Protein-tyrosine-phosphatase-mediated epidermal growth factor (EGF) receptor transinactivation and EGF receptor-independent stimulation of mitogen-activated protein kinase by bradykinin in A431 cells

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Transactivation of the epidermal growth factor (EGF) receptor (EGFR) has been proposed to represent an essential link between G-protein-coupled receptors and the mitogen-activated protein kinase (MAPK) pathway in various cell types. In the present work we report, in contrast, that in A431 cells bradykinin transinactivates the EGFR and stimulates MAPK activity independently of EGFR tyrosine phosphorylation. Both effects of bradykinin are mediated by a pertussis-toxin-insensitive G-protein. Three lines of evidence suggest the activation of a protein tyrosine phosphatase (PTP) by bradykinin: (i) treatment of A431 cells with bradykinin decreases both basal and EGF-induced EGFR tyrosine phosphorylation, (ii) this effect of bradykinin can be blocked by two different PTP inhibitors, and (iii) bradykinin significantly increased the PTP activity in total A431 cell lysates when measured *in vitro*. The transmembrane receptor PTP σ was identified as a putative mediator of bradykinin-induced downregulation of EGFR autophosphorylation. Activation of MAPK in response to bradykinin was insensitive towards

AG 1478, a specific inhibitor of EGFR tyrosine kinase, but was blocked by wortmannin or bisindolylmaleimide, inhibitors of phosphatidylinositol 3-kinase (PI3-K) and protein kinase C (PKC) respectively. These results also suggest that the bradykinin-induced activation of MAPK is independent of EGFR and indicate a pathway involving PI3-K and PKC. In addition, bradykinin evokes a rapid and transient increase in Src kinase activity. Although Src does not participate in bradykinin-induced stimulation of PTP activity, inhibition of Src by 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo(3,4-*d*)pyrimidine leads to an increase in MAPK activation by bradykinin. Our results suggest that in A431 cells the $G_{q/11}$ -protein-coupled bradykinin B_2 receptor may stimulate PTP activity and thereby transinactivate the EGFR, and may simultaneously activate MAPK by an alternative signalling pathway which can bypass EGFR.

Key words: A431 cells, bradykinin, EGF receptor transinactivation, MAPK activation, RPTP σ .

INTRODUCTION

In recent years, the pathways linking G-protein-coupled receptors (GPCRs) to mitogen-activated protein kinases (MAPKs) [specifically, the extracellular-signal-regulated kinases (ERK)1 and ERK2] have been studied extensively [1,2]. Although these pathways reveal a high degree of complexity and cell specificity, several principal mechanisms have been identified, which include a set of key regulatory signalling molecules, such as tyrosine kinases, adaptor molecules, phosphoinositide 3-kinase (PI3-K) and protein kinase C (PKC). Thus G_i -coupled receptors are thought to activate MAPK via their $\beta\gamma$ -subunit Src-family kinases and/or receptor tyrosine kinases (RTKs), resulting in the tyrosine phosphorylation of Shc and its association with Grb2. Depending on the cell type, the different PI3-K isoforms α , β or γ may be involved upstream or downstream of Src or RTKs [3–6]. G_q -coupled receptors appear to activate MAPK either via a pathway involving PKC and Raf, which can bypass Ras [7] or, alternatively, via a Ras-dependent pathway involving the stimulation of Src [8]. In certain cells, cytosolic protein tyrosine kinases such as Pyk2 may act with Src upstream of Ras [9].

Recently, ligand-independent tyrosine phosphorylation (transactivation) of RTKs, such as the epidermal growth factor (EGF) receptor (EGFR) [5,10] or the platelet-derived growth

factor receptor [11] has been suggested to represent an essential event for MAPK activation by both G_i - and G_q -coupled receptors [5].

Transactivation of EGFR was found in response to a variety of stimuli, such as endothelin-1, lysophosphatidic acid (LPA), thrombin, bombesin or carbachol (via M2 receptors) in Rat-1 fibroblasts or COS-7 cells [5,10], carbachol (via M1 receptors) in HEK-293 cells [12], angiotensin II in GN4 rat liver endothelial cells [13], or bradykinin in PC-12 cells [14] or COS-7 cells [15]. The molecular mechanism of ligand-independent tyrosine phosphorylation of RTKs induced by a GPCR is still poorly understood. For example, LPA-induced MAPK activation in COS-7 cells was found to be dependent on Src-mediated EGFR transactivation [16], whereas, in Rat-1 fibroblasts, Src is not required for MAPK activation by LPA [17]. Furthermore, in PC-12 cells the bradykinin-stimulated EGFR transactivation is critically dependent on the presence of extracellular Ca^{2+} [14], but in HEK-293 cells transactivation of EGFR by carbachol does not require Ca^{2+} [12]. In these cells, PKC was found to mediate EGFR transactivation [12], whereas in GN4 cells PKC suppressed the angiotensin II-induced EGFR transactivation [13]. At present it also remains unclear whether activation of Src, stimulation of the intrinsic kinase activity of EGFR or suppression of tyrosine phosphatase activity might be

Abbreviations used: EGF, epidermal growth factor; EGFR, EGF receptor; GPCR, G-protein-coupled receptor; RTK, receptor tyrosine kinase; PTP, protein tyrosine phosphatase; RPTP, receptor protein tyrosine phosphatase; SHP1, SH2-containing PTP1; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal-regulated kinase; PKC, protein kinase C; PI3-K, phosphatidylinositol 3-kinase; LPA, lysophosphatidic acid; MBP, myelin basic protein; DTT, dithiothreitol; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo(3,4-*d*)pyrimidine.

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responsible for an increase in EGFR tyrosine phosphorylation in response to an agonist-activated GPCR. Recently, it was demonstrated for HeLa cells and NIH 3T3 cells that the intrinsic tyrosine kinase activity of EGFR contributes to the LPA-stimulated MAPK activation [18]. On the other hand, there is also increasing evidence that tyrosine phosphatases may participate in GPCR-induced signalling events [19,20].

The human epidermoid carcinoma cell line A431 that over-express EGFR is a favoured model for studying EGFR and its signal transduction. Beside the EGFR, A431 cells also endogenously express, for instance, the purinergic P2 receptor [21], the LPA receptor [22] and the bradykinin B2 receptor [23,24]. Although the mitogenic as well as the antimitogenic effect of EGF and its relation to MAPK activation by EGF in this cell line is well studied [25,26], there are no investigations of GPCR-induced MAPK activation in A431 cells.

In the present work, we investigated the effect of bradykinin on EGFR tyrosine phosphorylation and MAPK activity in A431 cells. Our results suggest that bradykinin stimulates protein tyrosine phosphatase (PTP) activity and decreases tyrosine phosphorylation of EGFR, on the one hand, and independently activates MAPK via a pathway that is sensitive towards inhibitors of PI3-K and PKC, on the other hand. Thus we provide evidence that, in certain cells, transactivation of EGFR is not necessarily a prerequisite of GPCR-induced activation of MAPK.

EXPERIMENTAL

Materials

[γ - 32 P]ATP (3000 Ci/mmol) was obtained from NEN Life Science Products (Bruxelles, Belgium). Bradykinin, LPA, EGF, pertussis toxin, aprotinin, leupeptin, bacitracin, BSA, myelin basic protein (MBP), sodium orthovanadate, PMSF, dithiothreitol (DTT), ATP, Nonidet-P40, Protein A-Sepharose, horseradish peroxidase-conjugated goat anti-rabbit IgG, Hepes, Tween 20 and diagnostic film (Biomax; Eastman Kodak) were obtained from Sigma (Deisenhofen, Germany). Bisindolylmaleimide was purchased from Boehringer (Mannheim, Germany). Wortmannin, AG 1478 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo(3,4-*d*)pyrimidine (PP1) and phenylarsine oxide were obtained from Calbiochem-Novabiochem (Bad Soden, Germany). Polyclonal antibodies against MAPK p44, EGFR, the PTPs, SH2-containing PTP1 (SHP-1) and PTP-1B, and c-Src were purchased from Santa Cruz (Santa Cruz, CA, U.S.A.). The monoclonal anti-phosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). The specific Src peptide substrate p-AEEIYGEF-EAKKKK was synthesized and kindly provided by Dr Torsten Steinmetzer (Institute of Biochemistry and Biophysics, University of Jena, Germany).

Cell culture

Human epidermoid carcinoma cells A431 (DSM, Braunschweig, Germany) were grown in RPMI 1640 medium supplemented with 10 % (v/v) fetal-calf serum, 100 units/ml penicillin, 10 μ g/ml streptomycin and 0.25 μ g/ml amphotericin B, in humidified air with 5 % CO₂ at 37 °C. For stimulation experiments, A431 cells were grown in 6-well dishes, preincubated in serum-free medium for 2 h and then treated as indicated in the Figure legends.

Cell lysis, immunoprecipitation and Western blotting

After stimulation, cells were scraped off the well and centrifuged for 1 min at 5000 g. The medium was removed and the pellets

were lysed in 1 ml of lysis buffer [20 mM Hepes, pH 7.5, 10 mM EGTA, 40 mM β -glycerophosphate, 1 % (v/v) Triton X-100, 2.5 mM MgCl₂, 2 mM orthovanadate, 1 mM DTT, 1 mM PMSF, 20 μ g/ml aprotinin and 20 μ g/ml leupeptin]. Precleared supernatants were immunoprecipitated using either rabbit polyclonal anti-ERK1 antibody (1 μ g/ml) or rabbit polyclonal anti-EGFR antibody (1 μ g/ml). For immunoprecipitation of tyrosine phosphatases, polyclonal anti-SHP-1 (2 μ g/ml), polyclonal anti-PTP-1B (2 μ g/ml) or affinity-purified polyclonal antibodies against a C-terminal sequence in RPTP σ and LAR [27] (6 μ g/ml) were used and orthovanadate was omitted from the lysis buffer. Antigen-antibody complexes were recovered using Protein A-Sepharose. The immunoprecipitates were washed three times with PBS containing 1 % Triton X-100 and 2 mM orthovanadate (omitted in the case of PTP immunoprecipitates). For ERK1 immunoprecipitates (MAPK assay), a separate washing protocol was used. The samples were boiled in 50 μ l of Laemmli buffer and subjected to SDS/PAGE (7.5 % gels or, for MAPK assay samples, 13 % gels). Separated proteins were transferred to PVDF membranes, blocked overnight with 3 % (w/v) non-fat dried milk in TBST (10 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 0.1 % Tween 20). For immunoblotting analysis of EGFR and ERK1, blots were probed with the respective antibodies. Tyrosine phosphorylation of EGFR was detected with a monoclonal anti-phosphotyrosine antibody 4G10. In this case, filters were blocked with BSA in TBST. For reblotting, a polyclonal anti-EGFR antibody (Santa Cruz) was used. Horseradish peroxidase-conjugated anti-rabbit IgG was used as secondary antibody and proteins were detected using a ECL[®] detection system (Amersham) and exposure to Biomax films (Kodak).

Measurement of p44^{mapk} (ERK1) activity

The ERK1 immunoprecipitates of appropriately stimulated cells were subsequently washed with PBS containing 1 % Triton X-100/2 mM orthovanadate, Tris/HCl, pH 7.5, containing 0.5 M LiCl, and kinase buffer (12.5 mM Mops, pH 7.5, 12.5 mM β -glycerophosphate, 7.5 mM MgCl₂, 0.5 mM EGTA, 0.5 mM sodium fluoride, and 0.5 mM orthovanadate). The phosphorylation assay was performed in 30 μ l of kinase buffer containing 1 μ Ci [γ - 32 P]ATP, 20 μ M ATP, 1.5 mg/ml MBP and 3.3 μ M DTT. After 20 min at 30 °C, the reaction was terminated by the addition of 10 μ l of Laemmli buffer and the samples were boiled for 5 min. Proteins were separated by SDS/PAGE (12.5 % gels) and the gels were cut into two. The lower part was dried, autoradiographed and radioactivity incorporated into MBP was quantified using a Phosphorimager. The upper part of the gel was blotted and the immunoprecipitation was analysed by immunoblotting using an anti-ERK 1 antibody.

PTP assay

For substrate labelling, the synthetic peptide Raytide (1 mg/ml) was dissolved in 50 mM Hepes (pH 7.5), 0.1 mM EDTA, 0.05 % (v/v) Triton X-100. The reaction mixture was prepared by mixing 10 μ l Raytide solution, 10 μ l assay buffer [50 mM Hepes (pH 7.5), 0.1 mM EDTA, 0.015 % Brij 35, 0.1 mg/ml BSA, 0.2 % (v/v) 2-mercaptoethanol], 10 μ l ATP solution (buffer containing 1 mM ATP and 15 μ Ci/ml [γ - 32 P]ATP), and 2 μ l (50 units/ μ l) of active Src kinase. The reaction mixture was incubated for 3 h at 30 °C and then applied to a 0.5 ml Dowex AG 1 $\times$ 8 column. [32 P]Raytide was eluted with 30 % acetic acid and stored at -80 °C for not longer than 4 weeks.

For measuring PTP activity, the reaction mixture contained assay buffer, labelled Raytide (approx. 50000 c.p.m. per assay)

and diluted samples of cell lysates or PTP immunoprecipitates in a total volume of 50 μ l. After incubation for 30 min at 37 °C, the reaction was terminated by adding 750 μ l of ice-cold stop solution [0.9 M HCl, 90 mM sodium pyrophosphate, 2 mM NaH₂PO₄, 4% (w/v) activated charcoal]. After centrifugation for 5 min at 10000 g, 400 μ l of the supernatants were counted for radioactivity.

Phosphorylation of Src peptide substrate

A431 cells were stimulated for the indicated times with 100 nM bradykinin or 100 ng/ml EGF. Cell lysates were immunoprecipitated with c-Src antibody according to the manufacturer's instructions. Immunoprecipitates were washed three times with PBS containing 1% Triton X-100 and resuspended in buffer [50 mM Hepes (pH 7.4), 2 mM DTT, 10 mM MnCl₂, 10 mM MgCl₂, 10 μ M ATP]. Kinase reactions were initiated by the addition of 2 μ Ci [γ -³²P]ATP in the presence of the highly specific Src peptide substrate p-AEEIYGEFEAKKKK [28] in a total volume of 20 μ l. After incubation for 20 min at 30 °C, the reaction was terminated by addition of Laemmli sample buffer (20 μ l) and heating for 2 min at 95 °C. The phosphorylation of the peptide was analysed by SDS/PAGE (16% gel) and autoradiography. Alternatively, phosphorylation was stopped by the addition of 100 μ l of 10% (v/v) phosphoric acid and the reaction mixture was applied to ion-exchange chromatography paper (P-81; Whatman). Papers were washed five times in 0.5% phosphoric acid, dried and counted for radioactivity.

RESULTS

Bradykinin-induced decrease in EGFR tyrosine phosphorylation and stimulation of MAP kinase activity via a pertussis-toxin-insensitive G-protein in A431 cells

In A431 cells overexpressing EGFR, a basal level of EGFR tyrosine phosphorylation was detectable in the absence of EGF. Stimulation with EGF strongly increased tyrosine phosphorylation of EGFR (4.6 ± 0.5 fold over basal; $n = 4$). In contrast, treatment of A431 cells with bradykinin did not result in tyrosine phosphorylation (transactivation) of EGFR, as in other cells,

Figure 1 Time course of bradykinin-induced decrease in basal EGFR tyrosine phosphorylation and of MAPK activation

Serum-starved A431 cells were incubated with either 100 nM bradykinin or, for control, with 50 nM EGF for the times indicated. Cells were lysed, EGFR was immunoprecipitated and analysed using anti-phosphotyrosine antibody as described in the Experimental section (A). The amounts of immunoprecipitated EGFR was controlled by reblotting with anti-EGFR antibody (B). Before immunoprecipitation, samples of cell lysates were subjected to PAGE, blotted, and activated MAPK was determined using a monoclonal p-ERK antibody (C). The results shown are representative of two similar experiments performed in duplicate. WB, Western blot.

Figure 2 Both the effect of bradykinin on EGFR tyrosine phosphorylation and bradykinin-induced activation of MAPK are mediated via a pertussis toxin-insensitive G-protein

A431 cells were preincubated with pertussis toxin (200 ng/ml) for 24 h (PTX, +). The medium was then changed and serum-starved A431 cells were untreated (–) or treated (+) for 5 min with either bradykinin (100 nM) or with LPA (1 μ M). (A) The effect of pertussis-toxin treatment on EGFR tyrosine phosphorylation after exposure of cells to bradykinin or LPA was determined. The Western blots (WB) with 4G10 anti-phosphotyrosine antibody are shown in the upper panel and the respective reblot with anti-EGFR antibody is presented in the lower panel. The immunoblot shown is representative of two separate experiments performed in duplicate. (B) The effect of pertussis toxin (PTX) on bradykinin-induced MAPK activation was assessed using the MBP phosphorylation assay, as described in the Experimental section. The quality of immunoprecipitates was determined by reblotting using anti-ERK-1 antibody. Shown is a representative autoradiogram of three independent experiments.

but rapidly diminished the amount of tyrosine-phosphorylated EGFR in absence of EGF (transinactivation) over a period of 20 min. Summarizing the results of several experiments, the treatment of A431 cells with bradykinin for 5 min resulted in a decrease in basal EGFR tyrosine phosphorylation to $27.8 \pm 19.7\%$ ($n = 10$). Within the same time scale, bradykinin induced a stimulation of MAPK activity (Figure 1), and the combined results of different experiments revealed a 4.8 ± 1.5 -fold stimulation ($n = 10$) of MAPK activity in response to bradykinin. These effects of bradykinin were not significantly affected by pretreatment with pertussis toxin (Figure 2). The apparent slight inhibitory effect of pertussis toxin on bradykinin-induced transinactivation resulted from the extremely potent effect of bradykinin in the experiment shown but this effect was not significant in other experiments [$27.8 \pm 19.7\%$ ($n = 10$) in the presence of bradykinin versus $16.1 \pm 9.0\%$ ($n = 4$) in presence of pertussis toxin and bradykinin]. In controls, the same concentration of pertussis toxin was used to inhibit the decrease in EGFR tyrosine phosphorylation induced by LPA. The action of LPA in this cell line is mainly mediated by pertussis-toxin-insensitive G-proteins [22]. As shown in Figure 3, not only the basal but also the EGF-induced autophosphorylation of EGFR was clearly decreased after pretreatment of A431 cells with bradykinin, reaching a value of $50 \pm 15.1\%$ ($n = 4$) of the basal value. The effect of bradykinin on EGF-stimulated tyrosine phosphorylation of EGFR was also not affected by pertussis toxin (results not shown). In addition, the notable increase in MAPK activity induced by a short stimulation with EGF (5 min) was reduced in presence of bradykinin to the level of bradykinin-induced activation of MAPK. These results suggest that

Figure 3 Bradykinin inhibits EGF-induced autophosphorylation of EGFR (A) as well as EGF-stimulated activation of MAPK (B)

Serum-deprived A431 cells were stimulated with EGF (100 ng/ml) or bradykinin (100 nM) alone or with bradykinin and EGF simultaneously for 5 min. The cells were lysed and both tyrosine phosphorylated EGFR and MBP phosphorylation by immunoprecipitated MAPK were detected as described in the Experimental section. These experiments were repeated twice in duplicate with comparable results.

Figure 4 Effects of PTP inhibitors on bradykinin-induced decrease in basal EGFR tyrosine phosphorylation

Serum-starved A431 cells were preincubated with sodium orthovanadate (500 µM) or phenylarsine oxide (30 µM) for 1 h. The PTP inhibitor-treated cells and untreated controls were then stimulated with bradykinin (100 nM) for 5 min. After cell lysis, EGFR was immunoprecipitated and analysed for tyrosine phosphorylation as described in the Experimental section. The results are representative of two (phenylarsine oxide) or three (orthovanadate) experiments with duplicate samples of different cell preparations.

bradykinin counteracts the effect of EGF at the level of EGFR tyrosine phosphorylation.

Stimulation of PTP activity by bradykinin in A431 cells

When A431 cells were treated with bradykinin in the presence of PTP inhibitors, such as orthovanadate or phenylarsine oxide, the bradykinin-induced decrease in EGFR tyrosine phosphorylation was prevented (Figure 4). Both inhibitors increased the basal (EGF-independent) EGFR tyrosine phosphorylation and this level was not affected by bradykinin. This is the first evidence indicating the involvement of PTP activity in bradykinin signalling in A431 cells. A second line of evidence came from experiments which demonstrated the stimulation of PTP activity by bradykinin *in vitro*. As shown in Table 1, bradykinin significantly increased PTP activity in total cell lysates, as measured by the dephosphorylation of [³²P]Raytide. The increase in PTP activity in response to bradykinin was comparable with that induced by EGF. Indeed, EGF stimulation of EGFR-directed PTP activity in A431 cells has been observed previously

Table 1 Effects of bradykinin and EGF on PTP activity in A431 cells

The labelled PTP substrate, [³²P]Raytide, was incubated with lysates prepared from untreated A431 cells (basal) or A431 cells after treatment with bradykinin (100 nM) or EGF (100 ng/ml) for 5 min each. The PTP assay was performed as described in the Experimental section. Data are expressed as percentages of released [³²P]P_i from the respective controls (basal). Each value is the mean ± S.E.M. of six separate experiments in triplicate determinations. *, significantly different from the controls (100 ± 12% for bradykinin and 100 ± 10% for EGF respectively) (*P* < 0.05; Student's *t* test).

Treatment	PTP activity (%)
None (basal)	100
Bradykinin (100 nM)	139 ± 12*
EGF (100 ng/ml)	137 ± 16*

Table 2 Effect of bradykinin on the activity of different PTPs in A431 cells

Lysates from untreated (basal) and bradykinin-stimulated (5 min) A431 cells were immunoprecipitated with anti-RPTPσ/LAR, anti-SHP-1 or anti-PTP 1B antibodies. The immunoprecipitates were then subjected to the [³²P]Raytide assay as described in the Experimental section. Data are expressed as a percentage of released [³²P]P_i obtained with the respective immunoprecipitates from untreated cells (basal). Each value is the mean ± S.E.M. of six separate experiments in triplicate determinations. *, significantly different from control values (basal) (*P* < 0.05, Student's *t* test).

Form of PTP	PTP activity (%)	
	Basal	Bradykinin (100 nM)
RPTPσ/LAR	100	128 ± 16*
SHP-1	100	95 ± 11
PTP 1B	100	100 ± 10

Figure 5 Blockade of EGFR tyrosine kinase does not influence the stimulatory effect of bradykinin towards MAPK

Serum-deprived A431 cells were preincubated with 100 nM AG 1478 for 30 min and then treated with 100 nM bradykinin or, for control, 50 nM EGF for 5 min. After cell lysis, MAPK activity was determined as described in the Experimental section. Shown are representative autoradiograms of two (EGF) or three (bradykinin) independent experiments performed in duplicate.

[29]. We next investigated whether one of three PTPs known to interact with the EGFR might be affected by bradykinin. Evidence was obtained that RPTPσ was stimulated after treatment of A431 cells with bradykinin, whereas no significant changes in the activities of PTP SHP-1 and PTP 1B were detected (Table 2).

Activation of MAPK by bradykinin is independent of EGFR and involves both PI3K and PKC

As shown in Figure 5, bradykinin-induced activation of MAPK was not affected by the specific EGFR tyrosine kinase inhibitor

Figure 6 Inhibition of PI3-K by wortmannin blocks bradykinin-induced activation of MAPK but not activation of PTP

Serum-starved A431 cells were preincubated with the PI3-K inhibitor wortmannin (100 nM) for 30 min and then treated with bradykinin (100 nM) for 5 min. The cells were lysed and tyrosine phosphorylation of EGFR (**A**) and MAPK activity (**B**) were assayed as described in the Experimental section. The autoradiograms shown are representative of four separate experiments performed in duplicate.

Figure 7 Inhibition of PKC blocks bradykinin-induced MAPK activation but not PTP activation

Serum-deprived A431 cells were pretreated with the PKC inhibitor bisindolylmaleimide (5 μM) for 30 min, followed by the addition of 100 nM bradykinin for 5 min. The cells were lysed, and EGFR tyrosine phosphorylation (**A**) and MAPK activity (**B**) were determined as described in the Experimental section. The autoradiograms shown are representative of four separate experiments performed in duplicate.

AG 1478, suggesting that EGFR is not involved in the pathway linking the bradykinin receptor to MAPK. For comparison, in our assay system, the same concentration of AG 1478 efficiently reduced EGF-induced MAPK activation ($476 \pm 40\%$) to $171 \pm 36\%$ of basal levels ($n = 3$). Wortmannin, a specific inhibitor of PI3 kinases, clearly abolished the stimulatory effect of bradykinin on MAPK activity (Figure 6B), whereas the bradykinin-induced decrease in EGFR tyrosine phosphorylation was insensitive to wortmannin (Figure 6A). Furthermore, activation of MAPK in response to bradykinin was likewise completely abolished in presence of the PKC inhibitor bisindolylmaleimide (Figure 7B). In contrast, stimulation of PTP activity by bradykinin was not influenced by bisindolylmaleimide (Figure 7A). These results indicate that the pathway linking the bradykinin receptor to the MAPK cascade depends on the

Figure 8 Bradykinin evokes a rapid stimulation of c-Src activity and c-Src counteracts bradykinin-induced activation of MAPK

(**A**) Serum-starved cells were stimulated for the indicated times with 100 nM bradykinin or, for a control, EGF (100 ng/ml). Cell lysates were immunoprecipitated with an anti-c-Src antibody. Activity of c-Src kinase was determined *in vitro* as described in the Experimental section. The phosphorylation of the Src peptide substrate was analysed by SDS/PAGE (16% gel). The autoradiogram shown is representative of two experiments with similar results. (**B**) Serum-starved A431 cells were preincubated with the Src inhibitor PP-1 (100 nM) for 30 min followed by stimulation with 100 nM bradykinin for 5 min. MAPK activity (MBP phosphorylation) was determined as described in the Experimental section. The autoradiogram and the respective control blot (WB) of immunoprecipitated MAPK are representative of three separate experiments with different cell preparations performed in duplicate.

activation of certain isoforms of PI3K as well as of PKC in A431 cells.

Stimulation of Src-family kinase activity by bradykinin and effects of the Src inhibitor PP1

To investigate the influence of bradykinin on the activity of Src kinase, lysates from A431 cells were immunoprecipitated with a polyclonal c-Src antibody. The immunoprecipitable kinase activity was determined *in vitro* by its ability to phosphorylate the synthetic peptide AEEIYGEFEAKKKK. As shown in Figure 8(A), the c-Src activity was rapidly increased by bradykinin treatment, reaching a maximum at 40 s and returning to basal levels after 3 min. No peptide phosphorylation was observed when non-immune serum was used for immunoprecipitation (results not shown). PP1, a selective inhibitor for the Src kinase family [30], failed to block the effect of bradykinin on EGFR tyrosine phosphorylation (results not shown). In contrast, inhibition of Src by PP1 lead to an increase in bradykinin-induced stimulation of MAPK activity, up to $118 \pm 29\%$, compared with the effect of bradykinin on MAPK without pretreatment with PP1 (Figure 8B).

DISCUSSION

Tyrosine phosphorylation, and thereby activation of the EGFR, may be controlled by EGF, agonists of various GPCRs and several PTPs. The GPCR-mediated increase in EGFR tyrosine phosphorylation (transactivation) is still poorly understood and might be mediated by effects on intrinsic tyrosine kinase activity of EGFR, Src-family tyrosine kinases or inhibition of PTP

activity. Whereas, up to the present, no receptor-induced inhibition of PTPs has been reported, there is mounting evidence for a GPCR-mediated activation of PTPs. For example, stimulation of the angiotensin II type-2 receptor leads to activation of SHP-1 (PTP1C) [19]. Activation of somatostatin receptor 1 results in an increase in SHP-2 (PTP1D) activity [31]. In contrast, stimulation of somatostatin receptor 2 is followed by activation of SHP-1 [32]. The cytosolic and Src homology domains containing PTPs, SHP-1 and SHP-2, are relatively well characterized. SHP-2 has been implicated as a positive mediator, whereas SHP-1 was found to act as an inhibitor of growth factor-induced mitogenic signalling [33]. Indeed, stimulation of SHP-1 by angiotensin II or by somatostatin leads to inactivation of MAPK and inhibition of cell proliferation [19,32], whereas activation of SHP-2 via somatostatin receptor 1 is accompanied by stimulation of MAPK activity [33].

In A431 cells, we obtained three lines of evidence suggesting the stimulation of PTP activity by bradykinin. First, treatment of A431 cells with bradykinin reduced both basal and EGF-induced tyrosine phosphorylation of EGFR. Secondly, this effect of bradykinin was diminished in the presence of PTP inhibitors. Thirdly, the PTP activity in lysates from bradykinin-triggered A431 cells was significantly higher compared with lysates from unstimulated cells, as measured *in vitro* with the specific PTP substrate [³²P]Raytide.

Among the various PTPs, the cytosolic PTP SHP-1 [34], the cytosolic PTP 1B, which does not contain Src-homology or phosphotyrosine-binding domains [35], and the transmembrane RPTP σ [27] are thought to negatively modulate EGFR. Whereas the activity of SHP-1 or PTP 1B remained unchanged after treatment of A431 cells with bradykinin, we detected elevated PTP activity in immunoprecipitates generated with an antibody recognizing RPTP σ and the closely related PTP LAR. Thus one, or both, of these PTPs may participate in bradykinin-induced downregulation of EGFR autophosphorylation. The increase in the [³²P]Raytide dephosphorylating activity of cell lysates in response to bradykinin, however, was higher compared with the increase in activity of immunoprecipitated RPTP σ /LAR. It is therefore possible that additional PTPs may be activated by bradykinin and contribute to reduced EGFR tyrosine phosphorylation. Very recently, another transmembrane PTP, RPTP α , was found to be involved in m1 muscarinic acetylcholine receptor signalling [20]. Obviously, RPTPs, as well as cytosolic PTPs, may be regulated by GPCRs. The mechanism by which GPCRs modulate PTP activity is not yet known. However, in A431 cells the bradykinin-induced PTP activation is mediated via a pertussis toxin-insensitive G-protein. In this cell line, bradykinin has been shown to bind to the bradykinin B₂ receptor subtype, and to induce both a rapid stimulation of phospholipase C activity via G_{q/11} and a slow increase in cAMP concentration via G_s [24]. Considering the fast kinetics of PTP activation by bradykinin, it seems more likely that G_{q/11}-protein activation mediates the effect of bradykinin on PTP activity.

The first evidence for GPCR-induced decrease in EGFR tyrosine phosphorylation in A431 cells was presented by Langgut and Ogilvie [36]. They reported that stimulation of P2 purinergic ATP receptors leads to an enhanced vanadate-sensitive phosphatase activity which requires phospholipase C β and PKC activity. In contrast, the increase in PTP activity in response to bradykinin in A431 cells was independent of PKC. Since PP1 and wortmannin failed to inhibit the effect of bradykinin on EGFR tyrosine phosphorylation, Src and PI3-K may also be excluded as mediators of bradykinin-induced activation of PTP. Recently, Suzuki et al. [22] demonstrated the LPA-induced stimulation of PTP activity in A431 cells, which is mediated by

pertussis-toxin-sensitive G-proteins of the G_i and/or G_o family. Compared with the pertussis-toxin-insensitive and PKC-independent effect of bradykinin on the activity of PTP, it appears that different signalling pathways may participate in PTP activation by GPCRs. Thus it may be assumed that not only transactivation but also transinactivation of RTKs by GPCRs requires a high degree of cell and receptor specificity.

As reported for various GPCRs and cellular models, transactivation of EGFR is frequently followed by subsequent stimulation of MAPK activity [5,10,12–15]. Stimulation of PTP activity accompanied by EGFR transinactivation has been shown to result in inhibition of MAPK [19], or of EGF-induced activation of transcription factors [22]. In the present work, we demonstrate EGFR transinactivation by bradykinin and a concurrent activation of MAPK. Thus in contrast to other cells [14,15], in A431 cells the bradykinin-induced activation of MAPK is independent of a concomitant EGFR transactivation. In contrast, it requires activation of PI3-K and PKC. Since A431 cells do not contain immunochemically detectable amounts of PI3-K γ , but instead p85 adaptor protein and p110 β (results not shown), the dimeric PI3-K β should be used to mediate the bradykinin effect towards MAPK. Indeed, not only PI3-K γ [4], but also PI3-K β , was shown to be activated by $\beta\gamma$ -complexes from pertussis-toxin-sensitive G-proteins *in vitro* [37]. Recently, we demonstrated the sequential activation of a G_{q/11}-protein, PI3-K β , PKC ϵ and MAPK by bradykinin in the human colon carcinoma cell line SW 480 [6]. At present, we cannot discriminate whether PI3-K is activated upstream of PKC or vice versa, and which of the different bradykinin-activated PKC isoforms is involved in mediating the bradykinin effect on MAPK in A431 cells. Nevertheless, the consecutive activation of PI3-K and PKC may represent an alternative signalling pathway to link GPCRs to MAPK. Furthermore, as in Swiss 3T3 cells [8], bradykinin is capable of inducing a rapid and transient increase in Src kinase activity in A431 cells. Whereas, Src is not important for the effect of bradykinin on PTP activity, it appears to be involved in the fine-tuning of MAPK activation by bradykinin. The mechanism whereby active Src counteracts the effect of bradykinin on MAPK is completely unknown and its exploration represents a separate story. It might be speculated, however, that Src induces tyrosine phosphorylation and, thereby, inactivation of a PKC isoform which participates in bradykinin signalling on MAPK. Indeed, inhibition of PKC δ by an EGFR- and Src-mediated tyrosine phosphorylation was found recently in keratinocytes [38].

Taken together, we present further evidence for a complex signalling network in A431 cells, consisting of the interconnected signalling pathways of the bradykinin B₂ receptor and the EGFR. In the present work, we have demonstrated a signalling route from the bradykinin B₂ receptor to MAPK, which involves the activation of PI3-K and PKC via a G_{q/11}-protein. Bradykinin is also known to activate PKC in A431 cells via G_{q/11} and phospholipase C β , resulting in threonine phosphorylation of EGFR and its desensitization towards EGF [21]. In the present study, we show that, in A431 cells, bradykinin also induces a G_{q/11}-mediated stimulation of PTP activity and, thereby, additionally counteracts the activation of EGFR by EGF. Obviously, bradykinin is capable of using at least three separate G_{q/11}-mediated pathways in A431 cells: one to activate MAPK and two which converge at the EGFR level to prevent stimulation of MAPK by EGF. Three different PKC isoforms (α , δ and ζ) appear to play a key role in bradykinin signalling in A431 cells [39]. They may be activated by coincident signals from separate biochemical routes and divert the signals to different targets, e.g. the EGFR or Raf [7]. These findings in A431 cells

may also reflect at least two basic principles in signal transduction. The dual activation of the PTP as well as the PKC pathway [21], which both affect the EGFR, may be an example of the potency of cells to regulate important signalling molecules by co-operative pathways. Further, the demonstration that transactivation of RTKs is not a general prerequisite for the activation of MAPK by GPCRs shows anew the high degree of cellular specificity of mitogenic signalling.

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REFERENCES

- Van Biesen, T., Luttrell, L. M., Hawes, B. E. and Lefkowitz, R. J. (1996) *Endocrinol. Rev.* **17**, 698–714
- Gutkind, J. S. (1998) *J. Biol. Chem.* **273**, 1839–1842
- Hawes, B. E., Luttrell, L. M., van Biesen, T. and Lefkowitz, R. J. (1996) *J. Biol. Chem.* **271**, 12133–12136
- Lopez-Illasaca, M., Crespo, P., Pellici, P. G., Gutkind, J. S. and Wetzker, R. (1997) *Science* **275**, 394–397
- Daub, H., Wallasch, C., Lankenau, A., Herrlich, A. and Ullrich, A. (1997) *EMBO J.* **16**, 7032–7044
- Graness, A., Adomeit, A., Heinze, R., Wetzker, R. and Liebmann, C. (1998) *J. Biol. Chem.* **273**, 32016–32022
- Schönwasser, D. C., Marais, R. M., Marshall, C. J. and Parker, J. P. (1998) *Mol. Cell. Biol.* **18**, 790–798
- Rodriguez-Fernandez, J. L. and Rozengurt, E. (1996) *J. Biol. Chem.* **271**, 27895–27901
- Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A. and Schlessinger, J. (1996) *Nature (London)* **383**, 547–550
- Daub, H., Weiss, F. U., Wallasch, C. and Ullrich, A. (1996) *Nature (London)* **379**, 557–560
- Herrlich, A., Daub, H., Knebel, A., Herrlich, P., Ullrich, A., Schultz, G. and Gudermann, T. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 8985–8990
- Tsai, W., Morielli, A. D. and Peralta, E. G. (1997) *EMBO J.* **16**, 4597–4605
- Li, X., Lee, J. W., Graves, L. M. and Earp, H. S. (1998) *EMBO J.* **17**, 2574–2583
- Zwick, E., Daub, H., Aoki, N., Yamaguchi-Aoki, Y., Tinhofer, I., Maly, K. and Ullrich, A. (1997) *J. Biol. Chem.* **272**, 24767–24770
- Adomeit, A., Graness, A., Gross, S., Seedorf, K., Wetzker, R. and Liebmann, C. (1999) *Mol. Cell. Biol.* **19**, 5289–5297
- Luttrell, L. M., Della Rocca, G. J., van Biesen, T., Luttrell, D. K. and Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272**, 4637–4644
- Kranenburg, O., Verlaan, I., Hordijk, P. L. and Moolenaar, W. H. (1997) *EMBO J.* **16**, 3097–3105
- Cunnick, J. M., Dorsey, J. F., Standley, T., Turkson, J., Kraker, A. J., Fry, D. W., Jove, R. and Wu, J. (1998) *J. Biol. Chem.* **273**, 14468–14475
- Bedecs, K., Elbaz, N., Sutren, M., Masson, M., Susini, C., Strosberg, A. D. and Nahmias, C. (1997) *Biochem. J.* **325**, 449–454
- Tsai, W., Morielli, A. D., Cachero, T. G. and Peralta, E. G. (1999) *EMBO J.* **18**, 109–118
- Hosoi, K. and Edidin, M. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4510–4514
- Suzuki, Y., Ozawa, Y., Murakami, K. and Miyazaki, H. (1998) *Biochem. Mol. Biol. Int.* **44**, 979–987
- Tilly, B. C., van Paridon, P. A., Verlaan, I., Wirtz, K. W. A., deLaat, S. W. and Moolenaar, W. H. (1987) *Biochem. J.* **244**, 129–135
- Liebmann, C., Graness, A., Ludwig, B., Adomeit, A., Boehmer, A., Boehmer, F.-D., Nürnberg, B. and Wetzker, R. (1996) *Biochem. J.* **313**, 109–118
- Chajry, N., Martin, P.-M., Pages, G., Cochet, C., Aldel, K. and Berthois, Y. (1994) *Biochem. Biophys. Res. Commun.* **203**, 984–990
- Chajry, N., Martin, P.-M., Cochet, C. and Berthois, Y. (1996) *Eur. J. Biochem.* **235**, 97–102
- Suárez-Pestana, E., Tenev, T., Gross, S., Stoyanov, B., Ogata, M. and Böhrer, F.-D. (1999) *Oncogene* **18**, 4069–4079
- Songyang, Z., Carraway, III, K. L., Eck, M. J., Harrison, S. C., Feldman, R. A., Mohammadi, M., Schlessinger, J., Hubbard, S. R., Smith, D. P., Eng, C. et al. (1995) *Nature (London)* **373**, 536–539
- Hernández-Sotomayor, S. M., Arteaga, C. L., Solor, C. and Carpenter, G. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7691–7695
- Hanke, J. H., Gardner, J. P., Dow, R. L., Changelian, P. S., Brissette, W. H., Weringer, E. J., Pollok, B. A. and Connelly, P. A. (1996) *J. Biol. Chem.* **271**, 695–701
- Florio, T., Yao, H., Casey, K. D., Dillon, T. J. and Stork, P. J. (1999) *Mol. Endocrinol.* **13**, 24–37
- Lopez, F., Esteve, J. P., Buscail, L., Delesque, N., Saint-Laurent, N., Theviniau, M., Nahmias, C., Vaysse, N. and Susini, C. (1997) *J. Biol. Chem.* **272**, 24448–24454
- Bennett, A. M., Hausdorff, S. F., O'Reilly, A. M., Freeman, Jr., R. M. and Neel, B. R. (1996) *Mol. Cell. Biol.* **16**, 1189–1202
- Tomic, S., Greiser, U., Lammers, R., Kharitonov, A., Imyaninov, E., Ullrich, A. and Boehmer, F. D. (1995) *J. Biol. Chem.* **270**, 21277–21284
- Liu, F. and Chernoff, J. (1997) *Biochem. J.* **327**, 139–145
- Langgut, W. and Ogilvie, A. (1995) *FEBS Lett.* **372**, 173–176
- Kurosu, H., Maehama, T., Okada, T., Yamamoto, T., Hoshino, S., Fukui, Y., Ui, M., Hazeki, O. and Katada, T. (1997) *J. Biol. Chem.* **272**, 24252–24256
- Denning, M. F., Dlugosz, A. A., Threadgill, D. W., Magnuson, T. and Yuspa, S. H. (1996) *J. Biol. Chem.* **271**, 5325–5331
- Lui, Y.-W., Asaoka, Y., Suzuki, H., Tanhiro, Y., Yamamoto, S. and Chang, W.-C. (1994) *J. Pharmacol. Exp. Ther.* **271**, 567–573

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