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Ca^{2+} increase and Ca^{2+}-influx in human tracheal smooth muscle cells: role of Ca^{2+} pools controlled by sarco-endoplasmic reticulum Ca^{2+}-ATPase 2 isoform

Yassine Amrani, *Clarice Magnier, *Jocelyne Enouf, †Frank Wuytack & [1]Christian Bronner

INSERM U 425, Université Louis Pasteur Strasbourg I, 74, route du Rhin, 67401 Illkirch; *INSERM U348, Hôpital Lariboisière, 8 rue Guy Patin, 75010 Paris, France and †Laboratorium voor Fysiologie, Katholieke Universiteit, Campus Gasthuisberg, 3000 Leuven, Belgium

1 The contribution of sarco-endoplasmic reticulum Ca^{2+}-ATPases (SERCA)-regulated Ca^{2+} stores to the increase in intracellular free calcium ($[Ca^{2+}]_i$) induced by bradykinin (BK) was investigated in fura-2 loaded human tracheal smooth muscle cells (TSMC). For this purpose, we used thapsigargin, a selective inhibitor of Ca^{2+}-ATPases of intracellular organelles.

2 Thapsigargin (10^{-9} to 10^{-6} M) induced a dose-dependent increase in $[Ca^{2+}]_i$ in the presence of external Ca^{2+} with an EC_{50} value of 7.33 ± 1.26 nM. In Ca^{2+}-free conditions, the addition of Ca^{2+} (1.25 mM) caused an increase in $[Ca^{2+}]_i$ which was directly proportional to the pre-incubation time of the cells with thapsigargin. Net increases of 60 ± 9, 150 ± 22 and 210 ± 27 nM were obtained after 1, 3 and 5 min, respectively.

3 In the presence of extracellular Ca^{2+}, BK induced a typical biphasic increase in $[Ca^{2+}]_i$ with a fast transient phase and a sustained phase. The sustained component was reversed by addition of a bradykinin B_2-receptor antagonist (Hoe 140, 10^{-6} M) to the buffer as well as by deprivation of Ca^{2+}. The transient phase induced by BK, histamine and carbachol was inhibited in a time-dependent way by pre-incubation of the cells with thapsigargin.

4 Comparative western blotting of human TSMC membranes using anti-$SERCA_2$ isoform-specific antibodies clearly showed the greater expression of the 100-kDa $SERCA_{2\text{-}b}$ isoform compared with the $SERCA_{2\text{-}a}$ isoform.

5 Our data show that thapsigargin-sensitive Ca^{2+} stores contribute significantly to the activation of human TSMC which suggests a role for these stores in the subsequent induction of Ca^{2+} influx. These stores appear to be controlled by the Ca^{2+}-ATPases ($SERCA_{2\text{-}b}$ isoform) which could also participate in the regulation of Ca^{2+} influx through the plasma membrane.

Keywords: Tracheal smooth muscle cells; calcium; thapsigargin; bradykinin; carbachol; calcium ATPase

Introduction

Studies on tracheal smooth-muscle cells (TSMC) in culture from a variety of species have shown that bradykinin, carbachol and histamine induce a biphasic (transient and sustained) increase in cytosolic free calcium ($[Ca^{2+}]_i$) (Murray & Kotlikoff, 1991; Marsh & Hill, 1993; Yang *et al.*, 1993a,b; 1994b; Amrani *et al.*, 1994). This increase in $[Ca^{2+}]_i$ may play a fundamental role in smooth muscle contraction observed in functional studies *in vitro* (Trifilieff *et al.*, 1993; Farmer *et al.*, 1994). The aim of the present study was to define the regulation of Ca^{2+}, paying particular attention to the role of intracellular Ca^{2+} stores for the reasons described below.

The rapid and transient Ca^{2+} peak is generally accepted to be due to the mobilization of Ca^{2+} by inositol 1,4,5-trisphosphate-mediated Ca^{2+} from intracellular Ca^{2+} stores located mainly in the sarcoplasmic reticulum (Tsunoda, 1993; Fasolato *et al.*, 1994). This is supported by several lines of evidence including the maintenance of the transient phase in the absence of extracellular Ca^{2+} (Murray & Kotlikoff, 1991; Marsh & Hill, 1993; Amrani *et al.*, 1994), its insensitivity to Ni^{2+} (Amrani *et al.*, 1994) and the generation of inositol trisphosphate upon activation of muscarinic and bradykinin receptors (Marsh & Hill, 1992; Pyne & Pyne, 1993; Yang *et al.*, 1994a). The secondary, sustained phase is caused by Ca^{2+} influx from the extracellular medium since the absence of external calcium or the use of polyvalent cations such as Mn^{2+}, Ni^{2+} abolished the sustained phase (Murray & Kotlikoff, 1991; Marsh & Hill, 1993; Yang *et al.*, 1993a; Amrani *et al.*, 1994).

The cellular mechanisms linking both phases are not yet known. An interesting hypothesis is based on the capacitative entry model described by Putney (1993). In this model, Ca^{2+} influx can be activated by a process shunting second messengers such as inositol phosphate metabolites, whereby the filling state of some Ca^{2+}-stores alone is sufficient to induce Ca^{2+} entry. In this way, evidence supporting the capacitative model comes mainly from studies using specific sarcoplasmic reticulum Ca^{2+}-ATPase inhibitors, such as thapsigargin which empties the intracellular Ca^{2+}-stores without generating any known second messenger (Takemura *et al.*, 1989; Thastrup, 1990; Thastrup *et al.*, 1989; 1990; Fasolato *et al.*, 1994). Thus, the subsequent increase in $[Ca^{2+}]_i$ induced by thapsigargin and observed in a broad spectrum of cells (Thastrup, 1990; Thastrup *et al.*, 1990) is generally followed by an activation of Ca^{2+} influx, as shown in mast cells (Dar & Pecht, 1992), human platelets (Malcolm & Fitzpatrick, 1992), lacrimal acinar cells (Kwan *et al.*, 1990) and neuronal cell lines (Takemura *et al.*, 1991). The finding that thapsigargin also activates Ca^{2+} influx following depletion of intracellular Ca^{2+} stores in vascular smooth muscle cells (Xuan *et al.*, 1992) led us to suggest the existence, in airway smooth muscle cells, of a similar signalling mechanism linking the filling state of intracellular Ca^{2+} stores and Ca^{2+} entry.

Here, we show the presence of thapsigargin-sensitive Ca^{2+} stores in human TSMC that are involved in the response of

[1] Author for correspondence at: INSERM U425, Neuroimmunopharmacologie Pulmonaire, Faculte de Pharmacie, 74 route du Rhin, B.P. 24, 67401 Illkirch Cedex, France.

three different bronchoconstrictor agents. By use of antibodies directed against $SERCA_2$ isoforms, we suggest that the sarco-endoplasmic reticulum Ca^{2+} $ATPase_{2-b}$ is the major isoform that regulates these stores. Depletion of Ca^{2+} from these stores seems to be a key signal for the induction of a Ca^{2+} entry into the cells.

Methods

Cell culture

Primary cultures of human TSMC were prepared as described previously (Twort & Van Breemen, 1989; Kullman *et al.*, 1993). TSMC were cultured in DMEM/F12 medium supplemented with 10% foetal calf serum, 2 mM glutamine, 1% non-essential amino-acids, insulin (5 μg ml^{-1}), penicillin (100 u ml^{-1}) and streptomycin (100 μg ml^{-1}). All products were obtained from Gibco BRL (Cergy Pontoise, France).

Immunofluorescent staining

Cells were observed by light microscopy and identified by their typical Hill-and-Valley morphology at confluence. The identity of the human cultured cells as being smooth muscle cells was confirmed by immunolabelling with anti-smooth muscle α-actin antibody as described previously for guinea-pig TSMC in culture (Amrani *et al.*, 1994). Briefly, cells from the third passage of subculture were grown on glass coverslips. TSMC were washed with HEPES [2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethansulphonic acid] buffer, fixed with formaldehyde solution, then washed in HEPES buffer containing (mM): NaCl 137.5, $CaCl_2$ 1.25, $MgCl_2$ 1.25, NaH_2PO_4 0.4, KCl 6, glucose 5.6, HEPES 10 and 0.1% bovine serum albumin (w/v). The cells were permeabilized in cold methanol (–20°C), washed and incubated with mouse anti-smooth muscle α-actin IgG (Sigma, St Louis, MO, U.S.A.) for 90 min at room temperature. The cells were then washed and incubated with a biotinylated goat anti-mouse IgG antibody (Amersham, Les Ulis, France) for 60 min. This step was followed by three washings in HEPES buffer and by 60 min incubation with FITC-conjugated extravidin (Sigma, St Louis, MO, U.S.A.). After washing, glass coverslips were mounted onto glass slides with glycerin, examined by epifluorescence microscopy (Nikon, Tokyo) and photographed.

Measurement of cytosolic Ca^{2+}

Ca^{2+} measurements were performed on cell suspensions as described for guinea-pig TSMC (Mikki *et al.*, 1992; Amrani & Bronner, 1993; Amrani *et al.*, 1994). Briefly, human TSMC cultured in 75 cm^2 flasks were incubated with 3 μM fura-2/AM (3 mM stock solution in dimethylsulphoxide) at 37°C for 45 min in HEPES buffer of the composition described above. The cells were then washed with this buffer and incubation was prolonged for 15 min in the absence of fura-2 to allow completion of the intracellular hydrolysis of the probe. The cells were detached with trypsin (2 min at 37°C) and washed with HEPES buffer. Fura-2-loaded cells were resuspended at 10^6 cells ml^{-1} and placed in 1 cm quartz cuvettes. Cells in cuvettes were pre-incubated for 2 min at 37°C with gentle stirring in a thermostated cuvette holder before measuring fluorescence intensities with a F-2000 Hitachi spectrofluorimeter. Each sample of cells was washed just before placing in the fluorimeter in order to remove any extruded fura-2 during processing of the preceding sample. Drugs were added in a volume of 20 μl to 1 ml cell suspension in order to minimize dye dilution (2%). $[Ca^{2+}]_i$ was calculated from the fluorescence intensities measured at 510 nm after excitation at 340 and 380 nm as described by Grynkiewicz *et al.* (1985). Maximum and minimum fluorescence intensities were obtained wih 0.1% Triton X-100 and 10 mM EGTA in 2 M Tris-HCl, pH 8.5, respectively.

Membrane preparation

Human TSMC membrane preparation was performed as described for aortic smooth muscle cells by Magnier *et al.* (1992). Smooth muscle cells in monolayers were rinsed with 17 mM HEPES pH 7, 160 mM KCl; 0.05 mM EGTA and lysed in an ice-cold buffer containing: 10 mM HEPES pH 7, 10 mM KCl; 0.05 mM EGTA; 0.05 mM DTT (dithiothreitol) supplemented with a mixture of protease inhibitors (0.1 mg ml^{-1} trypsin inhibitor, 0.05 mg ml^{-1} aprotinin, 0.01 mg ml^{-1} leupeptin and 25 μM PMSF). The homogenate was sonicated and centrifuged for 10 min at 3500 *g* at 4°C. The supernatant was ultracentrifuged for 60 min at 4°C and 100,000 *g* and the resulting pellet resuspended in 17 mM HEPES pH 7, 160 mM KCl and 0.1 mM DTT. The microsomes were frozen and stored at –80°C. As controls, $SERCA_{2-a}$ and $SERCA_{2-b}$ enriched membrane preparations were obtained from rat heart and human platelets, respectively, according to the same procedure.

SDS-PAGE and Western Blot

Membrane fractions were solubilized and reduced for 30 min at room temperature in 50 mM Tris pH 6.8, containing 2% (w/v) SDS, 0.01% (v/v) bromophenol blue, 25% (v/v) glycerol, 10 mM DTT. Samples were analysed on an 8% SDS-PAGE and blotted onto nitrocellulose membranes as described previously (Magnier *et al.*, 1992). The membranes were first blocked overnight at room temperature in Tris buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl) containing 5% non fat dry milk, 0.1% (v/v) Tween 20, then incubated for 1 h at room temperature with the rabbit polyclonal isoform-specific anti-$SERCA_{2-a}$ and anti-$SERCA_{2-b}$ (1/100 dilution) in the blocking buffer. After three washes, the membranes were incubated for 1 h at room temperature with a donkey peroxidase-conjugated anti-rabbit IgG (1 : 50000 dilution) (Jackson, West Grove, PA, U.S.A.). The control of the immunostaining procedure was determined by using $SERCA_{2-a}$ and $_{2-b}$ isoform containing-membrane preparations from rat heart and human platelets respectively. For immunostaining detection, membranes were incubated for 1 min with the Enhanced Chemi-Luminescence (ECL) detection reagents (Amersham, Les Ulis, France) and then autoradiographed using Kodak X-OMAT AR films.

Drugs and chemicals

Bradykinin, thapsigargin, histamine, Triton X-100, leupeptin, aprotinin, PMSF, soybean trypsin inhibitor and fura-2/AM were purchased from Sigma Chemical Co (St Louis, MO, U.S.A.). The B_2 antagonist D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]BK (Hoe 140) was a kind gift from Hoechst (Frankfurt, Germany). Anti-$SERCA_{2-a}$ and -$SERCA_{2-b}$ isoform-specific antibodies were prepared as described previously (Wuytack *et al.*, 1989; Eggermont *et al.*, 1990).

Results

Confirmation of smooth muscle cell identity

Figure 1 shows immunofluorescent staining of α-smooth muscle actin which is arranged in fibres running parallel to the long axis of the cells. More than 95% of the cells bound anti-smooth muscle α actin antibodies.

Effect of thapsigargin on $[Ca^{2+}]_i$ in human TSMC

Exposure of human TSMC to thapsigargin, a Ca^{2+}-ATPases inhibitor, induced a rise in $[Ca^{2+}]_i$ in human TSMC. This rise was slow and uniform for 10^{-9} M and 10^{-8} M thapsigargin, reaching a plateau within 60–70 s ($n=5$). As the concentration of thapsigargin increased (10^{-7}–10^{-6} M), the rise became biphasic and was characterized with a fast and transient rise in

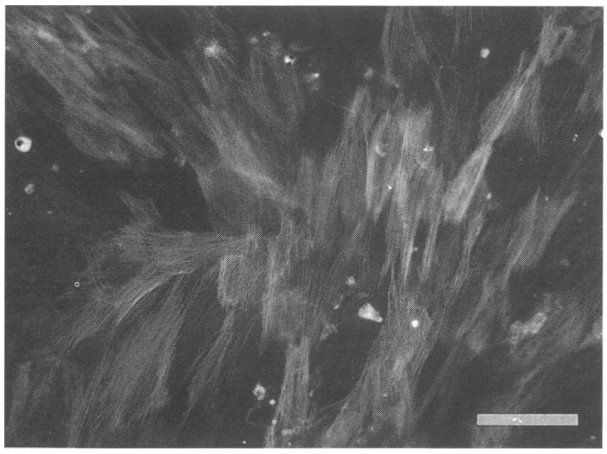

Figure 1 Epifluorescence photomicrographs of human tracheal smooth muscle cells in culture, labelled with mouse anti-smooth muscle α-actin. Calibration bar = 100 μm.

$[Ca^{2+}]_i$ which increased within 30–35 s, followed by a subsequent sustained plateau phase (Figure 2a, $n=4$). Considering the maximal net increase for each trace (at the transient or sustained phase), thapsigargin induced a concentration-dependent increase in $[Ca^{2+}]_i$ between 10^{-9} M and 10^{-6} M with an EC_{50} value of 7.33 ± 1.26 nM (Figure 2a and b, $n=4$).

Effect of thapsigargin on carbachol-, histamine- and BK-induced increases in $[Ca^{2+}]_i$

In order to identify the intracellular Ca^{2+} pools involved in agonist-induced activation of human TSMC, we investigated the effect of pretreatment with thapsigargin. Concentrations of 10^{-8} M and 10^{-6} M were chosen. The former concentration did not induce a transient rise and was close to the EC_{50} whereas the latter had a maximal effect (see Figure 2). Figure 3 and Table 1 show that pretreatment of human TSMC in culture with thapsigargin (10^{-8} M) significantly inhibited the increase in $[Ca^{2+}]_i$ induced by BK (Figure 3a), histamine (Figure 3b) and carbachol (Figure 3c) in a time-dependent manner. Moreover, increasing the thapsigargin concentration resulted in a greater reduction of the BK-induced Ca^{2+} peak. After 1 and 2 min preincubation with thapsigargin (10^{-6} M) the percentage inhibition was 52 ± 8 and $66 \pm 6\%$, respectively, ($n=4$, $P<0.05$). Similarly, for carbachol and histamine the degree of inhibition by thapsigargin was dependent upon the time of preincubation and on the concentration of thapsigargin (Table 1). Interestingly, the sustained phase disappeared concomitantly with the disappearance of the transient phase. These results suggest that histamine, carbachol and BK activate human TSMC by mobilizing Ca^{2+} from intracellular Ca^{2+} pools sensitive to thapsigargin. Furthermore, these results suggest that the sustained phase is dependent on the transient phase.

Role of extracellular Ca^{2+} in the thapsigargin-induced rise in $[Ca^{2+}]_i$

To determine whether one or both phases of the thapsigargin-induced response were dependent upon the presence of extracellular Ca^{2+}, comparisons of the thapsigargin-induced Ca^{2+} signals were made in the presence and absence of extracellular Ca^{2+} (Ca^{2+}-free and EGTA-containing medium). Figure 4a shows that thapsigargin, at 10^{-7} M, induced a rise in $[Ca^{2+}]_i$ in which the transient phase was slightly affected by the absence of extracellular Ca^{2+} ($70 \pm 8\%$ of the thapsigargin-response in the presence of 1.25 mM Ca^{2+}, $n=4$, $P<0.01$). Conversely, the sustained phase was drastically diminished compared with the control obtained in the presence of 1.25 mM Ca^{2+}, ($23 \pm 6\%$ of

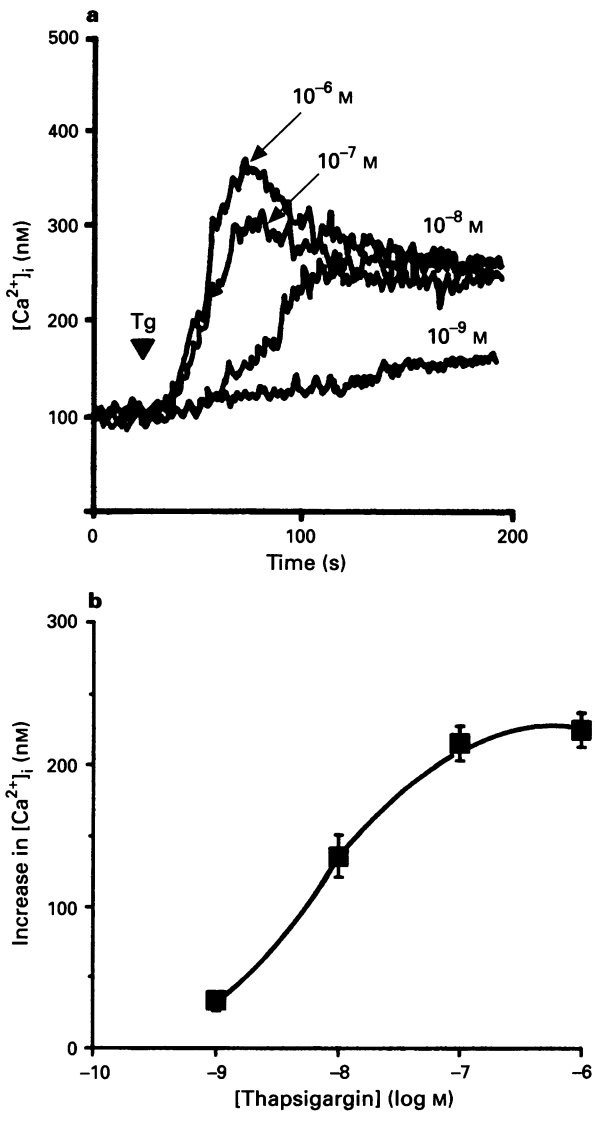

Figure 2 Effect of increasing concentrations of thapsigargin on $[Ca^{2+}]_i$ in fura-2-loaded human TSMC in Ca^{2+}-containing HEPES buffer. (a) Typical traces showing the concentration-related increase in $[Ca^{2+}]_i$ at various concentrations of thapsigargin. Arrows indicate the addition of thapsigargin. (b) Log concentration-response curve to thapsigargin. Values representing net maximal increase in intracellular Ca^{2+} over basal level are the mean ± s.e.mean of 4 separate experiments.

the thapsigargin-response in the presence of 1.25 mM Ca^{2+} $n=4$, $P<0.01$). The dependency of the sustained phase on extracellular Ca^{2+} was confirmed by the addition of 1 mM EGTA which decreased the sustained phase from 240–260 to 180–200 nM (Figure 4b, $n=5$). Increasing the concentration of EGTA (2 mM) resulted in a further reduction in the magnitude of the thapsigargin-induced sustained phase (from 240–260 to 110–130 nM, $n=5$, Figure 4b).

Re-introduction of 1.25 mM extracellular Ca^{2+} to human TSMC deprived of extracellular Ca^{2+} in the presence of thapsigargin (10^{-7} M) caused a rapid increase in $[Ca^{2+}]_i$ (Figure 5a). The magnitude of this increase in $[Ca^{2+}]_i$ was dependent upon the stimulation time with thapsigargin (60 ± 9, 150 ± 22 and 210 ± 27 nM after 1, 3 and 5 min respectively, $n=4$) (Figure 5b). Since our results suggested the occurrence of a Ca^{2+}-influx induced by thapsigargin, we tried to measure and quantify such an influx using Mn^{2+}-quenching of fura-2. However, no quenching of fura-2 at 360 nm (at the isobestic point) by Mn^{2+} was observed following the addition of either thapsigargin or agonists such

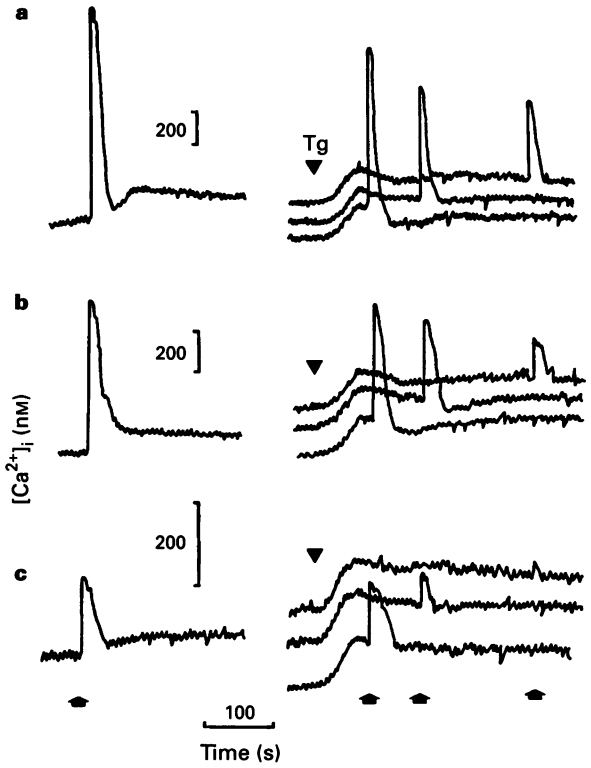

Figure 3 Typical traces showing the effects of (a) bradykinin (10^{-8} M), (b) histamine (10^{-4} M) and (c) carbachol (10^{-4} M) on $[Ca^{2+}]_i$ in TSMC in the absence (left panel) or in the presence (right panel) of thapsigargin (Tg). Cells in the presence of 1.25 mM Ca^{2+} were first exposed to thapsigargin (10^{-8} M) and bronchoconstrictor agents were then added at various incubation times (1, 2 and 5 min).

as bradykinin (results not presented), confirming previous findings by Murray & Kotlikoff (1991) that human TSMC in culture are impermeable to Mn^{2+}.

Effect of Hoe 140, thapsigargin and BK on $[Ca^{2+}]_i$ in human TSMC

Figure 6 shows the effect of BK and of Hoe 140 on $[Ca^{2+}]_i$ in human TSMC in the absence or the presence of external Ca^{2+} (1.25 mM). BK induced an increase in $[Ca^{2+}]_i$ composed of a fast transient phase (about 20 s) and a sustained phase occurring about 45 s after the addition of BK (Figure 6a). In the absence of external Ca^{2+} the sustained phase was abolished (Figure 6a). Hoe 140 (10^{-6} M), a B_2-receptor antagonist also suppressed the sustained phase in the presence of external Ca^{2+} (Figure 6b). Addition of thapsigargin prevented the effect of Hoe 140 (Figure 6b), suggesting that reduction of the plateau phase following removal of BK from its receptor requires functional Ca^{2+}-ATPases in the endoplasmic reticulum.

Characterization of the isoforms of Ca^{2+} ATPases present in human TSMC

Since thapsigargin is a potent and specific inhibitor of SERCA, western blotting was performed in order to characterize the SERCA isoforms present in human TSMC. Membrane preparations from TSMC were electrophoresed on 8% SDS-PAGE and immunoblotted with either polyclonal anti-$SERCA_{2\text{-a}}$ (lane 2) or anti-$SERCA_{2\text{-b}}$ (lane 4) antibody as described in the Methods section. As shown in Figure 7, the expression of the SERCA isoforms were detected at 100 kDa, as was the case for pig vascular smooth muscle cells (Magnier *et al.*, 1992). Membranes from rat heart (lane 1) and human platelets (lane 3) were also immunodetected respectively for $SERCA_{2\text{-a}}$ and $SERCA_{2\text{-b}}$, as positive control. The $SERCA_{2\text{-b}}$ isoform

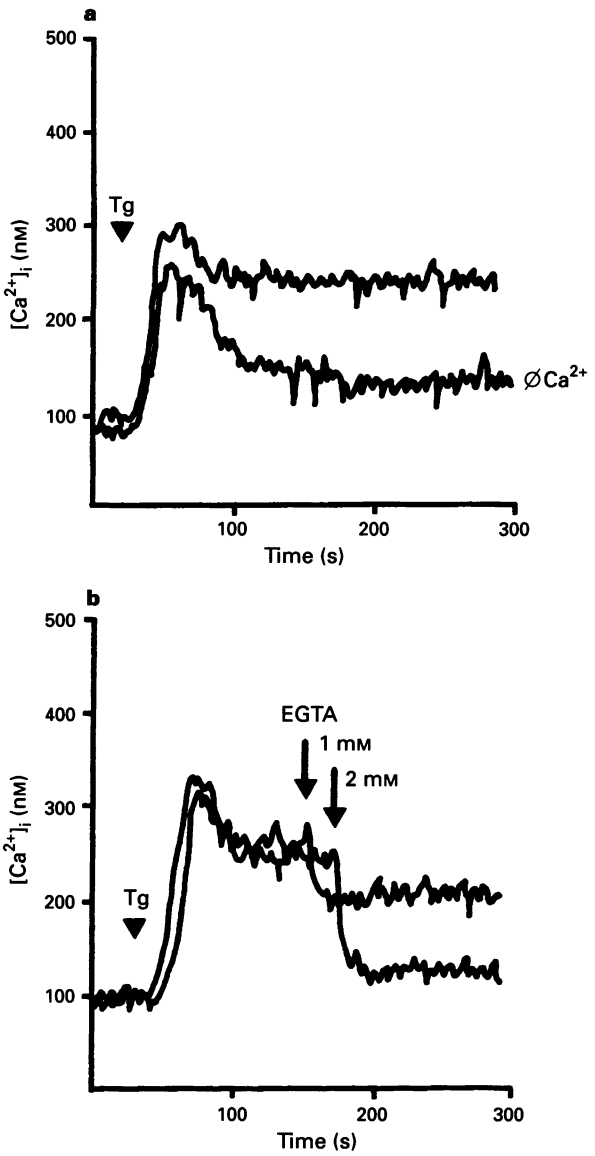

Figure 4 Typical traces showing the effect of thapsigargin-induced Ca^{2+} mobilization in Ca^{2+}-free conditions in human TSMC. (a) Thapsigargin (10^{-7} M) response in Ca^{2+}-containing HEPES buffer and a buffer without extracellular Ca^{2+} (ø Ca^{2+}), (i.e. in the presence of 50 μM EGTA). (b) Effect of EGTA (1 and 2 mM) on the plateau phase of the thapsigargin (10^{-7} M) response. Traces are representative of four to six separate experiments.

Table 1 Time course of inhibition by thapsigargin (Tg) of agonist-induced Ca^{2+} response in TSMC

	% inhibition (at the indicated time of pre-incubation with Tg)		
Thapsigargin (10^{-8} M)	*60s*	*120s*	*300s*
Bradykinin (10^{-8} M)	28 ± 5*	37 ± 6**	49 ± 7**
Carbachol (10^{-4} M)	39 ± 3*	60 ± 5**	86 ± 7**
Histamine (10^{-5} M)	20 ± 4*	36 ± 7**	56 ± 5**
Thapsigargin (10^{-6} M)			
Bradykinin (10^{-8} M)	51 ± 8*	60 ± 7**	70 ± 5**
Carbachol (10^{-4} M)	73 ± 4*	93 ± 5**	ND
Histamine (10^{-5} M)	68 ± 5*	72 ± 4**	83 ± 6**

Data represent the percentage inhibition (%) of agonist-induced transient rise in $[Ca^{2+}]_i$ obtained for various times of incubation of human TSMC with thapsigargin (10^{-8} or 10^{-6} M). Values are expressed as the mean ± s.e.mean of four separate experiments. *$P < 0.05$; **$P < 0.01$ as compared with thapsigargin-untreated cells stimulated with the agonist for the same time of incubation. ND, not determined.

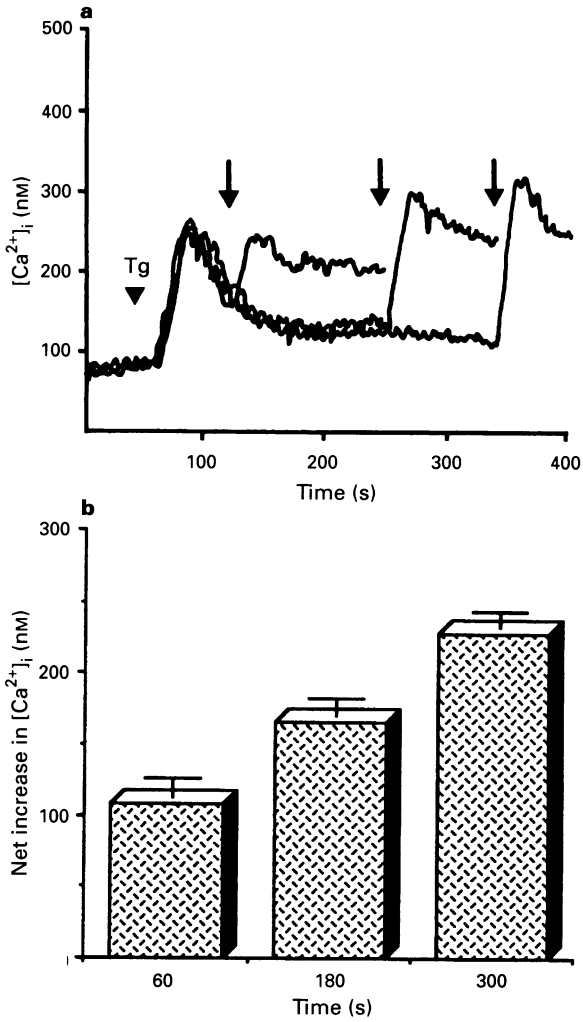

Figure 5 Effect of extracellular Ca^{2+} on thapsigargin-induced increase in $[Ca^{2+}]_i$. TSMC in Ca^{2+}-free medium (i.e. in the presence of 50 μM EGTA) were first stimulated with thapsigargin (10^{-7} M) for various times (60, 180 and 300 s) then Ca^{2+} (1.25 mM) was added (as indicated by the arrows). (a) Typical traces showing the time-dependence of thapsigargin-induced intracellular Ca^{2+} stores depletion on the magnitude of Ca^{2+} entry. (b) Values representing the net increase in $[Ca^{2+}]_i$ at the various times of Ca^{2+} addition. Each column is the mean ± s.e.mean of four separate experiments.

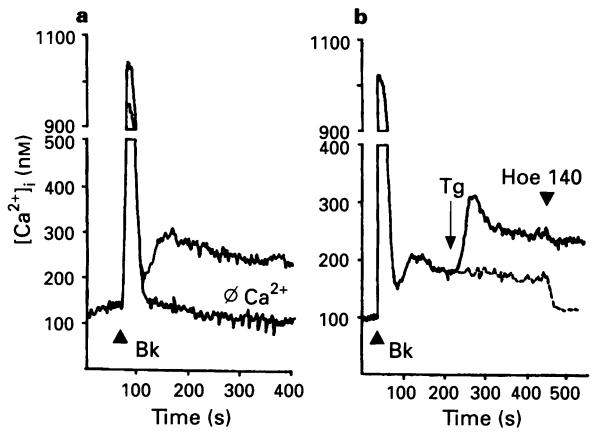

Figure 6 Effect of Ca^{2+}, thapsigargin and Hoe 140 on the increase in $[Ca^{2+}]_i$ induced by bradykinin (BK). (a) Human TSMC were in Ca^{2+}-free HEPES buffer (ø Ca^{2+}), (i.e. in the presence of 50 μM EGTA) or in Ca^{2+}-containing buffer (1.25 mM). (b) Human TSMC were in Ca^{2+}-containing medium. Thapsigargin, Hoe 140 and BK were added at 10^{-8} M, 10^{-6} M and 10^{-8} M, respectively.

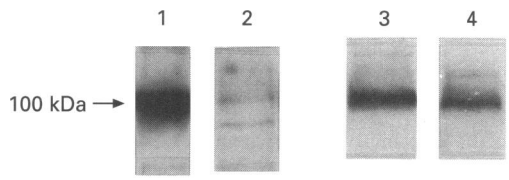

Figure 7 Comparative western blotting of the 100-kDa SERCA type Ca^{2+} ATPases in human TSMC using anti-$SERCA_2$ antibodies. TSMC membrane proteins (100 μg) were separated on 8% SDS-PAGE, electrotransferred onto nitrocellulose membranes and treated with the appropriate anti-$SERCA_2$ antibody. Lanes 1 and 2, isolated membranes from rat heart and human TSMC respectively, were treated with the antibody against the $SERCA_{2\text{-a}}$ isoform. In lanes 3 and 4, isolated membranes from human platelet and human TSMC respectively, were treated with the antibody against the $SERCA_{2\text{-b}}$ isoform. This blot is representative of five separate experiments.

protein was expressed at a higher level than the $SERCA_{2\text{-a}}$ isoform in TSMC (representative of five separate experiments).

Discussion

In the present study, we show that the biphasic rise in $[Ca^{2+}]_i$ induced by contractile agents bradykinin, carbachol and histamine i.e. the transient and sustained phase, was inhibited by thapsigargin. This supports the involvement of thapsigargin-sensitive Ca^{2+} stores in human TSMC that are also involved in the biphasic response as reported for guinea-pig TSMC (Amrani *et al.*, 1994). We found that the inhibition of the transient rise in $[Ca^{2+}]_i$ induced by these contractile agonists was dependent upon the incubation time with thapsigargin, suggesting a time-dependent depletion of intracellular Ca^{2+}-stores. The transient phase was poorly affected by the absence of extracellular Ca^{2+} showing that this phase is mainly dependent on the integrity of intracellular Ca^{2+} pools. The sustained phase was also abolished in the presence of thapsigargin suggesting it to be dependent upon the amplitude of the transient rise. We therefore propose that mobilization of Ca^{2+} from thapsigargin-sensitive intracellular Ca^{2+} pools is a key signal for initiating the occurrence of the sustained phase. This sustained phase could therefore result from a combination of the depletion of intracellular Ca^{2+} pools and subsequent Ca^{2+} influx. This was confirmed by our studies of human TSMC in Ca^{2+}-free solution using thapsigargin in order to stimulate the release of Ca^{2+} from the intracellular stores. Subsequent addition of Ca^{2+} to the extracellular medium induced a rapid transient increase in $[Ca^{2+}]_i$, suggesting that the depletion of intracellular Ca^{2+} pools is a signal sufficient for the activation of Ca^{2+} entry. This is consistent with previous studies in rat parotid acinar cells (Takemura *et al.*, 1989) and in vascular smooth-muscle cells (Xuan *et al.*, 1992) as well as in a large number of cells (reviewed in Thastrup, 1990 and in Putney, 1993) indicating a putative role of thapsigargin-sensitive Ca^{2+} stores in the regulation of Ca^{2+} influx. In addition, the magnitude of $[Ca^{2+}]_i$ increase after addition of Ca^{2+} to the buffer was closely dependent upon the duration time of stimulation with thapsigargin. A similar relationship between the magnitude of the $[Ca^{2+}]_i$ increase and the duration of stimulation with thapsigargin was described previously in human platelets (Malcolm & Fitzpatrick, 1992). This would suggest that in TSMC the Ca^{2+} influx pathway is intimately linked to the filling state of internal Ca^{2+} stores. Therefore, our findings in human TSMC are consistent with the capacitative Ca^{2+} entry model described previously by Putney (1993). Considering that thapsigargin-sensitive Ca^{2+} stores are involved in the agonist-induced biphasic Ca^{2+} response, we propose that the sustained phase results from a Ca^{2+} influx initiated by the depletion of

these intracellular Ca^{2+} stores.

However, the nature of the Ca^{2+} pools involved in the regulation of Ca^{2+} entry have not been identified. Several reports indicate the ability of thapsigargin to deplete the inositol trisphosphate-sensitive Ca^{2+} pools. In rat parotid acinar cells (Takemura *et al.*, 1991; Foskett *et al.*, 1992), lacrimal acinar cells (Kwan *et al.*, 1990), macrophages (Randriamampita & Trautman, 1990), endothelial cells (reviewed by Schilling & Eliott, 1992) and vascular smooth-muscle cells (Xuan *et al.*, 1992), thapsigargin-induced stimulation of Ca^{2+} entry appears to be a consequence of the depletion of inositol trisphosphate-sensitive Ca^{2+} stores. The present results suggest that this is also the case in human TSMC, since thapsigargin abolishes the rise in $[Ca^{2+}]_i$ induced by BK, carbachol and histamine, agents that induce inositol trisphosphate production in TSMC (Marsh & Hill, 1992; Pyne & Pyne, 1993; Daykin *et al.*, 1993; Yang *et al.*, 1994a). By sharing the same inositol trisphosphate-sensitive Ca^{2+} pools, thapsigargin and bronchoconstrictor agents may activate the same Ca^{2+} entry mechanism. The mechanism by which a decrease in Ca^{2+} content in the endoplasmic reticulum induces the opening of the plasma membrane Ca^{2+} channels is still unknown in human TSMC. In other cell systems it has been suggested that the depletion of these Ca^{2+} stores induces the release of a soluble mediator called 'Calcium Influx Factor' (Parekh *et al.*, 1993; Randriamampita & Tsien, 1993). In human neutrophils and in rat hepatocytes, it has been shown that thapsigargin is able to activate receptor-operated Ca^{2+} channels (Foder *et al.*, 1989; Kass *et al.*, 1990). Interestingly, the opening of receptor-operated Ca^{2+} channels following activation of human TSMC by BK during the sustained phase has been proposed by Murray & Kotlikoff (1991). These authors showed that removal of agonists during the sustained phase led to the disappearance of this phase. This is in agreement with our results for TSMC, where the bradykinin B_2 antagonist, Hoe 140 abolishes the sustained phase induced by BK. One could imagine that Ca^{2+} entry is activated as long as depletion of the Ca^{2+} stores occurs, which is itself governed by BK receptor occupancy. In the presence of thapsigargin subsequent addition of Hoe 140 did not abolish the sustained phase induced by BK, suggesting that receptor occupancy does not directly control Ca^{2+} entry. We propose that activation of the BK receptor leads to the activation of Ca^{2+}-entry via the depletion of intracellular Ca^{2+} stores.

The characterization of the Ca^{2+}-ATPases involved was then investigated. Thapsigargin specifically inhibits sarco endoplasmic reticulum Ca^{2+}-ATPases (SERCA), and does not affect plasma membrane Ca^{2+}-ATPases (Lytton *et al.*, 1991). Our results suggest the existence of SERCA in human TSMC sensitive to thapsigargin. They may be an important regulatory factor in the maintenance of Ca^{2+} homeostasis in TSMC. Since the $SERCA_2$-type Ca^{2+}ATPase is expressed in various types of smooth-muscle cells (Zarain-Herzberg *et al.*, 1990; Eggermont *et al.*, 1990; Magnier *et al.*, 1992) the expression of these $SERCA_2$ pumps was studied in human TSMC. Using specific polyclonal antibodies raised against the $SERCA_2$-type Ca^{2+}ATPases and in agreement with our previous work (Magnier *et al.*, 1992), we show here that human TSMC contain the two Ca^{2+}-ATPase isoforms, $SERCA_{2\text{-}a}$ and $SERCA_{2\text{-}b}$. However, $SERCA_{2\text{-}b}$ was the major protein expressed in human TSMC. This confirms our previous reports of greater expression of $SERCA_{2\text{-}b}$ isoform both in intestinal and in vascular smooth muscle cells (Eggermont *et al.*, 1990; Magnier *et al.*, 1992). This suggests that in human TSMC $SERCA_{2\text{-}b}$ associated Ca^{2+} pools may be the molecular target of thapsigargin, whose inhibition leads to depletion of intracellular Ca^{2+} stores. In vascular smooth muscle cells, recent work suggested that the $SERCA_2$ gene is regulated by a variety of factors. For example, during cell differentiation (Zarain-Herzberg *et al.*, 1990) where a concomitant switch from one isoform to the other has been reported (De Smedt *et al.*, 1991). An increased expression of the minor isoform $SERCA_{2\text{-}a}$ can be also induced by PDGF (Magnier *et al.*, 1992). In airway smooth muscle, changes in $SERCA_2$ gene expression is an interesting hypothesis to explore which may be involved in airway hyperresponsiveness. In that way, the effect of tumour necrosis factor α should be investigated on the $SERCA_2$ gene expression in human TSMC, since we have recently shown that the pretreatment of these cells with tumour necrosis factor α led to an increase Ca^{2+} response induced by carbachol and bradykinin (Amrani *et al.*, 1995).

Taken together, our data show that in human TSMC, the thapsigargin-sensitive Ca^{2+} stores may contribute significantly to the activation of Ca^{2+} influx via a capacitative Ca^{2+} entry model. These stores may be the same as those activated by the bronchoconstrictor agents, suggesting that reloading of the stores following cell stimulation could be mediated by the Ca^{2+}-ATPases (mainly $SERCA_{2\text{-}b}$ isoform) present in these stores.

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References

AMRANI, Y. & BRONNER, C. (1993). Tumor necrosis factor alpha potentiates the increase in cytosolic free calcium induced by bradykinin in guinea-pig tracheal smooth muscle cells. *C.R. Acad. Sci. Paris/Life Sci.*, **316**, 1489–1494.

AMRANI, Y., DA SILVA, A., KASSEL, O. & BRONNER, C. (1994). Biphasic increase in cytosolic free calcium induced by bradykinin and histamine in cultured tracheal smooth muscle cells: Is the sustained phase artifactual? *Naunyn-Schmied. Arch. Pharmacol.*, **350**, 662–668.

AMRANI, Y., MARTINET, N. & BRONNER, C. (1995). Potentiation by Tumour necrosis factor α of calcium signals induced by bradykinin and carbachol in human tracheal smooth muscle cells. *Br. J. Pharmacol.*, **114**, 4–5.

DAR, O. & PECHT, I. (1992). Fcε receptor mediated calcium influx into mast cells is modulated by the concentration of cytosolic free Ca^{2+} ions. *FEBS Lett.*, **2**, 123–128.

DAYKIN, K., WIDDOP, S. & HALL, I.P. (1993). Control of histamine induced inositol phospholipid hydrolysis in cultured human tracheal smooth muscle cells. *Eur. J. Pharmacol. (Mol. Pharmacol. Sec.)*, **246**, 135–140.

DE SMEDT, H., EGGERMONT, J.A., WUYTACK, F., PARYS, J.B., VAN DEN BOSCH, L., MISSIEN, L. & CASTEELS, R. (1991). Isoform switching of the sarcoplasmic reticulum Ca^{2+} pump during differenciation of BC3H1 myoblasts. *J. Biol. Chem.*, **266**, 7092–7095.

EGGERMONT, J.A., WUYTACK, F., VERBIST, J. & CASTEELS, R. (1990). Expression of endoplasmic-reticulum Ca^{2+} pump isoforms and of phospholamban in pig smooth-muscle tissues. *Biochem. J.*, **271**, 649–653.

FARMER, S.G. & DE SIATO, M.A. (1994). Effects of a novel nonpeptide bradykinin B2 receptor antagonist on intestinal and airway smooth muscle: further evidence for the tracheal B3 receptor. *Br. J. Pharmacol.*, **112**, 461–464.

FASOLATO, C., INNOCENTI, B. & POZZAN, T. (1994). Receptor-activated Ca^{2+} influx: how many mechanisms for how many channels. *Trends Pharmacol. Sci.*, **15**, 77–83.

FODER, B., SCHARFF, O. & THASTRUP, O. (1989). Ca^{2+} transients and Mn^{2+} entry in human neutrophils induced by thapsigargin. *Cell Calcium*, **10**, 477–490.

FOSKETT, J.K. & WONG, D. (1992). Calcium oscillations in parotid acinar cells induced by microsomal Ca^{2+}-ATPases inhibition. *Am. J. Physiol.*, **262,** C656–C663.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, Y.R. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260,** 3340–3450.

KASS, G.E.N., LIOPIS, J., CHOW, S.C., DUDDU, S.K. & ORRENIUS, S. (1990). Receptor-operated calcium influx in rat hepatocytes. *J. Biol. Chem.*, **265,** 17486–17492.

KULLMANN, A., VAILLANT, P., MULLER, V., MARTINET, Y. & MARTINET, N. (1993). In vitro effects of pentoxifylline on smooth muscle cell migration and blood monocyte production of chemotactic activity for smooth muscle cells: potential therapeutic benefit in the adult respiratory distress syndrome. *Am. J. Respir. Cell Mol. Biol.*, **145,** 332–336.

KWAN, C.Y., TAKEMURA, H., OBIE, J.F., THASTRUP, O. & PUTNEY, J.W.Jr. (1990). Effects of Mech, thapsigargin and La^{3+} on plasmalemmal and intracellular Ca^{2+} transport in lacrimal acinar cells. *Am. J. Physiol.*, **258,** C1006–1015.

LYTTON, J., WESTLIN, M. & HANLEY, M.R. (1991). Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase family of calcium pumps. *J. Biol. Chem.*, **266,** 17067–17071.

MAGNIER, C., PAPP, B., CORVAZIER, E., BREDOUX, R., WUYTACK, F., EGGERMONT, J., MACLOUF, J. & ENOUF, J. (1992). Regulation of Sarco-plasmic reticulum Ca^{2+}-ATPases during platelet-derived growth factor induced smooth muscle cell proliferation. *J. Biol. Chem.*, **267,** 15808–15815.

MALCOLM, K.C. & FITZPATRICK, F.P. (1992). Epoxieicosatrienoic acids inhibit Ca^{2+} entry into platelets stimulated by thapsigargin and thrombin. *J. Biol. Chem.*, **267,** 19854–19858.

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

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

MIKKI, I., NONAKA, H. & ISHII, I. (1992). Characterization of thromboxane A2/prostaglandin H2 receptors and histamine H1 receptors in cultured guinea-pig tracheal smooth-muscle cells. *Biochim. Biophys. Acta.*, **1137,** 107–115.

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

PAREKH, A.B., TERLAU, H. & STUHMER, W. (1994). Depletion of InsP3 stores activates a Ca^{2+} and K^+ current by means of a phosphatase and diffusible messenger. *Nature*, **364,** 814–818.

PUTNEY, J.W. (1993). Excitement about calcium signaling in inexcitable cells. *Science*, **262,** 676–678.

PYNE, S. & PYNE, N.J. (1993). Differential effects of B2 receptor antagonists upon bradykinin-stimulated phospholipase C and D in guinea-pig cultured tracheal smooth muscle. *Br. J. Pharmacol.*, **110,** 477–481.

RANDRIAMAMPITA, C. & TRAUTMANN, A. (1990). Arachidonic acid activates Ca^{2+} extrusion in macrophages. *J. Biol. Chem.*, **265,** 18059–18062.

RANDRIAMAMPITA, C. & TSIEN, R.Y. (1993). Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. *Nature*, **364,** 809–814.

SCHILLING, W.P. & ELLIOTT, S.J. (1992). Ca^{2+} signaling mechanisms of vascular endothelial cells and their role in oxidant-induced endothelial cell dysfunction. *Am. J. Physiol.*, **262,** H1717–H1730.

TAKEMURA, H., HUGHES, G.H., THASTRUP, O. & PUTNEY, J.W.Jr. (1989). Activation of calcium entry by tumor promoter thapsigargin in parotid acinar cells. *J. Biol. Chem.*, **264,** 12266–12271.

TAKEMURA, H., OHSHIDA, H., YOKOSAWA, N., OGUMA, K. & THASTRUP, O. (1991). The thapsigargin-sensitive intracellular Ca^{2+} pool is more important in plasma membrane Ca^{2+} entry than the IP_3-sensitive intracellular Ca^{2+} pool in neuronal cell lines. *Biochem. Biophys. Res. Commun.*, **180,** 1518–1526.

THASTRUP, O. (1990). Role of Ca^{2+}-ATPases in regulation of cellular Ca^{2+} signalling, as studied with the selective microsomal Ca^{2+}-ATPase inhibitor, thapsigargin. *Agents Actions*, **29,** 8–15.

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+}-ATPases. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 2466–2470.

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

TRIFILIEFF, A., AMRANI, Y., LANDRY, Y. & GIES, J.-P. (1993). Comparative action of new highly potent bradykinin receptor antagonists in the guinea-pig trachea. *Eur. J. Pharmacol.*, **239,** 227–229.

TSUNODA, Y. (1993). Receptor-operated Ca^{2+} signaling and cross-talk in stimulus secretion coupling. *Biochim. Biophys. Acta*, **1154,** 105–156.

TWORT, C.H.C. & VAN BREEMEN, C. (1989). Human airway smooth muscle in cell culture: control of the intracellular calcium store. *Pulmon. Pharmacol.*, **2,** 45–53.

WUYTACK, F., EGGERMONT, J.A., RAEYMAEKERS, PLESSERS, L. & CASTEELS, R. (1989). Antibodies against the non-muscle isoform of the endoplasmic reticulum Ca^{2+}-transport ATPase. *Biochem. J.*, **264,** 765–769.

XUAN, Y.-T., WANG, O.-L. & WHORTON, R. (1992). Thapsigargin stimulates Ca^{2+} entry in vascular smooth cells: nicardipine-sensitive and -insensitive pathways. *Am. J. Physiol.*, **262,** C1258–C1265.

YANG, C.M., CHOU, S.-P., WANG, Y.-Y., HSIEH, J.T. & ONG, R. (1993a). Muscarinic regulation of cytosolic free calcium in canine tracheal smooth muscle cells: Ca^{2+} requirement for phospholipase C activation. *Br. J. Pharmacol.*, **110,** 1239–1247.

YANG, C.M., HSIA, H.-C., CHOU, S.-P., ONG, R., HSIEH, J.T. & LUO, S.F. (1994a). Bradykinin stimulated phosphoinositide metabolism in cultured canine tracheal smooth muscle cells. *Br. J. Pharmacol.*, **111,** 21–28.

YANG, C.M., HSIA, H.-C., LUO, S.-F., ONG, R., HSIEH, J.T. & ONG, R. (1994b). The effect of cyclic AMP elevating agents on bradykinin- and carbachol-induced signal transduction in canine cultured tracheal smooth muscle cells. *Br. J. Pharmacol.*, **112,** 781–788.

YANG, C.M., YO, Y.-L. & WANG, Y.-Y. (1993b). Intracellular calcium in canine cultured tracheal smooth muscle cells is regulated by M3 muscarinic receptors. *Br. J. Pharmacol.*, **110,** 983–988.

ZARAIN-HERZBERG, A., MACLENNAN, D.H. & PERIASAMY, M. (1990). Characterization of rabbit cardiac sarcoendoplasmic reticulum Ca^{2+}-ATPase gene. *J. Biol. Chem.*, **263,** 4813–4819.

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