Characteristics of the bradykinin-induced changes in intracellular calcium ion concentration of single bovine tracheal smooth muscle cells

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1 Single bovine tracheal smooth muscle (BTSM) cells were cultured and used to measure bradykinin-induced changes in $[Ca^{2+}]_i$ by dynamic video imaging.

2 Bradykinin (10 pM–10 μ M)-induced an increase in $[Ca^{2+}]_i$ over basal levels (69 ± 2 nM; $n = 353$) which was concentration-dependent ($\log EC_{50} = -8.7$ M) in the presence of extracellular calcium ions (2 mM). The bradykinin B_2 receptor antagonist, D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin, produced a parallel shift to the right of the bradykinin concentration-response curve ($\log EC_{50} = -7.1$ M and -5.8 M in the presence of 1 μ M and 10 μ M antagonist respectively) yielding an apparent K_D of 26 nM.

3 In the absence of extracellular calcium ions (with 0.1 mM EGTA), bradykinin (10 pM–10 μ M) produced a uniform increase in $[Ca^{2+}]_i$ from a basal level of 33 ± 2 nM ($n = 140$) to approximately 180 nM in BTSM cells indicating an 'all-or-nothing' release of intracellular calcium ions. In the presence of 10 μ M D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin no responses could be induced by bradykinin at concentrations below 100 nM. However, at 100 nM and 1 μ M bradykinin there was no change in the uniform increase in $[Ca^{2+}]_i$ in these cells previously observed.

4 In both the absence or presence of D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin, there was a concentration-dependent increase in the percentage of cells responding to bradykinin (frequency) under calcium-rich or calcium-free conditions. Individual cells also demonstrated a difference in the sensitivity to any particular concentration of bradykinin.

5 A latency in the response of cells to bradykinin was observed both in calcium-containing and calcium-free conditions.

6 We conclude that bradykinin B_2 receptors are expressed by BTSM cells and are involved in the bradykinin-induced increase in $[Ca^{2+}]_i$. It appears that the increase in $[Ca^{2+}]_i$ can be mediated via a graded influx of calcium ions from the extracellular space or an 'all-or-nothing' release from intracellular stores.

Keywords: Bradykinin; calcium ion mobilisation; trachea; cultured smooth muscle

Introduction

The involvement of intracellular calcium ions in smooth muscle contraction and relaxation is a well recognized phenomenon; however, the mechanisms by which changes in intracellular calcium ion concentration ($[Ca^{2+}]_i$) are obtained within the cell are not completely understood, particularly in the smooth muscle of the airways. An increase in $[Ca^{2+}]_i$ can be mediated either via a release of calcium from intracellular stores (Somylo *et al.*, 1988; Berridge & Irvine, 1989; Irvine, 1990) or via an influx of calcium from the extracellular fluid (Benham & Tsien, 1987; Murray & Kotlikoff, 1991). The release of calcium ions from intracellular stores can be effected by a spasmogen-induced activation of phospholipase C leading to an increase in the production and action of inositol 1,4,5-triphosphate (IP₃; Berridge & Irvine, 1989). It has recently been proposed that, in a number of cell types, including pancreatic acini (Muallem *et al.*, 1989), hepatocytes (Taylor & Potter, 1990; Oldershaw *et al.*, 1991) and HeLa cells (Bootman *et al.*, 1992), the IP₃-mediated release of calcium from intracellular stores is a quantal process where these stores differ in their sensitivity to IP₃.

Bradykinin is a nonapeptide which has been suggested to have an involvement in the pathogenesis of allergic asthma. In addition, elevated levels of this peptide have been observed in asthmatic patients (Christiansen *et al.*, 1987), although the physiological role of bradykinin is unclear. It

has been demonstrated that bradykinin can induce an increase in $[Ca^{2+}]_i$ in both human (Murray & Kotlikoff, 1991) and guinea-pig (Farmer *et al.*, 1991a,b) airway smooth muscle cells, but the characteristics of these responses remain to be elucidated. Bradykinin receptors have classically been divided into two subtypes, those of B_1 and B_2 , according to the relative potencies of antagonists and agonists (Regoli & Barabe, 1980). However, the receptor by which bradykinin exerts its intracellular effect is unclear. Using cultured cells from the guinea-pig tracheal smooth muscle, Farmer *et al.* (1991b) suggested that the activation of a putative B_3 receptor (Farmer *et al.*, 1989) was responsible for the bradykinin-induced increase in $[Ca^{2+}]_i$ they observed which was insensitive to both B_1 and B_2 receptor antagonists.

We have previously reported that bradykinin can induce an increase in phosphoinositide hydrolysis in cultures of bovine tracheal smooth muscle cells which appears to be mediated via the activation of the bradykinin B_2 receptor (Marsh & Hill, 1992a). In addition, initial studies on single bovine tracheal smooth muscle cells indicated that bradykinin also induced an increase in $[Ca^{2+}]_i$ in the presence and absence of extracellular calcium ions (Marsh & Hill, 1992b,c). We have therefore extended these studies to investigate the characteristics of the bradykinin-induced increase in $[Ca^{2+}]_i$ of single bovine tracheal smooth muscle cells in both calcium-free and calcium-rich conditions using dynamic video imaging. This paper describes the involvement of the bradykinin B_2 receptor in mediating a bradykinin-induced increase

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in $[Ca^{2+}]_i$ and the characteristics of this increase by use of calcium-free and calcium-rich experimental conditions. A preliminary account of part of this work has been presented to the British Pharmacological Society (Marsh & Hill, 1993).

Methods

Cell culture

Cultures of bovine tracheal smooth muscle cells were obtained as described previously (Marsh & Hill, 1992a,b,c). Briefly, fresh tracheal smooth muscle denuded of mucosa and connective tissue was chopped into 1 mm³ pieces and transferred to tissue culture-treated plastic flasks. These explants were then covered with a D-Val-substituted minimum essential medium containing 10% foetal calf serum (FCS) and antibiotics (100 u ml⁻¹ penicillin G; 100 µg ml⁻¹ streptomycin; 250 ng ml⁻¹ amphotericin B) then maintained at 37°C in 10% CO₂. Smooth muscle cells grew to confluency from the explants and were routinely subcultured by treatment with trypsin (0.05% in versene, Glasgow formula). Using the Hoechst 33258 staining method of Chen (1977), mycoplasma contamination was shown to be negative. Immunocytochemical analysis using the monoclonal antibody to alpha smooth actin was performed as described previously (Marsh & Hill, 1992a) confirming the identity of the cells as smooth muscle. For image analysis purposes, cells from passages 4 to 10 were seeded at a split ratio of 1:10 onto 22 mm circular glass coverslips in Dulbecco's modified Eagle's medium with 10% FCS and grown for 72 h under the above conditions.

Image analysis

Coverslips with attached cells were washed three times in a physiological saline solution (PSS) containing (in mM): NaCl 145, KCl 5, MgSO₄ 1, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 10, glucose 10 and CaCl₂ 2, pH 7.4. The cells were then incubated at 37°C for 30 min with PSS containing 10% FCS and 5 µM fura-2 acetoxymethylester; then excess dye was removed by washing with PSS a further 3 times. Coverslips were transferred to a metal holder which was mounted in a heated chamber maintained at 37°C. A volume of 900 µl of PSS was added to the chamber and agents were added directly in a volume of 100 µl PSS. For experiments requiring calcium-free conditions, any PSS added to the chamber was deficient of CaCl₂ and supplemented with 0.1 mM EGTA. All coverslips were used within 90 min of the end of the loading time.

Image analysis was performed with 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 MagiCal hardware where the images underwent analogue to digital conversion. 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 with a 2-D look up table utilising the Grynkiewicz (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, thus allowing 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 (Figure 1a) and whole cell intracellular calcium ion quantification was obtained by outlining each individual cell with a light pen (Figure 1b). 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 by use of the non-linear programme ALLFIT (DeLean *et al.*, 1978). The equation fitted was

$$\% \text{ of maximal response} = E_{max} \times D^n / D^n + (EC_{50})^n$$

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 stimulation. Apparent dissociation constant (K_D) of the receptor antagonist was determined, assuming competitive antagonism, from shifts in the agonist concentration-response curves using the relationship

$$K_D = D / (K_2 / K_1 - 1)$$

where D is the concentration of antagonist, K_1 is the concentration of agonist producing half maximal response and K_2 is the concentration of agonist producing the same response in the presence of antagonist. The data point at each concentration of bradykinin was calculated from accumulated data from a single field of view (containing 4–10 cell) from each of at least six different coverslips each arising from different animals.

Chemicals

D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin, bradykinin acetate and Hoechst 22358 were obtained from Sigma. Anti-alpha smooth muscle actin monoclonal antibody was purchased from Dako Ltd.

Results

Figure 1a demonstrates the time sequence of changes in $[Ca^{2+}]_i$ of cells from a single field of view. An interval of approximately 3 s separates each image and bradykinin (10 nM) was added between the first two frames. The graphical analysis from Figure 1a is shown in Figure 1b where each line on the upper graph represents the changes in intracellular calcium ion concentration from the individual cell defined by the outline of the same colour.

In the presence of extracellular calcium, bradykinin produced a sharp increase in the $[Ca^{2+}]_i$ of bovine tracheal smooth muscle (BTSM) cells which fell to a lower yet elevated over basal level that was maintained for several minutes (Figure 1). On analysis of data from between 26 and 45 cells at each concentration, bradykinin was found to produce a concentration-dependent increase in $[Ca^{2+}]_i$ over basal levels (mean basal level in calcium-rich conditions = 69 ± 2 nM; $n = 353$; maximum increase =

Figure 1 Bradykinin-induced changes in intracellular calcium ion concentration in single bovine tracheal smooth muscle cells in the presence of extracellular calcium ions. (a) Montage of a time sequence of images obtained from the 'MagiCal' image analysis system. A time interval of approximately 3 s separates each image and the different colours represent different intracellular calcium ion concentrations (indicated to the left of the graph in Figure 1b). For evaluation of changes in calcium ion concentration within single cells, one field of view (4–10 cells) was monitored as a representative area from each coverslip. (b) Intracellular calcium ion concentrations for each of the cells were then measured individually as a function of time and are represented by separate traces on the graph. Each coloured trace of the graph (top) represents the data obtained from the cell (shown in the bottom left image) which is outlined by the same colour (bottom right image). Intracellular calcium ion concentrations are represented by the colours indicated to the left of the graph. Bradykinin (100 nM) was added to the bathing medium 15 s after the start of image capture (0 s).

428 ± 45 nM at 1 µM bradykinin; $n = 45$; Figure 2). It became increasingly apparent that not all the cells in any one field of view were responding to bradykinin, particularly at the lower concentrations of this agonist. We therefore analysed further this phenomenon with respect to the frequency of responding cells. This was done by calculating the number of cells which responded to a particular concentration of bradykinin as a percentage of the total number of cells observed at that concentration. By analysing the data in this way we found a concentration-dependent increase in the frequency of response of BTSM cells to bradykinin (Table 1). In light of this observation, data have been calculated from the bradykinin-sensitive cells only in order to evaluate the results as actual cell responses rather than as a function of the frequency of response of the cells. One field of view, containing 4–10 cells, was observed from each of six different coverslips (arising from different animals) for each concentration of bradykinin. The mean increase in $[Ca^{2+}]_i$ of all the cells which responded to each concentration of bradykinin was then calculated.

Evaluating the results in this way we found that the concentration-response curve for bradykinin in the presence of extracellular calcium ions revealed a log EC_{50} value of -8.7 M (Figure 3). On addition of the bradykinin B_2 receptor antagonist, D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin, 2 min prior to the addition of bradykinin, there was a parallel shift to the right of the bradykinin concentration-response curve

Figure 2 Concentration-response curve to bradykinin (10 pM–10 µM). Values represent the mean increase in intracellular calcium ion concentration over basal levels of all BTSM cells (i.e. bradykinin (BK)-responsive and non-responsive cells) observed. Means of the cell responses ($n = 26$ –45 at each concentration of bradykinin) were calculated from at least six fields of view from separate coverslips each arising from different animals. Points show means with s.e.mean.

Figure 3 Concentration-response curves to bradykinin (10 pM–10 µM) in the absence (○) and presence of 1 µM (x) or 10 µM (●) D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin added 2 min prior to bradykinin in the presence of extracellular calcium ions. Values represent the mean increase in intracellular calcium ion concentration over basal levels of only the BTSM cells which responded to bradykinin. Means of responding cells ($n = 21$ –38 at each concentration) were calculated from at least six fields of view from separate coverslips each originating from different animals. Points show means with s.e.mean.

(Figure 3) indicating competitive antagonism resulting in log EC_{50} values of -7.1 and -5.8 in the presence of 1 µM and 10 µM antagonist respectively. These values yielded an apparent K_D of 26 nM for D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin. Table 1 demonstrates the differences in the frequency of response of the cells to bradykinin in the absence and the presence of antagonist where no cell responses were observed below 10 nM bradykinin in the presence of 1 µM antagonist and below 100 nM bradykinin in the presence of 10 µM D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin. No increase in $[Ca^{2+}]_i$ was observed on addition of D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin to the cells (results not shown).

In the absence of extracellular calcium ions there was an increase in $[Ca^{2+}]_i$ above basal levels (mean basal level in calcium-free conditions = 33 ± 2 nM; $n = 140$) in response to bradykinin which returned to basal levels within 90 s (Figure 4). However, as in the presence of extracellular calcium ions, not all cells responded to bradykinin (Figure 4, Table 1). On analysing the data from bradykinin-sensitive cells only, it was revealed that there was a uniform increase in $[Ca^{2+}]_i$ of approximately 180 nM on addition of concentrations of bradykinin between 10 pM and 10 µM bradykinin ($n = 4$ –22 for each concentration of bradykinin) indicating the absence of any concentration-response relationship. The

Table 1 Frequency of response of single BTSM cells to various concentrations of bradykinin in calcium-containing (+Ca) and calcium-free conditions (-Ca)

log [BK] (M)	Frequency of response (%)						
	- 11	- 10	- 9	- 8	- 7	- 6	- 5
+ Calcium							
BK alone	65 (24/37)	67 (26/39)	63 (27/43)	75 (21/28)	84 (38/45)	84 (38/45)	96 (25/26)
+ 1 µM antagonist	–	0 (0/20)	0 (0/31)	41 (14/34)	90 (30/33)	96 (26/27)	100 (32/32)
+ 10 µM antagonist	–	0 (0/15)	0 (0/16)	0 (0/30)	15 (4/26)	86 (30/35)	95 (19/20)
- Calcium							
BK alone	18 (7/38)	15 (5/34)	39 (14/36)	34 (13/38)	27 (9/33)	55 (22/40)	67 (20/30)
+ 10 µM antagonist	–	0 (0/8)	0 (0/17)	0 (0/24)	31 (18/59)	39 (22/56)	53 (25/47)

Experiments, as described in the text, were performed in the absence or presence of 1 µM or 10 µM of D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin as indicated. The frequency indicated is calculated from a sample of cells taken from at least six separate coverslips each originating from different animals and is defined as the percentage of the total number of cells observed which respond to bradykinin. Numbers in parentheses indicate the number of cells responding to bradykinin/the total number of cells observed.

Figure 4 Bradykinin-induced changes in intracellular calcium ion concentration of single BTSM cells in calcium-free conditions (with 0.1 mM EGTA). The arrow indicates the addition of 100 nM bradykinin. Note that one of the four cells was not responsive to 100 nM bradykinin. Intracellular calcium ion concentrations were calculated as in Figure 1.

slope of the linear regression line fitted to the data points did not differ significantly from zero (Figure 5). However, the frequency of response of the cells under calcium-free conditions are shown in Table 1 and although these frequencies are lower than those observed in the presence of extracellular calcium, a concentration-dependent increase in frequency is still apparent. In the presence of 10 μ M D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin no responses were observed below a concentration of bradykinin of 100 nM in calcium-free conditions (Table 1). At concentrations of bradykinin of 0.1 and 1 μ M there was no statistically significant difference (unpaired *t* test) between the increases in $[Ca^{2+}]_i$ obtained in the absence or presence of D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin. However, on addition of 10 μ M bradykinin a significantly greater increase in $[Ca^{2+}]_i$ was induced in the presence of 10 μ M D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin (338 ± 44 , $n = 25$) than in the absence of antagonist (193 ± 20 , $n = 20$; $P < 0.01$).

In addition to the above observations, it was also noted that there was a difference in the latency of response to bradykinin between cells in any one field of view particularly

Figure 5 Bradykinin (BK) (10 pM–10 μ M)-induced increases in intracellular calcium ion concentrations in calcium-free medium in the absence (○) and presence (●) of 10 μ M D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin added 2 min before bradykinin. Means of responding cells ($n = 4$ –22 at each concentration) were calculated as for Figure 3. Points are means with s.e.mean. The dashed line represents the concentration-response curve to bradykinin in the presence of extracellular calcium taken from Figure 3 for purposes of comparison. The linear regression line fitted through the data points obtained in the absence of antagonist reveals a Y intercept of 189 nM and a slope of 1.5 which did not differ significantly from zero.

at the lower concentrations of bradykinin. Figure 6 demonstrates the different latencies of response of 3 cells to 1 nM bradykinin under calcium-free conditions which is representative of the phenomenon observed in both calcium-free and calcium-containing conditions. We do not attribute these latencies to the diffusion of bradykinin across the monolayer as the spatial arrangement of the cells and the temporal aspect of the response are not consistent with a diffusion pattern (results not shown).

A difference in sensitivity was observed between cells to any particular concentration of bradykinin. Figure 7a demonstrates the responses of 2 cells to 0.1, 1, 10 and 1000 pM bradykinin in the presence of extracellular calcium. The cell response depicted by the solid line responds to all four concentrations of bradykinin with increasing amplitude. In contrast the cell represented by the dashed line shows a marginal increase in $[Ca^{2+}]_i$ to 0.1 pM bradykinin, no further increase at 1 pM but additional increases at 10 and 1000 pM bradykinin. In the absence of extracellular calcium ions differences in sensitivity were also observed in response to 0.01, 0.1, 1 and 10 μ M bradykinin; however, under these conditions no further increases in $[Ca^{2+}]_i$ were seen on addition of higher concentrations of bradykinin.

Discussion

This study demonstrates that in the presence of extracellular calcium ions, bradykinin can induce a concentration-dependent increase in the $[Ca^{2+}]_i$ of single bovine tracheal smooth muscle (BTSM) cells. This increase can be attenuated by the bradykinin B₂ receptor antagonist D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin suggesting that the receptor via which bradykinin exerts its effects is the B₂ receptor. This would concur with our previous report that the B₂ receptor mediates the bradykinin-induced increase in phosphoinositide (PI) hydrolysis in these cells (Marsh & Hill, 1992a,b) in that stimulation of the B₂ receptor increases the production of IP₃ which itself can lead to the release of calcium from intracellular stores (Berridge & Irvine, 1989). One of the more striking findings of this study is the uniform increase in $[Ca^{2+}]_i$ induced by all concentrations of bradykinin used in the absence of extracellular calcium. These results would suggest that, in this cell type, bradykinin is able to induce an 'all-or-nothing' release of calcium from the intracellular stores. This therefore suggests that the influx of calcium from the extracellular space (plateau phase of Figure 1b) appears to have a graded component and that the concentration-response curve obtained in the presence of extracellular calcium is a product

Figure 6 Latencies of individual cells responding to bradykinin in calcium-free conditions. Each trace represents the changes in intracellular calcium ion concentration of individual cells in the same field of view. The three cells represented in the graph responded approximately 7 s, 21 s and 34 s after the addition of 1 nM bradykinin (arrow).

Figure 7 Different sensitivities of individual BTSM cells within a single field of view to bradykinin in (a) calcium-containing medium and (b) calcium-free medium. Arrows represent the sequential additions of (a) 0.1, 1, 10 and 100 pM bradykinin or (b) 0.01, 0.1, 1 and 10 μ M bradykinin.

of the graded influx of ions from the extracellular space and an 'all-or-nothing' release of calcium from internal stores.

The characteristics of the 'all-or-nothing' release of calcium in the present study appears to be different from that observed in other cell types (Muallem *et al.*, 1989; Taylor & Potter, 1990; Oldershaw *et al.*, 1991; Bootman *et al.*, 1992). These groups have proposed that the intracellular calcium stores consist of several smaller stores which differ in their sensitivity to IP_3 , resulting in the release of only a portion of the stores at low concentrations followed by a further emptying of more stores on application of higher concentrations of IP_3 . This is not a phenomenon observed in the present study where it is quite clearly shown in Figure 7b that, in the absence of extracellular calcium ions, once a cell has responded to bradykinin it will not repeat this increase on further additions of higher concentrations of bradykinin. We do not believe this to be a result of desensitization to bradykinin as multiple responses to bradykinin are observed under calcium-rich conditions. It is possible that the calcium release mechanism is sufficiently cooperative within individual BTSM cells to enable a complete emptying of the calcium stores which is consistent with the observation that IP_3 -stimulated calcium release can be highly cooperative (Meyer *et al.*, 1988; 1990; Bootman *et al.*, 1992). Interpretation of the results obtained in the present study in calcium-free conditions leads to several suggestions, one being that the intracellular calcium stores within BTSM cells are cooperative but are not compartmentalized. A particular concentration of IP_3 may then trigger a complete emptying of the entire store. If the sensitivity to IP_3 differs from cell to cell, then this could produce the apparent difference in sensitivity to bradykinin.

Alternatively, the stores may indeed be compartmentalized yet the bradykinin triggers an 'all-or-nothing' production of IP_3 which releases only a portion of the stores and the different sensitivities observed are due to the differences in sensitivities of the cells to bradykinin.

Another possibility is that the constant amplitude of the bradykinin response seen in this study in calcium-free media may be a consequence of the interaction between IP_3 -sensitive and IP_3 -insensitive calcium stores which leads to the filling and subsequent transient calcium-induced release of calcium from the latter stores. This mechanism is one which has been proposed to account for the oscillations in $[Ca^{2+}]_i$ observed in a number of cell types (Berridge *et al.*, 1988; Berridge, 1990). Indeed the latency of response of BTSM cells to bradykinin observed in this study would be consistent with this latter hypothesis (Figure 6). If this is the case, then the graded increases observed in the presence of extracellular calcium induced by the higher concentrations of bradykinin must therefore be able to increase $[Ca^{2+}]_i$ directly or to act via IP_3 -insensitive stores which are not accessible to calcium released from IP_3 -sensitive stores. The graded influx of calcium ions from the extracellular space may also interact synergistically with IP_3 to cause channel opening (Finch *et al.*, 1991) resulting in the calcium mobilized from the stores promoting further calcium release within the cell. However, the exact mechanisms involved in the bradykinin-induced increase in $[Ca^{2+}]_i$ require further intracellular investigation for complete elucidation.

It has been established that the receptor mediating the bradykinin-induced increase in PI hydrolysis is the B_2 receptor (Marsh & Hill, 1992a) and from the present study it is now apparent that this same receptor is involved in the bradykinin-induced increase in $[Ca^{2+}]_i$ in cultured BTSM cells. We have found that in this assay system, as in the PI hydrolysis system, there is no inherent partial agonist activity produced by D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin as has been observed in the guinea-pig lung and tracheal strips (Farmer *et al.*, 1989). The K_D value obtained in BTSM cells in this study for D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin (26 nM) is of the same order of magnitude as those obtained in functional studies using the B_2 receptor-rich system, the rabbit jugular vein (10 nM; Regoli *et al.*, 1990).

We suggested in our previous paper (Marsh & Hill, 1992a) that a species difference exists in the coupling of bradykinin receptors to PI hydrolysis and calcium ion mobilization in airway smooth muscle cells. This was due to the sensitivity of the bradykinin-induced increase in PI hydrolysis to B_2 receptor antagonists in bovine cells (Marsh & Hill, 1992a) and the insensitivity of calcium ion mobilization in guinea-pig cells to B_2 receptor antagonists (Farmer *et al.*, 1991b). The results presented here support the view that there are indeed species differences and bradykinin B_2 receptors in BTSM cells are coupled to both second messenger systems whereas the receptor mediating calcium ion mobilization in guinea-pig cells is not a B_2 receptor and may be a B_3 receptor as suggested by Farmer *et al.* (1991a,b).

The results we obtained of the responses to bradykinin in the presence of 10 μ M D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin in calcium-free media (Figure 5) are in the main consistent with what we would expect for the antagonism of an 'all-or-nothing' response. In this case the antagonist abolished the responses to low concentrations of bradykinin (<100 nM) but did not significantly alter the magnitude of the calcium response of the cells sensitive to 100 nM or 1 μ M bradykinin (circa 200 nM). The only possible exception was that 10 μ M bradykinin induced an increase in $[Ca^{2+}]_i$ which was significantly higher in the presence of D-Arg[Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin. This response may be a result of the high and perhaps non-specific concentration of bradykinin used or may reflect a further quantal release of intracellular calcium ions. Another possibility is the involvement of B_1 receptors becoming apparent at that concentration of bradykinin under these conditions.

The involvement of B_1 receptors in the bradykinin-induced increase in PI hydrolysis of BTSM cells was found to be very small in that the relative potency of the B_1 agonist des-Arg⁹-bradykinin (Regoli & Barabé, 1980) was only 16% of that produced by bradykinin itself (Marsh & Hill, 1992a). However it is of interest to note that initial studies investigating the involvement of the B_1 receptor, using des-Arg⁹-bradykinin, in the calcium response of BTSM cells indicates a possible role for the B_1 receptor in calcium ion mobilization. The presence of this receptor in airway smooth muscle from other species is doubted (Farmer *et al.*, 1989) and we are therefore extending our studies to evaluate the possible involvement of this receptor in mediating the bradykinin-induced increase in $[Ca^{2+}]_i$ of BTSM cells.

One curious result produced by this present study is the concentration-dependent change in the frequency of response of the cells to bradykinin in both the absence and presence of extracellular calcium ions. This could be easily explained solely by a difference in the sensitivity of individual cells to bradykinin. This would involve there being few highly sen-

sitive cells in a population which respond to lower concentrations of bradykinin yielding a lower frequency of response, whereas in comparison, the higher frequency of response result from the more abundant, less sensitive cells requiring higher concentrations of bradykinin to produce a detectable response. One possible reason for this heterogeneity of sensitivity could be that the cultures are taken from a full cross section of tracheal smooth muscle which itself may not be homogeneous in its distribution of bradykinin-sensitive cells. To prove or disprove this theory would require precise sectioning of the tracheal smooth muscle and differential culturing of the cells in order to establish whether or not homogeneous cultures can be obtained from a particular section of smooth muscle which may give us some insight into the physiological role of bradykinin in the airways.

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