Br. J. Pharmacol. (1994), **112**, 934–938 © Macmillan Press Ltd, 1994

Des-Arg^9-bradykinin-induced increases in intracellular calcium ion concentration in single bovine tracheal smooth muscle cells

[1]Katrina A. Marsh & Stephen J. Hill

Department of Physiology and Pharmacology, University of Nottingham Medical School, Queen's Medical Centre, Nottingham, NG7 2UH

1 Dynamic video imaging was used to measure the des-Arg^9-bradykinin-induced changes in the intracellular free calcium ion concentration ($[Ca^{2+}]_i$) of single bovine tracheal smooth muscle (BTSM) cells.

2 In the presence of extracellular calcium ions, des-Arg^9-bradykinin (1 nM – 10 μM) produced a concentration-dependent increase in the $[Ca^{2+}]_i$ over basal levels yielding an EC_{50} value of 316 nM. The percentage of cells responding to each concentration of des-Arg^9-bradykinin also increased in a concentration-dependent manner (from 9% to 100%).

3 The bradykinin B_2 receptor antagonist, D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin (10 μM), was without effect on the calcium response of the cells when added 2 min prior to des-Arg^9-bradykinin (100 nM). However, the B_1 receptor antagonist, des-Arg^{9}Leu^8-bradykinin (10 μM), completely abolished the des-Arg^9-bradykinin-induced response.

4 Under calcium-free conditions, des-Arg^9-bradykinin induced an increase in $[Ca^{2+}]_i$ at concentrations of 1 μM and 10 μM. The response to 10 μM des-Arg^9-bradykinin was reduced by preincubation of either D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin (10 μM) or des-Arg^{9}Leu^8-bradykinin (10 μM).

5 We conclude that bradykinin B_1 receptors are expressed by cultured BTSM cells and mediate the des-Arg^9-bradykinin-induced influx of calcium ions at low agonist concentrations (< 1 μM). At higher concentrations, des-Arg^9-bradykinin (1 μM and 10 μM) can stimulate both B_1 and B_2 receptors to effect intracellular calcium release under calcium-free conditions.

Keywords: Des-Arg^9-bradykinin; smooth muscle; intracellular calcium; trachea; bradykinin

Introduction

The involvement of kinins in inflammatory airway disease is not entirely understood although the nonapeptide, bradykinin, has been implicated in the pathogenesis of allergic asthma (Christiansen *et al.*, 1987). The receptors mediating responses to bradykinin have typically been divided into two subtypes, B_1 and B_2 receptors (Regoli & Barabé, 1980). Although present in a variety of tissues, B_2 receptors and not B_1 receptors, are found to be in great abundance in the rabbit jugular vein whereas the less commonly expressed B_1 receptors are found in the rabbit aorta (Regoli *et al.*, 1981). Bradykinin has been shown to elicit an increase in intracellular calcium ion concentration ($[Ca^{2+}]_i$) of human (Murray & Kotlikoff, 1991) and guinea-pig (Farmer *et al.*, 1991a,b) airway smooth muscle cells although the characteristics of these responses remain unclear. An increase in $[Ca^{2+}]_i$ is a mechanism necessary for the contraction of smooth muscle and can be mediated either via an influx of calcium ions from the extracellular space (Benham & Tsien, 1987; Murray & Kotlikoff, 1991) or via a release of calcium from the intracellular stores which may result from an increase in production of inositol 1,4,5-trisphosphate (Somlyo *et al.*, 1988; Berridge & Irvine, 1989).

In previous studies we have shown that bradykinin B_2 receptor subtype-stimulation can cause an increase in phosphoinositide hydrolysis in cultures of bovine tracheal smooth muscle (BTSM) cells (Marsh & Hill, 1992). In the same study we noted that the bradykinin B_1 receptor agonist, des-Arg^9-bradykinin, caused a small increase in the phosphoinositide hydrolysis (16% of that produced by bradykinin) of cultured BTSM cells (Marsh & Hill, 1992) indicating the presence of B_1 receptors in this system. In addition to these studies, we have demonstrated that bradykinin, via activation of the B_2 receptor, can also induce an increase in $[Ca^{2+}]_i$ both in the presence and absence of extracellular calcium ions (Marsh & Hill, 1993).

The presence of the B_1 receptor coupled to calcium ion mobilisation in airway smooth muscle cells from other species, such as guinea-pig, is doubted (Farmer *et al.*, 1989). We have therefore extended our studies to evaluate the ability of this receptor, which upon stimulation mediates an increase in phosphoinositide turnover, to mediate increases in $[Ca^{2+}]_i$ of cultured BTSM cells. This has been performed by use of dynamic video imaging of single BTSM cells in both calcium-containing and calcium-free conditions in order to evaluate the characteristics of the response. The present study demonstrates the presence of functional B_1 receptors coupled to intracellular calcium ion mobilisation in single BTSM cells.

Methods

Cell culture

BTSM cell cultures were established from fresh tissue as described previously (Marsh & Hill, 1992). Briefly, tracheal smooth muscle, dissected free of surrounding connective tissue and mucosa, was chopped into 1 mm^3 pieces. The explant tissue was then transferred to tissue culture-treated plastic flasks and covered with a D-Val-substituted minimum essential medium supplemented with 10% foetal calf serum (FCS) and antibiotics (100 u ml^{-1} penicillin G; 100 μg ml^{-1} streptomycin; 250 ng ml^{-1} amphotericin B). Smooth muscle cells were then maintained at 37°C in a humidified 10% CO_2 atmosphere until confluency and were routinely subcultured using trypsin (0.05% w/v in versene, Glasgow formula). Mycoplasmal contamination was shown to be negative by the Hoechst 33258 staining method of Chen (1977). Identity of smooth muscle cells was confirmed by indirect immunocyto-

[1] Author for correspondence.

chemical analysis using the monoclonal antibody to alpha smooth muscle actin as described previously in detail (Marsh & Hill, 1992). For the image analysis procedure, smooth muscle cells from passages 4 to 10 were seeded onto 22 mm circular glass coverslips at a 1:10 split ratio. These were maintained under the above conditions in Dulbecco's modified Eagle's medium with 10% FCS for 72 h until use.

Image analysis

Cells attached to the glass coverslips were washed three times in a physiological saline solution (PSS) containing (in mM): NaCl 145, KCl 5, $MgSO_4$ 1, N-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) 10, glucose 10 and $CaCl_2$ 2, pH 7.4. Cells were then incubated at 37°C for 30 min with PSS containing 10% FCS and 5 μM fura-2 acetoxymethylester. After washing a further three times, coverslips were transferred to a metal holder which was then mounted in a heated chamber in order to maintain the temperature at 37°C. A volume of 900 μl of PSS was then added to the chamber and agents were added directly to the cells in a volume of 100 μl PSS. For experiments requiring calcium-free conditions, any PSS added to the chamber was deficient in $CaCl_2$ and supplemented with 0.1 mM EGTA. All coverslips were used within 90 min of the end of loading time.

Image analysis was performed by use of MagiCal hardware and TARDIS software supplied by Applied Imaging International Ltd. (Hylton Park, Sunderland, Tyne & Wear) as described previously in detail (Neylon *et al.*, 1990). Briefly, fluorescent images were detected by a Nikon Diaphot epifluorescence microscope with a 10 × quartz objective lens then relayed through an image intensifying charged couple device camera (Photonic Science) to the TARDIS software where the images underwent analogue to digital conversion.

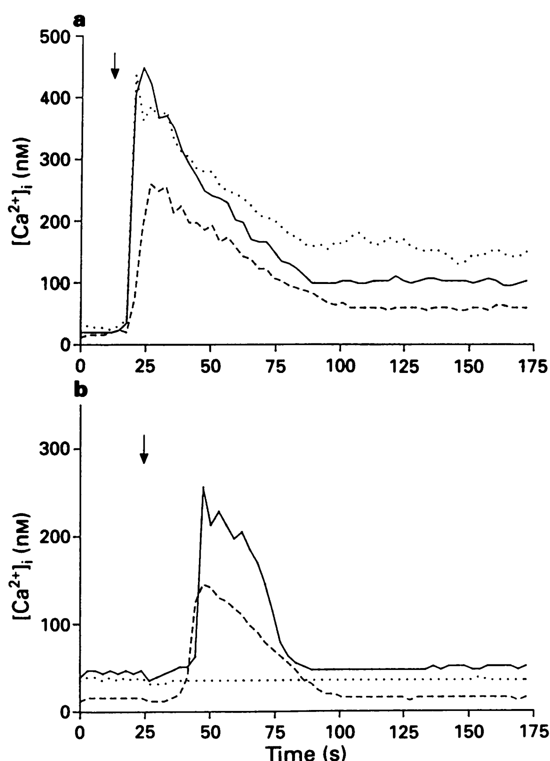

Figure 1 Des-Arg^9-bradykinin-induced changes in intracellular calcium ion concentration in single bovine tracheal smooth muscle cells. Individual traces represent the changes in intracellular calcium ion concentration of individual cells within the same field of view and are representative of responses from other cells measured. The arrow indicates the addition of des-Arg^9-bradykinin (1 μM) (a) in the presence of extracellular calcium ions (2 mM $CaCl_2$) or (b) in the absence of extracellular calcium ions (with 0.1 mM EGTA).

Images captured were 256 × 256 pixels in size and each frame was averaged 8 times with analogue hardware averaging to reduce camera noise. Incident light of alternating 340 and 380 nm wavelength was supplied to the sample by means of a rotating filter wheel so that the time between image pairs was 1.5 s. Once a sequence of images had been captured they were subjected to a background subtraction. For this purpose, an averaged image of each of the 340 and 380 nm types was captured from an area of the coverslip devoid of any cells using the same parameters as for cell measurements. The 340 nm background was then subtracted from each of the 340 nm images on a pixel-by-pixel basis and similar processing was performed with the 380 nm background and images.

After digital conversion background-corrected image pairs were ratioed (340/380) on a pixel-by-pixel basis and calcium ion concentration was calculated using a 2-D look up table utilising the Grynkiewicz *et al.* (1985) equation below

$$[Ca] = K_D \beta [(R - R_{min})/(R_{max} - R)]$$

where R is the measured ratio and β is the fluorescence ratio at 380 nm of R_{min} to R_{max}. R_{max} and R_{min} values were calculated for calibration purposes by exposing the cells firstly to 20 μM ionomycin in the presence of 10 mM calcium allowing for flooding of the cell with calcium and a maximum fluorescence ratio (R_{max}) to be obtained. R_{min} (minimum fluorescence ratio) was calculated by chelation of the free calcium ions with 6 mM EGTA. A dissociation constant (K_D) of 224 nM for fura-2 and calcium at 37°C was incorporated into the 2-D look up table.

Image analysis software performed quantification of mean calcium ion concentration as a function of time (e.g. Figure 1) and whole cell intracellular calcium ion quantification was obtained by outlining each individual cell using a light pen. Graphical representation was automatically produced from the pixel data contained within the defined region.

Data analysis

Concentration-response curves were fitted to a Hill equation using the non-linear curve-fitting programme ALLFIT (DeLean *et al.*, 1978). The equation fitted was

$$Response = E_{max} \times D^n/(D^n + (EC_{50})^n)$$

where D is the agonist concentration, n is the Hill coefficient, EC_{50} is the concentration of agonist giving half maximal response and E_{max} is the maximal effect. The data point at each concentration of bradykinin was calculated from accumulated data from a single field of view (containing 4–10 cells) from each of at least five different coverslips each arising from different animals.

Chemicals

D-Val minimum essential medium and the antibiotic/antimycotic solution used in culture were obtained from Gibco. Dulbecco's modified Eagle's medium and foetal calf serum were purchased from Northumbrian Biologicals Ltd. Powdered trypsin (3 × crystalized and dialysed) was bought from Worthington Biochemicals. Fura-2 acetoxymethylester was obtained from Calbiochem. D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin, des-Arg^{9}Leu^8-bradykinin, des-Arg^{9}bradykinin and Hoechst 33258 were obtained from Sigma. Anti-alpha smooth muscle actin monoclonal antibody was purchased from Dako Ltd.

Results

In the presence of extracellular calcium ions, addition of the bradykinin B_1 receptor agonist, des-Arg^9-bradykinin, to BTSM cells produced a sharp increase in $[Ca^{2+}]_i$ which fell to a level above basal that was maintained for the duration of the experiment (Figure 1a). The increase in $[Ca^{2+}]_i$ observed

appeared to be uniform across the cell throughout the duration of the response. On analysis of data from 27 to 43 cells at each concentration, des-Arg⁹-bradykinin was found to produce a concentration-dependent increase in $[Ca^{2+}]_i$ over basal levels (mean basal level = 51 ± 3 nM; n = 107; maximum increase = 373 ± 28 nM at 10 μM des-Arg⁹-bradykinin; n = 29; Figure 2a). As we have noted previously, when using bradykinin as an agonist in this system (Marsh & Hill, 1993), not all the cells in any one field of view were responsive to

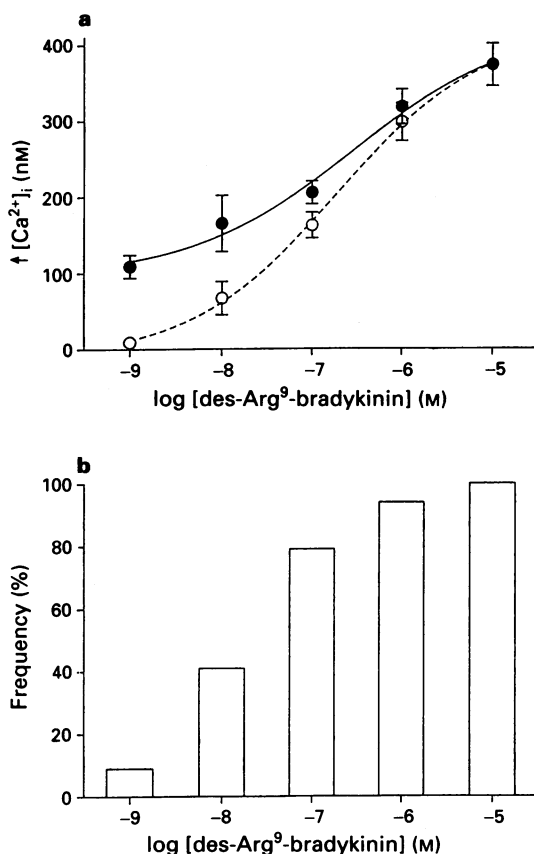

Figure 2 Concentration-response relationships to des-Arg⁹-bradykinin (1 nM–10 μM) in the presence of extracellular calcium. (a) Values represent the mean increase in intracellular calcium ion concentration over basal levels of all bovine tracheal smooth muscle (BTSM) cells observed (O) or responding cells only (●). Means ± s.e.means at each concentration were calculated from at least five separate coverslips each arising from different animals. (b) Columns represent the frequency of response of BTSM cells. Frequency is defined as the percentage of the total number of cells observed which respond to des-Arg⁹-bradykinin.

des-Arg⁹-bradykinin. Responding cells were identified by an increase in $[Ca^{2+}]_i$ above their basal levels and conversely, non-responders showed no variation in the baseline response on addition of the agonist (e.g. one cell in Figure 1b). We have therefore calculated the number of cells which responded to a particular concentration of des-Arg⁹-bradykinin as a percentage of the total number of cells observed at that concentration (frequency). By evaluating data in this manner it was found that des-Arg⁹-bradykinin also caused a concentration-dependent increase in the frequency of response of the BTSM cells from 9% at 1 nM to 100% at 10 μM des-Arg⁹-bradykinin (Figure 2b). A randomised pattern of response to des-Arg⁹-bradykinin was observed in the cells of any one field of view. Cellular contact did not appear to be a contributing factor to the nature of the responses of the cells.

In order to establish the nature of the mean cellular response (rather than the mean population response) to des-Arg⁹-bradykinin, the remaining results have been calculated from data obtained from des-Arg⁹-bradykinin-sensitive cells only. As a result of these calculations, the EC_{50} value for des-Arg⁹-bradykinin was found to be 316 nM in the presence of extracellular calcium ions (Figure 2a).

Addition of the bradykinin B_1 receptor antagonist, des-Arg⁹Leu⁸-bradykinin (10 μM), 2 min prior to the addition of agonist completely abolished the increase in $[Ca^{2+}]_i$ induced by 100 nM des-Arg⁹-bradykinin (Table 1). However, there was no significant difference between the response to des-Arg⁹-bradykinin alone and that obtained in the presence of the bradykinin B_2 receptor antagonist, D-Arg[Hyp³,Thi⁵,⁸,D-Phe⁷]-bradykinin (10 μM; Table 1). We have previously shown that this concentration of D-Arg[Hyp³,Thi⁵,⁸,D-Phe⁷]-bradykinin attenuates the bradykinin-induced calcium response in these cells (Marsh & Hill, 1993). The frequency of response of the cells to 100 nM des-Arg⁹-bradykinin was reduced to 0% by the B_1 receptor antagonist but was of a similar magnitude in the presence of the B_2 receptor antagonist (Table 1).

In the absence of extracellular calcium ions, des-Arg⁹-bradykinin was found to elicit a calcium response characterized by a rise in $[Ca^{2+}]_i$ followed by a fall to basal level (mean basal level = 32 ± 2 nM; n = 37) within 60–80 s (Figure 1b). However, only concentrations of des-Arg⁹-bradykinin equal to or greater than 1 μM were found to induce a calcium response in BTSM cells under these conditions. Des-Arg⁹-bradykinin, 1 μM, produced a mean increase of 184 ± 24 nM (n = 8) at a frequency of 18% and 10 μM des-Arg⁹-bradykinin elicited an increase of 210 ± 21 nM (n = 29) at a frequency of 62%. These two responses were found not to be significantly different from each other (unpaired t test). Table 1 also demonstrates that, in a separate series of experiments, des-Arg⁹Leu⁸-bradykinin (10 μM) and D-Arg[Hyp³,Thi⁵,⁸,D-Phe⁷]-bradykinin (10 μM) were each able to attenuate the calcium response of the cells

Table 1 Responses of single bovine tracheal smooth muscle cells to des-Arg⁹-bradykinin in calcium-containing and calcium-free conditions

	$\Delta[Ca^{2+}]_i$ (nM)	*Frequency of response (%)*
+ Calcium		
des-Arg⁹-bradykinin 100 nM	190 ± 21	62 (18/29)
+ B_1 antagonist 10 μM	0	0 (0/25)
+ B_2 antagonist 10 μM	149 ± 16	59 (13/22)
– Calcium		
des-Arg⁹-bradykinin 10 μM	239 ± 30	79 (19/24)
+ B_1 antagonist 10 μM	145 ± 13*	46 (12/26)
+ B_2 antagonist 10 μM	138 ± 10*	52 (12/23)

Experiments were performed as described in the text in the absence or presence of des-Arg⁹Leu⁸-bradykinin (B_1 antagonist) and D-Arg[Hyp³,Thi⁵,⁸,D-Phe⁷]-bradykinin (B_2 antagonist). The results are calculated from a sample of cells taken from at least four different coverslips each originating from different animals. Matching coverslips were used for the three different conditions (agonist ± antagonist) on each experimental day. Numbers in parentheses indicate the number of cells responding to des-Arg⁹-bradykinin/the total number of cells observed. *P < 0.05 (unpaired t test).

to des-Arg[9]-bradykinin (10 μM) to a similar extent under calcium-free conditions.

Discussion

The present study demonstrates the presence of functional bradykinin B_1 receptors on cultured BTSM cells which can elicit an increase in $[Ca^{2+}]_i$. The bradykinin B_1 receptor agonist, des-Arg[9]-bradykinin, can induce a concentration-dependent increase in $[Ca^{2+}]_i$ of BTSM cells in the presence of extracellular calcium ions suggesting activation of the bradykinin B_1 receptor. The pattern of increase of $[Ca^{2+}]_i$ induced by the agonist demonstrates an initial peak followed by the much lower plateau component of the response which is likely to be due to a continued influx of calcium ions from the extracellular environment. The EC_{50} value of 316 nM obtained under these conditions is consistent with that previously calculated in this laboratory from phosphoinositide studies on cultured BTSM cells (EC_{50} = 199 nM; Marsh & Hill, 1992). These values are similar to that obtained from calcium studies of the B_1 receptor in rat mesangial cells (560 nM; Bascands *et al.*, 1993) and are in the same concentration-range as those observed in the 'classic' B_1 receptor system, contractile responses of the rabbit aorta, where the EC_{50} was found to be 50 nM (Regoli *et al.*, 1981).

We have also demonstrated in this study that the calcium response to des-Arg[9]-bradykinin (100 nM) in calcium-containing medium was insensitive to the B_2 receptor antagonist (D-Arg[Hyp[3],Thi[5,8],D-Phe[7]]-bradykinin) but was completely abolished by the B_1 receptor antagonist used (des-Arg[9]Leu[8]-bradykinin). These data further suggest that des-Arg[9]-bradykinin is acting via stimulation of B_1 receptors as des-Arg[9]Leu[8]-bradykinin has previously been shown to be selective for B_1 receptors since it is active in the rabbit aorta (B_1 system) yet without effect in the rabbit jugular vein (B_2 system; Regoli *et al.*, 1990). It is possible, however, that the responses observed in the presence of extracellular calcium ions to concentrations of des-Arg[9]-bradykinin above 100 nM are in part due to activation of B_2 receptors. This suggestion has arisen from the observation in this study that the responses to des-Arg[9]-bradykinin at these concentrations obtained under calcium-free conditions are sensitive to the B_2 receptor antagonist. Furthermore a recent study demonstrated that the cDNA from the murine B_2 receptor encodes for two populations of receptors which exhibit the pharmacological properties of a B_1-like receptor and the B_2 receptor; however, it is noteworthy that receptors cloned from human and rat sources in the same study demonstrated pharmacological properties of B_2 receptors only (McIntyre *et al.*, 1993).

The results obtained in this study are consistent with the observation made in our previous studies where des-Arg[9]-bradykinin elicited an increase in phosphoinositide hydrolysis in these cells (Marsh & Hill, 1992) demonstrating the presence of functional bradykinin B_1 receptors on cultured BTSM cells. The mean data represented by Figure 4b of that paper demonstrate that des-Arg[9]-bradykinin was able to increase significantly the phosphoinositide hydrolysis over basal levels in BTSM cells at concentrations of 1 μM and 10 μM. It is only at these concentrations that, in the absence of extracellular calcium ions, an increase in $[Ca^{2+}]_i$ of BTSM cells was observed in this study. This increase observed in the absence of extracellular calcium must be due to a release of calcium from intracellular stores which, from the evidence available (Marsh & Hill, 1992 and present study), is likely to occur via an increase in phosphoinositide turnover and production of IP_3.

In a previous study we demonstrated that bradykinin, through activation of B_2 receptors, can induce an 'all-or-nothing' release of calcium from intracellular stores (Marsh & Hill, 1993). From the results obtained in the present studies it is possible that des-Arg[9]-bradykinin also induces an 'all-or-nothing' release of intracellular calcium in experiments conducted in the absence of extracellular calcium ions. Consistent with this view is the fact that no response in any cell was observed below a concentration of 1 μM des-Arg[9]-bradykinin yet the responses elicited by 1 μM and 10 μM agonist were of a similar magnitude in each cell and the mean responses were not significantly different from each other, i.e. no intermediate response was obtained between zero and approximately 200 nM even though the frequency of responding cells increased from 18% to 62% as the concentration rose from 1 μM to 10 μM antagonist. This value is similar to that obtained in our previous study where, in the presence of extracellular calcium ions, bradykinin stimulated B_2 receptors to produce an 'all-or-nothing' increase in $[Ca^{2+}]_i$ of approximately 180 nM (Marsh & Hill, 1993). It is of interest to note that the concentration of des-Arg[9]-bradykinin required to elicit a response under calcium-free conditions (1 μM) is much higher than that of bradykinin shown to produce a similar increase (10 pM; Marsh & Hill, 1993).

In the present study both the B_1 and B_2 receptor antagonists used (des-Arg[9]Leu[8]-bradykinin and D-Arg[Hyp[3],-Thi[5,8],D-Phe[7]]-bradykinin) were found to be equiactive in attenuating the calcium response of BTSM cells to 10 μM des-Arg[9]-bradykinin under calcium-free conditions. This suggests that B_2 receptors are also stimulated by this high concentration of B_1 agonist in the absence of extracellular calcium ions. However, the fact that both receptors are activated by the high concentrations of des-Arg[9]-bradykinin needed to release intracellular calcium, makes the pharmacology of the B_1 receptor, stimulated under these conditions, very difficult to evaluate. Furthermore, this partial antagonism of the response to des-Arg[9]-bradykinin, in the absence of intracellular calcium ions, by both B_1 and B_2 antagonists is not readily consistent with the 'all-or-nothing' release of intracellular calcium observed with bradykinin (Marsh & Hill, 1993). Nevertheless, the data obtained at the lower concentrations of des-Arg[9]-bradykinin suggest that the calcium response observed is mediated via stimulation of B_1 receptors to produce an influx of calcium ions from the extracellular environment.

The present study also demonstrates that the latency of response of the cells to des-Arg[9]-bradykinin in the presence of extracellular calcium ions (Figure 1a) is much shorter than that under calcium-free conditions (Figure 1b). The latency present in intracellular calcium release may, as we have suggested previously (Marsh & Hill, 1993), be a result of the interaction between IP_3-sensitive and IP_3-insensitive calcium stores. This would lead to the filling of IP_3-insensitive calcium stores and subsequent transient calcium-induced calcium release from these stores leading to the observed delay in response. The most likely explanation for the short latency of response observed at low agonist concentrations in the presence of extracellular calcium ions is that des-Arg[9]-bradykinin can stimulate calcium entry directly and/or be able to act via IP_3-insensitive stores that are not accessible to calcium released from IP_3-sensitive stores.

An interesting study using populations of rat mesangial cells demonstrated that the profiles of the calcium response to B_1 stimulation (des-Arg[9]-bradykinin) and to B_2 stimulation (bradykinin) were different in that the plateau, or influx, phase of the response was much greater upon B_1 stimulation (Bescands *et al.*, 1993). This led the authors to suggest that B_1 stimulation resulted in a larger part of the response being due to calcium influx whereas B_2 activation preferentially stimulated the release of calcium from intracellular stores. In our studies to date we have observed no apparent differences in the plateau phase of the calcium response upon B_2 stimulation (Marsh & Hill, 1993) or B_1 stimulation (present study). However, our data also suggest that des-Arg[9]-bradykinin predominantly causes an influx of calcium ions from the extracellular space, particularly at the lower concentrations of the agonist where no response was observed in the absence of extracellular calcium ions. Our results also dem-

onstrate that only the high and non-specific concentrations of des-Arg[9]-bradykinin are able to induce a release of calcium from intracellular stores which is mediated via activation of both B_1 and B_2 receptors. The results presented in this study provide us with a unique system with which to study the effects of B_1 receptor activation on airway smooth muscle tissue.

We would like to thank the AFRC for their financial support of this work.

References

BASCANDS, J.L., PECHER, C., RAUOUD, S., EMOND, C., TACK, J.L., BASTIE, M.J., BURCH, R., REGOLI, D. & GIROLAMI, J.-P. (1993). Evidence for the existence of two distinct bradykinin receptors on rat mesangial cells. *Am. J. Physiol.*, **264**, F548–F556.

BENHAM, C.D. & TSIEN, R.W. (1987). A novel receptor-operated Ca^{2+}-permeable channel activated by ATP in smooth muscle. *Nature*, **328**, 275–278.

BERRIDGE, M.J. & IRVINE, R.F. (1989). Inositol phosphates and cell signalling. *Nature*, **341**, 197–205.

CHEN, T.R. (1977). *In situ* detection of mycoplasma contamination in cell cultures by fluorescent Hoechst stain 33258. *Exp. Cell Res.*, **104**, 255–259.

CHRISTIANSEN, S.C., PROUD, D. & COCHRANE, C.G. (1987). Detection of tissue kallikrein in the bronchoalveolar lavage fluid of asthmatic patients. *J. Clin. Invest.*, **79**, 188–197.

DELEAN, A., MUNSON, P.J. & RODBARD, D. (1978). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay and physiological dose-response curves. *Am. J. Physiol.*, **235**, E97–E102.

FARMER, S.G., BURCH, R.M., KYLE, D.J., MARTIN, J.A., MEEKER, S.N. & TOGO, J. (1991a). D-Arg[Hyp[3]-Thi[5]-D-Tic[8]]-bradykinin, a potent antagonist of smooth muscle BK_2 receptors and BK_3 receptors. *Br. J. Pharmacol.*, **102**, 785–787.

FARMER, S.G., BURCH, R.M., MEEKER, S.A. & WILKINS, D.E. (1989). Evidence for a pulmonary B_3 bradykinin receptor. *Mol. Pharmacol.*, **36**, 1–8.

FARMER, S.G., ENSOR, J.E. & BURCH, R.M. (1991b). Evidence that cultured airway smooth muscle cells contain bradykinin B_2 and B_3 receptors. *Am. J. Respir. Cell Mol. Biol.*, **4**, 273–277.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence indicators. *J. Biol. Chem.*, **260**, 3440–3450.

MARSH, K.A. & HILL, S.J. (1992). Bradykinin B_2 receptor-mediated phosphoinositide hydrolysis in bovine cultured tracheal smooth muscle cells. *Br. J. Pharmacol.*, **107**, 443–447.

MARSH, K.A. & HILL, S.J. (1993). Characteristics of the bradykinin-induced changes in intracellular calcium ion concentration of single bovine tracheal smooth muscle cells. *Br. J. Pharmacol.*, **110**, 29–35.

MCINTYRE, P., PHILLIPS, E., SKIDMORE, E., BROWN, M. & WEBB, M. (1993). Cloned murine bradykinin receptors exhibit a mixed B_1 and B_2 pharmacological selectivity. *Mol. Pharmacol.*, **44**, 346–355.

MURRAY, R.K. & KOTLIKOFF, M.I. (1991). Receptor-activated calcium influx in human airway smooth muscle cells. *J. Physiol.*, **435**, 123–144.

NEYLON, C.B., HOYLAND, J., MASON, W. & IRVINE, R.F. (1990). Spatial dynamics of intracellular calcium in agonist-stimulated vascular smooth muscle cells. *Am. J. Physiol.*, **259**, C657–C686.

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

REGOLI, D.C., MARCEAU, F. & LAVIGNE, J. (1981). Induction of B_1 receptors for kinins in the rabbit by a bacterial lipopolysaccharide. *Eur. J. Pharmacol.*, **71**, 105–115.

REGOLI, D., RHALEB, N.-E., DRAPEAU, G. & DION, S. (1990). Kinin receptor subtypes. *J. Card. Pharmacol.*, **15**, S30–S38.

SOMLYO, A.P., WALKER, J.W., GOLDMAN, Y.E., TRENTHAM, D.R., KOBAYASHI, S., KITAZAWA, T. & SOMLYO, A.V. (1988). Inositol triphosphate, calcium and muscle contraction. *Phil. Trans. R. Soc. B.*, **320**, 399–404.

*(Received November 15, 1993
Revised March 7, 1994
Accepted March 14, 1994)*