British Journal of Pharmacology (2000) 131, 1553–1560

www.nature.com/bjp

In various tumour cell lines the peptide bradykinin B_2 receptor antagonist, Hoe 140 (Icatibant), may act as mitogenic agonist

[1]Sebastian Drube & *,[1]Claus Liebmann

[1]Institute of Biochemistry & Biophysics, Biological and Phamaceutical Faculty, Friedrich-Schiller-University Jena, Philosophenweg 12, D-07743 Jena, Germany

1 This study examined the mitogenic effects of bradykinin (BK, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), the peptide bradykinin B_2 receptor antagonist Hoe 140 (D-Arg^0[Hyp^3-Thi^6-D-Tic^7-Oic^8]BK, and the orally active, nonpeptide B_2 receptor antagonist FR 173657 ((E)-3-(6-acetamido-3-pyridyl)-N-[N-2-4-dichloro-3-[(2-methyl-8-quinolinyl) oxymethyl]phenyl]-N-methylaminocarbonyl-methyl]acrylamide) in three different human tumour cell lines: the small cell lung carcinoma (SCLC) cell line H-69, the breast carcinoma cell line EFM-192A, and the colon carcinoma cell line SW-480.

2 In these cell lines activation of mitogen-activated protein kinase (MAPK) is involved in BK-induced stimulation of cell proliferation and may be mediated by both G_q proteins (SW-480) and G_i proteins (EFM-192A; H-69).

3 In these cells BK as well as Hoe 140 increased the rate of DNA synthesis measured with the [^{3}H]-thymidine uptake assay. Hoe 140 did neither antagonize nor potentiate the effect of BK. FR 173657 did not stimulate [^{3}H]-thymidine incorporation but clearly antagonized the mitogenic effects of BK as well as Hoe 140. In H-69 cells, FR 173657 induced a decrease in the basal rate of DNA synthesis.

4 In all three cell lines BK and Hoe 140 stimulated the activity of MAPK. Their effect on MAPK activity was completely abolished by FR 173657 which itself did not increase the activity of MAPK. In H-69 cells, the basal activity of MAPK was slightly inhibited by FR 173657.

5 In the cell lines SW-480 and H-69 both BK and Hoe 140 but not FR 173657 stimulated phosphatidylinositol hydrolysis. In H-69 cells, FR 173657 decreased basal inositol phosphate formation.

6 Our results show that in certain tumour cell lines the classical peptide B_2 receptor antagonist, Hoe 140, may act as mitogenic B_2 receptor agonist whereas the nonpeptide B_2 receptor antagonist, FR 173657, does not. In H-69 cells FR 173657 was found to exhibit properties of an inverse agonist. *British Journal of Pharmacology* (2000) **131,** 1553–1560

Keywords: Bradykinin; tumor cell lines; B_2 receptor antagonists; Hoe 140; mitogenic agonist; FR 173657; inverse agonist.

Abbreviations: BK, bradykinin; B_2 receptor, bradykinin B_2 receptor; COS-7, transformed african green monkey kidney cell line; DAG, diacylglycerol; EFM-192A, human breast carcinoma cell line EFM-192A; EGF, epidermal growth factor; Erk1, extracellular-signal regulated kinase; FR 173657, ((E)-3-(6-acetamido-3pyridyl)-N-[N-[dichloro-3-[[2-methyl-8-quinolyl)oxymethyl]phenyl]-N-methylaminocarbonyl-methyl]acrylamide); G protein, guanine nucleotide binding regulatory protein; G_i, inhibitory G protein; G_q, G protein that activates phospholipase Cβ; H-69, human small cell lung carcinoma (SCLC) cell line H-69; Hoe 140, (D-Arg^0[Hyp^3-Thr^5-Tic^7-Oic^8]BK; IP_3, inositoltrisphosphate: MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; PD 098059, 2′-Amino-3′-methoxyflavone, inhibitor of MAP kinase kinase (MEK); PI3-kinase, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C, PTX, pertussis toxin; SW-480, human colon carcinoma cell line SW-480

Introduction

The peptide hormone bradykinin (BK; Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) may produce a variety of biological effects such as vasodilation, smooth muscle contraction or relaxation, bronchoconstriction, inflammation, pain and oedema (Regoli & Barabé, 1980; Hall, 1992; Stewart, 1995). Recently, BK was also shown to stimulate cell proliferation (Sethi & Rozengurt, 1991; Bunn *et al*., 1992; Graness *et al*., 1998) and/or to induce activation of mitogen-activated protein kinase (MAPK) (Clerk *et al*., 1996; Graness *et al*., 1998) in different cell types. Both physiological and pathophysiological effects of BK are mediated *via* two subtypes of G protein-coupled receptors designated B_1 and B_2 which have been pharmacologically characterized and identified by molecular cloning (Regoli & Barabé, 1980; McEachern *et al*., 1991; Hess *et al*., 1994; Menke *et al*., 1994). Almost all of the biological responses to BK are mediated *via* B_2 receptors which have a high affinity for BK and are prevalent in normal tissues (Regoli & Barabé, 1980; Hall, 1992). In most cells, the main signalling pathway of the B_2 receptor is the G_q protein-mediated stimulation of phosphatidylinositol metabolism resulting in the formation of inositoltrisphosphate (IP_3) and diacylglycerol (DAG) as second messengers (Yano *et al*., 1984). In contrast, stimulation of MAPK by BK appears to be highly cell specific and may be mediated *via* different pathways including transactivation of EGF receptor and protein kinase C (PKC) (Adomeit *et al*., 1999), phosphoinositide 3-kinase (Graness *et al*., 1998); or the cytosolic tyrosine kinase Pyk2 (Dikič *et al*., 1996).

To obtain useful tools for studying the biological actions of BK as well as putative therapeutic agents many bradykinin B_2 receptor antagonists have been developed.

*Author for correspondence; E-mail: b9licl@rz.uni-jena.de

Among the so-called 'second generation' BK antagonists, Hoe 140 (D-Arg^0[Hyp^3, Thi^5, D-Tic^7, Oic^8]BK), is one of the most selective and specific B_2 receptor antagonist in the tissues tested and was used successfully in basic pharmacology and even in clinical studies (Hock *et al*., 1991; Wirth *et al*., 1991; Lembeck *et al*., 1991; Griesbacher & Lembeck, 1992; Rhaleb *et al*., 1992; Regoli *et al*., 1998). In the past, extensive studies of Hoe 140 revealed some unusual and unexpected properties of this compound. Thus, although Hoe 140 was shown to be devoid of agonistic activity in nearly all human and animal preparations (Regoli *et al*., 1998) in sheep femoral artery Hoe 140 acts with high efficacy and potency as partial agonist (Félétou *et al*., 1994). Recently, Hoe 140 was also described to act as a full agonist at the chicken kinin receptor designated ornithokinin receptor (Schroeder *et al*., 1997). Furthermore, Hoe 140 is a competive antagonist in most biological assays but displays also non-competitive properties in several tissues (Rhaleb *et al*., 1992; Liebmann *et al*., 1993; Félétou *et al*., 1994). In the rabbit jugular vein, Hoe 140 behaves as an insurmontable and irreversible antagonist (Marceau *et al*., 1994). In rat myometrial cells, Hoe 140 was found to act as inverse agonist producing a decrease in basal phosphoinositide hydrolysis (Leeb-Lundberg *et al*., 1994). Finally, it has been shown very recently that Hoe 140 may act as both competitive antagonist and inverse agonist of the human B_2 receptor expressed in COS-7 cells but became a potent agonist in two constitutively active mutants of the B_2 receptor (Marie *et al*., 1999).

Here we describe equally agonistic effects of bradykinin as well as Hoe 140 on cell proliferation and MAPK activity in three different tumour cell lines, the human small cell lung carcinoma cell line (SCLC) H-69, the human breast carcinoma cell line EFM-192A, and the human colon carcinoma cell line SW-480. In H-69 cells and SW-480 cells, Hoe 140 also acts as agonist on phosphatidylinositol turnover. In contrast, the nonpeptide antagonist, FR173657((E)-3-(6-acetamido-3-pyridyl)-N-[N-[2-4-dichloro-3-[(2-methyl-8-quinolyl)oxymethyl]-phenyl]-N-methylaminocarbonyl-methyl]acrylamide) (Asano *et al*., 1997a, b), abolished these mitogenic effects of BK and Hoe 140 in all three cell lines and displayed properties of an inverse agonist in H-69 cells.

Methods

Cell culture

The SCLC (small cell lung carcinoma) cell line H-69 (ATCC, American Type Culture Collection), the human breast carcinoma cell line EFM-192A and the human colon carcinoma cell line SW-480 (both German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were grown in RPMI 1640 medium supplemented with 10% (v v^{-1}) calf serum, 100 u ml^{-1} penicillin, 10 μg ml^{-1} streptomycin, and 0.25 μg ml^{-1} amphotericin B in humidified air with 5% CO_2 at 37°C. For stimulation experiments, cells were grown in 6-well dishes, and for [^{3}H]-thymidine incorporation, they were grown in 24-well dishes and treated as indicated in the figure legends.

Measurement of DNA synthesis

Subconfluent cells were deprived of serum for 24 h and then treated with bradykinin, Hoe 140, and/or FR 173657 as indicated. The cells were incubated for another 24 h, followed by the addition of [^{3}H]-thymidine (1 μCi ml^{-1}) for 12 h. For screening measurement of [^{3}H]-thymidine uptake, cells were filtered through Whatman GF/C glass-fibre filters using a Brandel harvester and washed three times with 5 ml of 10 mM HEPES, pH 7.4. The filters were dried, and the cells were counted for incorporated radioactivity by liquid scintillation counting. Alternatively, [^{3}H]-thymidine incorporation was measured by washing of cells sequentially twice with ice-cold phosphate-buffered saline, 5% trichloracetic acid (TCA), and 95% ethanol. Then, the DNA was extracted with 1 N NaOH and the [^{3}H]-thymidine incorporation into the TCA precipitate was assayed by liquid scintillation counting.

Measurement of p44 MAPK (Erkl) activity

The cells were preincubated in serum-free RPMI 1640 medium for 2 h and then either treated with BK, Hoe 140, or FR 173657 for 5 min or preincubated with Hoe 140 or FR 173657 for 10 min followed by treatment with BK or Hoe 140 for 5 min as indicated. The concentration used for all compounds was 100 nM. After stimulation, cells were scraped off and centrifuged for 1 min at $5000 \times g$. The medium was removed, and the pellets were lysed in 1 ml of lysis buffer (in mM): HEPES 20 (pH 7.5), EGTA 10, β-glycerophosphate 40 (1% Triton X-100), $MgCl_2$ 2.5, orthovanadate 2, dithiothreitol 1, phenylmethylsulfonylfluoride 1, aprotinin 20 μg ml^{-1} and leupeptin 20 μg ml^{-1}). After 30 min on ice, the lysates were centrifuged (10 min, $15{,}000 \times g$, at 4°C) to pellet insoluble material. The supernatants were transferred into new tubes, and Erk1 was immunoprecipitated using a rabbit polyclonal antibody (1 μg ml^{-1} lysate) from Santa Cruz Biotechnology. The immunoprecipitates were subsequently washed with phosphate-buffered saline containing 1% Triton X-100 and 2 mM orthovanadate; Tris-HCl, pH 7.5, containing 0.5 mM LiCl; and kinase buffer (in mM) MOPS 12.5 (pH 7.5), β-glycerophosphate 12.5, $MgCl_2$ 7.5, EGTA 0.5, sodium fluoride 0.5 and orthovanadate 0.5. Phosphorylation of immunoprecipitates was performed in 30 μl of kinase buffer supplemented with 1 μCi of [γ-^{32}P]-ATP, 20 μM ATP, 1.5 mg ml^{-1} myelin basic protein (MBP), and 3.3 μM dithiothreitol. After 20 min at 30°C, the reaction was terminated by the addition of 30 μl of Laemmli buffer. The samples were boiled for 5 min and analysed by SDS gel electrophoresis on 12% (w v^{-1}) gels. Phosphorylated MBP was visualized by autoradiography and quantified using a Phosphorimager.

Determination of inositol phosphate formation

Measurement of phosphatidylinositol turnover was performed as decribed previously (Graness *et al*., 1998). Briefly, cells in 24-well plates were prelabelled with 4 μCi ml^{-1} *myo*-[^{3}H]-inositol for 24 h. At 2 h prior to stimulation, the cells were incubated in serum-free medium containing 20 mM HEPES, pH 7.4, and 1 μM captopril. The cells were stimulated with BK or Hoe 140, as indicated, in the presence of 10 mM LiCl for 10 min. For termination, the medium was replaced by 1 ml of 10% trichloracetic acid. After 10 min, the extracts were collected, and the trichloracetic acid was removed by washing four times with 2 volumes of water-saturated diethyl ether. After neutralization by adding Tris base, the samples were diluted to 4 ml with distilled water. The inositol phosphate fractions containing total inositol phosphates were obtained by eluting five times with 2 ml

of 1.0 M ammonium formate and 0.1 M formic acid from AG 1×8 columns (200–400 mesh, formate form, Bio-Rad). Radioactivity of the inositol-containing fractions was determined by liquid scintillation counting using Flo-Scint IV scintillator (Packard Bioscience B.V., Groningen, The Netherlands).

Materials

[γ-^{32}P]-ATP (3000 Ci mmol^{-1}) and *myo*-[^{3}H]-inositol (20.5 Ci mmol^{-1}) were obtained from NEN Life Science Products. [^{3}H]-Thymidine (2.0 Ci mmol^{-1}) and all reagents for SDS-polyacrylamide gel electrophoresis were purchased from Amersham Pharmacia Biotechnology. Bradykinin, captopril, Pertussis toxin, aprotinin, leupeptin, β-glycerophosphate, myelin basic protein, sodium orthovanadate, phenylmethylsulfonyl fluoride, dithiothreitol, EGTA, ATP, Triton X-100, protein A-Sepharose, HEPES, MOPS, and diagnostic film (Biomax, Eastman Kodak Co.) were obtained from Sigma (Deisenhofen, Germany). Ammonium formate and sodium tetraborate were from Serva (Heidelberg, Germany) Wortmannin and PD 098059 were obtained from Calbiochem (Bad Soden, Germany). Polyclonal antibodies against p44 MAPK (Erk1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Hoe 140 (icatibant) was kindly provided by Professor B. Schölkens (Hoechst AG, Frankfurt, Germany). FR 173657 was a generous gift of Dr N. Inamura (Fujisawa Pharmaceutical Co., Osaka, Japan).

Results

Signalling pathways from B_2 receptor to MAPK in the tumour cell lines investigated

In SW-480 cells, BK activates MAPK *via* a Pertussis toxin (PTX)-insensitive $G_{q/11}$ protein and subsequently by activation of PI3-kinase β and PKCε. This connection of a G protein-coupled receptor to MAPK is uncommon and was recently characterized in detail (Graness *et al*., 1998). In EFM-192A cells, the pathway linking the B_2R to MAPK is not yet described. We found a biochemical route which is sensitive towards PTX and wortmannin, an inhibitor of PI3-kinase (Figure 1) as well as bisindolylmaleimide, an inhibitor of PKC (not shown). This finding indicates a pathway which might be similar to that in SW-480 cells involving PI3-kinase and PKC but is mediated *via* a G_i protein. In H-69 cells BK also activates MAPK *via* a PTX-sensitive G_i protein but the effect of BK is insensitive to wortmannin excluding the involvement of a PI3-kinase (Figure 1). In contrast, in H-69 cells BK stimulates inositol phosphate formation whereas in EFM-192A cells phospholipase C activity remained unchanged in presence of BK.

Similar to bradykinin the B_2 receptor antagonist, Hoe 140, stimulates both DNA synthesis and MAP kinase activity

In the three different human tumour cell lines investigated the peptide bradykinin B_2 receptor antagonist Hoe 140 produced a significant increase in DNA synthesis. Figure 2A reveals the increase in [^{3}H]-thymidine incorporation in response to Hoe 140 at concentrations of 10 nM and 1 μM. As shown in Figure 2B, Hoe 140 also stimulates the activity of MAPK (Erk1) in these cell lines even at concentrations of 1–10 nM reaching a maximum at 1 μM.

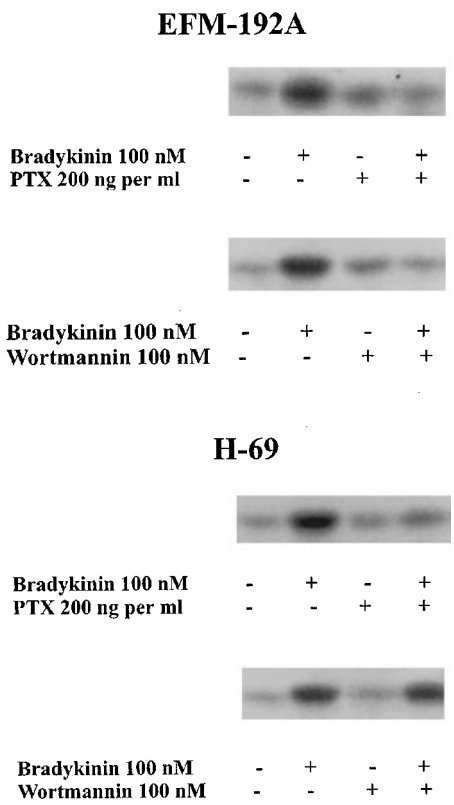

Figure 1 Bradykinin-induced activation of MAPK in the human tumour cell lines EFM-192A and H-69: effects of Pertussis toxin (PTX) and wortmannin. Cells were preincubated with either PTX (200 ng ml^{-1}) for 24 h or wortmannin (100 nM) for 30 min. Then, serum-starved cells were treated with 100 nM bradykinin for 5 min. After lysis of the cells, MAPK activity was assessed using the myelin basic protein (MBP) phosphorylation assay as described under Methods. Results from one of three similar experiments are shown.

Activation of MAPK is involved in the mitogenic action of both BK and Hoe 140

To clarify whether activation of the MAPK pathway is required for the induction of cell division in our cellular models, we measured the effects of BK and Hoe 140 on [^{3}H]-thymidine incorporation in the presence of PD 098059, which inhibits the activation of MAPK by blocking the activity of MAPK kinase (MEK).

In Figure 3 is shown that PD 098053 inhibits both [^{3}H]-thymidine incorporation and activation of MAPK induced by BK or Hoe 140. It may be concluded that the proliferation in all three cell lines used in response to both BK and Hoe 140 depends on the activation of the MAPK pathway.

The nonpeptide B_2 receptor antagonist, FR 173657, blocks the agonistic effects of BK and Hoe on DNA synthesis as well as MAPK activity

As shown in Figure 4, at equal concentrations bradykinin and Hoe 140 and stimulate the DNA synthesis in a very similar range. Furthermore, the effects of Hoe 140 and BK on thymidine incorporation are not additive suggesting that both peptides act as agonists at the same type of B_2 receptor in the cells studied. In EFM-192A and SW-480 cells the compound FR 173657 did not change the [^{3}H]-thymidine incorporation. In the SCLC cell line H-69, in contrast, FR 173657 significantly decreased the basal level of [^{3}H]-

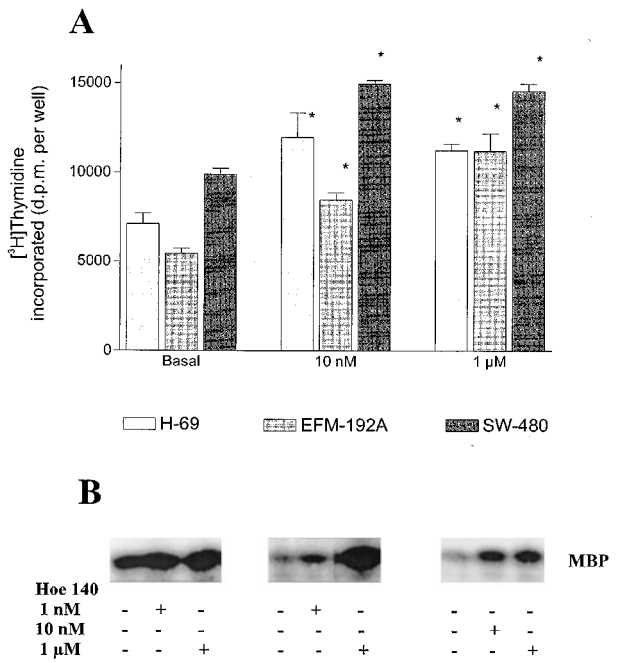

Figure 2 (A) Effect of different concentrations of Hoe 140 on [^{3}H]-thymidine uptake in H-69 cells, EFM-192A cells, and SW-480 cells compared with the respective basal rates of DNA synthesis. Serum-deprived cells were treated with Hoe 140 in the concentrations of 10 nM and 1 μM as indicated for 36 h and [^{3}H]-thymidine for 12 h of incubation. The results obtained with the TCA precipitation assay represent the means $\pm$ s.e.mean of 12 wells in three independent experiments. *Significantly different compared with the basal value ($P<0.05$; Student's t-test). (B) Stimulation of MAPK activity by different concentrations of Hoe 140. Serum-deprived cells were incubated with increasing concentrations of Hoe 140 for 5 min., lysed, and MAPK activity was determined as outlined under Methods. Shown is a representative autoradiogram from two experiments with similar results.

thymidine incorporation. In all three human tumour cell lines FR 173657 clearly antagonized the stimulatory effects of BK as well as Hoe 140 on DNA synthesis (Figure 4). This is a second line of evidence suggesting that the mitogenic action of both peptides is mediated *via* B_2 receptors.

In H-69 cells BK as well as Hoe 140 induced activation of p44 MAPK (Figure 5). In contrast, FR 173657 slightly decreased the relatively high basal activity of MAPK cells to approximately 78% (Figure 5). The apparent stronger increase in MAPK activity in response to Hoe 140 compared with BK in the autoradiogram shown is not significant throughout the repeated experiments. The increase in basal activity of MAPK induced by both peptides varied between 300 and 320%. The stimulatory effects of BK as well as Hoe 140 on MAPK were clearly abolished by pretreatment of H-69 cells with FR 173657. In contrast, in cells pretreated with Hoe 140 followed by the addition of BK there was no significant change in MAPK activity compared with the single effects of BK or Hoe 140. Similar patterns of MAPK activation by BK and Hoe 140 were obtained in EFM-192A cells and SW-480 cells (Figure 5). In EFM-192A cells both BK and Hoe 140 stimulated MAPK activity to approximately 330–340% and in SW-480 cells to approximately 540–610% each compared with the respective basal activities (100%). In SW-480 cells, the higher increase in MAPK activity results from the extremely low basal activity. In both cases FR 173657 itself was without any effect on MAPK but prevented the stimulation of MAPK by BK and Hoe 140.

Bradykinin and Hoe 140 stimulate inositol phosphate formation

In parallel studies we investigated whether Hoe 140 may also act as an agonist in conventional BK signalling pathways such as the stimulation of phosphatidylinositol metabolism. As shown in Figure 6, in H-69 cells as well as in SW-480 cells an increase in inositol phosphate accumulation in response to Hoe 140 was obtained which is comparable with that induced by BK. Whereas in SW-480 cells FR 173657 was without effect on phosphatidylinositol turnover we observed a slight but significant decrease in basal inositol phosphate formation in presence of FR 173657 in H-69 cells. In EFM-192A cells neither BK nor Hoe 140 induced a measurable effect on inositol phosphate formation. This may be explained by our finding that in EFM-192A cells activation of MAPK by BK is mediated *via* a G_i protein (Figure 1). Thus, in this cell line the activation of PLCβ is apparently not involved in B_2R signalling.

Discussion

Bradykinin B_2 receptor antagonists have been mainly characterized with respect to inhibition of the responses of isolated smooth muscle preparations to bradykinin (Vavrek & Stewart, 1985; Hock *et al*., 1991; Lembeck *et al*., 1991; Griesbacher & Lembeck, 1992; Rhaleb *et al*., 1992; Aramori *et al*., 1997; Asano *et al*., 1997a,b), in animal models of nociception (Lembeck *et al*., 1991) or inflammation (Asano *et al*., 1997a,b), and in receptor binding studies (Hock *et al*., 1991; Liebmann *et al*., 1993; Aramori *et al*., 1997; Asano *et al*., 1997a). There are only a few reports that Hoe 140 was used to block mitogenic activities of BK. Thus, Hoe 140 was reported to inhibit BK-induced activation of MAPK in cultured ventricular myocytes (Clerk *et al*., 1996) or in vascular smooth muscle cells (Velarde *et al*., 1999). Here, in contrast, we present the three different human tumour cell lines H-69, EFM-192A, and SW-480 where Hoe 140 did not inhibit BK-induced mitogenic responses but itself acts as mitogenic B_2 receptor agonist. This is supported by several lines of evidence: (i) similar to BK, Hoe 140 stimulates at nanomolar concentrations DNA synthesis as well as activation of MAPK in all three cell lines investigated; (ii) the nonpeptide B_2 receptor antagonist, FR 173657, which did neither stimulate DNA synthesis nor MAPK activity abolished the agonistic effects of both BK and Hoe 140; (iii) the effects of BK and Hoe 140 are not additive suggesting that both peptides act *via* the same type of receptor and (iv) similar to BK, Hoe 140 stimulates phosphatidylinositol metabolism in H-69 cells and SW-480 cells. In EFM-192A cells, stimulation of phospholipase C is apparently not involved in the mitogenic B_2 receptor signalling. Comparable agonistic effects of both BK and Hoe 140 on [^{3}H]-thymidine incorporation were additionally detected in the human lung carcinoma cell line A-427 and in the human small cell lung cancer cell line COLO-677 (results not shown).

Taken together, these findings suggest that Hoe 140 may act as mitogenic agonist *via* the B_2 receptor and may probably use the same mitogenic signalling pathways to MAPK as BK. The pathways linking G protein coupled receptors (GPCRs) such as the bradykinin B_2 receptor to the MAPK cascade may be highly diverse and cell specific (Luttrell *et al*., 1999). For the human B_2 receptor transiently expressed in COS-7 cells, for example, a $G_{q\alpha}$ mediated dual pathway of MAPK activation has been detected which

involves both EGF receptor transactivation and the activation of the PKC isoforms α and ε (Adomeit *et al.*, 1999). In contrast, in several tumour cell lines different connections between B_2 receptor and MAPK may be found. Thus, for SW-480 cells we have recently shown that the BK-induced stimulation of MAPK activity is mediated *via* the consecutive

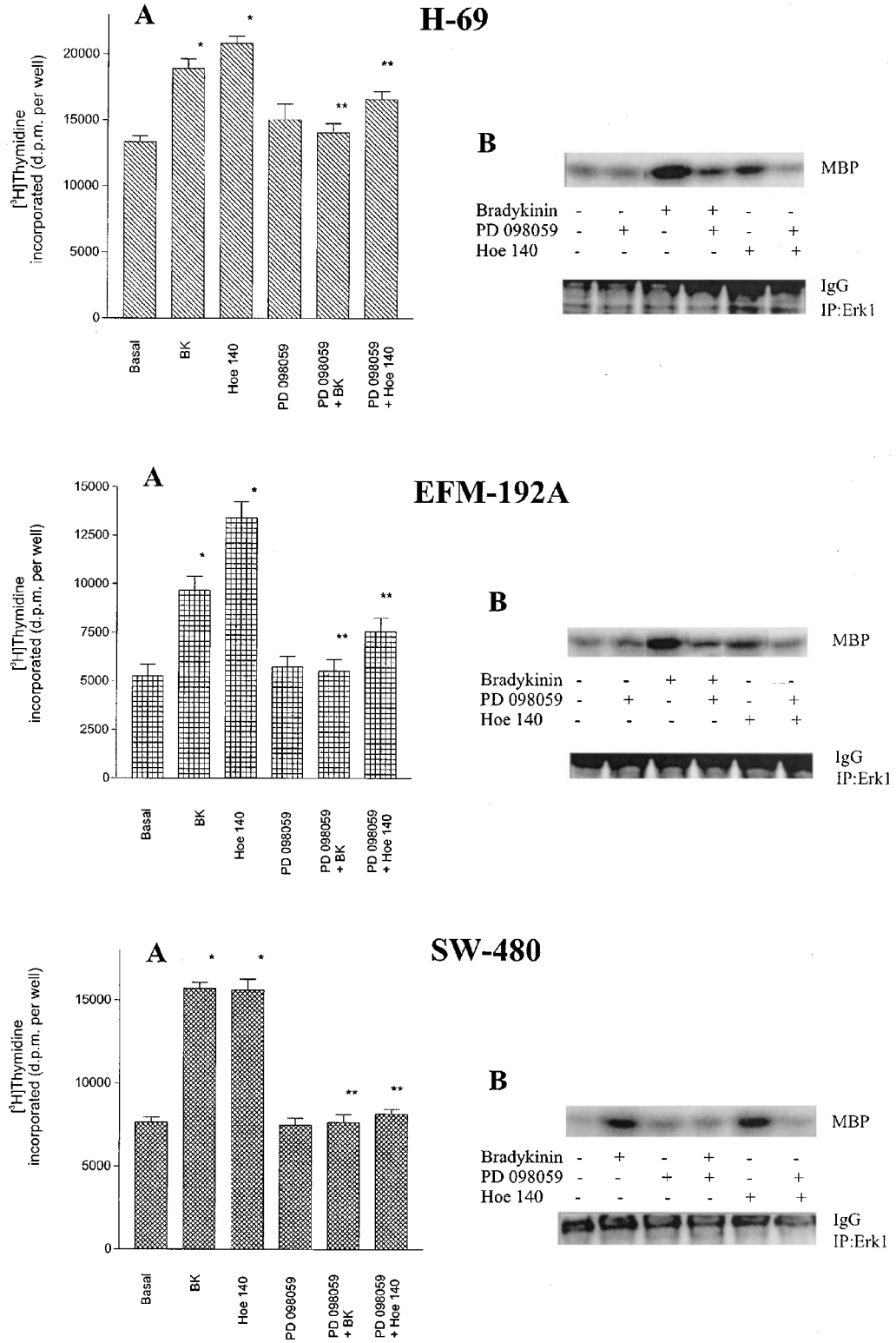

Figure 3 Effects of both Bradykinin and Hoe 140 on DNA synthesis is dependent on MAPK activation. (A) Cells were treated with 1 μM BK or Hoe 140 for 24 h in the absence or presence of the MEK-inhibitor PD 098059 (30 μM) and then DNA synthesis was assessed by measuring [^{3}H]-thymidine incorporation using the TCA precipitation assay. Each value is the mean $\pm$ s.e.mean of 12 wells representative of two independent experiments. *Significantly higher compared with the basal values; **Significantly different compared with the effects without the inhibitor ($P<0.05$; Student's t-test). (B) Lysates from the respective cells after stimulation with 1 μM BK or Hoe 140 in absence or presence of 30 μM PD 098059 were analysed for MAPK activity as described. Shown are representative autoradiograms of two independent experiments. Representative control blots of immunoprecipitated MAPK (IP:Erk1) after Western blotting with anti-Erk1 antibodies are shown in the panels below the MAPK blots.

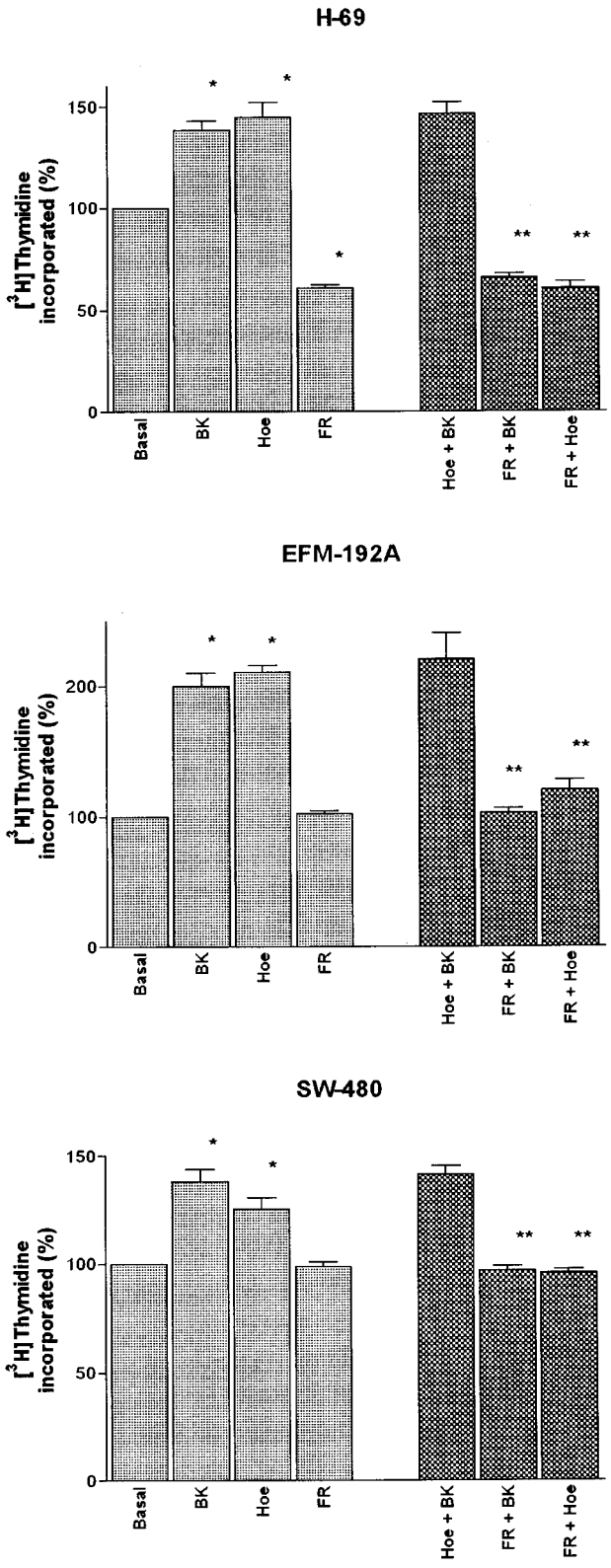

Figure 4 Effects of bradykinin, Hoe 140, and FR 173657 on the rate of DNA synthesis in the human tumour cell lines H-69, EFM-192A, and SW-480. For the additive treatments, serum-deprived cells were preincubated with the putative antagonists Hoe 140 (1 μM) or FR 173657 (1 μM) for 1 h. Then, cells were stimulated with BK, Hoe 140, or FR 173657 (1 μM each) and assayed for [^{3}H]-thymidine uptake or incorporation as described under Methods. The results are expressed as the means$\pm$s.e.mean from 16 wells in four separate experiments. *Significantly different, compared with the respective basal values; **Significantly different compared with the respective singular effects ($P<0.05$; Student's t-test).

activation of $G_{q\alpha}$, PI3-kinase β, and PKC ε (Graness *et al*., 1998). Here we present evidence that in two other cell lines, EFM-192A cells and H-69 cells, BK activates MAPK *via* a PTX-sensitive G protein of the G_i family. In H-69 cells but not in EFM-192A cells the B_2 receptor is capable of stimulating inositol phosphate formation *via* a G_i protein. In both cell lines, the pathways of B_2 receptor to MAPK involve activation of PI3-kinase and PKC (not shown) but are independent of EGFR transactivation (not shown). To the present knowledge the binding sites of agonists and antagonists in the bradykinin B_2 receptor are not identical but appear to overlap partially (Jarnagin *et al*., 1996). It may be speculated, therefore, that in certain tumour cell lines cell specific mutations in the B_2 receptor or alterations in its conformation or palmitoylation might switch the antagonist contact site to agonist binding site positions and couple the antagonist binding site to the signal transduction machinery. This assumption is supported by the recently published finding that the antagonist Hoe 140 which behaved as inverse agonist of the wild-type receptor was a potent agonist at two constitutively active B_2 receptor mutants (Marie *et al*., 1999). Furthermore, the property of Hoe 140 and of several other

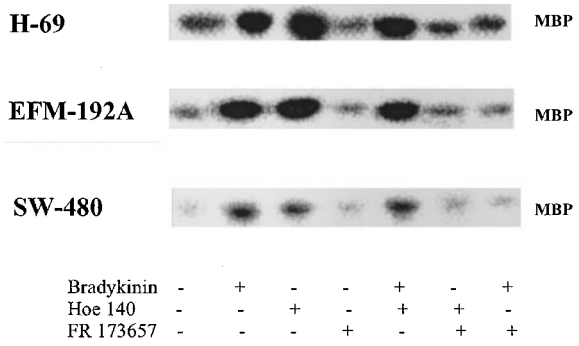

Figure 5 Effects of BK, Hoe 140, and FR 173657 on the activity of MAPK in the cell lines H-69, EFM-192A, and SW-480. Serum-starved cells were preincubated with either Hoe 140 or FR 173657 (100 nM each) as indicated for 10 min. Then, the respective treatments with BK, Hoe 140 or FR 173657 (100 nM each) were performed for 5 min. MAPK activity was assessed as described under Methods. Shown are representative autoradiograms from three separate experiments with similar results.

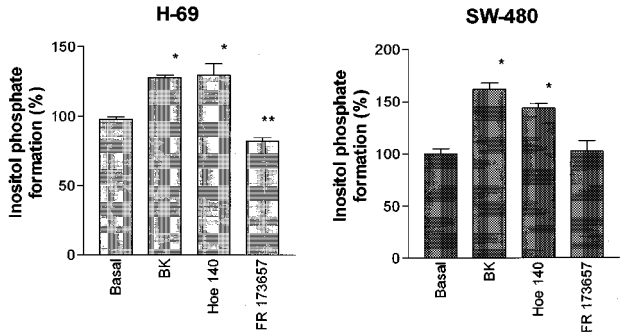

Figure 6 Effects of BK, Hoe 140, and FR 173657 on inositol phosphate formation in SW-480 cells and in H-69 cells. Serum-starved cells prelabelled with *myo*[^{3}H]-inositol (4 μCi ml^{-1}) were treated with BK, Hoe 140 or FR 173657 (100 nM each) for 10 min and then assayed for inositol phosphates as described under Methods. Shown are the means$\pm$s.e.mean from three separate experiments performed in quadruplicate determinations. *Significantly higher and **Significantly lower compared with the basal values ($P<0.05$; Student's t-test).

peptide B_2 receptor antagonists to produce a decrease in basal phosphoinositide hydrolysis thereby acting as inverse agonist (negative antagonist) is a well-known phenomenon (Leeb-Lundberg *et al*., 1994). The detection of an inverse agonist is often difficult and needs a cell system with a sufficiently high number of receptors that exist spontaneously in an activated (G protein-uncoupled) state such as, for example, naturally occurring constitutively active mutants. In our study the nonpeptide B_2 receptor antagonist FR 173657 exhibited inverse agonistic activity which was obtained in the SCLC line H-69 as a decrease in basal [^{3}H]-thymidine incorporation, a decrease in basal MAPK activity and a decrease in basal inositol phosphate formation in presence of FR 173657. In the other cell lines investigated no inverse agonism of FR 173657 was detectable reflecting either an insufficient amount of spontaneously active B_2 receptors or a high degree of cell specificity of this effect of FR 173657. In all three human tumour cell lines tested FR 173657 exhibited strong antagonistic properties with respect to both BK and Hoe 140. It may be assumed that in the cell lines used, the B_2 receptor displays a different pattern of binding sites for the peptidic antagonist Hoe 140 which represents a derivative of BK and the nonpeptidic antagonist FR 173657.

In conclusion, our study shows that in different tumour cell lines the signalling pathways from bradykinin B_2 receptor to MAPK are highly cell specific and may be mediated by G_q as well as G_i proteins. We also demonstrate that in these tumour cell lines the classical peptide B_2 receptor antagonist Hoe 140 may act as a potent mitogenic agonist. This finding suggests that in tumour cells G protein-coupled receptors such as the B_2 receptor may generate novel properties towards antagonists compared with non-cancer cells. Furthermore, the orally active nonpeptide B_2 receptor antagonist FR 173657 was found to be devoid of mitogenic side effects. This finding supports its putative role as a useful drug for inflammatory diseases.

The authors thank Mrs Carmen Mertens and Mrs Britta Haarseim for excellent technical assistance and Mrs Heidi Traber for help in preparation of figures. This work was supported by the Deutsche Forschungsgemeinschaft.

References

ADOMEIT, A., GRANESS, A., GROSS, S., SEEDORF, K., WETZKER, R. & LIEBMANN, C. (1999). Bradykinin B_2 receptor-mediated mitogenactivated protein kinase activation in COS-7 cells requires dual signaling via both protein kinase C pathway and epidermal growth factor receptor. *Mol. Cell. Biol.,* **19,** 5289–5297.

ARAMORI, I., ZENKOH, J., MORIKAWA, N., O'DONELL, N., ASANO, M., NAKAMURA, K., IWAMI, M.K., KOJO, H. & NOTSU, Y. (1997). Novel subtype-selective nonpeptide bradykinin receptor antagonists FR 167344 and FR 173657. *Mol. Pharmacol.,* **51,** 171–176.

ASANO, M., HATORI, C., INAMURA, N., SAWAI, H., HIROSUMI, J., FUJIWARA, T. & NAKAHARA, K. (1997b). Effects of a nonpeptide bradykinin B_2 receptor antagonist, FR 167344, on different *in vivo* animal models of inflammation. *Br. J. Pharmacol.,* **122,** 1436–1440.

ASANO, M., INAMURA, N., HATORI, C., SAWAI, H., FUJIWARA, T., KATAYAMA, A., KAYAKIRI, H., SATOH, S., ABE, Y., INOUE, T., SAWADA, Y., NAKAHARA, K., OKU, T. & OKUHARA, M. (1997a). The identification of an orally active, nonpeptide bradykinin B_2 receptor antagonist, FR 173657. *Br. J. Pharmacol.,* **120,** 617–624.

BUNN, P.A., CHAN, D.C., DIENHART, D.G., TOLLEY, R., TAGAWA, M. & JEWETT, P. (1992). Neuropeptide signal transduction in lung carcinoma: clinical implications of bradykinin and overall heterogeneity. *Cancer Res.,* **52,** 24–31.

CLERK, A., GILLESPIE-BROWN, J., FULLER, S.J. & SUDDEN, P.H. (1996). Stimulation of phosphatidylinositol hydrolysis, protein kinase C translocation, and mitogen-activated protein kinase activity by bradykinin in rat ventricular monocytes: dissociation from the hypertrophic response. *Biochem. J.,* **317,** 109–118.

DIKIĈ, I., TOKIWA, G., LEV, S., COURTNEIDGE, S.A. & SCHLESSINGER, J. (1996). A role for Pyk2 and Src in linking G protein-coupled receptors to MAP kinase activation. *Nature,* **383,** 547–550.

FELETOU, M., GERMAIN, M., THIURIEAU, C., FAUCHERE, J.L. & CANET, E. (1994). Agonistic and antagonistic properties of the bradykinin B_2 receptor antagonist, Hoe 140, in isolated blood vessels from different species. *Br. J. Pharmacol.,* **112,** 683–689.

GRANESS, A., ADOMEIT, A., HEINZE, R., WETZKER, R. & LIEBMANN, C. (1998). A novel mitogenic signaling pathway of bradykinin in the human colon carcinoma cell line SW-480 involves sequential activation of a $G_{q/11}$ protein, phosphatidylinositol 3-kinase β, and protein kinase C ε. *J. Biol. Chem.,* **273,** 32016–32022.

GRIESBACHER, T. & LEMBECK, F. (1992). Analysis of the antagonistic actions of Hoe 140 and novel bradykinin analogues on the guinea-pig ileum. *Eur. J. Pharmacol.,* **211,** 393–398.

HALL, J.M. (1992). Bradykinin receptors: Pharmacological properties and biological roles. *Pharmacol. Ther.,* **56,** 131–190.

HESS, J.F., BORKOWSKI, J.A., MACNEIL, T., STONESIEFER, G.Y., FRAHER, J., STRADER, C.D. & RANSOM, R.W. (1994). Differential pharmacology of cloned human and mouse B_2 bradykinin receptors. *Mol. Pharmacol.,* **45,** 1–8.

HOCK, F.J., WIRTH, K., ALBUS, U., LINZ, W., GERHARDS, H.J., WIEMER, G., HENKE, S., BREIPOHL, G., KÖNIG, W., KNOLLE, J. & SCHÖLKENS, B.A. (1991). Hoe 140 a new potent and long-acting bradykinin antagonist: *in vitro* studies. *Br. J. Pharmacol.,* **102,** 769–773.

JARNAGIN, K., BHAKTA, S., ZUPPAN, P., YEE, C., HO, T., PHAN, T., TAHILRAMANI, R., PEASE, J.H.B., MILLER, A. & FREEDMANN, F. (1996). Mutations in the B_2 bradykinin receptor reveal a different pattern of contacts for peptidic agonists and peptidic antagonists. *J. Biol. Chem.,* **271,** 28277–28286.

LEEB-LUNDBERG, L.M.F., MATHIS, S.A. & HERZIG, M.C.S. (1994). Antagonists of bradykinin that stabilize a G-protein-uncoupled state of the B_2 receptor act as inverse agonists in rat myometrial cells. *J. Biol. Chem.,* **269,** 25970–25973.

LEMBECK, F., GRIESBACHER, T., ECKHARDT, M., HENKE, S., BREIPOHL, G. & KNOLLE, J. (1991). New, long-acting, potent bradykinin antagonists. *Br. J. Pharmacol.,* **102,** 297–304.

LIEBMANN, C., NAWRATH, S., LUDWIG, B. & PAEGELOW, I. (1993). Pharmacological and molecular actions of the bradykinin B_2 receptor antagonist, Hoe 140, in the rat uterus. *Eur. J. Pharmacol.,* **235,** 183–188.

LUTTRELL, L.M., DAAKA, Y. & LEFKOWITZ, R.J. (1999). Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr. Opin. Cell Biol.,* **11,** 177–183.

MARCEAU, F., LEVESQUE, L., DRAPEAU, G., RIOUX, F., SALVINO, J.M., SEOANE, P.R. & SAWUTZ, D.G. (1994). Effects of peptide and nonpeptide antagonists of bradykinin B2 receptors in the venoconstrictor action of bradykinin. *J. Pharmacol. Exp. Ther.,* **269,** 1136–1143.

MARIE, J., KOCH, C., PRUNEAU, D., PAQUET, J.L., GROBLEWSKI, T., LARGUIER, R., LOMBARD, C., DESLAURIERS, B., MAIGRET, B. & BONNAFOUS, J.C. (1999). Constitutive activation of the human bradykinin B_2 receptor induced by mutations in transmembrane helices III and VI. *Mol. Pharmacol.,* **55,** 92–101.

MCEACHERN, A.E., SHELTON, E.R., BHAKTA, S., OBERNOLTE, R., BACH, C., ZUPPAN, P., FUJISAKI, J., ALDRICH, R.W. & JARNAGIN, K. (1991). *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 7724–7728.

MENKE, J.G., BORKOWSKI, J.A., BIERILO, K.K., MACNEIL, T., DERRICK, A.W., SCHNECK, K.A., RANSOM, R.W., STRADER, C.D., LINEMEYER, D.L. & HESS, J.F. (1994). Expression cloning of a human B_1 bradykinin receptor. *J. Biol. Chem.,* **269,** 21583–21586.

REGOLI, D. & BARABÉ, J. (1980). Pharmacology of bradykinin and related peptides. *Pharmacological Rev.,* **32,** 1–46.

REGOLI, D., NSA-ALLOGHO, S., RIZZI, A. & GOBEIL, F.J. (1998). Bradykinin receptors and their antagonists. *Eur. J. Pharmacol.,* **348,** 1 – 10.

RHALEB, N.-E., ROUISSI, N., JUKIC, D., REGOLI, D., HENKE, S., BREIPOHL, G. & KNOLLE, J. (1992). Pharmacological characterization of a new highly potent B_2 receptor antagonist (Hoe 140: D-Arg-[Hyp^3, Thi^{5,8}, D-Tic^7, Oic^8]bradykinin). *Eur. J. Pharmacol.,* **210,** 115 – 120.

SCHRÖDER, C., BEUG, H. & MÜLLER-ESTERL, W. (1997). Cloning and functional characterization of the ornithokinin receptor. *J. Biol. Chem.,* **272,** 12475 – 12481.

SETHI, T. & ROZENGURT, E. (1991). Multiple neuropeptides stimulate clonal growth of small cell lung cancer: effects of bradykinin, vasopressin, cholecystokinin, galanin, and neurotensin. *Cancer Res.,* **51,** 3621 – 3623.

STEWART, J.M. (1995). Bradykinin antagonists: development and applications. *Biopolymers,* **37,** 143 – 155.

VAVREK, R. & STEWART, J.M. (1985). Competitive antagonists of bradykinin. *Peptides,* **6,** 161 – 164.

VELARDE, V., ULLIAN, M.E., MORINELLI, T.A., MAYFIELD, R.U. & JAFFA, A.A. (1999). Mechanisms of MAPK activation by bradykinin in vascular smooth muscle cells. *Am. J. Physiol.,* **227,** C253 – C261.

WIRTH, K., HOCK, J.J., ALBUS, U., LINZ, W., ALPERMANN, H.G., ANAGNOSTOPOULUS, H., HENKE, S., BREIPOHL, G., KÖNIG, W., KNOLLE, J. & SCHÖLKENS, B.A. (1991). Hoe 140 a new potent and long acting bradykinin antagonist: *in vivo* studies. *Br. J. Pharmacol.,* **102,** 774 – 777.

YANO, K., HIGASHIDA, H., INOUE, R. & NOZAWA, Y. (1984). Bradykinin-induced rapid breakdown of phosphatidylinositol-4,5-bisphosphate in neuroblastoma × glioma hybrid NG108-15 cells. *J. Biol. Chem.,* **259,** 10201 – 10207.

(Received July 27, 2000
Revised October 10, 2000
Accepted October 10, 2000)