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Ca^{2+} signalling by endothelin receptors in rat and human cultured airway smooth muscle cells

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1 The aim of the current study was to characterize the ET receptor subtypes in cultured airway smooth muscle cells derived from rat trachea and human bronchus using radioligand binding techniques and to investigate the coupling of ET receptors to intracellular calcium signalling mechanisms using endothelin receptor-selective agonists (sarafotoxin S6c) and antagonists (BQ-123, BQ-788) and digital image fluorescence microscopy.

2 Confluent rat airway smooth muscle cells in culture possessed a mixed ET receptor population (30% ET_A : 70% ET_B), with a density of approximately 3400 ± 280 ET_A and 8000 ± 610 ET_B receptors/cell ($n = 3$ experiments). The density of ET_B, but not ET_A receptors increased substantially in serum-containing medium. However, a 2-day period of serum deprivation, which inhibited cellular growth, substantially reduced ET_B receptor density such that the ET receptor subtype proportions were approximately equal (55% ET_A; 45% ET_B) and similar to those previously observed in intact rat tracheal smooth muscle.

3 Challenge of rat airway smooth muscle cells in culture with endothelin-1 elicited a concentration-dependent biphasic increase in $[Ca^{2+}]_i$ (EC_{50}: 16 nM), that comprised an initial transient peak $[Ca^{2+}]_i$ increase (typically 350 nM) followed by a modest sustained component. The endothelin-1-induced biphasic $[Ca^{2+}]_i$ increase was primarily due to ET_A receptor activation, although a modest and inconsistent ET_B response was observed. The ET_A-mediated $[Ca^{2+}]_i$ increase was due primarily to the mobilization of IP_3-sensitive and to a lesser extent ryanodine-sensitive intracellular calcium stores. In contrast, ET_B receptor activation was exclusively coupled to extracellular calcium influx.

4 Somewhat surprisingly, human airway smooth muscle cells in culture contained a homogeneous population of ET_A receptors at a density of 6100 ± 800 receptors $cell^{-1}$ ($n = 3$ experiments). Serum deprivation was without effect on either ET receptor subtype proportion or ET_A receptor density. Challenge of human airway smooth muscle cells with endothelin-1 provoked a concentration-dependent increase in $[Ca^{2+}]_i$ (EC_{50}: 15 nM), with a peak $[Ca^{2+}]_i$ increase to greater than 700 nM. Furthermore, the ET_A-mediated calcium response in these human airway smooth muscle cells in culture was entirely dependent upon the mobilization of calcium from intracellular stores.

5 In summary, rat cultured tracheal airway smooth muscle cells contained both ET_A and ET_B receptors. ET_A receptors, the numbers of which remained constant during cell growth, were linked to the release of Ca^{2+} from intracellular stores and a strong rise in $[Ca^{2+}]_i$ in the majority of airway smooth muscle cells. In stark contrast, the numbers of ET_B receptors increased significantly during cell growth, an effect that was diminished substantially by incubation in serum-free medium. Moreover, despite the greater number of ET_B receptors, their activation in a small number of airway smooth muscle cells produced only a weak rise in $[Ca^{2+}]_i$, which appeared to be attributable to the influx of extracellular Ca^{2+}. In contrast, the populations of ET receptors and their linkage to $[Ca^{2+}]_i$ were markedly different in the human cultured airway smooth muscle cells used in the current study compared to that previously observed in intact human isolated bronchial smooth muscle.

Keywords: Endothelin-1; endothelin receptors; airway smooth muscle cells; calcium; bronchus; trachea; cultured cells

Introduction

In human isolated bronchus, endothelin-1 caused contractions that were mediated primarily *via* ET_B receptors (Goldie *et al*., 1995; Fukuroda *et al*., 1996). Consistent with this, radioligand binding and quantitative autoradiographic studies revealed that the majority (90%) of ET receptors in human isolated bronchial smooth muscle were of the ET_B receptor subtype (Goldie *et al*., 1995). The smaller population of ET_A receptors may also be linked to contractile (Fukuroda *et al*., 1996) and proliferative (Panettieri *et al*., 1996) responses in human bronchial airway smooth muscle. Neither Ca^{2+}-free medium nor pretreatment with the dihydropyridine calcium channel antagonist verapamil inhibited endothelin-1-induced contraction of human isolated bronchi (McKay *et al*., 1991). Furthermore, endothelin-1 induced a concentration-dependent biphasic elevation in $[Ca^{2+}]_i$ in human isolated bronchial smooth muscle cells that was preceded by inositol trisphosphate production (Mattoli *et al*., 1991). These findings suggest that endothelin-1-induced contractions were mediated *via* the activation of the phosphoinositide pathway and the release of Ca^{2+} from intracellular stores. Unfortunately, these early studies could not elucidate the identity of the ET receptor subtype(s) that were linked to intracellular Ca^{2+} mobilization, since selective agonists (sarafotoxin S6c, Williams *et al*., 1991) and antagonists (BQ-123, Ihara *et al*., 1992; BQ-788, Ishikawa *et al*., 1994) for ET_A and ET_B receptors were not widely available.

In both mouse and rat trachea, the airway smooth muscle band contained ET_A and ET_B receptors in approximately equal proportions (Henry, 1994; Carr *et al*., 1996) and each receptor

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subtype was linked to smooth muscle contraction. Consequently, endothelin-1-induced contractions were inhibited in the combined presence of the ET_A receptor-selective antagonist, BQ-123 and the ET_B receptor-selective antagonist BQ-788, but not by either antagonist alone. In rat tracheal smooth muscle, stimulation of ET_A and ET_B receptors appeared to induce contraction by activating different signal transduction pathways (Henry, 1993). ET_A receptor-mediated contractions were associated with increased phosphoinositide turnover and were not inhibited by Ca^{2+}-free medium, providing indirect and circumstantial evidence that stimulation of ET_A receptors was linked to contraction *via* the release of intracellular Ca^{2+}. In contrast, ET_B receptor-mediated contractions were markedly inhibited in Ca^{2+}-free medium and were not associated with marked activation of the phosphoinositide pathway, indicating that the source of activator Ca^{2+} was extracellular. However, direct measurements of changes in intracellular Ca^{2+} in response to ET_A and ET_B receptor activation in airway smooth muscle from rat trachea and human bronchus have not hitherto been reported.

Functional studies in intact preparations have highlighted the primary role of calcium in endothelin-1-induced contraction of airway smooth muscle, although the pathways through which endothelin-1 induces this change and the duration and degree of $[Ca^{2+}]_i$ perturbation has not been extensively examined. However, with the advent of fluorescent Ca^{2+}-indicator dyes that can be loaded non-invasively into live cells and highly sensitive imaging techniques able to monitor the Ca^{2+}-dependent changes in the dye's fluorescent properties, it is now possible to study these events at the cellular level. Thus, the primary thrust of the present study was to examine ET_A and ET_B receptor-mediated $[Ca^{2+}]_i$ signalling in rat and human airway smooth muscle cells. In the first instance, this involved the characterization of the ET receptor population and culminated in the examination of ET receptor subtype coupling to mechanisms of calcium signalling.

Methods

Rat airway smooth muscle cell culture

Rat airway smooth muscle band dissection and digestion Male Wistar rats (10–12 weeks of age) were anaesthetized (pentobarbitone, 120 mg kg^{-1}, i.p.) and killed by carotid artery exsanguination. The trachea was removed and placed in sterile ice-cold 'low calcium' Krebs Henseleit buffer containing (in mM): NaCl (139), KCl (5.4), $MgSO_4$ (1.47), glucose (11), KH_2PO_4 (1.47), Na_2HPO_4 (2.8), $NaHCO_3$ (1.4) and $CaCl_2$ (0.2). The trachea was cut longitudinally through the cartilage, the epithelial layer removed mechanically by rubbing with a cotton bud and the basement membrane teased away from the underlying smooth muscle band. Under a dissection microscope, the smooth muscle was carefully dissected free of any remaining connective tissue on the serosal side and the cartilage removed. Dissected muscle from five trachea was placed in 2 ml of 'low calcium' Krebs containing 2.5 mg ml^{-1} bovine serum albumin (0.25% BSA), 2 mg ml^{-1} collagenase I and 10 U ml^{-1} elastase IV and incubated for 30 min at 37°C in a shaking water bath. The smooth muscle bands were washed and centrifuged twice (10 min, 1000 *g*) in 'low calcium' Krebs and placed in 2 ml of 'low calcium' Krebs containing 0.25% BSA, 1 mg ml^{-1} collagenase I and 20 U ml^{-1} elastase IV, and incubated for 45–60 min at 37°C. Following the second digest, the digestion mix was washed twice in DMEM/F12 (containing 10% foetal calf serum (FCS), 100 U ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin). A Pasteur pipette with a heat-smoothed tip (internal diameter <0.6 mm) was used to gently dissociate the loosely attached airway smooth muscle cells (approximately 15–30 aspirations). Viable airway smooth muscle cells were counted (haemocytometer, Trypan blue exclusion method) and seeded at a density of 5×10^4 viable cells cm^{-2} onto Falcon Primaria plates (33 mm × 10 mm) (Becton Dickenson & Co., U.S.A.) in a 2 ml volume of DMEM/F12 (with 10% FCS). Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Culture medium was completely replaced the following day and thereafter, approximately 75% of the existing medium was aspirated every 2 days and replaced with the same volume of fresh DMEM/F12 (with 10% FCS). Greater than 95% of cells were α-actin smooth muscle immunopositive.

Human airway smooth muscle cell culture

Human airway smooth muscle cells derived from the bronchus of a 13-year-old male donor were purchased from Clonetics (San Diego, U.S.A.), grown to confluence and passaged to provide cell stocks. The identity of human airway smooth muscle cells was verified following incubation with a fluorescently labelled monoclonal antibody to α-smooth muscle actin (Skalli *et al*., 1986). Greater than 95% of cells were α-actin smooth muscle immunopositive.

Radioligand binding in cultured airway smooth muscle cells

Radioligand binding experiments were performed on airway smooth muscle cells seeded onto 24-well culture dishes at various stages of culture and where appropriate, deprived of serum for 2 days prior to the experiment. Airway smooth muscle cells at a predetermined stage of culture were repeatedly washed in PBS containing (in mM) NaCl (145), KH_2PO_4 (1.8), Na_2HPO_4 (8.2), BSA (0.25% w/v) and the protease inhibitor phenylmethylsulphonyl fluoride (10 μM) (binding medium) and left to air dry for 3 h. Air-dried cells were incubated in binding medium (0.5 ml) containing 0.2 nM [^{125}I]-endothelin-1 for 30–180 min at 22°C, either alone, in the presence of 1 μM BQ-123 (to prevent ET_A receptor-specific binding) or 200 nM sarafotoxin S6c (to prevent ET_B receptor-specific binding), or in the combined presence of 1 μM BQ-123 and 200 nM sarafotoxin S6c (to assess non-specific binding). The aforementioned concentrations of BQ-123 and sarafotoxin S6c have previously been demonstrated to completely prevent specific binding of [^{125}I]-endothelin-1 to ET_A and ET_B receptor sites respectively (Goldie *et al*., 1994; Knott *et al*., 1995). Cells were then washed (1.5 ml) twice for 10 min with ice-cold binding medium and then once for 20 min (2 ml). Airway smooth muscle cells were treated over 15 min with 200 μl NaOH (1 M) and removed from the wells by wiping with glass microfibre filter paper (Whatman GF/A). Airway smooth muscle cell-associated radioactivity was counted in a Packard Auto-Gamma counter (Model 5650) and the resultant data expressed as binding sites per cell. The average cell number per well was determined by haemocytometer in wells that were set aside earlier. The binding of endothelin-1 to its receptors is pseudo-irreversible and thus radioligand binding data were analysed using an appropriate kinetic model (Waggoner *et al*., 1992). The maximum binding capacity of [^{125}I]-endothelin-1 (B_{max}) to endothelin receptors was calculated using nonlinear least squares regression analysis of specific [^{125}I]-endothelin-1 binding data obtained from association time-course experiments fitted to the equation, $RL_t = B_{max}(1 - e^{-k_1 t})$, where RL_t

is the bound receptor concentration at time t and k_1 is the association rate constant (Waggoner *et al*., 1992). The numbers of ET_A and ET_B receptors was determined by analysis of [^{125}I]-endothelin-1 binding data obtained in the presence of 200 nM S6c (to occlude ET_B receptors) and 1 μM BQ-123 (to occlude ET_A receptors), respectively.

Calcium imaging in airway smooth muscle cells in culture

Calcium imaging experiments were performed using airway smooth muscle cells seeded onto 22-mm square coverslips and housed in sterile culture plates (33 mm × 10 mm) (Falcon Primaria, Becton Dickenson & Co., U.S.A.). Cells were left overnight in 2 ml DMEM/F12 to allow them to attach and flatten onto the coverslip and they were then deprived of serum for a further 2 days prior to experimentation. Cells were loaded with the calcium indicator dye fura-2 (2.5 μM fura-2 AM in DMEM/F12 culture medium for 60 min at 37°C) and washed three times with 1.5 ml of buffer containing (in mM); NaCl (137), KCl (5.4), $CaCl_2$ (2.5), $MgSO_4$ (1.47), D-glucose (11), KH_2PO_4 (1.47), Na_2HPO_4 (2.8), $NaHCO_3$ (1.4) and BSA (0.25% w/v) (imaging medium). The coverslip with attached cells was then placed face up in a circular chamber (Metaltek, Raleigh, NC, U.S.A.) bathed in 0.5 ml of imaging medium, mounted on the temperature controlled stage (37°C) of an inverted microscope (Olympus, model IMT2-RFL, Olympus Optical Co. Ltd., Tokyo, Japan) and a cover placed over the chamber to limit evaporation of the bathing medium. Estimation of $[Ca^{2+}]_i$ in single cells that had minimal contact with neighbouring cells was achieved by ratiometric analysis of fura-2 fluorescence images elicited by excitation at 340 nm and 380 nm that were captured approximately 700 ms apart as previously described (Maxwell *et al*., 1998). For each experiment, a single region was nominated for recording of a cell free area within the field of view, to assess the background levels of fluorescence at each excitation wavelength. Up to nine additional regions could be engaged for selective acquisition of the fluorescence from individual cells. Background images for both 340 nm and 380 nm fluorescence signals were subtracted from each pixel value recorded in subsequent 340 nm and 380 nm images for that experiment, respectively. Following background subtraction, each 340 nm image was divided by its corresponding 380 nm fluorescence image on a pixel by pixel basis and the individual pixel ratios averaged to provide a $F_{340}:F_{380}$ ratio value for that time point. Calculation of $[Ca^{2+}]_i$ from the $F_{340}:F_{380}$ ratio was according to Grynkiewicz *et al*. (1985).

Cellular autofluorescence and background fluorescence at routine excitation intensities were below threshold detection levels. Furthermore, quenching of calcium sensitive fura-2 fluorescence by manganese revealed there to be only a small residual and diffuse fluorescence signal that represented less than 3% of the original fura-2 fluorescence intensity. The diffuse and relatively even distribution of residual 340 nm and 380 nm fluorescence images indicated that minimal compartmentalization or localization of indicator to distinct regions within the cell had occurred.

Agonist challenge The effects of agonist challenge on $[Ca^{2+}]_i$ were examined in fura-2 loaded cells which had been equilibrated for 15 min in calcium containing imaging buffer (0.5 ml). Following establishment of a stable $[Ca^{2+}]_i$ baseline (acquisition of five ratio image pairs at 30 s intervals), image capture was temporarily halted to allow the exchange of imaging buffer for prewarmed fresh imaging buffer (0.5 ml), containing the appropriate agonist concentration, with minimal disturbance to the cells. Ratio image pair capture was resumed at 3 s intervals and continued for 1 min, followed by image acquisition at 30 s intervals for a further 8–10 min. Receptor antagonists of other modulators of function were introduced to the cells 20 min prior to agonist challenge and included in subsequent changes of medium. In many experiments, cells were challenged with bradykinin (BK), as a reference agonist after responses to the primary agonist were assessed. In these instances, cells were washed twice with imaging buffer at the end of the acquisition period concerning the primary agonist and left for a washout period of 20 min before challenge with BK. A short period of image acquisition was performed at 3 s intervals to assess the responsiveness of the cells. Where agonist challenge was to occur in calcium-free conditions, cells were maintained in calcium-containing imaging buffer prior to introduction of agonist and during the acquisition of baseline $[Ca^{2+}]_i$. Cells were then washed twice in prewarmed calcium-free imaging buffer (0.5 ml) prior to the addition of prewarmed calcium-free buffer (0.5 ml), containing the appropriate agonist and antagonist concentrations, and resumption of image acquisition.

Drugs

Drugs and chemicals utilized were: endothelin-1, [^{125}I]-endothelin-1, sarafotoxin S6c, BQ-123 (cyclo[D-Trp-D-Asp-L-Pro-D-Val-L-Leu]) (Auspep, Melbourne, Australia), phenyl-

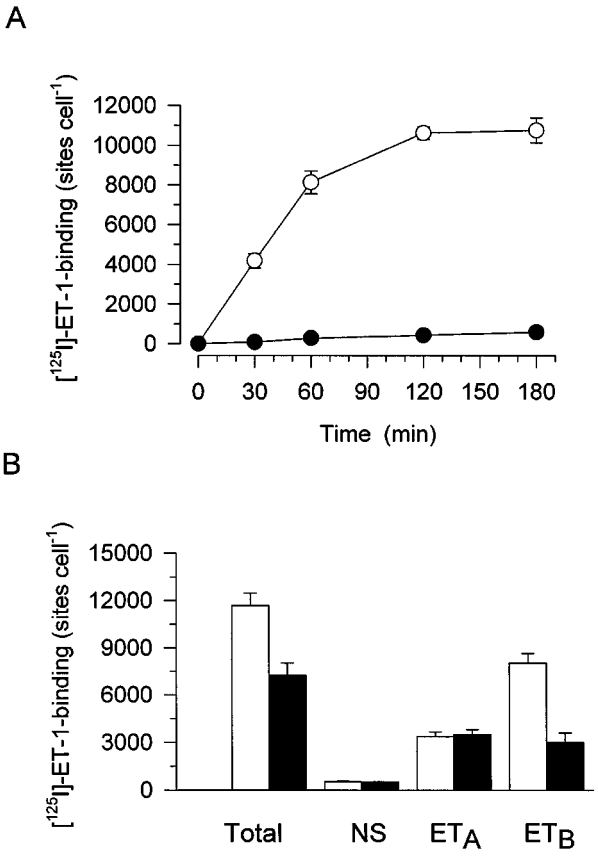

Figure 1 (A) Time dependence of total (○) and non-specific (●) [^{125}I]-endothelin-1 binding in confluent monolayer cultures of rat airway smooth muscle cells (7th passage). (B) Number of total and non-specific (NS) [^{125}I]-endothelin-1 binding sites and of ET_A and ET_B receptors in rat airway smooth muscle cells (7th passage) grown in 10% foetal calf serum (open columns) or for a 2-day period in serum-free media (filled columns). Data are expressed as means ±s.e.mean of triplicate determinations from three separate studies.

methylsulphonyl fluoride (Calbiochem, La Jolla, CA, U.S.A.), bovine serum albumin, bradykinin, caffeine, lanthanum, nicardipine, ryanodine, thapsigargin (Sigma Chemical Co., St. Louis, MO, U.S.A.), BQ-788 (N-*cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine) (gift from Banyu Pharmaceutical Co., Tsukuba, Japan). Stock solutions of endothelin-1 and sarafotoxin S6c were prepared in 0.1 M acetic acid, whereas BQ-123 was prepared in 100 mM Na_2CO_3 and BQ-788 in dimethyl sulfoxide. All drugs were kept on ice throughout an experiment and subsequent dilutions performed in saline.

Results

Radioligand binding studies in rat airway smooth muscle cells

[^{125}I]-Endothelin-1 binding in confluent rat airway smooth muscle cells Specific [^{125}I]-endothelin-1 (0.2 nM) binding to confluent monolayer cultures of rat airway smooth muscle cells increased rapidly in a time-dependent manner (Figure 1A) and plateaued between 120 and 180 min at a maximum density (B_{max}) of 10,770 $\pm$ 630 binding sites cell^{-1}. Non-specific binding, determined in the presence of BQ-123 (1 μM) and sarafotoxin S6c (200 nM), increased in a linear fashion over time and represented no more than 10% of specific binding at any given time point. Sarafotoxin S6c (200 nM) and BQ-123 (1 μM) reduced specific [^{125}I]-endothelin-1 binding by 70 and 30% respectively, indicating that ET_A and ET_B receptors co-existed in these rat airway smooth muscle cells in culture (open columns, Figure 1B).

Effect of serum deprivation on [^{125}I]-endothelin-1 binding in confluent rat airway smooth muscle cells Serum deprivation reduced the levels of total specific [^{125}I]-endothelin-1 binding by approximately 40%, although non-specific binding levels remained unaffected (filled columns, Figure 1B). This decrease in total specific binding was reflected in the 60% reduction of ET_B-specific binding site numbers following serum-free medium incubation, while ET_A-specific binding site numbers

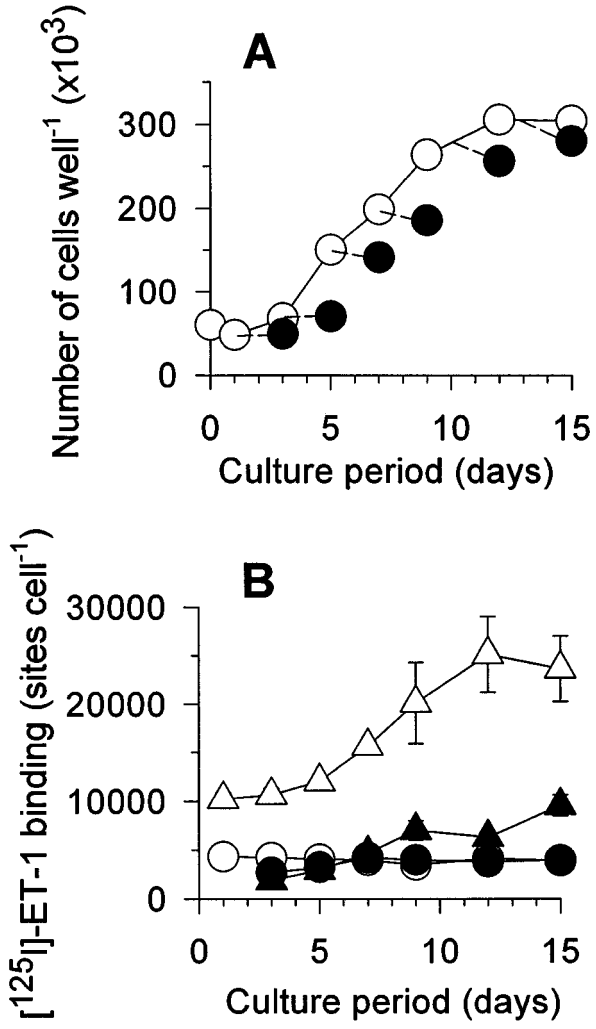

Figure 2 Effect of serum deprivation on (A) cell growth and (B) ET receptor subtype number in rat airway smooth muscle cells in culture (8th passage). (A) Time course for the growth of rat airway smooth muscle cells in serum-containing media (○) and after a 2-day period in serum-free media (●). (B) Number of ET_A (○, ●) and ET_B (Δ, ▲) receptors in rat airway smooth muscle cells grown in serum-containing medium (open symbols) and after a 2-day period in serum-free medium (filled symbols). Data are expressed as means $\pm$s.e.mean from three separate studies.

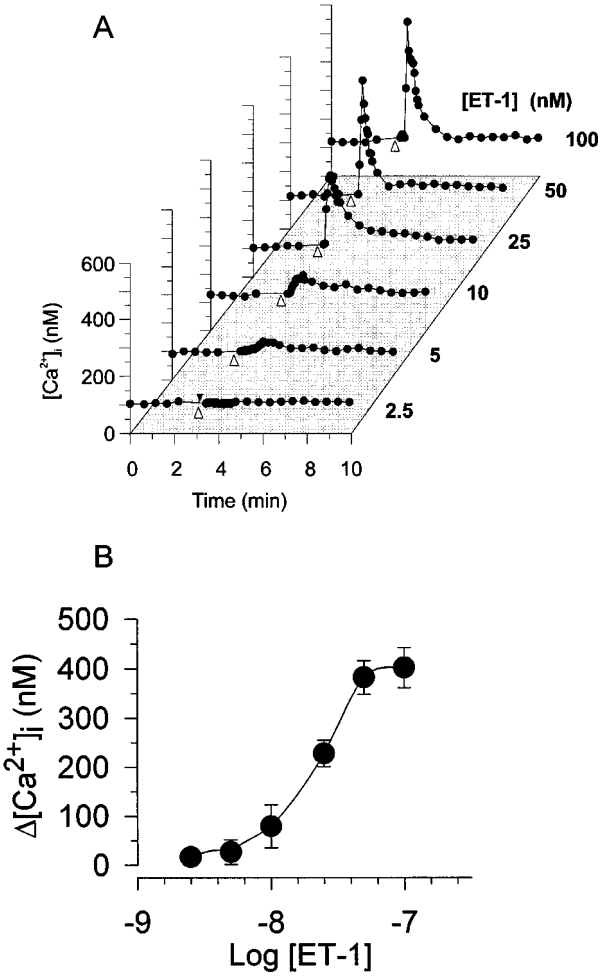

Figure 3 Concentration-dependence of endothelin-1-induced increases in $[Ca^{2+}]_i$ in rat airway smooth muscle cells in culture. (A) Representative curves illustrating the effects of increasing [endothelin-1] on $[Ca^{2+}]_i$. The open triangle indicates the moment at which endothelin-1 was added to the cells. (B) Concentration-response relationship between [endothelin-1] and the difference between baseline and peak $[Ca^{2+}]_i$. Data are the means $\pm$ s.e.mean from a single experiment (where $n = 37-45$ cells for each [endothelin-1]), and is representative of two separate studies.

remained unchanged (Figure 1B). Thus, serum deprivation induced a change in the proportions of ET receptors in rat tracheal smooth muscle cells from one containing predominantly ET_B receptors to a population with approximately equal numbers of ET_A (55%) and ET_B receptors (45%).

[^{125}I]-endothelin-1 binding during cell growth of rat airway smooth muscle cells Cells seeded at approximately 6×10^4 cells well^{-1} grew rapidly to achieve confluence between days 9 and 12, with cell numbers reaching a plateau of approximately 3×10^5 cells well^{-1} (Figure 2A). Throughout this growth period the number of ET_A receptor-specific binding sites remained relatively constant at around 5000 sites cell^{-1}. However, ET_B receptor-specific binding sites more than doubled from 10,000 sites cell^{-1} on days 1 and 3, to over 20,000 sites cell^{-1} between days 9 and 12 as the cells reached confluence (Figure 2B). An additional 3–6 days in culture appeared to have no further effect upon ET_B receptor-specific binding levels. Thus, the population of ET_A and ET_B receptors seen on days 1 and 3 (30% ET_A: 70% ET_B) approached a new steady state as cells achieved confluence, such that ET_B receptors represented an increased majority of the total ET receptor population (16% ET_A: 84% ET_B).

Effect of serum deprivation on [^{125}I]-endothelin-1 binding during rat airway smooth muscle cell growth Exposure of 8th passage rat airway smooth muscle cells to serum-free medium for 2 days inhibited cell growth at all time points examined, such that cell numbers were consistently lower than for cells exposed to 10% FCS (Figure 2A). Serum deprivation had a negligible effect on ET_A-specific binding site levels throughout the culture period of 15 days, with numbers remaining relatively stable at around 5000 sites cell^{-1} for both serum free and 10% serum conditions (Figure 2B). The number of ET_B receptor-specific binding sites was substantially reduced following serum deprivation at all time points examined, although a small increase in ET_B receptor-specific binding sites was still observed over the 15 days of culture.

Endothelin-1-induced $[Ca^{2+}]_i$ mobilization in rat airway smooth muscle cells

Concentration-response relationship to endothelin-1 Endothelin-1 elicited a concentration-dependent increase in $[Ca^{2+}]_i$ (Figure 3A). As the concentration of endothelin-1 was increased, a dramatic increase in magnitude of the transient $[Ca^{2+}]_i$ peak was observed. In addition, a slight increase in the subsequent sustained elevation in $[Ca^{2+}]_i$ was also observed with $[Ca^{2+}]_i$ typically returning to prestimulation levels within 2–5 min of endothelin-1 challenge (Figure 3A). The dose-dependence of the endothelin-1-induced peak $[Ca^{2+}]_i$ response is clearly illustrated in Figure 3B. The EC_{50} for the endothelin-1-mediated peak increase in $[Ca^{2+}]_i$ was estimated to be 16 nM.

Involvement of ET receptor subtypes Endothelin-1 (50 nM) caused a substantial increase in $[Ca^{2+}]_i$ which subsided to near baseline levels within 3–5 min (Figure 4A). Inclusion of BQ-788 (1 μM), did not significantly alter the response of cells to endothelin-1 (Figure 4C). In contrast, the ET_B-receptor-selective agonist sarafotoxin S6c (10 nM) induced a gradual change in $[Ca^{2+}]_i$ of much smaller magnitude than that linked to ET_A activation (Figure 4B). Occlusion of ET_A receptors with BQ-123 (3 μM) substantially attenuated the endothelin-1-induced $[Ca^{2+}]_i$ peak increase and resulted in a more gradual and diminished $[Ca^{2+}]_i$ response (Figure 4D). Challenge of rat airway smooth muscle cells with BK (10 μM) induced a substantial increase in $[Ca^{2+}]_i$ in all cells examined. In excess of 80% of cells responded to non-specific ET_A and ET_B

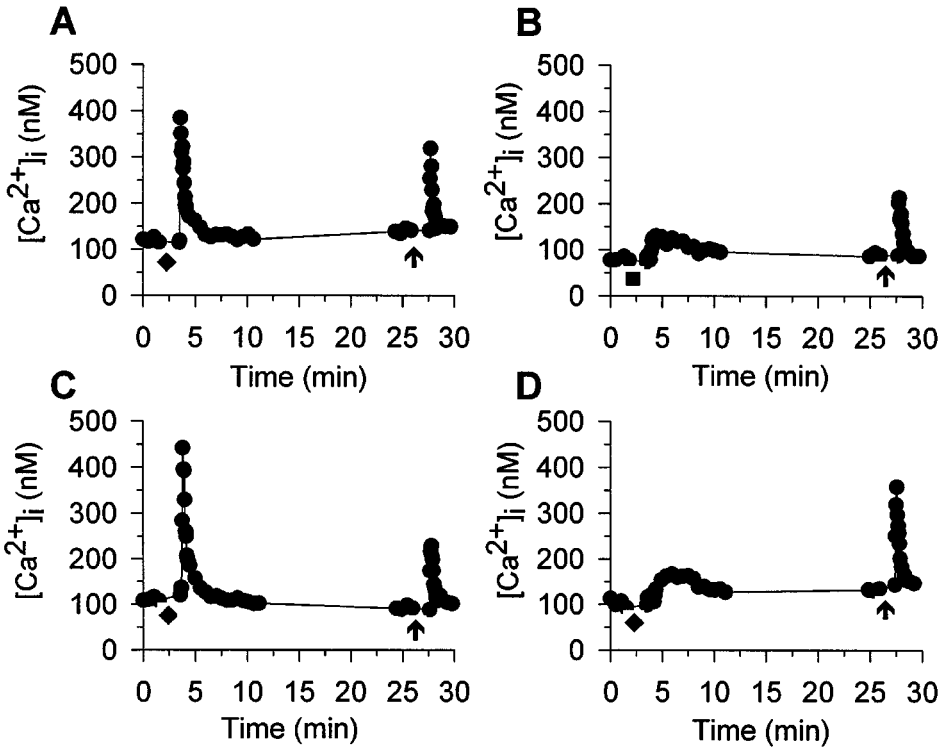

Figure 4 Representative curves of the effects of ET receptor activation by endothelin-1 (◆) or sarafotoxin S6c (■) and of subsequent challenge by BK (↑) on $[Ca^{2+}]_i$ in single rat airway smooth muscle cells in culture. (A) endothelin-1 and BK. (B) sarafotoxin S6c and BK. (C) endothelin-1 + BQ-788 and BK. (D) endothelin-1 + BQ-123 and BK. The experiment was conducted using 45–48 cells in each group and is representative of two separate studies.

receptor challenge with endothelin-1 and selective ET_A receptor activation by endothelin-1 in the presence of BQ-788 revealed a similar proportion of responder cells. In contrast, sarafotoxin S6c elicited a change in $[Ca^{2+}]_i$ in only approximately 30% of cells examined, as was the case for selective ET_B stimulation by endothelin-1 in the presence of BQ-123.

Linkage of ET receptor subtypes to extracellular sources of calcium The mode of endothelin-1-induced calcium entry in rat airway smooth muscle cells linked to ET_A and ET_B receptor subtypes was examined by specific blockade of calcium influx mechanisms and subsequent assessment of the effect on the 'peak increase' and 'sustained increase' components of the endothelin-1-induced $[Ca^{2+}]_i$ response. The linkage of intracellular calcium release in the initial transient $[Ca^{2+}]_i$ increase to ET_A receptors, was highlighted by the inability of calcium entry blockade, by either lanthanum (30 μM) or calcium-free medium, to affect the endothelin-1-induced peak increase in the presence of BQ-788 (Figure 5A). However, removal of calcium from the incubation medium substantially reduced the magnitude of the subsequent sustained $[Ca^{2+}]_i$ increase ($P<0.05$). Although complicated somewhat by the irregularity of the ET_B-mediated $[Ca^{2+}]_i$ response in rat airway smooth muscle cells, both peak (Figure 5C) and sustained (Figure 5D) increases were found to be abolished by blockade of calcium entry, both by calcium-free medium and lanthanum ($P<0.05$), indicating a dependence of this response upon the influx of extracellular calcium.

Discrimination of intracellular calcium sources Thapsigargin (1 μM), caffeine (25 mM) and ryanodine (10 μM) were without effect upon $[Ca^{2+}]_i$ (Figure 5E). Moreover, preincubation with either ryanodine or caffeine did not affect the magnitude of the ET_A receptor-mediated $[Ca^{2+}]_i$ transient. However, in combination ryanodine and caffeine significantly attenuated the magnitude of the subsequent ET_A-mediated peak $[Ca^{2+}]_i$ increase (approximately 30%) ($P<0.05$). In addition, depletion of IP_3 sensitive stores by thapsigargin abolished the peak $[Ca^{2+}]_i$ response (Figure 5E).

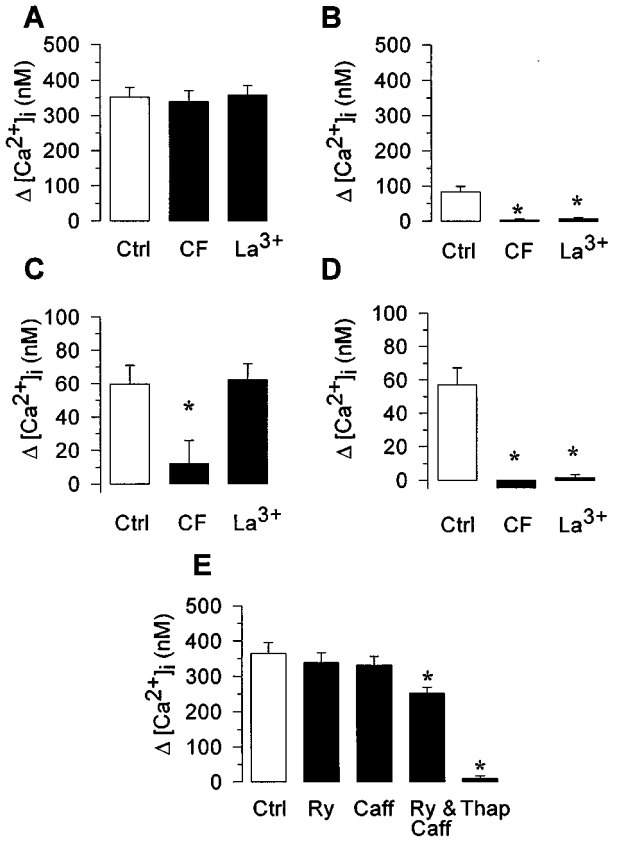

Figure 5 (A–D) Effect of calcium entry blockade (La^{3+}) and calcium-free solution (CF) on ET_A and ET_B receptor-mediated increases in peak and sustained levels of $[Ca^{2+}]_i$ in rat airway smooth muscle cells. ET_A receptor-mediated responses were induced by endothelin-1 in the presence of BQ-788, and ET_B receptor-mediated responses were induced by endothelin-1 in the presence of BQ-123. Open columns depict responses in calcium-containing medium, whereas filled columns depict responses in La^{3+} or CF. (A) ET_A receptor-mediated increases in peak levels of $[Ca^{2+}]_i$. (B) ET_B receptor-mediated increases in peak levels of $[Ca^{2+}]_i$. (C) ET_A receptor-mediated increases in sustained levels of $[Ca^{2+}]_i$. (D) ET_B receptor-mediated increases in sustained levels of $[Ca^{2+}]_i$. (E) Effect of ryanodine (Ry), caffiene (Caff), ryanodine and caffiene (Ry and Caff) and thapsigargin (Thap) on ET_A receptor-mediated increases in peak $[Ca^{2+}]_i$ in rat airway smooth muscle cells. Shown are responses to ET_A receptor stimulation (endothelin-1 in the presence of BQ-788) in calcium-free medium (Ctrl; open column) and following intracellular calcium store depletion (filled columns). Data are expressed as the means ± s.e.mean ($n=47-49$) and are representative of two separate studies (*$P<0.05$).

Human airway smooth muscle cells in culture

The growth characteristics of human airway smooth muscle cells cultures were examined on 8th passage cells. Cells were

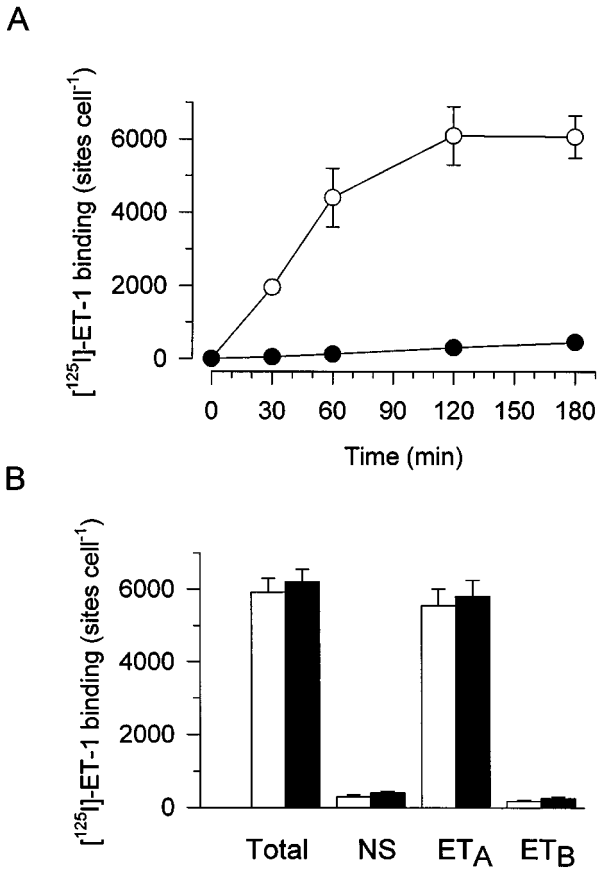

Figure 6 (A) Time dependence of total (○) and non-specific (●) [^{125}I]-endothelin-1 binding in confluent monolayer cultures of human airway smooth muscle cells (8th passage). (B) Number of total and non-specific (NS) [^{125}I]-endothelin-1 binding sites and of ET_A and ET_B receptors in human airway smooth muscle cells (8th passage) grown in 10% foetal calf serum (open columns) or for a 2-day period in serum-free media (filled columns). Data are expressed as means ± s.e.mean of triplicate determinations from three separate studies.

seeded at a density of approximately 8×10^4 cells well^{-1} and a small increase in cell number to 12×10^4 cells well^{-1} was observed by day 3, followed by a period of rapid growth with the culture reaching confluence between 7 and 9 days after seeding. Minimal growth occurred with a further 6 days in culture and cell numbers plateaued at an approximate density of 3.5×10^5 cells well^{-1}. The identity of 8th passage human airway smooth muscle cells seeded at subconfluent densities was confirmed as being airway smooth muscle by immunofluorescent staining with an antibody specific for α-smooth muscle actin. Immunoreactive α-actin was observed in long fibres traversing the length of the cells and was present in the vast majority of cells examined, although an occasional lone cell exhibited a weak, diffuse fluorescence that did not appear to be localized to any structure or organelle. Non-specific fluorescent staining or cellular autofluorescence was undetectable under the conditions employed to view specific fluorescence. The effect of serum deprivation for 2 days on cell number was investigated throughout the growth period of 8th passage human airway smooth muscle cells, from seeding to 6 days post-confluence. A 2 day incubation in serum-free medium inhibited cell growth at all time points examined, such that serum-free medium cell numbers were consistently lower than the corresponding cell density of those exposed to 10% serum (data not shown).

Radioligand binding studies in human airway smooth muscle cells

[^{125}I]-endothelin-1 binding to confluent human airway smooth muscle cells Specific [^{125}I]-endothelin-1 binding to confluent monolayer cultures of human airway smooth muscle cells (8th passage) increased rapidly in a time-dependent manner and plateaued after 120 min. The maximum density (B_{max}) of specific binding to human airway smooth muscle cells was 6100 ± 800 binding sites cell^{-1} (Figure 6A). Non-specific binding, determined in the presence of BQ-123 (1 μM) and sarafotoxin S6c (200 nM), increased in a linear fashion over time, but represented no more than 10% of specific binding at any given time point. The ET_B receptor-selective ligand sarafotoxin S6c (200 nM) was essentially without effect upon [^{125}I]-endothelin-1 binding, whereas the ET_A receptor-selective ligand BQ-123 (1 μM) completely abolished specific [^{125}I]-endothelin-1 binding (open columns, Figure 6B), indicating that the ET receptor population in these human airway smooth muscle cells in culture was almost exclusively of the ET_A subtype. Serum deprivation for 2 days did not affect the levels of total specific binding or non-specific binding, nor were ET_A- and ET_B-specific binding site densities affected. Thus, ET_A remained the predominant receptor population in human airway smooth muscle cells in culture under serum free conditions (filled columns; Figure 6B).

Endothelin-1-mediated $[Ca^{2+}]_i$ mobilization in human airway smooth muscle cells

Endothelin-1 elicited a dose-dependent increase in $[Ca^{2+}]_i$, with the most dramatic aspect of the concentration-dependence of the endothelin-1 response being the increase in magnitude of the transient $[Ca^{2+}]_i$ peak (Figure 7A,B). The EC_{50} value was estimated to be 15.5 nM.

Involvement of ET receptor subtypes Endothelin-1 (50 nM) caused a substantial increase in $[Ca^{2+}]_i$ which subsided to near baseline levels within 5–10 min (Figure 8A), whereas sarafotoxin S6c (10 nM) was without effect (Figure 8B).

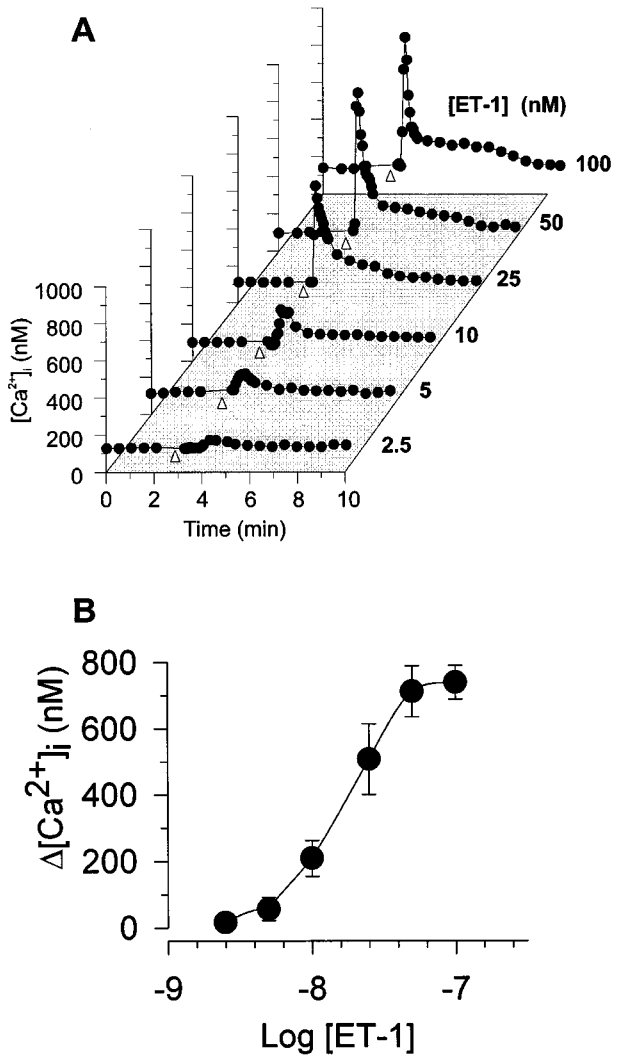

Figure 7 Concentration-dependence of endothelin-1-induced $[Ca^{2+}]_i$ increase in human airway smooth muscle cells in culture. (A) Representative curves illustrating the effects of increasing [endothelin-1] on $[Ca^{2+}]_i$. The open triangle indicates the moment at which endothelin-1 was added to the cells. (B) Concentration-response relationship between [endothelin-1] and the peak $[Ca^{2+}]_i$ increase. Data are the means ± s.e.mean from a single day's experiment (where $n = 42-46$ for each [endothelin-1]) and is representative of two separate studies.

Furthermore, inclusion of BQ-788 (1 μM) did not alter the response of cells to endothelin-1 (Figure 8C), whereas BQ-123 (3 μM) abolished the endothelin-1-induced $[Ca^{2+}]_i$ increase (Figure 8D). In all instances, BK (10 μM) was able to induce a substantial increase in $[Ca^{2+}]_i$ indicating that failure to respond to ET receptor-selective activation was due either to specific receptor blockade or to the absence of a receptor subtype. The cells retained responsiveness to agonists for up to 30 min under the conditions employed. Although, 100% of cells responded to challenge by endothelin-1 and to selective ET_A receptor activation, ET_B receptor activation elicited no response. Thus, ET_A receptors exclusively mediated the effects of endothelin-1 on $[Ca^{2+}]_i$ in human airway smooth muscle cells in culture.

Extracellular sources of calcium The mode of endothelin-1-induced calcium entry in human airway smooth muscle cells was examined by blockade of calcium influx and subsequent assessment of the effect of the 'peak increase' and 'sustained increase' components of the endothelin-1-induced $[Ca^{2+}]_i$

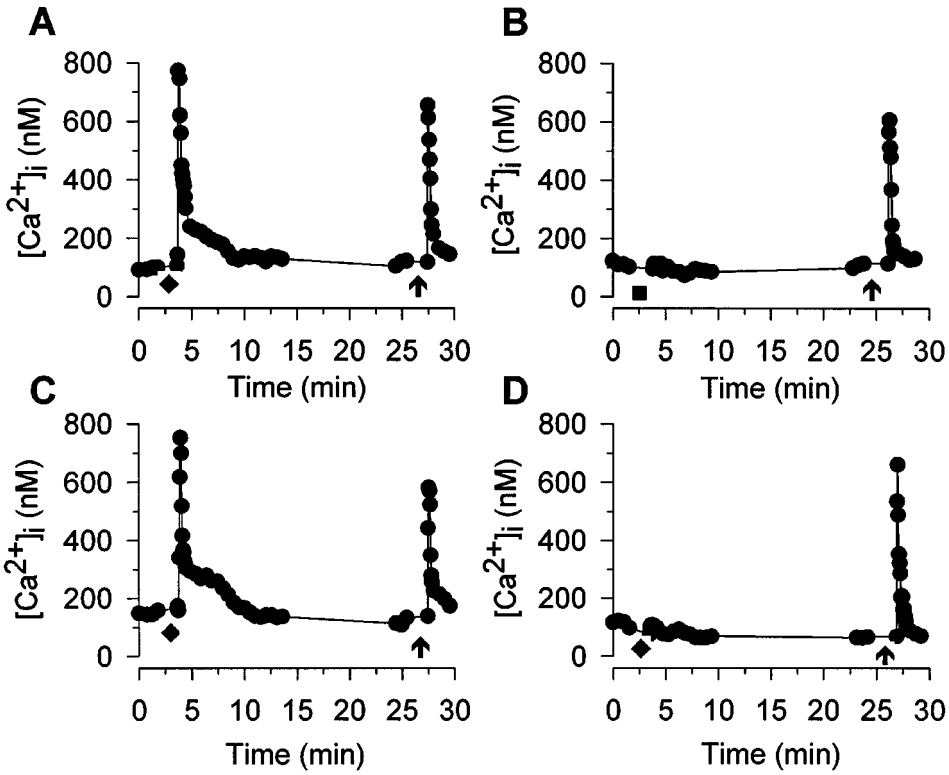

Figure 8 Representative curves of the effects of ET receptor activation by endothelin-1 (◆) or sarafotoxin S6c (■) and of subsequent challenge by BK (↑) on $[Ca^{2+}]_i$ in single human airway smooth muscle cells in culture. (A) endothelin-1 and BK. (B) sarafotoxin S6c and BK. (C) endothelin-1 + BQ-788 and BK. (D) endothelin-1 + BQ-123 and BK. The experiment was conducted using 47–50 cells in each group and is representative of two separate studies.

response. The exclusive contribution of intracellular calcium stores to the initial transient $[Ca^{2+}]_i$ increase was highlighted by the inability of calcium entry blockade to affect the endothelin-1-induced peak increase (Figure 9). Moreover, calcium-free medium was without effect upon the modest sustained component of the endothelin-1-mediated $[Ca^{2+}]_i$ response. Therefore, it is apparent that extracellular calcium influx makes little contribution to the endothelin-1-induced $[Ca^{2+}]_i$ response in these human airway smooth muscle cells in culture (Figure 9).

Discussion

This study has characterized the ET_A and ET_B receptor populations in human and rat cultured airway smooth muscle cells and examined ET receptor subtype coupling to Ca^{2+} signalling mechanisms. Rat airway smooth muscle cells contained a mixture of both ET_A and ET_B receptors and challenge with endothelin-1 elicited a concentration-dependent biphasic increase in $[Ca^{2+}]_i$, that comprised an initial transient peak $[Ca^{2+}]_i$ increase (typically 350 nM) followed by a smaller, sustained increase. Endothelin-1-induced increases in $[Ca^{2+}]_i$ were primarily due to ET_A receptor-mediated mobilization of IP_3-sensitive and to a lesser extent ryanodine-sensitive intracellular calcium stores. In contrast, a modest and inconsistent ET_B receptor-mediated response was exclusively coupled to extracellular calcium influx. Surprisingly, the human airway smooth muscle cells used in the current study contained a homogeneous population of ET_A receptors, quite different from the predominantly ET_B receptor population typically seen in intact human bronchus. Endothelin-1 elicited a concentration-dependent increase in $[Ca^{2+}]_i$ in single human airway smooth muscle cells in culture, which was exclusively linked to ET_A receptor-mediated mobilization of intracellular calcium stores.

Rat tracheal airway smooth muscle cells grown to confluence in serum-containing medium contained a clear predominance of ET_B receptors, which is in marked contrast with previous findings of approximately equal proportions of ET_A and ET_B receptors in intact rat tracheal smooth muscle (Henry, 1994). Nevertheless, the inclusion of a 2-day period of serum deprivation caused a reduction in the density of ET_B receptors from over 20,000 sites $cell^{-1}$ to around 5000 sites $cell^{-1}$, without any noticeable change in ET_A receptor density which remained constant at approximately 5000 sites $cell^{-1}$. Thus, maintenance of rat tracheal airway smooth muscle cells in a serum-free medium, which may well mimic the *in vivo* environment more closely than serum-containing medium, resulted in cultured cells which contained equal proportions of ET_A and ET_B receptors and which were similar to the proportions previously observed in intact rat tracheal smooth muscle (Henry, 1994). The selective alteration of ET_B receptor numbers during cell culture in serum-containing medium is consistent with recent findings in lamb airway smooth muscle cells (Maxwell *et al*., 1998) and with functional reports of enhanced ET_B-mediated responses when subjected to short term cell culture conditions (Woodcock & Land, 1992; Adner *et al*., 1995). Although the ability of serum deprivation to modify receptor expression is not without some precedent, as it has been shown to selectively alter receptor subunit mRNA levels and the density and proportion of NMDA receptor subtypes (Zhong *et al*., 1994), this has not been directly demonstrated before in relation to ET receptors, either in non-smooth muscle or smooth muscle tissues. Nevertheless, phenotypic changes in vascular smooth muscle in culture have

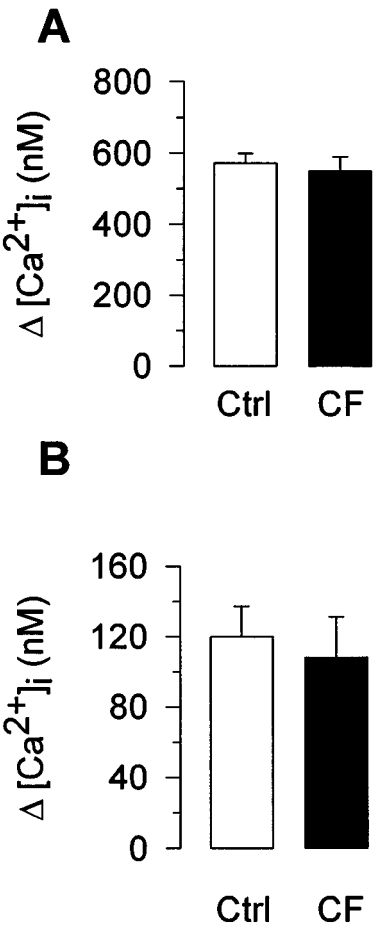

Figure 9 Effect of calcium entry blockade on endothelin-1-induced (A) peak and (B) sustained $[Ca^{2+}]_i$ increases in human airway smooth muscle cells. Shown are responses induced by endothelin-1 in calcium containing medium (Ctrl; open columns) and endothelin-1 in calcium-free (CF) medium (filled columns). Data are expressed as the means ± s.e.mean ($n = 47-49$) and are representative of two separate studies.

been associated with changes in the ET receptor subtypes (Eguchi *et al*., 1994). It has been suggested that switching the ET receptor subtype from ET_A to ET_B during phenotypic change may in part contribute to the development of vascular lesions, such as atherosclerosis (Eguchi *et al*., 1994). An interesting, but as yet untested, possibility is that the use of late passage airway smooth muscle cells in culture might mimic a phenotype seen in remodelled airways following exposure to injurious stimuli or chronic inflammation.

Human airway smooth muscle cells cultured in serum-containing medium possessed an homogeneous population of ET_A receptors, which contrasts sharply with the predominance of ET_B receptors reported previously in human cultured tracheal airway smooth muscle cells (35% ET_A: 65% ET_B; Panettieri *et al*., 1996), in intact human bronchial airway smooth muscle (12% ET_A: 88% ET_B; Goldie *et al*., 1995) and in human bronchial membrane preparations (40% ET_A: 60% ET_B; Fukuroda *et al*., 1996; Hay *et al*., 1998). Moreover, and quite distinct from rat airway smooth muscle cells, incubation of these human airway smooth muscle cells in serum-free medium did not have any effect on the density of ET receptors and did not change the ET receptor subtype proportions towards that previously reported in intact human bronchial tissue. The reasons for the predominance of ET_A receptors in these cultured airway smooth muscle cells is unknown. Perhaps the single donor from which the airway smooth muscle cells culture was derived had bronchial smooth muscle cells which contained a higher proportion of ET_A receptors than has previously been observed in other subjects. It seems more likely that the extensive culturing required to generate sufficient quantities of human bronchial airway smooth muscle cells to complete these studies has led to a change in the expression of ET_A and ET_B receptors. Interestingly, recent studies have reported that ET_A receptors, rather than ET_B receptors mediated the co-mitogenic effects of endothelin in human airway smooth muscle cells (Panettieri *et al*., 1996). In the current studies, specific [^{125}I]-endothelin-1 binding to the human bronchial smooth muscle cells was prevented by traditional ET_A and ET_B receptor ligands such as BQ-123 and sarafotoxin S6c. Thus, the current study provided no evidence for the presence of novel ET receptor subtypes, which have recently been reported by Hay and coworkers (1998) in human bronchial membrane preparations.

Rat airway smooth muscle cells responded to endothelin-1 challenge with a biphasic elevation in $[Ca^{2+}]_i$, in what has become a characteristic of agonist-mediated calcium signalling in most cell types, including smooth muscle (reviews: Hall & Kotlikoff, 1995; Pollock *et al*., 1995). The endothelin-1-induced biphasic rise in $[Ca^{2+}]_i$ was characterized by a rapid and substantial transient increase in $[Ca^{2+}]_i$ (approximately 400 nM), which was followed by a modest sustained phase of elevated Ca^{2+}. The potency of endothelin-1 in increasing peak $[Ca^{2+}]_i$ in rat airway smooth muscle cells corresponded closely to the potency of endothelin-1 in contracting rat isolated tracheal segments (Henry, 1993, 1994). Endothelin-1-induced increases in $[Ca^{2+}]_i$ were inhibited by BQ-123 but not BQ-788, indicating an ET_A receptor-mediated response. Furthermore, selective ET_A receptor stimulation (induced by endothelin-1 in the presence of BQ-788) elicited a large response in the majority of cells examined. In contrast, selective ET_B receptor stimulation (elicited by sarafotoxin S6c or by endothelin-1 in the presence of BQ-123) caused a small increase in $[Ca^{2+}]_i$ in only about 30% of cells. There appeared to be three distinct sub-populations of rat airway smooth muscle cells in this culture, defined as possessing either (a) ET_A, (b) ET_B or (c) both ET_A and ET_B receptors, based upon the ability of single cells to respond to ET_A stimulation following prior exposure to the ET_B agonist sarafotoxin S6c. Whether the presence of these three distinct sub-populations is indicative of the existence of a single type of cell which contains different ET receptor subtype distribution or of the existence of a heterogeneous population of cells in this culture is not known. Nevertheless, the heterogeneous expression of ET receptors within an airway smooth muscle cell culture has been described previously (Inui *et al*., 1994). The extent to which the two receptors may interact with respect to $[Ca^{2+}]_i$ signalling is not apparent from these data.

In rat airway smooth muscle cells, both the transient and sustained increases in $[Ca^{2+}]_i$ induced by ET_A receptor activation were found to rely exclusively upon mobilization of intracellular calcium stores. Lanthanum was without effect on either of these parameters and the transient ET_A receptor-mediated increase in $[Ca^{2+}]_i$ was abolished by thapsigargin, which causes irreversible emptying of sarcoplasmic reticulum calcium stores (Thastrup *et al*., 1989, 1990; Witcome *et al*., 1991). However, selective stimulation of the sarcoplasmic reticulum-resident ryanodine calcium channel, by ryanodine or caffeine and subsequent depletion of calcium from the calcium-sensitive compartment, was without effect in rat airway smooth muscle cells. In light of the fact that ryanodine activation of the channel has been suggested to be use-

dependent, due to a strong requirement to act on the activated state of the channel (Vites & Pappano, 1994), and that caffeine has been demonstrated to substantially enhance binding of ryanodine to sarcoplasmic reticulum fragments (Zhang *et al*., 1993), the effect of caffeine and ryanodine in combination was examined. In rat airway smooth muscle cells, the modest attenuation of the transient $[Ca^{2+}]_i$ response in the presence of ryanodine and caffeine was consistent with the observation that ET_A receptors appear to be linked to intracellular calcium mobilization, through both IP_3-dependent and -independent mechanisms in rat trachea (Henry, 1993, 1994). Consistent with this, preincubation of rat trachea with ryanodine prior to endothelin-1 challenge has been shown to affect contraction (Henry, 1994).

ET_B receptor-mediated increases in transient and sustained $[Ca^{2+}]_i$ in rat airway smooth muscle cells were abolished by calcium-free medium or lanthanum. Thus, the ET_B-mediated response was principally dependent upon the influx of calcium, although further characterization of the mode of entry of calcium was complicated by the fickle nature of the response and it was not possible to ascertain the mechanisms by which ET_B receptors initiate calcium influx in rat airway smooth muscle cells. Nevertheless, these data are in agreement with studies performed on intact rat trachea which established that ET_B receptor-mediated contractions in this tissue involved the influx of extracellular calcium through non-L-type calcium channels, whereas ET_A-mediated contraction was primarily dependent upon intracellular calcium mobilization (Henry, 1993, 1994).

In human airway smooth muscle cells, endothelin-1 challenge induced a concentration-dependent biphasic increase in $[Ca^{2+}]_i$ that was strikingly similar to that observed in rat airway smooth muscle cells and the EC_{50} value for the observed dose-dependent peak $[Ca^{2+}]_i$ increase was well within the range reported for endothelin-1-induced contractions of human isolated bronchus (Review: Goldie *et al*., 1996). Endothelin-1-mediated increases in $[Ca^{2+}]_i$ were abolished by BQ-123, indicative of ET_A receptor activation. Consistent with this, selective ET_B receptor stimulation by sarafotoxin S6c failed to induce an increase in $[Ca^{2+}]_i$ and ET_B receptor blockade by BQ-788 was without effect on endothelin-1-induced increases in $[Ca^{2+}]_i$. Although at odds with data indicating a primary role for ET_B receptor activation in endothelin-1-mediated contraction of intact bronchus, these results reflect the exclusive ET_A receptor population observed in these human cultured airway smooth muscle cells. Moreover, not only do these data indicate that the cells had lost all ET_B receptors, but the 100% responder rates to ET_A-selective challenge demonstrate that every human airway smooth muscle cells examined had acquired ET_A receptors in culture. Both the transient and modest sustained increase in $[Ca^{2+}]_i$ elicited by endothelin-1 challenge in human airway smooth muscle cells remained unaffected in calcium-free medium, indicating that both were entirely due to the release of intracellular calcium stores. In contrast, a substantial sustained $[Ca^{2+}]_i$ component of the endothelin-1 response that was entirely dependent upon extracellular calcium influx was observed in the study by Mattoli and coworkers (1991). However, it is notable that this study employed HEPES as a buffer, which has been demonstrated to result in an artificial elevation of $[Ca^{2+}]_i$ following agonist challenge, particularly in the sustained phase (Ganz *et al*., 1990). Certainly, endothelin-1-induced contraction of human isolated bronchi has been shown to be unaffected by calcium-free conditions (McKay *et al*., 1991) and sarafotoxin S6c activated ET_B receptors do not appear to be coupled to calcium influx in human bronchus (Hay *et al*., 1994). Therefore, calcium influx is by no means critical to endothelin-1-induced contraction and data from the present study and intact tissue support the notion that ET receptors are coupled primarily to intracellular calcium mobilization.

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References

ADNER, M., ERLINGE, D., NILSSON, L & EDVINSSON, L. (1995). Upregulation of a non-ET_A receptor in human arteries *in vitro*. *J. Cardiovasc. Pharmacol.,* **26** (suppl 3), S314–S316.

CARR, M.J., GOLDIE, R.G. & HENRY, P.J. (1996). Time course of changes in ET_B receptor density and function in tracheal airway smooth muscle during respiratory tract viral infection in mice. *Br. J. Pharmacol.,* **117,** 1222–1228.

EGUCHI, S., HIRATA, Y., IMAI, T., KANNO, K. & MARUMO, F. (1994). Phenotypic change of endothelin receptor subtype in cultured rat vascular smooth muscle cells. *Endocrinology,* **134,** 222–228.

FUKURODA, T., OZAKI, S., IHARA, M., ISHIKAWA, K., YANO, M., MIYAUCHI, T., ISHIKAWA, S., ONIZUKA, M., GOTO, K. & NISHIKIBE, M. (1996). Necessity of dual blockade of endothelin ET(A) and ET(B) receptor subtypes for antagonism of endothelin-1-induced contraction of human bronchi. *Br. J. Pharmacol.,* **117,** 995–999.

GANZ, M.B., RASMUSSEN, J., BOLLAG, W.B. & RASMUSSEN, H. (1990). Effect of buffer systems and pH_i on the measurement of $[Ca^{2+}]_i$ with fura-2. *FASEB J.,* **4,** 1638–1644.

GOLDIE, R.G., GRAYSON, P.S., KNOTT, P.G., SELF, G.J. & HENRY, P.J. (1994). Predominance of endothelinA (ET_A) receptors in ovine airway smooth muscle and their mediation of ET-1-induced contraction. *Br. J. Pharmacol.,* **112,** 749–756.

GOLDIE, R.G., HENRY, P.J., KNOTT, P.G., SELF, G.J., LUTTMANN, M.A. & HAY, D.W.P. (1995). Endothelin-1 receptor density, distribution and function in human isolated asthmatic airways. *Am. J. Respir. Crit. Care Med.,* **152,** 1653–1658.

GOLDIE, R.G., KNOTT, P.G., CARR, M.J., HAY, D.W.P. & HENRY, P.J. (1996). The endothelins in the pulmonary system. *Pulm. Pharmacol.,* **9,** 69–93.

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

HALL, I.P. & KOTLIKOFF, M. (1995). Use of cultured airway myocytes for study of airway smooth muscle. *Am. J. Physiol.,* **268,** L1–L11.

HAY, D.W.P., LUTTMANN, M.A. & GOLDIE, R.G. (1994). Calcium (Ca^{2+}) translocation mechanisms mediating endothelin-1 (ET-1)- and sarafotoxin S6c (S6c)-induced contractions in isolated human bronchus. *Am. J. Resp. Crit. Care Med.,* **149,** A1083.

HAY, D.W.P., LUTTMANN, M.A., PULLEN, M.A. & NAMBI, P. (1998). Functional and binding characterization of endothelin receptors in human bronchus: Evidence for a novel endothelin B receptor subtype? *J. Pharmacol. Exp. Ther.,* **284,** 669–677.

HENRY, P.J. (1993). Endothelin-1 (ET-1)-induced contraction in rat isolated trachea: involvement of ET_A and ET_B receptors and multiple signal transduction systems. *Br. J. Pharmacol.,* **110,** 435–441.

HENRY, P.J. (1994). Inhibitory effects of nordihydroguaiaretic acid on ET_A-receptor-mediated contractions to endothelin-1 in rat trachea. *Br. J. Pharmacol.,* **111,** 561–569.

IHARA, M., NOGUCHI, K., SAEKI, T., FUKURODA, T., TSCHIDA, S., KIMURA, S. & YANO, M. (1992). Biological profiles of highly potent novel endothelin antagonists selective for the ET_A receptor. *Life Sci.,* **50,** 247–255.

INUI, T., JAMES, A.F., FUJITANA, Y., TAKIMOTO, M., OKADA, T., YAMAMURA, T. & URADE, Y. (1994). ET_A and ET_B receptors on single smooth muscle cells cooperate in mediating guinea pig tracheal contraction. *Am. J. Physiol.,* **266,** L113–L124.

ISHIKAWA, K., IHARA, M., NOGUCHI, K., MASE, T., MINO, M., SAEKI, T., FUKURODA, T., FUKAMI, T., OZAKI, S., NAGASE, T., NISHIKIBE, M. & YANO, M. (1994). Biochemical and pharmacological profile of a potent and selective ET_B receptor antagonist, BQ-788. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 4892–4896.

KNOTT, P.G., D'APRILE, A.C., HENRY, P.J., HAY, D.W.P. & GOLDIE, R.G. (1995). Receptors for endothelin-1 in asthmatic human peripheral lung. *Br. J. Pharmacol.,* **114,** 1–3.

MATTOLI, S., SOLOPERTO, M., MEZZETTI, M. & FASOLI, A. (1991). Mechanisms of calcium mobilization and phosphoinositide hydrolysis in human bronchial smooth muscle cells by endothelin 1. *Am. J. Respir. Cell Mol. Biol.,* **5,** 424–430.

MAXWELL, M.J., GOLDIE, R.G. & HENRY, P.J. (1998). Altered ET_B- but not ET_A-receptor density and function in sheep airway smooth muscle cells in culture. *Am. J. Physiol.,* **274,** L951–L957.

MCKAY, K.O., BLACK, J.L. & ARMOUR, C.L. (1991). The mechanism of action of endothelin in human lung. *Br. J. Pharmacol.,* **102,** 422–428.

PANETTIERI, R.A. JR., GOLDIE, R.G., RIGBY, P.J., ESZTERHAS, A.J. & HAY, D.W. (1996). Endothelin-1-induced potentiation of human airway smooth muscle proliferation: an ET_A receptor-mediated phenomenon. *Br. J. Pharmacol.,* **118,** 191–197.

POLLOCK, D.M., KEITH, T.L. & HIGHSMITH, R.F. (1995). Endothelin receptors and calcium signalling. *FASEB J.,* **9,** 1196–1204.

SKALLI, O., ROPRAZ, P., TRZECIAK, A., BENZONANA, G., GILLESSEN, D. & GABBIANI, G. (1986). A monoclonal antibody against α-smooth muscle actin: a new probe for smooth muscle differentiation. *J. Cell Biol.,* **103,** 2787–2796.

THASTRUP, O., DAWSON, A.P., SCHARFF, O., FODER, B., CULLEN, P.J., DROBAK, B.K., BJERRUM, P.J., CHRISTENSON, S.B. & HANLEY, M.R. (1989). Thapsigargin, a novel molecular probe for studying intracellular calcium release and storage. *Agents Actions,* **27,** 17–23.

THASTRUP, O., CULLEN, P.J., DROBAK, B.K., HANLEY, M.R. & DAWSON, A.P. (1990). Thapsigargin, a tumor promotor, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc. Natl. Acad. Sci. U.S.A.,* **87,** 2466–2470.

VITES, A.-M. & PAPPANO, A.J. (1994). Distinct modes of inhibition by ruthenium red and ryanodine of calcium-induced calcium release in avian atrium. *J. Pharmacol. Exp. Ther.,* **268,** 1476–1484.

WAGGONER, W.G., GENOVA, S.L. & RASH, V.A. (1992). Kinetic analyses demonstrate that the equilibrium assumption does not apply to [^{125}I]-endothelin-1 binding data. *Life Sci.,* **51,** 1869–1876.

WILLIAMS, D.L., Jr, JONES, K.L., PETTIBONE, D.J., LIS, E.V. & CLINESSCHIDT, B.V. (1991). Sarafotoxin S6c: an agonist which distinguishes between endothelin receptor subtypes. *Biochem. Biophys. Res. Commun.,* **175,** 556–561.

WITCOME, M., MICHELANGELI, F., LEE, A.G. & EAST, M. (1991). The inhibitor thapsigargin and 2,5-di(tert-butyl)-1,4-benzohydroquinone favour the E2 form of the Ca^{2+}, Mg^{2+}-ATPase. *FEBS Lett.,* **304,** 109–113.

WOODCOCK, E.A. & LAND, S. (1992). Interaction between vasopressin and endothelin in renal papillary tubules: uncoupling following cell isolation and culture. *Clin. Exp. Pharmacol. Physiol.,* **19,** 384–387.

ZHANG, Z.-D., KWAN, C.-Y. & DANIEL, E.E. (1993). Subcellular-membrane characterisation of [^{3}H]ryanodine-binding sites in smooth muscle. *Biochem. J.,* **290,** 259–266.

ZHONG, J., RUSSELL, S.L., PRITCHETT, D.B., MOLINOFF, P.B. & WILLIAMS, K. (1994). Expression of mRNAs encoding subunits of the N-methyl-D-aspartate receptor in cultured cortical neurons. *Mol. Pharmacol.,* **45,** 846–853.

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