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# Capacitative $Ca^{2+}$ entry in vascular endothelial cells is mediated *via* pathways sensitive to 2 aminoethoxydiphenyl borate and xestospongin C

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**1** Agonists increase endothelial cell intracellular $Ca^{2+}$, in part, by capacitative entry, which is triggered by the filling state of intracellular $Ca^{2+}$ stores. It has been suggested that depletion of endoplasmic reticulum (ER) $Ca^{2+}$ stores either leads to a physical coupling between the ER and a plasma membrane channel, or results in production of an intracellular messenger which affects the gating of membrane channels. As an axis involving the $IP_3$ receptor has been implicated in a physical coupling mechanism the aim of this study was to examine the effects of the putative $IP_3$ receptor antagonists/modulators, 2 aminoethoxydiphenyl borate (2APB) and xestospongin C, on endothelial cell $Ca^{2+}$ entry.

**2** Studies were conducted in fura 2 loaded cultured bovine aortic endothelial cells and endothelial cells isolated from rat heart.

**3** 2APB (30 – 300 $\mu$M) inhibited $Ca^{2+}$ entry induced by both agonists (ATP 1 $\mu$M, bradykinin 0.1 $\mu$M) and receptor-independent mechanisms (thapsigargin 1 $\mu$M, ionomycin 0.5 and 5 $\mu$M). 2APB did not diminish endothelial cell ATP-induced production of $IP_3$ nor effect *in vitro* binding of $[^3H]$-$IP_3$ to an adrenal cortex binding protein. Capacitative $Ca^{2+}$ entry was also blocked by disruption of the actin cytoskeleton with cytochalasin (100 nM) while the initial $Ca^{2+}$ release phase was unaffected.

**4** Similarly to 2APB, xestospongin C (3 – 10 $\mu$M) inhibited ATP-induced $Ca^{2+}$ release and capacitative $Ca^{2+}$ entry. Further, xestospongin C inhibited capacitative $Ca^{2+}$ entry induced by thapsigargin (1 $\mu$M) and ionomycin (0.5 $\mu$M).

**5** The data are consistent with a mechanism of capacitative $Ca^{2+}$ entry in vascular endothelial cells which requires (a) $IP_3$ receptor binding and/or an event distal to the activation of the ER receptor and (b) a spatial relationship, dictated by the cytoskeleton, between $Ca^{2+}$ release and entry pathways.

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**Keywords:** Calcium; capacitative $Ca^{2+}$ entry; $Ca^{2+}$ stores; 2 aminoethoxydiphenyl borate; xestospongin C; inositol trisphosphate receptor; cytoskeleton

**Abbreviations:** 2APB, 2 aminoethoxydiphenyl borate; ATP, adenosine triphosphate; CICR, $Ca^{2+}$-induced $Ca^{2+}$ release; DMEM, Dulbecco's modified essential medium; DMSO, dimethyl sulphoxide; EHS, Engelberth Holm-Swarm; ER, endoplasmic reticulum; $IP_3$, inositol trisphosphate; Trp, transient receptor potential

## Introduction

The production of vasodilator factors such as nitric oxide (*via* constituitive NO synthase) and prostacyclin (*via* cyclo-oxygenase) by endothelial cells represent $Ca^{2+}$-dependent processes (for example see references Martin & Michaelis, 1990; Lin *et al*., 2000; Mizuno *et al*., 2000). The increase in intracellular $Ca^{2+}$ typically occurs following the activation of membrane receptors which initiate signal transduction pathways that lead to $Ca^{2+}$ release from the endoplasmic reticulum (ER) and entry from the extracellular compartment.

In many cell types, including endothelial cells, release of intracellular $Ca^{2+}$ has been shown to be coupled to subsequent $Ca^{2+}$ entry by store depletion or capacitative $Ca^{2+}$ entry (Putney, 1990; Schilling *et al*., 1992; Parekh & Penner, 1997; Fasolato & Nilius, 1998). Such coupling has been demonstrated in response to receptor-mediated stimuli (Putney, 1990; Schilling *et al*., 1992; Parekh & Penner, 1997; Fasolato & Nilius, 1998) and receptor-independent emptying of intracellular stores by agents such as thapsigargin and ionomycin (Putney, 1990; Schilling *et al*., 1992; Parekh & Penner, 1997; Fasolato & Nilius, 1998). While the exact mechanism by which the filling state of the ER is linked to $Ca^{2+}$ entry is uncertain, it has been proposed that either a factor(s) modulating $Ca^{2+}$ current across the cell membrane is released on emptying of the stores (Randriamampita & Tsien, 1993; Thomas & Hanley, 1995), or that store-depletion leads to a physical coupling between the ER and the plasma membrane (Irvine, 1990; Berridge, 1995). With respect to the former, evidence has been provided for the involvement of a number candidate signalling molecules including a small molecular weight phosphate-containing compound (Randriamampita & Tsien, 1993), a cytochrome P450 epoxygenase

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metabolite of arachidonic acid (Rzigalinski *et al*., 1999), and events requiring tyrosine phosphorylation (Fleming *et al*., 1996; Babnigg *et al*., 1997). In recent studies of clonal embryonic kidney cells Ma *et al*. (2000) have provided evidence supporting a direct coupling mechanism, and further, that this coupling requires the inositol trisphosphate ($IP_3$) receptor or a related molecule (Ma *et al*., 2001). This conclusion was based on the observations that both rearrangement of the actin cytoskeleton and blockade of the $IP_3$ receptor with 2 aminoethoxydiphenyl borate (2APB) uncoupled store depletion from $Ca^{2+}$ entry. Similar results have been presented for other cell types, including human platelets, where an alternate $IP_3$ receptor blocker, xestospongin C, inhibited both the association of $IP_3$ receptors with the membrane $Ca^{2+}$ channel protein Trp1, and capacitative $Ca^{2+}$ entry (Rosado & Sage, 2001). However, cell or species specific differences may exist as Ribeiro *et al*. (1997) reported that NIH-3T3 cells do not require an intact cytoskeleton to demonstrate capacitative $Ca^{2+}$ entry as thapsigargin-induced $Ca^{2+}$ entry was unaffected by cytochalasin D treatment. Further, this group also reported differences in the regulation of capacitative $Ca^{2+}$ entry between 3T3 and pancreatic acinar cells (Louzao *et al*., 1996). In addition to cell specific differences, *per se*, variation may occur as a result of the heterogeneity within the family of protein subunits which constitute the $Ca^{2+}$ influx channels (Hofmann *et al*., 1999; Putney, 1999; Mery *et al*., 2001). In this regard some seven mammalian homologues of the Trp proteins have been identified (Putney, 1999).

The present study aimed to demonstrate capacitative $Ca^{2+}$ entry pathways in bovine aortic and rat heart endothelial cells and determine specifically whether such $Ca^{2+}$ entry is dependent on an axis involving the $IP_3$ receptor-mediated mechanisms. Dependence on the $IP_3$ receptor was examined using the cell permeable, small molecular weight, inhibitors 2APB as described by Maruyama *et al*. (1997) and xestospongin C (Gafni *et al*., 1997).

# Methods

## *Cell culture*

Bovine aortae, obtained from a local slaughterhouse, were placed in cold physiological salt solution and transported to the laboratory. Vessels were then trimmed of adherent tissue, washed, and filled with serum free DMEM (5.6 mM glucose) containing 0.2 mg collagenase and incubated for 20 mins at 37°C. Endothelial cells were detached from the vessel wall by gentle agitation and the resulting cell suspension centrifuged. The cell pellet was then resuspended in fresh DMEM containing 10% heat-inactivated foetal calf serum and incubated at 37°C in an atmosphere of 5% $CO_2$. Cells were studied between passages 3 and 5.

For rat heart endothelial cells, hearts were removed from euthanized rats (protocol approved by the Animal Experimentation and Ethics Committee, RMIT University) and a polyethylene cannula exerted into the left ventricle. The cannulated preparation was connected to a re-circulating perfusion system and perfused with Joklik's medium containing heparin (1 u $ml^{-1}$) and collagenase (0.7 mg $ml^{-1}$) for 30 min, 37°C. Hearts were then mechanically disrupted and endothelial cells obtained by differential sieving and centrifugation (modified from Ford & Rovetto, 1987). Endothelial cells were then incubated in DMEM (5.6 mM glucose) with 20% foetal bovine serum at 37°C, 10% $CO_2$.

Endothelial cells were characterized by positive staining for endothelial cell nitric oxide synthase and negative staining for $\alpha$ smooth muscle actin.

## *Measurement of intracellular $Ca^{2+}$ ($Ca^{2+}{}_i$).*

Following removal of endothelial cells from culture flasks ($Ca^{2+}$ free solution containing 0.02% EDTA and 0.25% trypsin) cells ($10^5$ cells $ml^{-1}$) were plated on glass coverslips which had been precoated with 50 $\mu$l of Engelberth Holm-Swarm (EHS) mouse sarcoma matrix (2.5 mg protein $ml^{-1}$). Cells were allowed to adhere for 16 h (37°C, 5% $CO_2$) prior to preparation for experiments. The coverslips were then transferred to a HEPES-buffered Kreb's solution containing the acetomethoxy ester of fura 2 (1 $\mu$M; 60 min). After loading cells were washed in fresh buffer to remove excess $Ca^{2+}$ indicator.

Changes in intracellular $Ca^{2+}$ related fluorescence were monitored using a video-based imaging system (Universal Imaging, PA, U.S.A.) coupled to an inverted microscope (20 × Nikon Fluor objective lens, N.A. 0.75). As an index of changes in $Ca^{2+}{}_i$, the ratio of emitted fluorescence (510 nm) intensities was calculated following excitation at 340 and 380 nm. Fluorescence excitation (340 and 380 nm) was obtained by passing light from a 75 W Xenon source through a computer-controlled filter wheel. In all experiments the responses in various protocols were averaged across 8–10 cells/coverslip. Preliminary experiments verified that similar responses were obtained in single cells and groups of cells.

## *Protocols*

Initial studies established the concentration–response relationships for the inhibitory effect of 2APB (10–300 $\mu$M) on ATP (1 $\mu$M)-induced increases in intracellular $Ca^{2+}$. The effects of 2APB were examined both in the presence of extracellular $Ca^{2+}$ and in separate experiments during re-addition of $Ca^{2+}$ following exposure to ATP in the absence of extracellular $Ca^{2+}$. Additional experiments were performed using bradykinin to mobilize intracellular $Ca^{2+}$ to determine whether the processes of capacitative $Ca^{2+}$ entry were common for different modes of receptor activation.

Following establishment of the inhibitory concentrations of 2APB, the effect of 100 $\mu$M 2APB on thapsigargin (1 $\mu$M) and ionomycin (0.5 and 5 $\mu$M)-induced $Ca^{2+}$ entry was determined.

To support studies conducted with 2APB, additional experiments were performed with the $IP_3$ receptor antagonist xestospongin C (Gafni *et al*., 1997). Initial studies examined the concentration-dependent effects of xestospongin C (3–10 $\mu$M) on ATP (1 $\mu$M)-induced $Ca^{2+}$ responses. The responses to xestospongin C were studied both in the presence of extracellular $Ca^{2+}$ and during $Ca^{2+}$ re-addition following ATP stimulation in the absence of extracellular $Ca^{2+}$. Further studies examined the effect of xestospongin C (6 and 10 $\mu$M) on thapsigargin (1 $\mu$M) and ionomycin (0.5 $\mu$M)-stimulated $Ca^{2+}$ entry.

An additional set of studies were performed to examine whether cytoskeletal disruption dissociated agonist-induced

$Ca^{2+}$ release from capacitative $Ca^{2+}$ entry, and whether this manoeuvre paralleled the effects of 2APB. Cells were prepared on EHS coated glass coverslips and loaded with fura 2 as described above. The cells were then treated with 100 nM cytochalasin D (60 min, 37°C) and ATP-induced changes in $Ca^{2+}{}_i$ determined as above. Disruption of the actin cytoskeleton was confirmed by FITC-phalloidin staining and confocal microscopy.

To determine whether 2APB exerted effects on production of $IP_3$, as opposed to an effect distal to generation of the second messenger, ATP-induced changes in cellular $IP_3$ were determined using a radioreceptor assay (Amersham). In brief, cells were plated on EHS-coated glass coverslips, as above, and placed in six-well culture plates containing DMEM. After the 16 h adherence period cells were washed and placed in Krebs buffer and stimulated with ATP (1 $\mu$M) in the presence and absence of 2APB (30 and 100 $\mu$M). In preparation for assay, $IP_3$ was extracted from cells using ice-cold 20% perchloric acid followed by neutralization in 60 mM HEPES, 1.5 M KOH solution. $IP_3$ production was measured at baseline and 10 s after the addition of ATP. This timepoint was chosen as preliminary studies have shown that in our endothelial cell preparations, $IP_3$ production in response to purinergic stimulation, is maximal at 10 s (data not shown) which is comparable with published studies (e.g. Purkiss *et al*., 1994).

### *Statistical methods*

Changes in $Ca^{2+}{}_i$ were assessed as changes in the 340 : 380 nm fluorescence ratio at baseline (i.e. in the absence of agonist/drug treatment). Baseline levels were designated as 100% and responses normalized to this value. Group data is shown as mean ± s.e.mean. Statistical differences between treatments has been determined by analysis of variance (ANOVA) with appropriate *post hoc* tests. Simple comparison of the means of two groups was determined using the Student *t*-test. Statistical significance was accepted at the $P<0.05$ level.

### *Chemicals and reagents*

2APB (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) was prepared as a 0.5 M stock solution in dimethysulphoxide; subsequent dilutions were made in physiological salt solution. ATP (Sigma Chemical Co., St Louis, MO, U.S.A.) and bradykinin (Sigma) were dissolved in physiological salt solution. Thapsigargin (Sapphire Bioscience, New South Wales, Australia) was dissolved in DMSO and subsequent dilutions made in physiological salt solution. Ionomycin (Sigma) was dissolved in chloroform and further diluted in physiological salt solution. Xestospongin C (Calbiochem) was prepared in DMSO as a 5.6 mM stock solution with subsequent dilution in physiological salt solution.

## Results

### *Effects of 2APB on endothelial cell capacitative $Ca^{2+}$ entry*

The effect of 2APB, in bovine aortic endothelial cells, on ATP-induced changes in $Ca^{2+}{}_i$ are detailed in Figure 1. Figure 1a illustrates that in the presence of extracellular $Ca^{2+}$ (2.5 mM) ATP (1 $\mu$M) stimulation results in a biphasic change in $Ca^{2+}{}_i$, with a rapid increase followed by a decline to a steady-state level which remains significantly above basal levels. 2APB alone had no effect on basal fluorescence but caused a concentration-dependent inhibition of both phases of $Ca^{2+}$ mobilization (30 – 300 $\mu$M; Figure 1a) with 100 $\mu$M 2APB abolishing the secondary phase (Figure 1a,b). To illustrate capacitative $Ca^{2+}$ entry, cells were exposed to ATP in the absence of extracellular $Ca^{2+}$, after which $Ca^{2+}$ was returned to the superfusate in the continued presence of the agonist (Figure 1b). 2APB (100 $\mu$M) added prior to ATP both inhibited the initial $Ca^{2+}$ release and prevented the influx of $Ca^{2+}$ following the return of the cation (Figure 1b). Similarly, when 2APB was added immediately prior to the re-addition of $Ca^{2+}$, capacitative $Ca^{2+}$ entry was inhibited consistent with the capacitive entry mechanism *per se* being inhibited in this condition rather than solely being a consequence of attenuated $IP_3$-mediated store release (Figure 1c).

To determine whether 2APB inhibits the $Ca^{2+}$ release and entry components in bovine endothelial cells, the effects of 10, 50 and 100 $\mu$M 2APB on ATP (1 $\mu$M)-induced $Ca^{2+}$ mobilization were compared (Figure 1d). While both components showed a 2APB concentration – dependent inhibition it appears that a residual release component persisted in the presence of 100 $\mu$M 2APB, whereas this concentration of 2APB abolished the influx component.

Endothelial cells cultured from rat heart showed a qualitatively similar response to ATP, with an initial $Ca^{2+}{}_i$ release phase followed by $Ca^{2+}$ entry (Figure 1e). As observed with the bovine cells, 2APB inhibited both phases of $Ca^{2+}$ mobilization (Figure 1e).

Similar results were obtained when bradykinin (0.1 $\mu$M) was used to mobilize $Ca^{2+}{}_i$. For example, 100 $\mu$M 2APB decreased the initial $Ca^{2+}$ release peak from $268\pm69\%$ of baseline to $107\pm5\%$ ($P<0.05$) and the secondary $Ca^{2+}$ entry phase from $224\pm47\%$ to $108\pm4\%$ ($P<0.05$; $n=5$). This suggests that the effects of 2APB are not specific to ATP-receptor mediated events.

To illustrate the effects of 2APB on receptor-independent mechanisms of stimulating capacitative $Ca^{2+}$ entry, separate preparations of bovine endothelial cells were treated with ionomycin (0.5 and 5 $\mu$M; Figure 2) or thapsigargin (1 $\mu$M; Figure 3). Ionomycin added to cells in the absence of extracellular $Ca^{2+}$ resulted in a release of intracellular $Ca^{2+}$, which was followed by an influx of $Ca^{2+}$ on re-addition of the cation to the superfusate (Figure 2a,b). Concentration-dependent effects of ionomycin on both phases of $Ca^{2+}$ mobilization were observed (Figure 2a,b). Addition of 2APB (100 $\mu$M) prior to ionomycin treatment prevented subsequent $Ca^{2+}$ influx (Figure 2a,b). When 2APB was added to ionomycin-treated cells during the $Ca^{2+}$ influx component there was a rapid decrease in intracellular $Ca^{2+}$, consistent with 2APB inhibiting the $Ca^{2+}$ entry process (Figure 2c). Washout of 2APB restored ionomycin-induced $Ca^{2+}$ influx (data not shown).

Exposure of bovine aortic endothelial cells to the ER $Ca^{2+}$ ATPase inhibitor thapsigargin resulted in an increase in $Ca^{2+}{}_i$ and $Ca^{2+}$ influx on re-addition of $Ca^{2+}$ to the superfusate (Figure 3a). As in the case of ionomycin, 2APB significantly inhibited the thapsigargin-induced $Ca^{2+}$ influx component (Figure 3a,b). Figure 3b further illustrates that washout of 2APB, in the presence of $Ca^{2+}$ and thapsigargin, restores $Ca^{2+}$ influx.

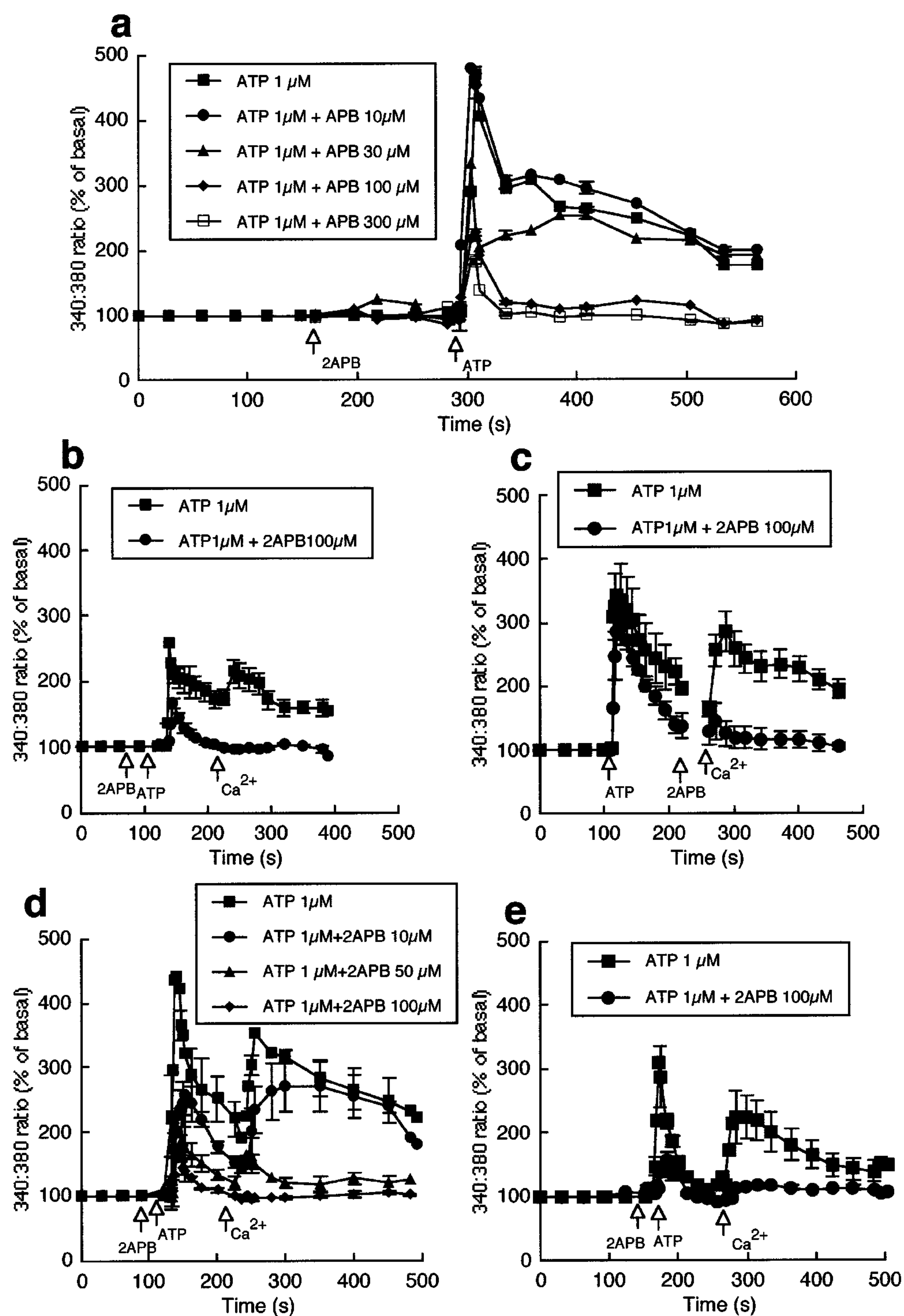


**Figure 1** Effects of 2APB on ATP-induced changes in intracellular $Ca^{2+}$. Studies shown in (a–d) were performed in bovine aortic endothelial cells and those in (e) in rat heart endothelial cells. (a) Shows the concentration-dependent effects of 2APB on ATP-induced changes in $Ca^{2+}{}_i$ in the presence of extracellular $Ca^{2+}$ ($n$=10). 2APB inhibits both the initial release of $Ca^{2+}$and the subsequent plateau phase. (b) Illustrates the effect of 100 $\mu$M 2APB on ATP-induced $Ca^{2+}$ release in the absence of extracellular $Ca^{2+}$ and the effect of subsequent re-addition of $Ca^{2+}$ (1 mM) to the superfusate ($n$=6). (c) Demonstrates the effect of 2APB specifically on the $Ca^{2+}$ entry phase; addition of 2APB after ATP-induced $Ca^{2+}$ release and immediately prior to the re-addition of extracellular $Ca^{2+}$ prevented capacitative $Ca^{2+}$ entry ($n$=6). (d) Shows the concentration dependence of the effects of 2APB (10–100 $\mu$M) on the ATP (1 $\mu$M)-induced $Ca^{2+}$ release and influx components ($n$=5). (e) Demonstrates that 2APB similarly inhibits ATP (1 $\mu$M)-induced $Ca^{2+}$ release and influx in cultured rat heart endothelial cells ($n$=6). Results are presented as mean±s.e.mean; $n$=number of coverslips. For clarity, representative error bars are shown.

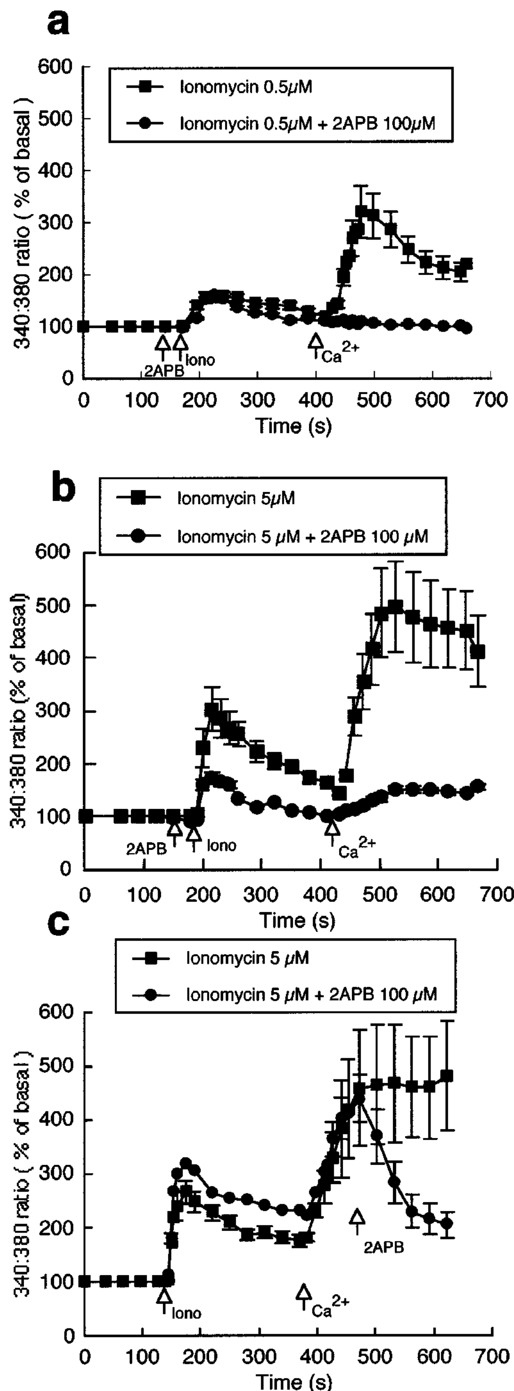


**Figure 2** Effect of 2APB (100 $\mu$M) on ionomycin (0.5, $n=6$ and 5 $\mu$M, $n=18$)-induced changes in intracellular $Ca^{2+}$ (a and b). (c ($n=6$)) Illustrates that addition of 2APB during the phase of capacitative $Ca^{2+}$ entry inhibits entry causing a decline in $Ca^{2+}_i$. Results are presented as mean ± s.e.mean. For clarity, representative error bars are shown.

## *Effect of xestospongin C on endothelial cell $Ca^{2+}$ responses to ATP and capacitative $Ca^{2+}$ entry*

The effects of xestospongin C, in bovine aortic endothelial cells, on ATP-induced changes in $Ca^{2+}_i$ are shown in Figure 4. Xestospongin C alone had no effect on basal fluorescence but appeared to cause a concentration-dependent inhibition of both phases of $Ca^{2+}$ mobilization (3–6 $\mu$M; Figure 4a). To specifically examine capacitative, $Ca^{2+}$ entry, cells were exposed to ATP in the absence of extracellular $Ca^{2+}$, after which $Ca^{2+}$ was returned to the superfusate in the continued presence of the agonist (Figure 4b). Xestospongin C added prior to ATP both inhibited the initial $Ca^{2+}$ release and prevented the influx of $Ca^{2+}$ following the return of the cation (Figure 4b). For example, in the control state re-addition of $Ca^{2+}$ caused a peak increase in fluorescence of 354 ± 23% ($n=7$), while in the presence of xestospongin C (10 $\mu$M) the change in fluorescence was reduced to 172 ± 23% ($n=3$; $P<0.05$).

To further confirm an inhibitory effect of xestospongin C on capacitative $Ca^{2+}$ entry, the response to $Ca^{2+}$ re-addition following thapsigargin (1 $\mu$M) treatment was compared in the absence and presence of the $IP_3$ receptor antagonist. Xestospongin C (6 and 10 $\mu$M) significantly inhibited $Ca^{2+}$ entry on re-addition of extracellular $Ca^{2+}$ (Figure 5a). Similarly, $Ca^{2+}$ entry induced by ionomycin (0.5 $\mu$M) was significantly attenuated by pretreatment with xestospongin C (10 $\mu$M) (Figure 5b). To specifically demonstrate an effect on the $Ca^{2+}$ entry component, xestospongin C (10 $\mu$M) was added to ionomycin (0.5 $\mu$M) treated cells at the time when $Ca^{2+}$ was returned to the superfusate (Figure 5c). As with xestospongin C pretreated cells, $Ca^{2+}$ entry was significantly ($P<0.05$) attenuated. Additional experiments demonstrated that xestospongin C treatment also reversed $Ca^{2+}$ entry induced by thapsigargin (1 $\mu$M) (data not shown). Collectively these data indicate that xestospongin C inhibits the capacitative $Ca^{2+}$ entry phase in bovine endothelial cells.

## *Effect of cytoskeletal disruption on endothelial cell capacitative $Ca^{2+}$ entry*

Cytochalasin D treatment, *per se*, had no apparent significant effect on baseline intracellular $Ca^{2+}$, as indicated by fluorescence ratio values measured in 0 mM extracellular $Ca^{2+}$ conditions, prior to the addition of ATP. Control, 0.42 ± 0.03; 100 nM cytochalasin 0.47 ± 0.05; 1 $\mu$M cytochalasin 0.51 ± 0.05 ($n=6$, ANOVA, $P<0.331$, n.s.).

Exposure of cells to cytochalasin D (100 nM and 1 $\mu$M) had no apparent effect on ATP-induced $Ca^{2+}$ release, while significantly inhibiting subsequent capacitative $Ca^{2+}$ entry (Figure 6). Effectiveness of cytochalasin in disrupting the actin cytoskeleton was confirmed by confocal microscopy of FITC-phalloidin labelled endothelial cells (data not shown). Consistent with published studies (Rosado & Sage, 2001), cytochalasin treatment appeared to cause retraction of the cytoskeleton from the plasma membrane with a clumping of phalloidin-staining material within deeper regions of the cells. In addition, cytochalasin appeared to cause concentration-dependent changes in cell morphology evident as an increased occurrence of cytoplasmic protrusions.

## *Effects of 2APB on ATP-induced inositol trisphosphate production*

ATP (1 $\mu$M) caused a significant increase in $IP_3$ production; 2.95 ± 2.22 pmol/well baseline compared to 9.86 ± 3.44 pmol/well after 10 s of ATP exposure ($P<0.05$ compared to baseline). In the presence of 2APB (100 $\mu$M) ATP caused a similarly significant increase in $IP_3$ production; baseline 3.63 ± 3.63 pmol well$^{-1}$ compared to 9.92 ± 2.97 pmol well$^{-1}$

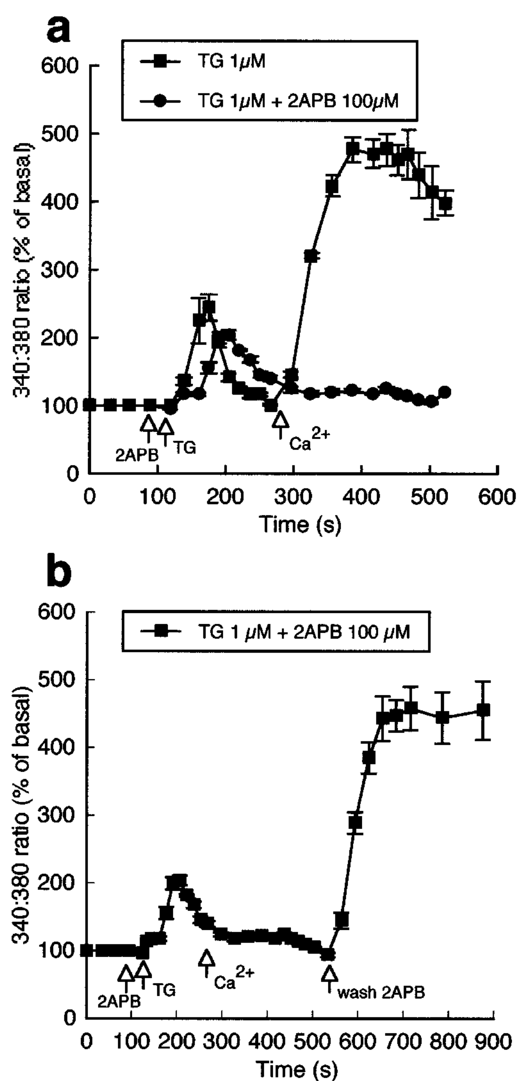


**Figure 3** Effect of 2APB (100 $\mu$M) on thapsigargin (1 $\mu$M)-induced changes in intracellular $Ca^{2+}$. In the absence of extracellular $Ca^{2+}$ thapsigargin induced a transient increase in $Ca^{2+}{}_i$; subsequent addition of $Ca^{2+}$ to the superfusate resulted in a large increase in $Ca^{2+}{}_i$ (a; $n=5$). (b) Illustrates that 2APB while not preventing the initial thapsigargin-induced increase in $Ca^{2+}$ prevented subsequent capacitative $Ca^{2+}$ entry ($n=5$). Washout of 2APB restored entry of $Ca^{2+}$. Results are presented as mean$\pm$s.e.mean. For clarity, representative error bars are shown.

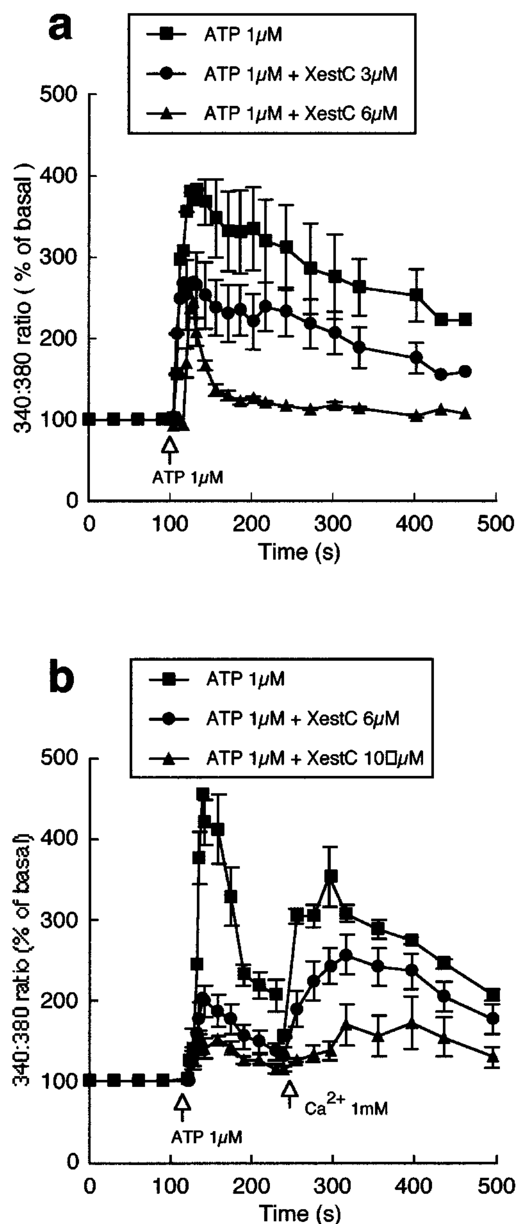


**Figure 4** Effects of xestospongin C on intracellular $Ca^{2+}$ in bovine endothelial cells. (a) Shows the concentration-dependent effects of xestospongin C on ATP-induced changes in intracellular $Ca^{2+}$. Studies were performed in the presence of extracellular $Ca^{2+}$. (b) Illustrates the effect of xestospongin C on ATP-induced $Ca^{2+}$ release in the absence of extracellular $Ca^{2+}$ and the effect of subsequent re-addition of $Ca^{2+}$ (1 mM) to the superfusate. Results are presented as mean$\pm$s.e.mean. For clarity, representative error bars are shown.

after 10 s of ATP exposure ($P<0.05$ compared to baseline). Results are presented as mean$\pm$s.e.mean for six coverslips from two independent experiments.

To verify, under cell free conditions, that 2APB did not inhibit $IP_3$ binding, concentration response curves for $IP_3$ and 2APB in displacing [$^3$H]-$IP_3$ from bovine adrenal cortex were prepared. 2APB (3–100 $\mu$M) did not result in a measurable alteration in the binding of [$^3$H]-$IP_3$ to the binding protein (data not shown).

## Discussion

The results of the present study are consistent with a mechanism of capacitative $Ca^{2+}$ entry in vascular endothelial cells which requires $IP_3$ receptor binding or an event distal to the activation of the ER receptor. This conclusion is based on the observation that 2APB and xestospongin C, putative $IP_3$ receptor antagonists or modulators, inhibit capacitative $Ca^{2+}$ entry induced by either agonists (ATP, bradykinin) or receptor-independent $Ca^{2+}$ mobilization (*via* ionomycin or thapsigargin). Control experiments demonstrated that 2APB did not lead to a reduction in $IP_3$ production or [$^3$H]-$IP_3$ binding. Further, the finding of similar effects of 2APB on $Ca^{2+}$ mobilization in endothelial cells from both bovine aorta and rat heart suggest that the findings are consistent across species and possibly between vascular sites.

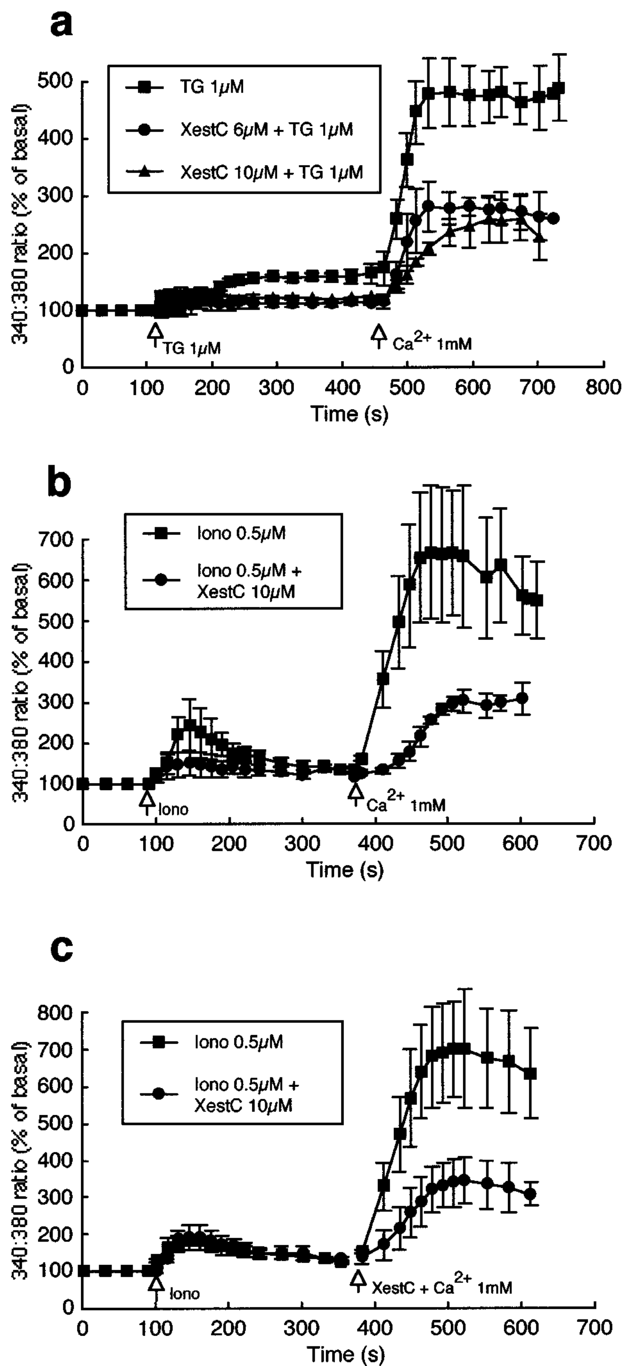


**Figure 5** Effects of xestospongin C on thapsigargin and ionomycin-induced $Ca^{2+}$ entry in bovine endothelial cells. (a) Illustrates the inhibitory effect of xestospongin C (6 and 10 $\mu$M; $n=5$ and 4 respectively) compared to control ($n=7$) on capacitative $Ca^{2+}$ entry induced by exposure of endothelial cells to thapsigargin (1 $\mu$M). (b) Shows the inhibitory effect of xestospongin C (10 $\mu$M) pretreatment on ionomycin (5 $\mu$M; $n=3$)-induced $Ca^{2+}$ entry compared with control ($n=3$). (c) Illustrates that xestospongin C (10 $\mu$M) added at the time of $Ca^{2+}$ addition to the superfusate also attenuates capacitative $Ca^{2+}$ entry ($n=4$ for each condition). Results are presented as mean$\pm$s.e.mean. For clarity, representative error bars are shown.

Consistent with a number of previous studies (for example Lynch *et al*., 1992; Vaca & Kunze, 1994; Wang & Van Breemen, 1997) exposure of endothelial cells to ATP or bradykinin resulted in a biphasic change in intracellular $Ca^{2+}$; an initial rapid increase that is a function of ER release and a sustained plateau that is, in part, dependent on $Ca^{2+}$ entry from the extracellular space. As endothelial cells lack voltage gated $Ca^{2+}$ channels, entry of this cation is considered to primarily occur through receptor/ligand gated channels and mechanisms related to the filling state of the ER, that is capacitative $Ca^{2+}$ entry (Barritt, 1999; Lin *et al*., 2000; Sedova *et al*., 2000). The existence of the latter in the present studies was suggested by the influx of $Ca^{2+}$ that occurred when the cation was returned to the superfusate of cells initially exposed to the agonists in the absence of extracellular $Ca^{2+}$. Further, when the ER $Ca^{2+}$ store was depleted by the ionophore, ionomycin, or the $Ca^{2+}$ ATPase inhibitor, thapsigargin, $Ca^{2+}$ entry was stimulated. As these latter compounds act on the filling state of the ER the data is consistent with a capacitative $Ca^{2+}$ entry mechanism.

Two principal mechanisms have been proposed for the coupling of the ER filling state to $Ca^{2+}$ entry; [1] that store depletion causes the release of a factor which acts to alter the gating properties of channels within the cell membrane (Randriamampita & Tsien, 1993; Thomas & Hanley, 1995) and [2] that store depletion results in a conformational change in an ER element which forms a direct or physical communication with the plasma membrane to allow $Ca^{2+}$ entry (Irvine, 1990; Berridge, 1995). Recent studies of Ma *et al*. (2000) have been used to support a model involving a physical association between the $IP_3$ receptor on the ER and a $Ca^{2+}$ entry channel on the plasma membrane (Berridge *et al*., 2000). The involvement of the $IP_3$ receptor was suggested from studies using both 2APB and xestospongin, while the physical association between the two compartments was suggested by studies altering the actin cytoskeleton. As in the present study, disruption of the cytoskeleton with cytochalasin D, in a cell line derived from pulmonary artery endothelium, was shown to inhibit capacitative $Ca^{2+}$ entry without an effect on the initial agonist-induced release of $Ca^{2+}$ (Holda & Blatter, 1997). Further support for a dynamic role of the actin cytoskeleton is provided by the studies of Rosado & Sage (2001) in human platelets, showing that both stabilization of the actin cytoskeleton with jasplakinolide and disruption by cytochalasin prevent capacitative $Ca^{2+}$ entry.

The exact role that the cytoskeleton appears to play in capacitative $Ca^{2+}$ entry appears to vary between cell types. In contrast to the findings of the present study, and those described above (Holda & Blatter, 1997; Rosado & Sage, 2001), Riberio *et al*. (1997) reported that in NIH-3T3 cells cytochalasin treatment specifically affects the agonist-induced release of $Ca^{2+}$ while having no effect on capacitative $Ca^{2+}$ entry. The differences, particularly in regard to the $Ca^{2+}$ release phase, may relate to the extent of cytochalasin treatment as Riberio *et al*. (1997) used a 10 $\mu$M concentration which markedly changed cell morphology while in the present study 100 nM and 1 $\mu$M concentrations of cytochalasin were employed. The lower concentrations were chosen to avoid gross changes in cell shape and detachment of the cells from the underlying matrix. In a study examining the role of capacitative $Ca^{2+}$ entry in regulation of adenylyl cyclase isoforms, of C6-2B rat glioma cells, Fagan *et al*. (1998) have suggested that

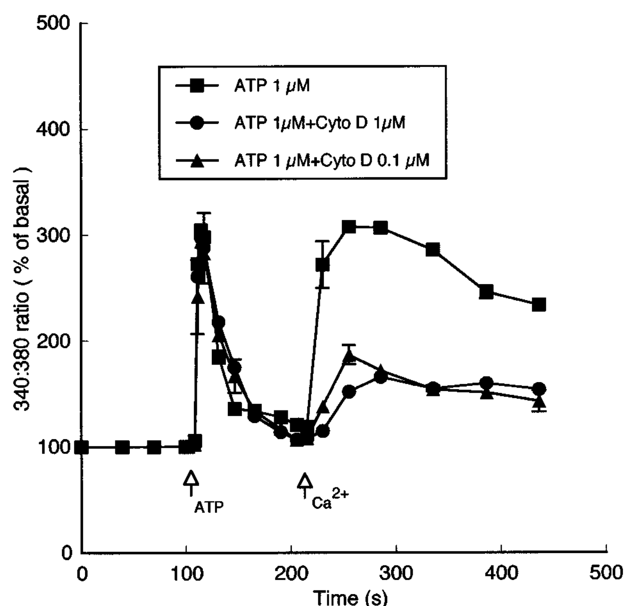


**Figure 6** Effects of cytoskeletal disruption on ATP-induced changes in intracellular $Ca^{2+}$. Cells were exposed to cytochalasin for 1 h at 37°C in a tissue culture incubator. As treatment did not cause a significant change in baseline flourescence ratio between groups, data are presented as normalized to per cent change from baseline prior to ATP stimulation. Exposure of cells to cytochalasin D (100 nM or 1 $\mu$M) had no apparent effect on the initial ATP-induced $Ca^{2+}$ release while significantly inhibiting the capacitative $Ca^{2+}$ entry phase ($n=6$ for each condition). Results are presented as mean$\pm$s.e.mean. For clarity, representative error bars are shown.

regulation of the cyclase requires an intimate association with the $Ca^{2+}$ entry pathway; although this relationship could not be altered by agents known to disrupt the cytoskeleton (cystochalasin, nocodozole, colchicine). Similarly to the studies of Riberio *et al.* (1997) cytoskeletal disruption did not impair capacitative $Ca^{2+}$ entry in response to thapsigargin treatment. In studies of embryonic kidney cells (HEK293) Ma *et al.* (2000) reported that condensation of cortical actin by treatment with the phosphatase inhibitor calyculin A impaired capacitative $Ca^{2+}$ entry while not affecting $IP_3$-mediated $Ca^{2+}$ release. Formation of a dense cortical actin layer was considered to impair the physical interaction between the plasma membrane and endoplasmic reticulum. This group, however, has previously reported that disassembly of the actin cytoskeleton with cytochalasin D did not impair coupling between $Ca^{2+}$ release and $Ca^{2+}$ entry (Patterson *et al.*, 1999). In addition to these studies of other investigators, we did not find cytochalasin treatment to markedly inhibit capacitative $Ca^{2+}$ entry in arteriolar smooth muscle (Potocnik & Hill, 2001). Thus, collectively the available data suggest that dependence of capacitative $Ca^{2+}$ entry on a functional cytoskeleton may vary between cell types. Conceivably the physical relationship between the endoplasmic reticulum and $Ca^{2+}$ entry channels is dependent on different structural elements in differing cell types. Alternatively contrasting results may reflect other factors such as differences in the $Ca^{2+}$ entry channels in the varying cell types (Putney, 1999) or variation in the amount/distribution of cytoskeletal elements.

The results of the present studies add to a growing body of evidence supporting a modulatory effect of 2APB on $IP_3$ receptor-mediated processes (Maruyama *et al.*, 1997; Ascher-Landsberg *et al.*, 1999; Gysembergh *et al.*, 1999; Ma *et al.*, 2000). With respect to ATP-induced $Ca^{2+}$ mobilization 2APB concentration-dependently attenuated both the initial release phase and subsequent $Ca^{2+}$ entry. 2APB did not, however, appear to have any effect on ATP-induced $IP_3$ production or basal $Ca^{2+}$ levels as assessed by changes in the 340 : 380 nm fluorescence ratio. In contrast to the latter observation Gysembergh *et al.* (1999) reported an effect of 2APB (1 – 10 $\mu$M) on rabbit cardiac myocytes, while Maruyama *et al.* (1997) found that concentrations greater than 90 $\mu$M 2APB were required to increase baseline $Ca^{2+}{}_i$ in a rat cerebral microsomal preparation and greater than 200 $\mu$M 2APB was required to increase basal $Ca^{2+}{}_i$ in human platelets. Whether these differences reflect tissue specific or methodological differences is currently uncertain.

Recent studies have suggested that 2APB may exert a direct inhibitory effect on capacitative entry at sites other than the $IP_3$ receptor. Consistent with this, 2APB has been shown to inhibit $Ca^{2+}$ entry in DT 40 B cells in which the $IP_3$ receptor has been deleted (Broad *et al.*, 2001), and in excised membrane patches from rat basophilic leukaemia cells (Braun *et al.*, 2001). In the present study, however, both putative $IP_3$ receptor antagonists, 2APB and xestospongin, inhibited endothelial cell capacitative $Ca^{2+}$ entry, suggesting that in this cell type the $IP_3$ receptor may indeed be a component of the capacitative $Ca^{2+}$ entry pathway. Consistent with these results, Rosado & Sage (2001) have recently shown that xestospongin inhibits capacitative $Ca^{2+}$ entry in human platelets and the association between $IP_3$ receptors and Trp1. No evidence currently exists for xestospongin exerting an effect directly at the level of Trp channels, although it has been suggested that in solution 2APB may dimerise thereby resembling the molecular shape of xestospongin C (Van Rossum *et al.*, 2000; Liu & Ambudkar, 2001). The apparently discrepant results may also relate to the particular $Ca^{2+}$ channel proteins (for example mammalian homologues of Trp channels (Putney, 1999)) or $IP_3$ receptor subtypes expressed in different cell types. With respect to the latter it has been shown in A7r5 cells that specific $IP_3$ receptor subtypes (namely the $IP_3R1$ subtype) are associated with capacitative $Ca^{2+}$ entry, while the $IP_3R3$ subtype appears unrelated to this mode of $Ca^{2+}$ entry (Wang *et al.*, 2001).

In more recent studies Ma *et al.* (2001) have suggested that 2APB, while being an $IP_3$ receptor antagonist, may also interact with a regulatory protein that exerts an action over both $IP_3$ receptors and capacitative $Ca^{2+}$ entry channels. Such an effect could be consistent with both the data from the present studies and those suggesting an inhibitory effect of 2APB proximal to the ER in this signalling pathway.

That 2APB inhibited store-depletion mediated $Ca^{2+}$ entry in the present studies was demonstrated firstly by the addition of 2APB to ATP-stimulated endothelial cells following the initial release peak (Figure 1) and secondly by inhibition of $Ca^{2+}$ entry following exposure of cells to ionomycin (Figure 2) and thapsigargin (Figure 3). In the latter case $Ca^{2+}$ depletion of the ER occurs independently of plasma membrane receptor activation. Somewhat surprisingly, 2APB reduced the initial $Ca^{2+}$ release caused by 5 $\mu$M ionomycin while not affecting the initial increase in $Ca^{2+}{}_i$

induced by either 0.5 $\mu$M ionomycin or thapsigargin (1 $\mu$M). Conceivably the higher concentration of the ionophore, as well as depleting intracellular $Ca^{2+}$ stores, exerts an additional effect at the level of the ER membrane. Alternatively, at the higher concentration of ionomycin unidentified effects are evident; perhaps related to the greater release of $Ca^{2+}$ seen under this condition (relative to 0.5 $\mu$M ionomycin). Regardless of this effect, the data presented are consistent with 2APB inhibiting store depletion mediated $Ca^{2+}$ entry in endothelial cells.

An alternate explanation of the data relates to the functional relationship between ryanodine release channels of the ER and plasma membrane $K_{Ca}$ channels. $Ca^{2+}$ release from the ER into the subplasmalemmal space has been shown to activate $K_{Ca}$ channels, resulting in membrane hyperpolarization (Frieden & Graier, 2000). This change in membrane potential subsequently increases the driving force for $Ca^{2+}$ entry and has been implicated in agonist-induced production of a number of $Ca^{2+}$-dependent vasoactive compounds (Luckhoff & Busse, 1990a). Support of this pathway has also been derived from the observation that $K^+$ channel activators lead to hyperpolarization and $Ca^{2+}$ entry in endothelial cells (Luckhoff & Busse, 1990b). Analogous to the data obtained in the present studies disruption of the normal relationship between the superficial ER and the plasma membrane with nocodozole prevented ryanodine-induced activation of $K_{Ca}$ channels in a human umbilical vein endothelial cell line (Frieden & Graier, 2000). While it is possible that such a mechanism would have been disrupted in the current studies, by exposure of cells to cytochalasin, it appears unlikely that both the 2APB (see also Maruyama *et al*., 1997) and xestospongin data could be explained simply by a ryanodine receptor-$Ca^{2+}$ entry pathway. Further, if the endothelial cells studied exhibit both $IP_3$ receptor-dependent mechanisms and $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) (Mozhayeva, 1996) the effectiveness of 2APB and xestospongin C in preventing $Ca^{2+}$ entry suggests that the $IP_3$ receptor dependent processes occur proximally to CICR.

In summary, the data presented demonstrate the effectiveness of 2APB and xestospongin C, putative modulators of the $IP_3$ receptor, in inhibiting agonist-induced $Ca^{2+}$ entry in vascular endothelial cells. Further, the fact that cytoskeletal disruption with cytochalasin specifically inhibited the $Ca^{2+}$ influx phase, as opposed to the release component, adds support to the requirement of a physical coupling, or spatial proximity, for the coupling of store depletion to $Ca^{2+}$ entry in endothelial cells. Differences in the effect of agents such as cytochalasin on $Ca^{2+}$ entry, however, suggest that variation in the exact coupling mechanism may exist between cell types. Given the ability of the endothelial cell in responding to physiological forces such as shear stress (Davies *et al*., 1997) and the fact that the cytoskeleton is implicated in mechanotransduction (Ingber, 1997), these results may be of direct relevance to physiological mechanisms of endothelial cell autacoid production.

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## References


ASCHER-LANDSBERG, J., SAUNDERS, T., ELOVITZ, M. & PHILLIPPE, M. (1999). The effects of 2-aminoethoxydiphenyl borate, a novel inositol 1,4,5-trisphosphate receptor modulator on myometrial contractions. *Biochem. Biophys. Res. Comm.*, **264,** 979–982.

BABNIGG, G., BOWERSOX, S.R. & VILLEREAL, S.R. (1997). The role of $pp60^{c\text{-}src}$ in the regulation of calcium entry via store-operated calcium channels. *J. Biol. Chem.*, **272,** 29434–29437.

BARRITT, G.J. (1999). Receptor-activated $Ca^{2+}$ inflow in animal cells: a variety of pathways tailored to meet different $Ca^{2+}$ signalling requirements. *Biochem. J.*, **337,** 153–169.

BERRIDGE, M.J. (1995). Capacitative calcium entry. *Biochem. J.*, **312,** 1–11.

BERRIDGE, M.J., LIPP, P. & BOOTMAN, M.D. (2000). The calcium entry pas de deux. *Science,* **287,** 1064–1065.

BRAUN, F-J., BROAD, L.M., ARMSTRONG, D.L. & PUTNEY, JR., J.W. (2001). Stable activation of single $Ca^{2+}$ release-activated $Ca^{2+}$ channels in divalent cation-free solutions. *J. Biol. Chem.,* **276,** 1063–1070.

BROAD, L.M., BRAUN, F-J., LIEVREMONT, J-P., BIRD, G.ST.J., KUROSAKI, T. & PUTNEY, JR., J.W. (2001). Role of the phospholipase C-inositol 1,4,5-trisphosphate pathway in calcium release-activated calcium current and capacitative calcium entry. *J. Biol. Chem.*, **276,** 15945–15952.

DAVIES, P.F., BARBEE, K.A., VOLIN, M.V., ROBOTEWSKYJ, A., CHEN, J., JOSEPH, L., GRIEM, M.L., WERNICK, M.N., JACOBS, E., POLACEK, D.C., DEPAOLA, N. & BARAKAT, A.I. (1997). Spatial relationships in early signaling events of flow-mediated endothelial mechanotransduction. *Annu. Rev. Physiol.*, **59,** 527–549.

FAGAN, K.A., MONS, N. & COOPER, D.M.F. (1998). Dependence of the $Ca^{2+}$-inhibitable adenylyl cyclase of C6-2B glioma cells on capacitative $Ca^{2+}$ entry. *J. Biol. Chem.*, **273,** 9297–9305.

FASOLATO, C. & NILIUS, B. (1998). Store depletion triggers the calcium release-activated calcium current (ICRAC) in macrovascular endothelial cells: a comparison with Jurkat and embyonic kidney cell lines. *Pflügers Arch.*, **436,** 69–74.

FLEMING, I., FISSLTHALER, B. & BUSSE, R. (1996). Interdependence of calcium signaling and protein tyrosine phosphorylation in human endothelial cells. *J. Biol. Chem.*, **271,** 11009–11015.

FRIEDEN, M. & GRAIER, W.F. (2000). Subplasmalemmal ryanodine-sensitive $Ca^{2+}$ release contributes to $Ca^{2+}$-dependent $K^+$ channel activation in a human umbilical vein endothelial cell line. *J. Physiol.*, **524,** 715–724.

FORD, D.A. & ROVETTO, M.J. (1987). Rat cardiac myocyte adenosine transport and metabolism. *Am. J. Physiol.*, **252,** H54–H63.

GAFNI, J., MUNSCH, J.A., LAM, T.H., CATLIN, M.C., COSTA, L.G., MOLINSKI, T.F. & PESSAH, I.N. (1997). Xestospongins: potent membrane permeable blockers of the inositol 1,4,5-trisphosphate receptor. *Neuron,* **19,** 723–733.

GYSEMBERGH, H., LEMAIRE, S., PIOT, C., SPORTOUCH, C., RICHARD, S., KLONER, R.A. & PRZYKLENK, K. (1999). Pharmacological manipulation of $Ins(1,4,5)P_3$ signaling mimics preconditioning in rabbit heart. *Am. J. Physiol.*, **277,** H2458–H2469.

HOFMANN, T., OBUKHOV, A.G., SCHAEFER, M., HARTENECK. C., GUDERMANN, T. & SCHULTZ, G. (1999) Diect activation of human TRPC6 and TRPC3 channels by diacylglycerol. *Nature,* **397,** 259–263.

HOLDA, J.R. & BLATTER, L.A. (1997). Capacitative calcium entry is inhibited in vascular endothelial cells by disruption of cytoskeletal microfilaments. *FEBS Lett.*, **403,** 191–196.

INGBER, D.E. (1997). Tensegrity: the architectural basis of cellular mechanotransduction. *Annu. Rev. Physiol.*, **59,** 575–599.

IRVINE, R.F. (1990). ''Quantal'' $Ca^{2+}$ release and the control of $Ca^{2+}$ entry by inositol phosphates – a possible mechanism. *FEBS Lett.*, **263,** 5–9.

LIN, S., FAGAN, K.A., LI, K-X., SHAUL, P.W., COOPER, D.M.F. & RODMAN, D.M. (2000). Sustained endothelial nitric oxide synthase activation requires capacitative $Ca^{2+}$ entry. *J. Biol. Chem.*, **275,** 17979–17985.

LIU, X. & AMBUDKAR, I.S. (2001). Characteristics of a store-operated calcium-permeable channel, SOCC: sarcoendoplasmic reticulum calcium pump function controls channel gating. *J. Biol. Chem.*, **276,** 29891–29898.

LOUZAO, M.C., RIBEIRO, C.M.P., BIRD, G.ST.J. & PUTNEY, JR., J.W. (1996). Cell type-specific modes of feedback regulation of capacitative calcium entry. *J. Biol. Chem.*, **271,** 14807–14813.

LUCKHOFF, A. & BUSSE, R. (1990a). Calcium influx into endothelial cells and formation of endothelium-derived relaxing factor is controlled by membrane potential. *Pflügers Arch.*, **416,** 305–311.

LUCKHOFF, A. & BUSSE, R. (1990b). Activators of potassium channels enhance calcium influx into endothelial cells as a consequence of potassium currents. *Naunyn Schmiedebergs Arch. Pharmacol.*, **342,** 94–99.

LYNCH, M., GILLESPIE, J.I., GREENWELL, J.R. & JOHNSON, C. (1992). Intracellular calcium 'signatures' evoked by different agonists in isolated bovine aortic endothelial cells. *Cell Calcium,* **13,** 227–233.

MA, H-T., PATTERSON, R.L., VAN ROSSUM, D.B., BIRNBAUMER, L., MIKOSHIBA, K. & GILL, D.L. (2000). Requirement of the inositol trisphosphate receptor for activation of store-operated $Ca^{2+}$ channels. *Science,* **287,** 1647–1651.

MA, H.-T., VENKATACHALAM, K., LI, H.-S., MONTELL, C., KUROSAKI, T., PATTERSON, R.L. & GILL, D.L. (2001). Assessment of the role of the inositol 1,4,5-trisphosphate receptor in the activation of transient receptor potential channels and store-operated $Ca^{2+}$ entry channels. *J. Biol. Chem.*, **276,** 18888–18896.

MARTIN, T.W. & MICHAELIS, K.C. (1990). $Ca^{2+}$-dependent synthesis of prostaglandin $I_2$ and mobilization of arachidonic acid from phospholipids in cultured endothelial cells permeabilized with saponin. *Biochim. Biophys. Acta.*, **1054,** 159–168.

MARUYAMA, T., KANAJI, T., NAKADE, S., KANNO,T. & MIKOSHIBA, K. (1997). 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of ins $(1,4,5)P_3$-induced $Ca^{2+}$ release. *J. Biochem (Tokyo),* **122,** 498–505.

MERY, L., MAGNINO, F., SCHMIDT, K., KRAUSE, K.H. & DUFOUR, J.F. (2001). Alternative splice variants of hTrp4 differentially interact with the C-terminal portion of the inositol 1,4,5-trisphosphate receptors. *FEBS Lett.*, **487,** 377–383.

MIZUNO, O., KOBAYASHI, S., HIRANO, K., NISHIMURA, J., KUBO, C. & KANAIDE, H. (2000). Stimulus-specific alteration of the relationship between cytosolic $Ca^{2+}$ transients and nitric oxide production in endothelial cells ex vivo. *Br. J. Pharmacol.,* **130,** 1140–1146.

MOZHAYEVA, M.G. (1996). $[Ca^{2+}]_i$ elevation evoked by $Ca^{2+}$ readdition to the medium after agonist-induced $Ca^{2+}$ release can involve both $IP_3$-, and ryanodine-sensitive $Ca^{2+}$ release. *Pflügers Arch.*, **433,** 180–187.

PAREKH, A.B. & PENNER, R. (1997). Store depletion and calcium influx. *Physiol. Rev.*, **77,** 901–930.

PATTERSON, R.L., VAN ROSSUM, D.B. & GILL, D.L. (1999). Store-operated $Ca^{2+}$ entry: evidence for a secretion-like coupling model. *Cell,* **98,** 487–499.

POTOCNIK, S.J. & HILL, M.A. (2001). Pharmacological evidence for capacitative $Ca^{2+}$ entry in cannulated and pressurized skeletal muscle arterioles. *Brit. J. Pharmacol.*, **134,** 247–256.

PURKISS, J.R., WILKINSON, G.F. & BOARDER, M.R. (1994). Differential regulation of inositol 1,4,5-trisphosphate by co-existing $P_2Y$-purinoceptors and nucleotide receptors on bovine aortic endothelial cells. *Br. J. Pharmacol.*, **111,** 723–728.

PUTNEY, JR., J.W. (1990). Capacitative calcium entry revisited. *Cell Calcium,* **11,** 611–624.

PUTNEY, JR., J.W. (1999). TRP, inositol 1,4,5-trisphosphate receptors, and capacitative calcium entry. *Proc. Natl Acad. Sci.*, **96,** 14669–14671.

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.

RIBERIO, C.M.P., REECE, J. & PUTNEY, JR., J.W. (1997). Role of the cytoskeleton in calcium signaling in NIH 3T3 cells: An intact cytoskeleton is required for agonist-induced $[Ca^{2+}]_i$ signaling, but not for capacitative calcium entry. *J. Biol. Chem.*, **272,** 26555–26561.

ROSADO, J.A. & SAGE, S.O. (2001). Activation of store-mediated calcium entry by secretion-like coupling between the inositol 1,4,5-trisphosphate receptor type II and human transient receptor potential (hTrp1) channels in human platelets. *Biochem. J.*, **256,** 191–198.

RZIGALINSKI, B.A., WILLOUGHBY, K.A., HOFFMAN, S.W., FALCK, J.R. & ELLIS, E.F. (1999). Calcium influx factor, further evidence it is 5,6,-epoxyeicosatrienoic acid. *J. Biol. Chem.*, **274,** 175–182.

SCHILLING, W.P., CABELLO, O.A. & RAJAN, L. (1992). Depletion of the inositol 1,4,5-trisphosphate-sensitive intracellular $Ca^{2+}$ store in vascular endothelial cells activates the agonist-sensitive $Ca^{2+}$ influx pathway. *Biochem. J.*, **284,** 521–530.

SEDOVA, M., KLISHIN, A., HUSER, J. & BLATTER, L.A. (2000). Capacitative $Ca^{2+}$ entry is graded with degree of intracellular $Ca^{2+}$ store depletion in bovine vascular endothelial cells. *J. Physiol.*, **523,** 549–559.

THOMAS, D. & HANLEY, M.R. (1995). Evaluation of calcium influx factors from stimulated Jurkat T-lymphocytes by microinjection into *Xenopus* oocytes. *J. Biol. Chem.*, **270,** 6429–6432.

VACA, L. & KUNZE, D.L. (1994). Depletion of intracellular $Ca^{2+}$ stores activates a $Ca^{2+}$ selective channel in vascular endothelium. *Am. J. Physiol.*, **267,** C950–C955.

VAN ROSSUM, D.B., PATTERSON, R.L., MA, H.-T. & GILL, D.L. (2000). $Ca^{2+}$ entry mediated by store depletion, s-nitrosylation, and TRP3 channels. *J. Biol. Chem.*, **275,** 28562–28568.

WANG, X. & VAN BREEMEN, C. (1997). Multiple mechanisms of activating $Ca^{2+}$ entry in freshly prepared rabbit aortic endothelial cells. *J. Vasc. Res.*, **34,** 196–207.

WANG, Y., CHEN, J., WANG, Y., TAYLOR, C.W., HIRATA, Y., HAGIWARA, H., MIKOSHIBA, K., TOYO-OKA, T., OMATA, M. & SAKAKI, Y. (2001). Crucial role of type 1, but not type 3, inositol 1,4,5-trisphosphate ($IP_3$) receptors in $IP_3$-induced $Ca^{2+}$ release, capacitative $Ca^{2+}$ entry, and proliferation of A7r5 vascular smooth muscle cells. *Circ. Res.*, **88,** 202–209.

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