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The tachykinin NK_1 receptor antagonist, RP67580, inhibits the bradykinin-induced rise in intracellular Ca^{2+} concentration in bovine pulmonary artery endothelial cells

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Abstract

The bradykinin-induced rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and the bradykinin receptor involved in this response were characterized in bovine pulmonary artery endothelial cells. It was found that bradykinin induces an intracellular biphasic Ca^{2+} response, consisting of a transient peak followed by an elevated plateau phase. Both bradykinin and the bradykinin B_1 receptor agonist, des-Arg^9-bradykinin, induced a concentration-dependent increase in $[Ca^{2+}]_i$, but the bradykinin-induced rise was much greater. Moreover, the bradykinin-induced $[Ca^{2+}]_i$ rise could be inhibited by the bradykinin B_2 receptor antagonists, D-Arg^0[Hyp^3, Thi^{5,8}, D-Phe^7]bradykinin and Hoe 140 (D-Arg[Hyp^3, Thi^5, D-Tic^7, Oic^8]bradykinin), but not by the bradykinin B_1 receptor antagonist, des-Arg^9-[Leu^8]bradykinin. From these results it can be concluded that a bradykinin B_2 receptor is involved in this response. Furthermore, we found that the tachykinin NK_1 receptor antagonist, RP67580 ([imino 1 (methoxy-2-phenyl)-2 ethyl]-2 diphenyl 7,7 perhydroisoindolone-4 (3aR, 7aR)), and its negative enantiomer, RP68651 (2-[1-imino 2-(2 methoxy phenyl) ethyl] 7,7 diphenyl 4-perhydroisoindolone (3aS–7aS)), could inhibit the bradykinin-induced $[Ca^{2+}]_i$ response, although no functional tachykinin NK_1 receptors were found. Binding studies evidenced no binding of RP67580 or RP68651 to the bradykinin receptor. We conclude that RP67580 inhibits the bradykinin-induced rise in $[Ca^{2+}]_i$ via a bradykinin B_2 receptor-independent mechanism. © 1998 Elsevier Science B.V.

Keywords: Bradykinin; Ca^{2+}, intracellular; Pulmonary artery endothelium; Tachykinin NK_1 receptor antagonist

1. Introduction

There is growing interest in the role of bradykinin as a mediator in asthma. In the airways, bradykinin causes bronchoconstriction, pulmonary and bronchial vasodilatation, mucus secretion and microvascular leakage (Trifilieff et al., 1993). Activation of the endothelium by bradykinin plays an important role in processes such as vasodilatation and leakage.

Most of the actions of bradykinin are mediated through at least two different receptors, termed bradykinin B_1 and bradykinin B_2 receptor (Bhoola et al., 1992; Farmer and Burch, 1992; Dutta, 1993; Regoli et al., 1993). In many cell types bradykinin receptors activate phosphoinositide hydrolysis. The resultant increase in inositol 1,4,5-trisphosphate (IP_3) releases Ca^{2+} from internal stores. Recent studies with fluorescent Ca^{2+} indicators, such as quin 2, fura 2 and indo 1, have demonstrated that endothelium-dependent vasodilators such as bradykinin can elevate the intracellular levels of Ca^{2+} concentration ($[Ca^{2+}]_i$) in endothelial cells derived from a variety of vascular sites (Himmel et al., 1993; Ricupero et al., 1993). The bradykinin-induced elevation of $[Ca^{2+}]_i$ is biphasic in nature, consisting of a large initial transient peak, followed by a lower but more sustained elevation of $[Ca^{2+}]_i$. The large initial peak is thought to be partly due to the IP_3-mediated release of Ca^{2+} from the intracellular Ca^{2+} stores. Ca^{2+} influx through activated Ca^{2+} channels may be the other component involved in this first phase. There is general agreement that the second phase is completely

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dependent upon Ca^{2+} influx into the cell, as it is abolished on removal of extracellular Ca^{2+} by pretreatment with calcium chelators such as EGTA or with inhibitors of Ca^{2+} influx such as Ni^{2+} (Buchan and Martin, 1991).

In the present study we investigated the effect of bradykinin on $[Ca^{2+}]_i$ in bovine pulmonary artery endothelial cells loaded with fura 2. The response was characterized using Ni^{2+} and thapsigargin, a specific inhibitor of Ca^{2+}-ATPase. With these compounds the importance of intracellular Ca^{2+} stores in the bradykinin-induced Ca^{2+} rise can be assessed. Using a number of agonists and antagonists, we then characterized the bradykinin receptor involved in the $[Ca^{2+}]_i$ rise.

Furthermore, we investigated the effect of the tachykinin NK_1 receptor antagonist, RP67580 ([imino 1 (methoxy-2-phenyl)-2 ethyl]-2 diphenyl 7,7 perhydroisoindolone-4 (3aR, 7aR)), on the bradykinin-induced Ca^{2+} response. Our interest in this compound originated from some recent reports claiming that RP67580 can produce effects unrelated to neurokinin (NK) antagonism (Wang et al., 1994). Also, it has been shown that RP67580 and other tachykinin NK_1 receptor antagonists can influence cellular Ca^{2+} concentrations in rat cerebral cortex and skeletal muscle cells (Guard et al., 1993; Lombet and Spedding, 1994, respectively). We found that the tachykinin NK_1 receptor antagonist, RP67580, inhibited the bradykinin-induced $[Ca^{2+}]_i$ increase. In order to explain the inhibitory effect of RP67580 two possible mechanisms of action were investigated: interference with the binding of bradykinin to its receptor and a possible influence on the Ca^{2+} release from intracellular stores.

2. Materials and methods

2.1. Cell culture

Bovine pulmonary artery endothelial cells were obtained (ATCC, Rockville, USA) and cultured in Minimal Essential Medium (MEM) containing fetal calf serum (20%) and 50 μg/ml gentamycin. Cells of passage 20–28 were used in this study. The cells were grown in an incubator at 37°C under an atmosphere of 5% CO_2 in air. The culture medium was replaced every three days. Upon reaching confluence (3–4 days) the cells were detached by 7-min exposure to trypsin (0.05%) and ethylenediaminetetraacetic acid (EDTA, 0.02%). The cells were seeded on glass coverslips (13 × 18 mm) and grown to confluence (3–4 days).

2.2. Measurement of $[Ca^{2+}]_i$

Upon reaching confluence the endothelial cell monolayers were washed in Hank's Balanced Salt Solution (HBSS) buffered with 10 mM HEPES, containing 1% fetal calf serum (slight modification of methods in Morgan-Boyd et al., 1987; Sage et al., 1989; Schaeffer et al., 1993). Then the monolayers were incubated in the same medium containing 3 μM fura 2 acetoxymethyl ester (fura 2-AM) for 30 min at 37°C. After the incubation the medium was removed and fresh HBSS/HEPES medium with 1% fetal calf serum was added to the coverslips. The cells were then incubated for 10–15 min at room temperature, in order to de-esterify the fura 2-AM in the cells to fura 2. In some experiments, antagonists were added to this medium during the incubation period. The coverslip was then inserted into a quartz cuvette containing fresh HBSS/HEPES with 0.05% gelatin. If antagonists were used, they were present throughout the measurements. The cuvette contained a special Teflon insert which held the coverslip at an angle of 45° to the excitation beam. The cuvette was placed in the temperature-controlled holder (37°C) of a PTI-ratio-fluorometer (Photon Technology Int., South Brunswick, USA) and stirred continuously.

Fluorescence of Ca^{2+}-bound and unbound fura 2 was measured by rapidly alternating the dual excitation wavelengths between 340 and 380 nm (filter rotation frequency of 100 Hz). The fluorescence emission was collected at 510 nm and fluorescence readings were integrated at 1 s intervals. The autofluorescence of a control monolayer from the same endothelial cell batch was subtracted from the fluorescence data. The ratio (R) of the fluorescence at the two wavelengths was computed and used to calculate changes in $[Ca^{2+}]_i$. At the end of each incubation time the ratios of maximum (R_{max}) and minimum (R_{min}) fluorescence of fura 2 were determined by subsequent addition of ionomycin (10 μM) and EGTA (4 mM in 3 M Tris-buffer, pH = 8). $[Ca^{2+}]_i$ was then calculated using the following equation (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_i = K_d \times \frac{(R - R_{min})}{(R_{max} - R)} \times \frac{S_{f2}}{S_{b2}}$$

The K_d of fura 2 for Ca^{2+} was assumed to be 224 nM at 37°C (Grynkiewicz et al., 1985). S_{f2} and S_{b2} are the fluorescent values obtained at 380 nm in the absence of Ca^{2+} and in the presence of saturating levels of Ca^{2+}, respectively.

One concentration of bradykinin was tested on each coverslip.

2.3. [^{3}H]bradykinin binding assay

The endothelial cells were removed from the culture flasks using EDTA. Cells were collected in PBS (phosphate-buffered saline) and spun down. The cell pellet was resuspended and homogenised in 10 ml Tris–HCl-buffer (5 mM, pH = 7.4) with a Polytron homogeniser. The cells were centrifuged for 20 min at 30 000 × g and washed once in Tris–HCl, 50 mM, pH = 7.4 by rehomogenisation and recentrifugation. The pellet obtained from one culture flask (162 cm^2) was suspended in 1.25 ml incubation buffer (composition: Tris–HCl, 50 mM, pH = 7.4; 1,10

phenanthrolin, 1 mM; bacitracin, 140 μl/mg; $MgCl_2$, 2 mM; dithiothreitol, 1 mM and bovine serum albumin, 0.1%.

For cell concentration binding experiments the membranes were diluted to a concentration of 340, 170, 85, 57, 43, 34, 28 and 17 μg protein/ml (determined using Bio-rad protein assay). Incubations were performed in a 0.5 ml total volume, adding 0.5 nM [^{3}H]bradykinin with a specific activity of 106 Ci/mmol for 30 min at 25°C.

After the cell concentration binding experiments, a saturation experiment was performed in order to calculate total receptor density (B_{max}) and dissociation constant (K_d). Various concentrations of [^{3}H]bradykinin (0.04–2.7 nM) were used and the mixtures were incubated for 30 min at 25°C. Non-specific binding was determined using 1 μM unlabelled bradykinin. The amount of specific binding was calculated as the total binding minus the binding in the presence of 1 μM unlabeled bradykinin.

In competition experiments, various non-labeled compounds were added at the same time as 0.5 nM [^{3}H]bradykinin to the endothelial cell membranes. The mixtures were incubated for 30 min at 25°C.

All reactions (from cell concentration, saturation and competition experiments) were terminated by rapid filtration of the mixtures under vacuum through Whatman GF/B filters. The filters were rinsed with 3 ml buffer. After the filtration step, each filter was transferred to a vial containing 5 ml scintillation solution and the radioactivity was determined in a liquid scintillation counter.

The competitive inhibition of the [^{3}H]bradykinin binding was evaluated using Hoe 140 (D-Arg[Hyp^3, Thi^5, D-Tic^7, Oic^8]bradykinin), RP67580 and RP68651 (2-[1-imino 2-(2 methoxy phenyl) ethyl] 7,7 diphenyl 4-perhydroisoindolone (3aS–7aS)). Half-maximal inhibitory concentration values (IC_{50}) were calculated from competition experiments and transformed to apparent inhibitory constant values (K_i) according to the Cheng–Prussoff formula, $K_i = IC_{50}/[1 + L_0/K_d]$.

2.4. Chemicals

EGTA, HBSS, $NiCl_2$, $MgCl_2$ and gelatin were obtained from Merck (Darmstadt, Germany). MEM, fetal calf serum, gentamycin and trypsin were purchased from Gibco (Paisley, UK). Bradykinin, des-Arg^9-bradykinin, D-Arg^0[Hyp^3, Thi^{5,8}, D-Phe^7]bradykinin, des-Arg^9-[Leu^8]bradykinin, Substance P, fura 2-AM, EDTA, thapsigargin, ionomycin, bovine serum albumin, bacitracin, phenantrolin and dithiothreitol were obtained from Sigma (St. Louis, USA). HEPES was obtained from Boehringer (Mannheim, Germany). [^{3}H]bradykinin was obtained from New England Nuclear (USA). Tris was obtained from Bio-Rad (Hercules, USA).

Hoe 140 was kindly provided by Dr. K. Wirth, Hoechst (Frankfurt am Main, Germany). RP67580 and RP68651 were kindly provided by Dr. C. Garret, Rhône Poulenc (Vitry, France).

2.5. Statistical analysis

The results are expressed as the means ± S.E.M. Statistical analysis of the data was performed using an analysis of variance (ANOVA). If appropriate, the statistical significance of differences between means was determined using the Newman–Keuls test. A probability of 0.05 or less was considered significant. Concentration–effect curves were obtained and EC_{50}, pD_2 and approximate pA_2 values were calculated by non-linear least squares regression, using the sigmoid concentration response relationship (GraphPad).

3. Results

3.1. Characterisation of the Ca^{2+} response induced by bradykinin

In the presence of 1.26 mM extracellular Ca^{2+}, the resting level of $[Ca^{2+}]_i$ in the bovine pulmonary artery endothelial cells was 116 ± 6.6 nM ($n = 82$). Bradykinin (1 nM–0.3 μM) induced a biphasic elevation, consisting of a very rapid initial rise which peaked within 20 s, followed by a sustained phase of elevated $[Ca^{2+}]_i$ (Fig. 1). No return to baseline level could be seen within 15 min of incubation. At a concentration of 0.1 μM, bradykinin caused an initial rise in $[Ca^{2+}]_i$ of 318 ± 39 nM ($n = 6$) and a sustained (increased) level of 110 ± 23 nM ($n = 6$) after 300 s (on top of the resting level of 116 nM). The biphasic response became less pronounced as the agonist concentration was reduced.

We used thapsigargin and Ni^{2+} to characterize the biphasic Ca^{2+} response. Thapsigargin interferes with the activity of microsomal Ca^{2+}-ATPase and thus depletes the intracellular Ca^{2+} stores (Gericke et al., 1993). When a high concentration of thapsigargin (2 μM) was added to the cells to insure maximal Ca^{2+} release, a small rise in

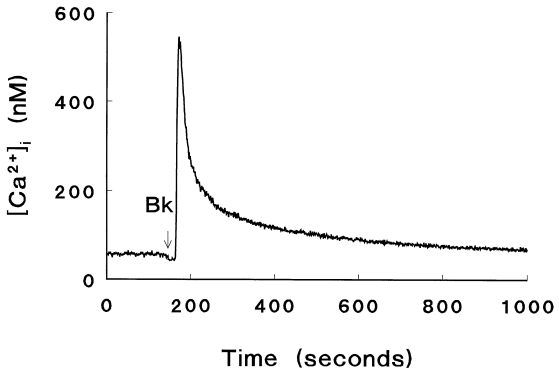

Fig. 1. Intracellular Ca^{2+} response induced by 0.3 μM bradykinin in fura 2-loaded bovine pulmonary artery endothelial cells. This figure depicts a representative tracing. The arrow indicates where bradykinin (Bk) was added to the endothelial cells.

Table 1
Influence of the calcium channel blocker, Ni^{2+} (5 mM), alone or in combination with the tachykinin NK_1 receptor antagonist, RP67580 (10 μM), on the 0.1 μM bradykinin-induced $[Ca^{2+}]_i$ response

Treatment	$[Ca^{2+}]_i$ response (ratio)	n[b]
BK[a]	1.32 ± 0.22	5
Ni^{2+}, BK	1.30 ± 0.18	8
RP67580, Ni^{2+}, BK	0.30 ± 0.18[c]	4

Fura 2-loaded endothelial cells were pretreated with Ni^{2+} (5 min before bradykinin addition) or a combination of Ni^{2+} with RP67580 (added 15 min before bradykinin). The bradykinin-induced $[Ca^{2+}]_i$ response was measured and the results were expressed as a ratio (R), since no calibration of the cellular calcium concentration can be performed in the presence of Ni^{2+}.
Each value represents the mean $\pm$ S.E.M.
[a] BK = bradykinin.
[b] n = number of experiments.
[c] Significant difference ($P < 0.05$, ANOVA) from the bradykinin-induced and bradykinin/Ni^{2+}-induced $[Ca^{2+}]_i$-response.

$[Ca^{2+}]_i$ could be detected. After 10-min incubation with thapsigargin the bradykinin-induced $[Ca^{2+}]_i$ peak was diminished to 6.0% $\pm$ 6.0 ($n = 5$) of the original response of 0.1 μM bradykinin. Apparently Ca^{2+} release from intracellular Ca^{2+} stores does play an important role in the first phase of the bradykinin-induced response.

We also investigated the influence of the Ca^{2+} channel blocker, Ni^{2+}. In the presence of 1.26 mM extracellular Ca^{2+}, treatment with Ni^{2+} (5 mM) reduced the resting $[Ca^{2+}]_i$. This treatment had no effect on the magnitude of the initial transient peak of $[Ca^{2+}]_i$ induced by bradykinin (0.1 μM) (see Table 1). However, the treatment with Ni^{2+} did abolish the sustained phase of the $[Ca^{2+}]_i$-induced rise with bradykinin. This result confirms that the first phase of the bradykinin-induced Ca^{2+} response is not due to the influx of extracellular Ca^{2+}, but is caused by the release of Ca^{2+} from intracellular Ca^{2+} stores.

3.2. Pharmacological characterisation of the bradykinin-induced rise in $[Ca^{2+}]_i$

Bradykinin, predominantly a bradykinin B_2 receptor agonist, induces a concentration-dependent Ca^{2+} response between 1 nM and 0.3 μM (Fig. 2A; only peak values are depicted). When the bradykinin B_1 receptor agonist, des-Arg^9-bradykinin, was added to the endothelial cells we also observed a concentration-dependent increase in $[Ca^{2+}]_i$, between 0.03 μM and 1 μM (Fig. 2A). The response was similar to the bradykinin-induced $[Ca^{2+}]_i$ rise, although the increases in $[Ca^{2+}]_i$ were smaller (maximally 47% of the highest bradykinin response). Concentration–effect curves were obtained and these curves yielded pD_2 values of 8.11 ± 0.20 and 7.17 ± 0.25 for bradykinin and des-Arg^9-bradykinin, respectively.

The effect of the bradykinin B_1 receptor antagonist, des-Arg^9-[Leu^8]bradykinin, and the bradykinin B_2 receptor antagonists, D-Arg^0[Hyp^3, Thi^{5,8}, D-Phe^7]bradykinin and Hoe 140, on the bradykinin-induced Ca^{2+} rise was assessed. The influence of the antagonists on bradykinin-induced $[Ca^{2+}]_i$ peak values and sustained phase was similar, but only the results obtained on peak values are shown to illustrate our findings. The effects of the antagonists on the bradykinin-induced peak values are depicted in Fig. 2B and Fig. 3A. Preincubation with 1 μM bradykinin B_1 receptor antagonist, des-Arg^9-[Leu^8]bradykinin, did not cause any significant change in the bradykinin-induced increase in $[Ca^{2+}]_i$ ($pD_2 = 8.18 \pm 0.29$). However, the bradykinin B_2 receptor antagonist, D-Arg^0[Hyp^3, Thi^{5,8}, D-Phe^7]bradykinin (0.1, 1 μM), inhibited the bradykinin-induced increase in $[Ca^{2+}]_i$; the concentration–effect curve was shifted in parallel to the right (Fig. 2B). From these results an approximate pA_2 value of 7.4 ± 0.7 could be calculated for the bradykinin B_2 receptor antagonist, D-Arg^0[Hyp^3, Thi^{5,8}, D-Phe^7]bradykinin, assuming the nature of antagonism to be competitive. The potent bradykinin B_2

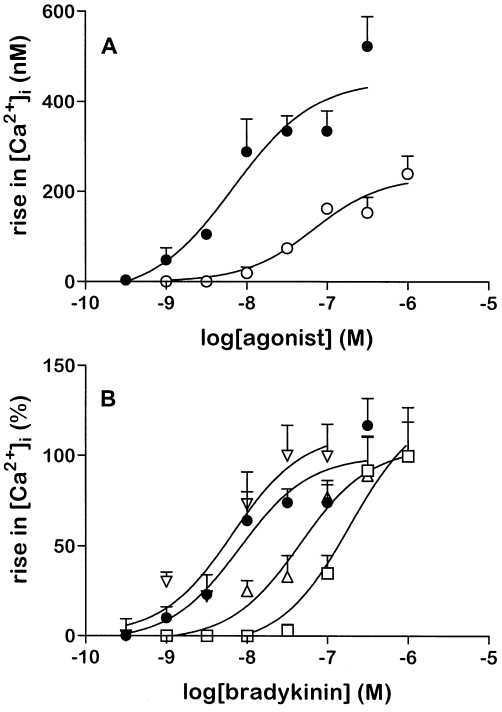

Fig. 2. Pharmacological characterization of the bradykinin-induced Ca^{2+} response. (A) Concentration–effect curves for bradykinin and the bradykinin B_1 receptor agonist, des-Arg^9-bradykinin. Fura 2-loaded bovine pulmonary artery endothelial cells were stimulated with different concentrations of bradykinin (●, $n = 6$) or des-Arg^9-bradykinin (○, $n = 3$). (B) Effect of preincubation with the bradykinin B_1 receptor antagonist, des-Arg^9-[Leu^8]bradykinin, or the bradykinin B_2 receptor antagonist, D-Arg^0[Hyp^3, Thi^{5,8}, D-Phe^7]bradykinin, on the bradykinin-induced concentration–effect curve. The fura 2-loaded cells were stimulated with different concentrations of bradykinin (control; ●, $n = 6$), in the presence of des-Arg^9-[Leu^8]bradykinin (1 μM; ▽, $n = 3$) or in the presence of D-Arg^0[Hyp^3, Thi^{5,8}, D-Phe^7]bradykinin (0.1 μM; △, $n = 3$ and 1 μM; □, $n = 4$). Only the increases in Ca^{2+} peak levels are depicted. In (B) the results are expressed as percentages of the maximal bradykinin-induced Ca^{2+} response. Data are shown as means $\pm$ S.E.M.

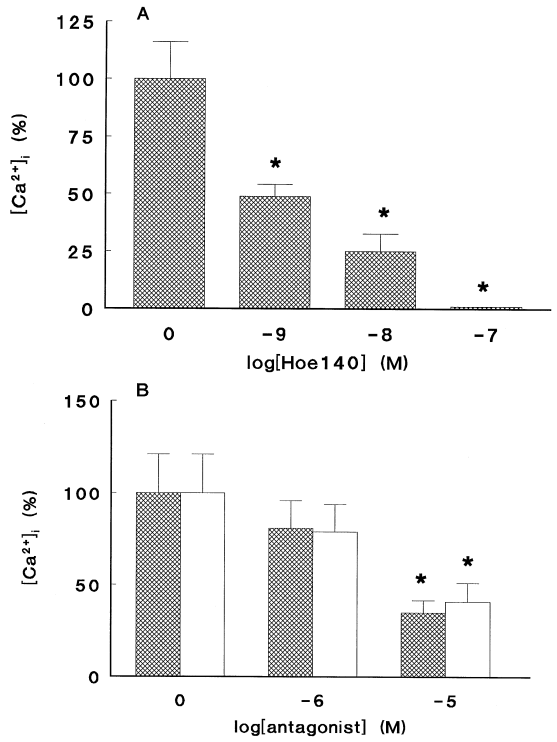

Fig. 3. Inhibition of the 0.1 μM bradykinin-induced $[Ca^{2+}]_i$ response by the bradykinin B_2 receptor antagonist, Hoe 140 (A; $n = 3$), and by the tachykinin NK_1 receptor antagonist, RP67580 (hatched bars), or its negative enantiomer, RP68651 (open bars) (B; $n = 3$–4). Data are shown as means ± S.E.M. * Significant difference ($P < 0.05$, ANOVA) from bradykinin-induced $[Ca^{2+}]_i$ response.

receptor antagonist, Hoe 140, was also capable of inhibiting the rise in $[Ca^{2+}]_i$ induced by 0.1 μM bradykinin in a concentration-dependent manner (Fig. 3A).

3.3. Effect of RP67580 on the bradykinin-induced rise in $[Ca^{2+}]_i$

The ability of the tachykinin NK_1 receptor antagonist, RP67580, to affect the Ca^{2+} response to bradykinin was examined. As can be seen in Fig. 3B, RP67580 (1, 10 μM) caused a significant, concentration dependent inhibition of the 0.1 μM bradykinin response. The negative enantiomer, RP68651 (1, 10 μM), caused a similar concentration-dependent inhibition of the bradykinin-induced increase in $[Ca^{2+}]_i$ (Fig. 3B). The effect of RP67580 on the first and second phase of the 0.1 μM bradykinin-induced $[Ca^{2+}]_i$ response was further analyzed, with measurements of slope values of the Ca^{2+} signals. The upward slope of the Ca^{2+} rise (first phase) was significantly inhibited by 10 μM RP67580 (bradykinin-induced response: 0.43 ± 0.04 ratio/s; $n = 6$, after preincubation with RP67580: 0.18 ± 0.03 ratio/s; $n = 3$). In contrast, no influence at all was found on the downward slope of the Ca^{2+} response (bradykinin-induced response: 0.078 ± 0.0070 ratio/s; $n = 6$), after preincubation with RP67580: 0.065 ± 0.0077; $n = 3$). This demonstrates that RP67580 influences only the first phase of the bradykinin-induced increase in $[Ca^{2+}]_i$, possibly via Ca^{2+} release from intracellular stores.

We could find no functional tachykinin NK_1 receptors on this cell line, as assessed by measuring changes in $[Ca^{2+}]_i$ after incubation with the tachykinin NK_1 receptor agonist, substance P (10 μM; data not shown).

3.4. Mechanism of action of RP67580 on the bradykinin response

One of the possibilities to explain the inhibitory effect of R67580 on the bradykinin-induced $[Ca^{2+}]_i$ rise could be the ability to bind to the bradykinin receptor. Binding studies were applied to examine this possibility. Specific [^{3}H]bradykinin binding increased linearly with increasing membrane protein concentrations (from 17 to 340 μg protein/ml), when the membrane fractions were incubated with 0.5 nM [^{3}H]bradykinin at 25°C for 30 min. In subsequent experiments a dilution of 42 μg protein/ml was

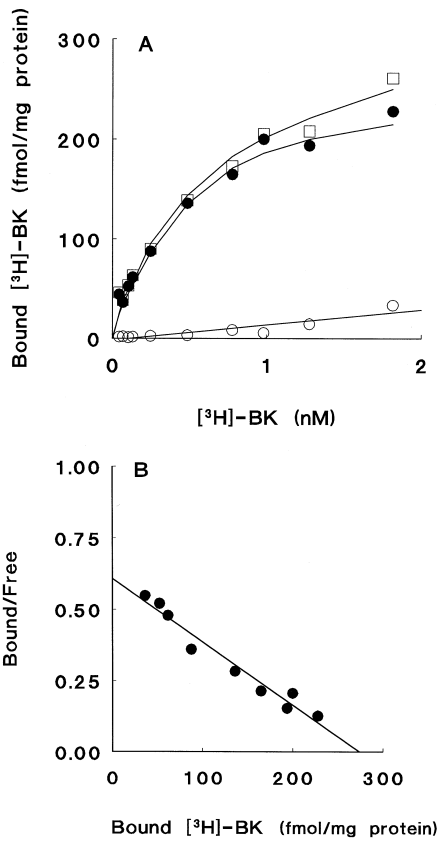

Fig. 4. Binding of [^{3}H]bradykinin ([^{3}H]BK) to bovine pulmonary artery endothelial cell membranes. (A) Endothelial cell membranes (42 μg protein/ml) were incubated with increasing concentrations of [^{3}H]bradykinin in the presence (non-specific binding; ○) or absence (total binding; □) of 1 μM non-labeled bradykinin. Specific binding (●) was determined by subtracting non-specific binding from total binding. Each point is the mean of 5 determinations in 1 experiment (duplicate experiments were performed). (B) Data for the Scatchard plot were calculated from specific binding data shown in (A).

used. The saturability of [^{3}H]bradykinin binding was measured by incubating the protein membrane fraction with various concentrations of [^{3}H]bradykinin, from 0.04 to 2.7 nM. The saturation curve is a rectangular hyperbola, suggesting that there is a single population of saturable high-affinity binding sites (Fig. 4A). Non-specific binding, on the other hand, increased linearly with increasing [^{3}H]bradykinin concentrations. Scatchard plot analysis (Fig. 4B) of specifically bound [^{3}H]bradykinin to the endothelial cell membrane fraction gave a dissociation constant (K_d) of 0.28 ± 0.04 nM ($n = 5$) and a maximal receptor density (B_{max}) of 211 ± 8.1 fmol/mg protein ($n = 5$).

The specificity of the binding was defined by studying the inhibition of [^{3}H]bradykinin binding by Hoe 140. It was found that Hoe 140 displaced [^{3}H]bradykinin binding in a concentration dependent manner. The concentration of Hoe 140 required to inhibit 50% of the specific binding (pIC_{50}) was 9.33 ± 0.03 ($n = 5$), and the inhibition constant (K_i) was 0.18 ± 0.001 nM ($n = 5$). In contrast, RP67580 and RP68651 did not displace [^{3}H]bradykinin binding up to a concentration of 10 μM, indicating that they do not bind to bradykinin B_2 receptors (Fig. 5).

Another possibility to explain the inhibitory effect of RP67580 on the bradykinin-induced $[Ca^{2+}]_i$ rise could be the ability to interact with the intracellular Ca^{2+} stores. Preincubation with Ni^{2+} was used to exclude Ca^{2+} influx through Ca^{2+} channels, in order to investigate this possible interaction with Ca^{2+} stores. As mentioned above, treatment with Ni^{2+} (5 mM), in the presence of 1.26 mM extracellular Ca^{2+}, reduced the resting $[Ca^{2+}]_i$. This treatment had no effect on the magnitude of the initial transient peak of $[Ca^{2+}]_i$ induced by bradykinin (0.1 μM), but did abolish the sustained phase of the $[Ca^{2+}]_i$ rise induced by bradykinin (Table 1). Nevertheless, in the presence of Ni^{2+} (5 mM), the inhibitory effect of RP67580 (10 μM) on the bradykinin-induced Ca^{2+} peak response was still present (see Table 1).

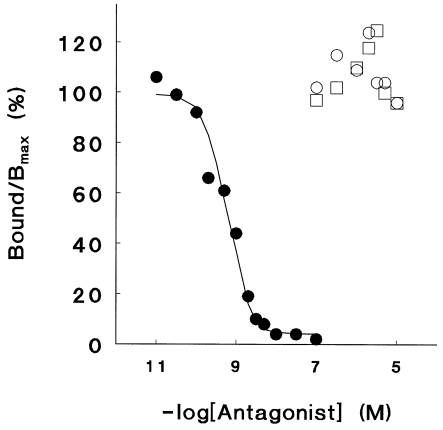

Fig. 5. Competition for [^{3}H]bradykinin binding to bovine pulmonary artery endothelial cell membranes by the bradykinin B_2 receptor antagonist, Hoe 140 (●), the tachykinin NK_1 receptor antagonist, RP67580 (○), and its negative enantiomer, RP68651 (□). Endothelial cell membranes (42 μg protein/ml) were incubated with various concentrations of the competing ligand in the presence of 0.5 nM [^{3}H]bradykinin. Each point is the mean of 5 determinations in 1 experiment (duplicate experiments were performed).

4. Discussion

In this study we showed that the bradykinin-induced rise in $[Ca^{2+}]_i$ in a bovine pulmonary artery endothelial cell line is biphasic, bradykinin B_2 receptor mediated and can be inhibited by the tachykinin NK_1 receptor antagonist, RP67581, and its negative enantiomer, RP68651.

Bradykinin was found to induce a biphasic elevation of $[Ca^{2+}]_i$ in bovine pulmonary artery endothelial cells, consisting of an initial rapid rise followed by a sustained phase of elevated $[Ca^{2+}]_i$. Our experiments indicate that the first rise in intracellular Ca^{2+} is dependent upon mobilization of Ca^{2+} from intracellular Ca^{2+} stores. Firstly, because the bradykinin-induced Ca^{2+} response was almost completely abolished after depletion of the Ca^{2+} stores (with thapsigargin). Secondly, when we preincubated the endothelial cells with the Ca^{2+} channel blocker, Ni^{2+}, the bradykinin-induced Ca^{2+} peak was still maintained, but the sustained phase was completely abolished. The finding of a bradykinin-induced biphasic response is in agreement with previous observations made in various endothelial cells and smooth muscle cells (Schilling et al., 1988, 1989; Sung et al., 1988; Buchan and Martin, 1991; Amrani et al., 1994).

After characterisation of the biphasic response, we investigated the bradykinin receptor involved in this Ca^{2+} response. Both bradykinin (bradykinin B_2 receptor agonist) and the bradykinin B_1 receptor agonist, des-Arg^9 bradykinin, caused a dose dependent increase in $[Ca^{2+}]_i$. Bradykinin, however, was much more potent than des-Arg^9-bradykinin. The EC_{50} value obtained for bradykinin in the present study was similar to those reported for bradykinin B_2 receptor-mediated responses in smooth muscle preparations and cell cultures, while the EC_{50} value obtained for des-Arg^9-bradykinin was similar to those reported for bradykinin B_1 receptor-mediated responses (Morgan-Boyd et al., 1987; Sung et al., 1988; Yang et al., 1994, 1995; Smith et al., 1995). Furthermore, the maximal response induced by des-Arg^9-bradykinin was less than that evoked by bradykinin. These results are consistent with the hypothesis that des-Arg^9-bradykinin acts via bradykinin B_1 receptors rather than bradykinin B_2 receptors.

The responses to bradykinin were blocked by the potent and selective bradykinin B_2 receptor antagonist Hoe 140 and the bradykinin B_2 receptor antagonist des-Arg^0[Hyp^3, Thi^{5,8}, D-Phe^7]bradykinin. The latter antagonist caused parallel shifts in the concentration–effect curve, with an approximate pA_2 value of 7.4. In vascular preparations pA_2 values between 6.2 and 7.9 have been reported for

this antagonist (Regoli et al., 1993). The bradykinin B_1 receptor antagonist, des-Arg^9[Leu^8]bradykinin, however, did not block the bradykinin-induced Ca^{2+} responses. These results clearly indicate that bradykinin acts via bradykinin B_2 receptors.

We also investigated the effect of the tachykinin NK_1 receptor antagonist, RP67580, on the bradykinin-induced Ca^{2+} elevation. Although RP67580 is claimed to be a potent and selective tachykinin NK_1 receptor antagonist (Garret et al., 1991), it was found to produce effects that are unrelated to tachykinin NK receptor antagonism, including non-specific inhibitory effects on neurotransmission (Wang et al., 1994). We found that this compound could inhibit the bradykinin-induced Ca^{2+} elevation, although we could not find any functional tachykinin NK receptors, as assessed by Ca^{2+} measurements (using substance P). These results suggest that this effect caused by RP67580 was not tachykinin NK receptor-mediated.

Moreover, the inhibition of the bradykinin-induced Ca^{2+} response was not stereoselective, since both RP67580 and its negative enantiomer, RP68651, showed a similar inhibition of the Ca^{2+} elevation. This non-stereoselectivity argues against a receptor mediated mechanism.

We then proceeded to investigate the mechanism involved in this RP67580-induced inhibition. Our binding studies showed that RP67580 and RP68651 are not capable of inhibiting [^{3}H]bradykinin binding to bovine pulmonary artery endothelial cells and thus again demonstrated that these compounds do not act via a bradykinin B_2 receptor-operated mechanism. Also, Garret et al. (1991) reported that 1 μM RP67580 was without antagonistic effect on the contractile response to bradykinin in a guinea pig ileum preparation.

RP67580 had so far been thought to interfere with L-type voltage-dependent Ca^{2+} channels (Guard et al., 1993; Lombet and Spedding, 1994). However, it is unlikely that RP67580 interacts with an L-type calcium channel on endothelial cells: electrophysiological studies have failed to show the existence of these channels on endothelial cells (Revest and Abbott, 1992). There are indications that endothelial cells contain IP_4-activated cation channels or receptor-operated calcium channels and leak channels (Adams et al., 1989; Curry, 1992; Fasolato et al., 1994). Interaction of RP67580 with one of these types of Ca^{2+} channels is conceivable.

We found that RP67580 only influenced the upward slope of the first phase of the bradykinin-induced response but not the downward slope, indicating that Ca^{2+} release from intracellular stores might be involved. Moreover, in the presence of the Ca^{2+} channel blocker, Ni^{2+}, the inhibitory effect of RP67580 (10 μM) on the bradykinin-induced Ca^{2+} peak response was still present. Therefore, RP67580 possibly interferes with the release of Ca^{2+} from intracellular Ca^{2+} stores.

In summary, RP67580 inhibits the bradykinin-induced rise in $[Ca^{2+}]_i$, via a bradykinin B_2 receptor-independent mechanism.

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