Wageningen, Netherlands), an immunogold reagent of the 2nd antibody, which had been diluted 1:25 with transfer buffer. Three washing steps with transfer buffer, 10 min each, and three steps rinsing with bidistilled water, each for 5 min followed.

For visualization of gold conjugated immunocomplexes, accompanying specific silver enhancement was done for 30 min and was then stopped by washing in bidistilled water.

To control for nonspecific binding, nonspecific mouse IgG was used in the case of 1st antibody, and incubation in buffer instead of 1st antibody, in the case of 2nd antibody.

Gel filtration chromatography of membrane proteins.

Solubilisates of membrane proteins (see above) were fractionated using a Bio-Sil TSK^R 250 or SEC (Size Exclusion Column) 250 column (Bio-Rad Laboratories, München, Germany) and the elution profile monitored at 280 nm. To evaluate kDa values, gel filtration standards (Bio-Rad Laboratories) were fractionated in the same way. Eluent was 20 mM PIPES buffer, 4 mM CHAPS, 0.85% NaCl, pH 6.8. Fractions of 1 ml were tested for tyrosine phosphorylation with the appropriate ELISA (see above).

RESULTS

Visualization of tyrosine phosphorylated proteins by immunofluorescence.

Initially the effect of different culture conditions was analyzed. It turned out, that only keratinocytes exposed to optimized concentrations of Ca^{2+} present in the culture medium (1.427 mM $CaCl_2x2H_2O$) and of EGF (10 ng/ml) showed an enhanced tyrosine phosphorylation upon stimulation by bradykinin (Fig 1A). At concentrations of bradykinin below 200 nM no effects were observed. In control cultures in the absence of bradykinin only extremely weak background fluorescence signals of the FITC conjugated anti-phosphotyrosine antibody were detectable (Fig 1B).

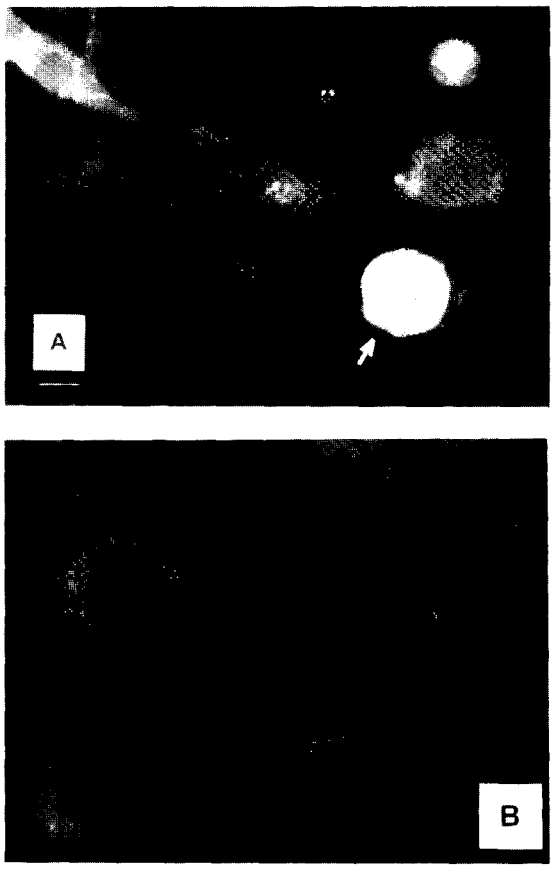

Fig 1. Tyrosine phosphorylation in cultivated human primary keratinocytes, in the presence (A) or absence of 200 nM bradykinin (B), incubation with bradykinin was for 30 s. Bar, 5 μm. Tyrosine phosphorylation was detected by immunofluorescence with anti-phosphotyrosine antibody, as described in Material and Methods.

After stimulation with 200 nM bradykinin for 30 s, those keratinocytes showing tyrosine phosphorylation by bright immunofluorescence are round-headed and just adherent. Other keratinocytes, which are spread over the slide surface and show pseudopodia, were not labelled. These G1 phase keratinocytes with bright immunofluorescence are well attached to the surface of the chamber slide but at the time they are spreading out immunofluorescence vanishes. G1 phase keratinocytes appear to have reached a distinct large diameter, whereas smaller ones show only a background tyrosine phosphorylation.

Specificity of binding was demonstrated by preincubation of FITC conjugated anti-phosphotyrosine antibody with ortho-phosphotyrosine for the purpose of competition.

SDS-PAGE and Western blotting of tyrosine phosphorylated proteins after stimulation with bradykinin.

The extent of total tyrosine phosphorylation of particulate proteins of keratinocytes was tested by ELISA. Cytosolic proteins did not significantly contribute to total tyrosine protein phosphorylation (not shown). Upon short-time stimulation with bradykinin, significantly increased (14% above control) phosphorylation of particulate proteins at tyrosine residues was detected by anti-phosphotyrosine antibody.

CHAPS solubilized proteins of particulate fraction of keratinocytes were used for SDS-PAGE. The increase in phosphorylation of proteins at tyrosine residues was much more pronounced during short-time stimulation and is reflected by a pattern of bands different from that of long-time stimulation or control incubation.

Discrete bands appeared at 82, 76, 70, 57, 54, 48, 40 and 39 kDa, and a diffuse band at 62 kDa (Fig 2, lane A). Tyrosine phosphorylation increased at long-time stimulation (lane B) for the 76 kDa band, the 48 and 40 kDa bands became more diffuse, the 39 kDa band remained unchanged, whereas the other bands disappeared. In the absence of bradykinin (lane C), the basal tyrosine phosphorylation is represented. Only a single band at 38

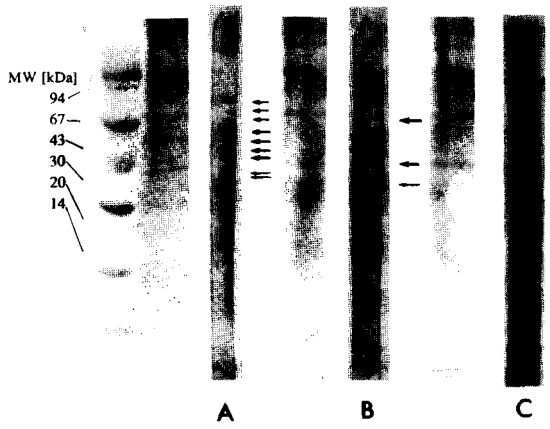

Fig 2. SDS-PAGE and Western blotting of solubilized particulate proteins of human keratinocytes. A, short-time stimulation, 200 nM bradykinin for 30 s. B, long-time incubation, 200 nM bradykinin for 4 h. C, control in the absence of bradykinin. Protein bands in 10-25% SDS polyacrylamide gradient gel were visualized after electrophoresis using Coomassie blue reagent. Tyrosine phosphorylated proteins transferred on polyvinylidendifluoride membrane slices (A, B, C) were detected by forming a gold conjugated immunocomplex with anti-phosphotyrosine as 1st antibody, followed by silver enhancement. For experimental details, see Material and Methods.

kDa is visible as compared to the doublet at 38/39 kDa after short-time and long-time stimulation. Diffuse broad bands are present at about 48, 54 and 62 kDa.

When bands of immunoreactivity are related to the Coomassie blue dyed SDS-PAGE bands, it turned out that –in general– tyrosine phosphorylated proteins are of low abundance, except for the 76, 62 and 40 kDa bands after short-time stimulation, and the 76 and 48 kDa bands after long-time incubation with bradykinin.

Tyrosine phosphatases might be responsible for transiency of bands induced by short-time stimulation. Long-time incubation with bradykinin for 4 h may reflect the effects and reactions in the signal transduction cascades downstream to an initial bradykinin action. Bradykinin itself is expected to be almost completely degraded within 4 h (Schremmer-Danninger *et al*, 1995).

Gel filtration chromatography of solubilized particulate proteins of keratinocytes.

The increase in phosphorylation of tyrosine residues of proteins upon stimulation with bradykinin was also visualized by gel filtration chromatography. During short-time stimulation this increase was much more pronounced and is reflected by two broad peaks in the elution profile (Fig 3A). The first broad peak due to tyrosine phosphorylation is visible between 35 and 92 kDa, with shoulders at 44 and 90 kDa. The specific tyrosine phosphorylation within these peaks was more pronounced at 44 kDa (54%) and at 60 kDa (58%), as compared to the shoulder at 90 kDa (39%). The second peak of immunoreactivity in the high molecular weight region might represent aggregates of tyrosine phosphorylated proteins.

The elution profile from long-time stimulated keratinocytes (Fig 3B) showed that the degree of tyrosine phosphorylation was reduced to about 50% as compared to short-time stimulated keratinocytes. However, a distinct peak at 180 kDa appeared which is not recognized after short-time stimulations. The specific phosphorylation of proteins at tyrosine residues represented in this peak is 92%.

Figure 3C shows basal tyrosine phosphorylation in the absence of bradykinin.

DISCUSSION

The signal transduction cascades induced by binding of bradykinin at its receptor finally cause phosphorylation of proteins at serine, threonine or tyrosine residues, thereby translating short-time stimulation into a long lasting effect. Tyrosine phosphorylation is a basal mechanism of cellular regulation in particular for cell growth. Various growth factors like EGF possess intrinsic tyrosine kinase activity involved in autophosphorylation of their receptors as well as in phosphorylation of other proteins which are thought to play a role in cell proliferation. A mitogenic effect of bradykinin is described to be associated with phosphorylation of tyrosine residues in proteins (Jaffa *et al*, 1997; Zwick *et al*, 1997; Fleming & Busse, 1997).

Tyrosine phosphorylation upon stimulation by bradykinin is only visible in keratinocytes which are in the G1 phase of

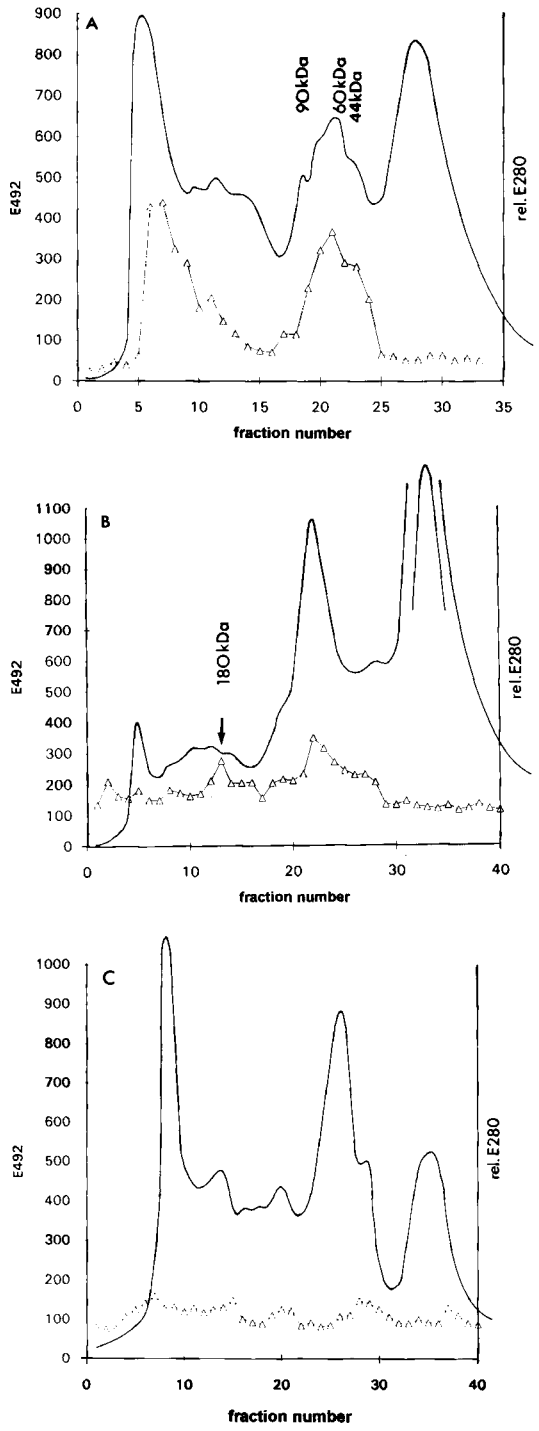

Fig 3. Gel filtration chromatography of solubilized membrane proteins of keratinocytes in primary culture. A, short-time stimulation, 200 nM bradykinin for 30 s. B, long-time stimulation, 200 nM bradykinin for 4 h. C, control without stimulation. Continuous line, extinction at 280 nm, elution profile of proteins. Triangles, extinction at 492 nm of 1 ml fractions tested with ELISA. Solubilization of particulate keratinocyte fractions, gel filtration chromatography and ELISA of tyrosine phosphorylated proteins were done as described in Material and Methods.

cell division (Fig 1A, white arrow). Morphologically, this represents the phase before the so called pan-cake spreading and keratinocytes build pseudopodia covering the surface of the flask or slide. In the G1 phase, protein synthesis occurs at a high rate.

Among the tyrosine phosphorylated bands detected by SDS-PAGE, Western blotting and immunodetection after short-time stimulation with bradykinin, the 40 kDa band most likely represents actin. Tyrosine phosphorylation of actin is strongly correlated with an alteration of the cell shape. Howard *et al* (1993) described this phenomenon in *Dictyostelium* where generated diacylglycerol is capable to induce polymerization of actin and to release Ca^{2+}. This might have some impact on binding of actin to the cell skeleton. In keratinocytes bradykinin is releasing secondary mediators such as inositolphosphates and diacylglycerol due to activation of phospholipase C and Ca^{2+} (Rosenbach *et al*, 1993; Haase *et al*, 1997a). Tyrosine phosphorylation of a number of cytoskeletal proteins is regulated by the actual Ca^{2+} concentration in human endothelial cells (Fleming *et al*, 1996). Ca^{2+} release in keratinocytes seems to be related to the alteration of the cell shape in cultivated cells. Within the intact epidermis the bradykinin receptor bearing basal keratinocytes (Schremmer-Danninger *et al*, 1995) may play a role in the strong association of epidermis and subepidermal layer. Bradykinin-induced tyrosine phosphorylation of keratinocyte actin, as we observed, might be involved in the cell shape alteration contributing to these phenomena.

It has previously been shown that the bradykinin receptor itself is phosphorylated at tyrosine residues leading to a 78 kDa protein band mass value (Jong *et al*, 1993). The band observed by us at 76 kDa, weak at short-time stimulation and enhanced at long-time stimulation, may represent the tyrosine auto-phosphorylated B2 bradykinin receptor. On the other hand, Blaukat *et al* (1996) previously reported that the unstimulated bradykinin B2 receptor is phosphorylated only at its serine and threonine residues. Tyrosine phosphorylation of bradykinin

receptors has been proposed to be involved in receptor function and regulation (Jong *et al*, 1993; Prado *et al*, 1997).

After bradykinin stimulation of endothelial cells, Fleming and Busse (1997) identified a 77 kDa protein as paxillin and a 42/44 kDa doublet as mitogen activated protein (MAP) kinase. Although of slightly different molecular weight, the doublet we observed at 38/39 kDa may represent MAP kinase induced by action of bradykinin. In nonstimulated keratinocytes only the 38 kDa band is visible. MAP kinase recognizes many different substrates in the cell, including growth factors receptors, microtubule-associated proteins and transcription factors. Therefore, tyrosine phosphorylation of MAP kinase may be a prerequisite for mitosis.

In bradykinin stimulated keratinocytes, Coutant *et al* (1995) characterized a 68 kDa protein as paxillin, which represents a focal adhesion associated protein. Lee and Villereal (1996) found proteins bands at 70 and 130 kDa, and identified the 70 kDa band as paxillin in bradykinin stimulated fibroblasts. Hence, paxillin is the most likely 70 kDa candidate protein being phosphorylated in primary human keratinocytes. The function of this focal adhesion associated protein has been reported to involve regulation of actin stress fibers in Swiss 3T3 fibroblasts (Coutant *et al*, 1995).

The above authors also found bands at 54 and 48 kDa in a HaCaT keratinocyte cell line, a spontaneously immortalized non-tumorigenic human keratinocyte cell line. These proteins may be substrates for bradykinin-induced tyrosine phosphorylation, similarly to those we observed in short-time (54 and 48 kDa) and long-time stimulated (48 kDa) primary keratinocytes. Tyrosine phosphorylation of these protein bands seems to be specific for bradykinin stimulation since in nonstimulated keratinocytes only diffuse broad bands were visible close to these kDa values. These proteins are not yet characterized in detail.

Long-time stimulated keratinocytes show a discrete peak of tyrosine phosphorylated proteins at 180 kDa (Fig 3B). Coutant *et al* (1995) identified the EGF receptor (175 kDa) as substrate for the tyrosine phosphorylation induced by stimulation with bradykinin. This finding provides a link between activation of the G protein-coupled bradykinin receptor and modulation of the tyrosine phosphorylation of a growth factor, perhaps mediating synergistic effects. Bradykinin synergistically enhances proliferative responses of fibroblasts to a variety of growth factors, including EGF (Olsen *et al*, 1988), and similar synergy has been observed in other cell types (Walsh & Fan, 1997). Bradykinin-induced modulation of growth factor action may also result in a paracrine action on neighboring cells, which do not express bradykinin receptors themselves. The net effect of bradykinin on cell proliferation *in vivo* depends not only on the expression and second messenger coupling of bradykinin receptors, but also on the presence of other growth factor producing cells in the skin. These effects of bradykinin might be involved in the repair of cutaneous lesions and/or in proliferative skin diseases (Thomas *et al*, 1992).

In summary, the substrates shown to be tyrosine phosphorylated by the action of bradykinin are known to be associated with cellular events such as mitosis, adhesion or variation of cell shape. Therefore, binding of bradykinin to specific B2 bradykinin receptors in keratinocytes might contribute to these actions.

ACKNOWLEDGEMENT

This work was supported by Sonderforschungsbereich 207 and 469 of the Ludwig-Maximilians-Universität, München, Germany.

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