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# Propofol regulation of calcium entry pathways in cultured A10 and rat aortic smooth muscle cells

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**1** We have investigated the effect of propofol, an intravenous anaesthetic, on the intracellular calcium concentration ($[Ca^{2+}]_i$), $Ca^{2+}$ entry pathways and on inositol phosphate formation in vascular smooth muscle cells. $[Ca^{2+}]_i$ and $Ca^{2+}$ flux were monitored with the $Ca^{2+}$-sensitive fluorescent dye, fura-2, and by $^{45}Ca^{2+}$ uptake. Production of labelled inositol phosphates was analysed by anion-exchange chromatography.

**2** Treatment of the cells with endothelin-1 (ET-1) increased formation of inositol phosphates and elevated $[Ca^{2+}]_i$ due to both release of $Ca^{2+}$ from intracellular pools and prolonged entry of $Ca^{2+}$ from outside the cell. Propofol reduced production of inositol phosphates mediated by ET-1 and arginine vasopressin which activate phospholipase C.

**3** The sustained $Ca^{2+}$ entry stimulated by ET-1 was found to occur through the activation of L-type Ca channels. This was inhibited by propofol in a dose-dependent manner.

**4** Activation of protein kinase C (PKC) by phorbol esters activated a pharmacologically-similar channel and produced a similar change in $[Ca^{2+}]_i$ due to $Ca^{2+}$ entry. The entry was blocked by an L-type channel antagonist, nicardipine and by the anaesthetic drug, propofol.

**5** Treatment of the cells with thapsigargin, a selective inhibitor of the sarcoplasmic reticulum $Ca^{2+}$-ATPase, also elevated $[Ca^{2+}]_i$ by inducing the release of intracellular $Ca^{2+}$ and the continued entry of extracellular $Ca^{2+}$ through a nicardipine-insensitive Ca channel. Neither release nor entry induced by thapsigargin was affected by propofol.

**6** These findings suggest that propofol selectively inhibits $Ca^{2+}$ entry through the L-type channel induced by ET-1 and phorbol esters but has no effects on $Ca^{2+}$ entry via the nicardipine-insensitive channel and on $Ca^{2+}$ release from intracellular pools initiated by thapsigargin. This may represent one of the mechanisms responsible for propofol-induced vasodilatation.

**Keywords:** Anaesthetics; endothelin-1; calcium channels; phospholipase C; protein kinase C; thapsigargin; vascular smooth muscle cell

## Introduction

Propofol (2,6-diisopropylphenol) is a widely-used intravenous anaesthetic with a rapid onset, short duration of action and rapid elimination (Sebel & Lowdon, 1989). Propofol also causes marked decreases in systemic arterial blood pressure which correlate with its plasma concentration (Coates *et al.*, 1987; Brussel *et al.*, 1989). It has been suggested that the hypotensive effect results from decreases in both cardiac output and peripheral vascular resistance (Bentley *et al.*, 1989; Rouby *et al.*, 1991; Cook & Housmans, 1994). The decreased resistance has been ascribed to its direct vasodilator effect on the vascular smooth muscle since propofol relaxes preconstricted arteries in endothelium-denuded aortic rings and attenuates $Ca^{2+}$-dependent contraction induced by $K^+$ depolarization (Bentley *et al.*, 1989) and by agonists (Park *et al.*, 1992; Yamanoue *et al.*, 1994). Thus, it has been suggested that propofol may relax smooth muscle by interfering with $Ca^{2+}$ mobilization. However, the direct effects of propofol on $Ca^{2+}$ mobilization, $Ca^{2+}$ entry and Ca channels in smooth muscle cells have not been investigated.

It is well known that intracellular free $Ca^{2+}$ is an important determinant in the regulation of smooth muscle contraction and relaxation. Increased intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) activates myosin light chain kinase through a calmodulin-dependent mechanism (Somlyo & Himpens, 1989; Van Breemen, 1989). The active kinase phosphorylates myosin light chain leading to initiation of contraction. The sustained elevation of $[Ca^{2+}]_i$ above basal levels is known to be coupled to elevated tone in vascular smooth muscle (Van Breemen, 1989). A number of mechanisms play a role in regulating $Ca^{2+}$ mobilization. These include the release of $Ca^{2+}$ from the sarcoplasmic reticulum, $Ca^{2+}$ entry across the plasma membrane and pumping $Ca^{2+}$ into the sarcoplasmic reticulum by $Ca^{2+}$-ATPase or out of the cell by the sarcolemmal $Ca^{2+}$ATPase (Rasmussen *et al.*, 1987; Van Breemen, 1989).

Endothelin-1 (ET-1) is a 21-amino acid peptide, synthesized primarily by the vascular endothelium, which produces a slowly-developing, sustained contraction (Yanagisawa *et al.*, 1988). In vascular smooth muscle, the ET-1 interaction at the $ET_A$ receptor elevates $[Ca^{2+}]_i$ due to both an initial release of $Ca^{2+}$ from the intracellular stores and a prolonged entry of extracellular $Ca^{2+}$ through L-type channels (Goto *et al.*, 1989; Xuan *et al.*, 1991). ET-1 stimulates phospholipase C, the formation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), the generation of diacylglycerol and PKC activity (Renterghem *et al.*, 1988; Xuan *et al.*, 1989; 1994). $Ins(1,4,5)P_3$ is known to regulate $Ca^{2+}$ release from the sarcoplasmic reticulum (Berridge & Irvine, 1989; Xuan *et al.*, 1991). The mechanisms involved in $Ca^{2+}$ influx are largely unknown, although $Ca^{2+}$ can enter the cell through both L-type Ca channels and receptor-operated Ca channels (Van Breemen, 1989). The L-type channel is inhibited by classic Ca channel blockers such as dihydropyridines. In vascular smooth muscle it has been suggested that membrane depolarization (Renterghem *et al.*, 1988) and PKC (Fish *et al.*, 1988; Xuan *et al.*, 1994) are involved in the activation of the L-type channel, although the exact mechanism remains unclear. Activation of PKC stimulates $Ca^{2+}$ influx

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and produces a slowly-developing, sustained contraction (Rasmussen *et al.*, 1987; Fish *et al.*, 1988; Xuan *et al.*, 1994). In other systems, inositol phosphates (Vilven & Coronado, 1988; Berridge & Irvine, 1989), $Ca^{2+}$ (Takemura *et al.*, 1989), GTP-binding proteins (Yatani *et al.*, 1987; Rosenthal *et al.*, 1988; Kitazama *et al.*, 1989) and PKC (Lacerda *et al.*, 1988) have been reported to modulate the Ca channel activity.

More recently it has been shown that thapsigargin, a selective inhibitor of the endoplasmic and sarcoplasmic reticulum $Ca^{2+}$-ATPases, activates $Ca^{2+}$ entry in many non-excitable cell types (Takemura *et al.*, 1989; Thastrup, 1990) and smooth muscle cells (Xuan *et al.*, 1992). Thapsigargin has proved to be a useful tool since it elevates $[Ca^{2+}]_i$ by mobilizing intracellular $Ca^{2+}$ and initiating $Ca^{2+}$ entry through dihydropyridine-insensitive Ca channels without stimulating inositol phosphate formation (Takemura *et al.*, 1989; Xuan *et al.*, 1992).

The aims of the present study were to determine the effects of propofol on $[Ca^{2+}]_i$ and inositol phosphate formation and to investigate $Ca^{2+}$ entry pathways through the L-type channel induced by ET-1 and phorbol esters and via the nicardipine-insensitive Ca channel initiated by thapsigargin in vascular smooth muscle cells.

## Methods

### *Cell culture*

A10 smooth muscle cells were purchased from American Type Culture Collection (Rockville, MD, U.S.A.). Primary cultures of smooth muscle cells were isolated from rat aortae and identified as smooth muscle by immunofluorescence staining by use of a smooth muscle-specific α-actin monoclonal antibody and a FITC anti-mouse IgG as previously described (Xuan *et al.*, 1992). Cells were grown in DMEM containing 10% FCS at 37°C in an atmosphere of 5% $CO_2$–95% air, and the medium was changed every 2–3 days. For subculture, the cells were removed from the flasks by incubating with 0.25% trypsin solution containing 0.25% ethylenediaminetetraacetic acid (EDTA) (pH 7.5). To monitor the changes in $[Ca^{2+}]_i$, cells were grown in glass coverslips (1.25 × 3.0 cm) by seeding with $5 \times 10^4$ cells/slide in DMEM containing 10% FCS and allowed to grow to confluence (Xuan *et al.*, 1991).

### *Measurement of cytosolic $Ca^{2+}$ concentration*

$[Ca^{2+}]_i$ was monitored by previously-described methods (Xuan *et al.*, 1991). Confluent monolayers of cells on glass coverslips were incubated with 20 μM fura-2 AM for 1 h at 37°C in a HEPES-buffered Hank's balanced salt solution (HHBSS composition, mM: NaCl 120, KCl 4.7, $CaCl_2$ 1, $MgCl_2$ 1, $KH_2PO_4$ 0.3, $Na_2HPO_4$ 0.3, $NaHCO_3$ 4, HEPES 10, pH 7.4) containing 0.1% BSA. The slides were then rinsed and placed in a 3-ml cuvette across the diagonal. Inlet and outlet lines were passed through a rubber stopper and positioned so that the inlet was at the bottom and the outlet at the top of the cuvette. The cuvette was placed in a heated (37°C) sample compartment of an LS-50s spectrofluorometer (Perkin Elmer) and perfused at 3 ml $min^{-1}$ with HHBSS or with a similar buffer which contained 1 mM EGTA but no $Ca^{2+}$ ($Ca^{2+}$-free HHBSS). The fura-2-loaded cells were excited alternately at 340 nm and 380 nm. Relative fluorescence at 510 nm was measured for each wavelength, and the ratio between the relative fluorescence at 340 nm and 380 nm determined. Cells were stimulated with ET-1 by stopping the perfusion and passing 20 ml of either buffer containing the agonist through the cuvette (20 ml $min^{-1}$) to bring the concentration of the agent rapidly to the desired value. The perfusion was restarted after 1 min. $[Ca^{2+}]_i$ was calculated by the procedures of Grynkiewicz *et al.* (1985). The use of these methods in monitoring changes in $[Ca^{2+}]_i$ in smooth muscle cells has been thoroughly discussed elsewhere (Somlyo & Himpens, 1989).

### *Determination of $^{45}Ca^{2+}$ uptake*

Confluent cells grown on six-well plates (9 $cm^2$ well) were incubated with HHBSS plus 0.25% BSA (pH 7.4) containing 3 μCi $ml^{-1}$ of $^{45}Ca^{2+}$ plus or minus agonists for the time periods indicated. To determine the effect of propofol on $^{45}Ca^{2+}$ influx, propofol was added to the cells 5 min prior to addition of agonists. $^{45}Ca^{2+}$ uptake was terminated by removing the incubation buffer, quickly washing the cells with ice-cold buffer, and adding 0.5 ml of 0.1 N HCl to disrupt the cells and solubilize the labelled $Ca^{2+}$. Aliquots were taken to determine $^{45}Ca^{2+}$ uptake as previously published procedures (Xuan *et al.*, 1992).

### *Production of inositol phosphates*

Production of inositol polyphosphates in response to agonists and propofol was determined by using a modification of our previously-described procedures (Xuan *et al.*, 1989). Cells grown on six-well plates were incubated in medium containing [$^3$H]-inositol (10 μCi $ml^{-1}$) for 24 h. Labelled cells were washed with HBSS containing 10 mM lithium chloride (pH 7.4) and preincubated with the same buffer for 10 min. Propofol was added to the cells 5 min prior to addition of agonists. After treatments, labelled inositol phosphates were extracted and analysed as previously described. Retention times were verified by use of labelled standards.

### *Materials*

ET-1 and arginine vasopressin (AVP) were purchased from Peninsula Laboratories (Belmont, CA, U.S.A.); thapsigargin from LC Services (Woburn, MA, U.S.A.); fura-2 acetoxymethyl ester (fura-2 AM) from Behring Diagnostics (San Diego, CA, U.S.A.) and Dulbecco's modified Eagle's medium (DMEM) from Grand Island Biological Co. (Grand Island, NY, U.S.A.). Propofol solution is a 100% pure preparation provided by Zeneca Pharmaceuticals (Wilmington, Delaware, U.S.A.). $^{45}Ca^{2+}$ (40 mCi $mg^{-1}$) was obtained from Amersham (Arlington Heights, IL, U.S.A.). Foetal calf serum (FCS), bovine serum albumin (BSA), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), nicardipine, phorbol 12-myristate 13-acetate (PMA), 4α-phorbol-12,13-didecanoate (4α-PDD), and 4β-phorbol-12,13-didecanoate (4α-PDD) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

### *Statistical analysis*

Data were analysed by either one- or two-way analysis of variance. Multiple comparisons were made with either Newman-Keuls' or Dunnett's *post hoc* tests. Data are given throughout as mean ± s.e.mean.

## Results

### *Effect of propofol on ET-1-mediated $Ca^{2+}$ entry through nicardipine-sensitive Ca channels*

As shown in Figure 1a, when fura-2-loaded cells were perfused with $Ca^{2+}$-free HHBSS, ET-1 induced a transient rise in $[Ca^{2+}]_i$, which rapidly returned to basal levels within 2 min. This represents release of $Ca^{2+}$ from intracellular $Ca^{2+}$ stores. When the cells were perfused with HHBSS, ET-1 induced a rapid rise of $[Ca^{2+}]_i$ which was followed by a sustained phase. During the sustained phase, $[Ca^{2+}]_i$ remained elevated above baseline for at least 18 min. The difference between the two curves represents entry of extracellular $Ca^{2+}$.

The $Ca^{2+}$ entry was further investigated by determining the effects of $Ni^{2+}$ (a non-specific Ca channel blocker) and the L-type channel antagonist, nicardipine, on ET-1-stimulated $^{45}Ca^{2+}$ influx. As shown in Figure 1b, both $Ni^{2+}$ and nicardipine effectively blocked ET-1-stimulated $^{45}Ca^{2+}$ entry,

suggesting that ET-1 activates entry primarily through the L-type channel. This confirmed our previous findings that ET-1stimulated $Ca^{2+}$ entry, especially the sustained entry, was dependent on L-type channels (Xuan *et al.*, 1991).

When propofol was added to cells during the sustained phase of ET-1-induced $Ca^{2+}$ responses, the elevated $[Ca^{2+}]_i$ was rapidly returned toward baseline (Figure 2a). Propofol alone did not change basal $[Ca^{2+}]_i$ in cells perfused with either HHBSS or $Ca^{2+}$-free HHBSS (data not shown). Furthermore, treatment of the cells with propofol inhibited ET-1-stimulated $^{45}Ca^{2+}$ entry (Figure 2b). Again, propofol alone did not modify basal $^{45}Ca^{2+}$ uptake. This inhibitory effect of propofol on ET-1-stimulated $^{45}Ca^{2+}$ uptake was dose-dependent (5.6–56 $\mu$M) with an $IC_{50}$ of 28 $\mu$M (Figure 3), which is closely in agreement with the range of serum levels during induction of clinical anaesthesia (Shafer *et al.*, 1988).

### *Effect of propofol on $Ca^{2+}$ entry through nicardipine-sensitive Ca channels activated by PKC*

To explore the mechanism involved in the inhibition of ET-1-stimulated $Ca^{2+}$ influx through the nicardipine-sensitive channel by propofol, we next studied the effect of this compound on $Ca^{2+}$ entry through the L-type channel activated by phorbol esters. Phorbol esters, through activation of PKC, induce $Ca^{2+}$ entry through the L-type channels without stimulating the formation of inositol phosphates (Xuan *et al.*, 1994). Treatment of the cells with the active isomer, 4$\beta$-PDD, induced a slow rise in $[Ca^{2+}]_i$ followed by a sustained elevation of $[Ca^{2+}]_i$ while the inactive isomer (4$\alpha$-PDD) did not (Figure 4a). As shown in Figure 4b, treatment of the cells with PMA also elevated $[Ca^{2+}]_i$ in cells bathing with HHBSS and this effect was inhibited by propofol. PMA did not elevate $[Ca^{2+}]_i$ in cells perfusing $Ca^{2+}$-free HHBSS (data not shown), indicating that the phorbol ester elevated $[Ca^{2+}]_i$ by activating $Ca^{2+}$ entry. In addition, propofol was found to reduce PMA-stimulated $^{45}Ca^{2+}$ entry in both A10 cells and primary cultures of rat aortic smooth muscle cells (Figure 5).

### *Effect of propofol on thapsigargin-initiated nicardipine-insensitive $Ca^{2+}$ entry*

As seen in Figure 6a, when cells were superfused with HHBSS and treated with 0.2 $\mu$M thapsigargin, we observed a slow rise in $[Ca^{2+}]_i$ which reached a peak value within 10 min and remained elevated for a long period of time. This response differed from the ET-1-induced elevation of $[Ca^{2+}]_i$ which rapidly reached a peak response (within 30 s). However, the sustained elevation of $[Ca^{2+}]_i$ seen following thapsigargin was also de-

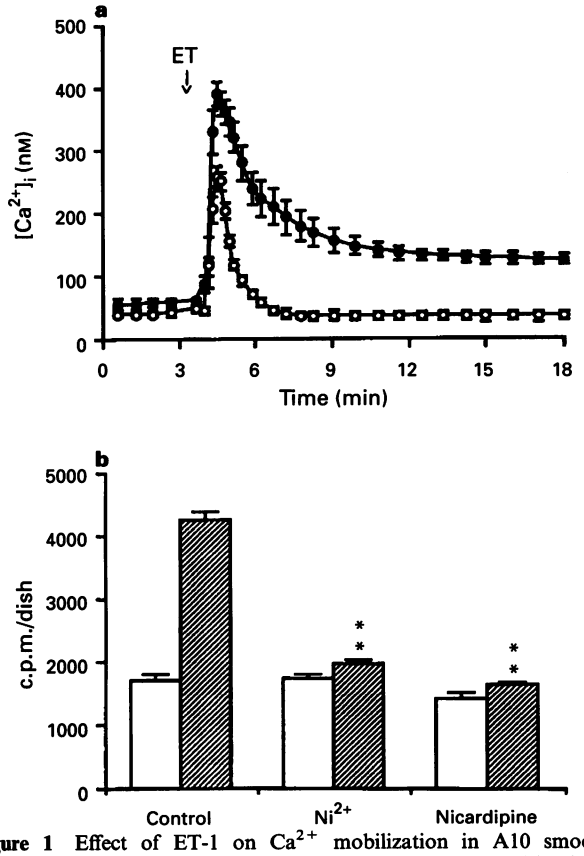


**Figure 1** Effect of ET-1 on $Ca^{2+}$ mobilization in A10 smooth muscle cells. (a) Fura-2-loaded cells were superfused with either HHBSS (●) or $Ca^{2+}$-free HHBSS (○). At the times indicated, the cells were rapidly exposed to 10 nM ET-1 (see Methods). After 1 min, perfusion was re-started and ET-1 was washed from the cuvette. Data are given as mean ± s.e.mean, $n=6$. (b) Effects of $Ni^{2+}$ and nicardipine on ET-1-stimulated $^{45}Ca^{2+}$ uptake in A10 cells. Cells were preincubated with HHBSS in the absence (Control) or presence of 1 mM $Ni^{2+}$ or 1 $\mu$M nicardipine as indicated. After 5 min, $^{45}Ca^{2+}$ (3 $\mu$Ci ml$^{-1}$) was added to the cells and $^{45}Ca^{2+}$ uptake was initiated by addition of 10 nM ET-1 (hatched columns) or without ET-1 (open columns). After an additional 5 min, $^{45}Ca^{2+}$ accumulation was determined. Data represent mean ± s.e.mean, $n=4$. Both $Ni^{2+}$ and nicardipine inhibited ET-1-stimulated $^{45}Ca^{2+}$ uptake when compared to controls (**$P<0.01$).

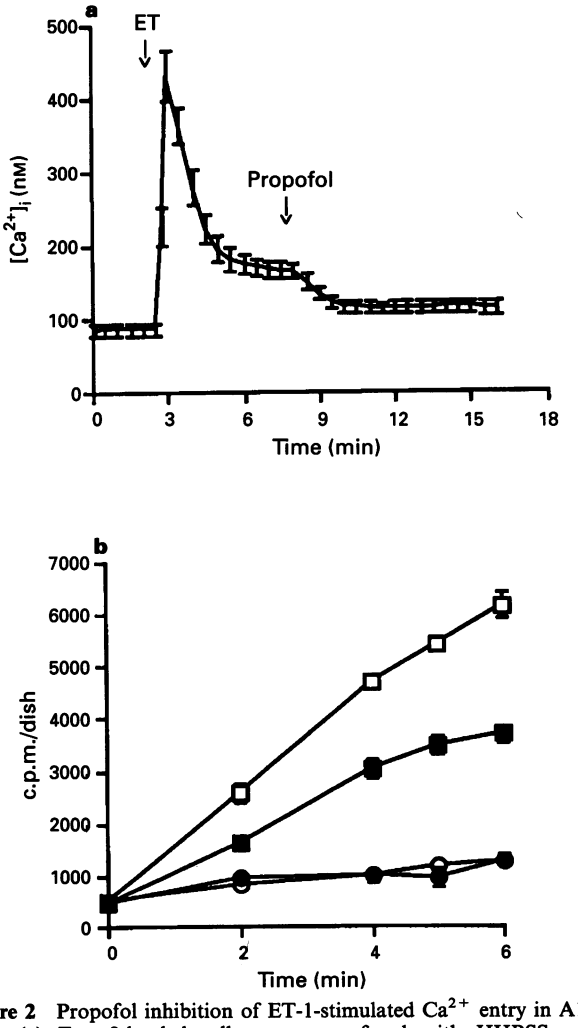


**Figure 2** Propofol inhibition of ET-1-stimulated $Ca^{2+}$ entry in A10 cells. (a) Fura-2-loaded cells were superfused with HHBSS and stimulated by 10 nM ET-1 as indicated by the arrow. At a time (about 8 min) where continued elevation of $[Ca^{2+}]_i$ represented continued entry of extracellular $Ca^{2+}$, propofol (28 $\mu$M) was added to the perfusion buffer. Data represent mean ± s.e.mean, $n=5$. (b) Cells were preincubated for 5 min with HHBSS containing $^{45}Ca^{2+}$ (3 $\mu$Ci ml$^{-1}$) in the absence (○ and □) or presence of propofol (28 $\mu$M; ● and ■). $^{45}Ca^{2+}$ entry was initiated by adding 10 nM ET-1 (□ and ■). $^{45}Ca^{2+}$ accumulation was determined as described in Figure 1b. Data represent mean ± s.e.mean, $n=4$.

pendent on extracellular $Ca^{2+}$ entry because it was greatly reduced in cells bathed in $Ca^{2+}$-free HHBSS (Figure 6c). The nature of the $Ca^{2+}$ entry mechanism is not known. In non-muscle cells it has been suggested that thapsigargin leads to activation of receptor-operated Ca channels (Dolor *et al.*, 1992). In our previous work, we found that the $Ca^{2+}$ entry pathway operated by thapsigargin in smooth muscle cells is nicardipine-insensitive and thus most likely not an L-type Ca channel (Xuan *et al.*, 1992).

We next investigated whether propofol regulates the nicardipine-insensitive $Ca^{2+}$ entry activated by thapsigargin. Exposure to propofol at a time when thapsigargin had activated the sustained $Ca^{2+}$ entry failed to block $Ca^{2+}$ entry (Figure 6b). At this time point, thapsigargin-initiated mobilization of $Ca^{2+}$ was solely dependent on entry of extracellular $Ca^{2+}$ and beyond those initial events associated with intracellular $Ca^{2+}$ mobilization (see Figures 6a and 6c). Because thapsigargin initiates $Ca^{2+}$ entry through mechanisms involving depletion of intracellular $Ca^{2+}$ pools (Dolor *et al.*, 1992; Xuan *et al.*, 1992), we further examined whether propofol alters thapsigargin-induced release of $Ca^{2+}$ from intracellular stores. As can be seen, when cells were perfused with $Ca^{2+}$-free HHBSS, propofol did not alter thapsigargin-mediated release of $Ca^{2+}$ from intracellular stores (compare Figures 6c and 6d). We followed $^{45}Ca^{2+}$ uptake 10 min after the initial stimulation of cells with thapsigargin to investigate the effect of propofol on the sustained $Ca^{2+}$ entry. As seen in Figure 7, exposure of the cells to thapsigargin led to a time-dependent increase of the sustained $^{45}Ca^{2+}$ uptake. Treatment of the cells with propofol at doses which blocked ET-1-mediated $Ca^{2+}$ entry through the L-type channel did not alter thapsigargin-initiated $^{45}Ca^{2+}$ uptake.

### *Effect of propofol on production of inositol phosphates*

Because ET-1 does not directly activate the L-type Ca channel, this peptide has been proposed as a modulator of the activity of this channel via a secondary mechanism, perhaps involving intracellular mediators (Inoue *et al.*, 1990). Therefore, we have investigated the effect of propofol on ET-1-mediated activation of phospholipase C by measuring the production of inositol phosphates. When [$^3$H]-inositol-labelled cells were pre-incubated with propofol, ET-1-stimulated production of IP, $IP_2$ and $IP_3$ was reduced (Figure 8a). Furthermore, propofol also partially inhibited the formation of each inositol phosphate isomer (Figures 8b, c, and d) stimulated by AVP.

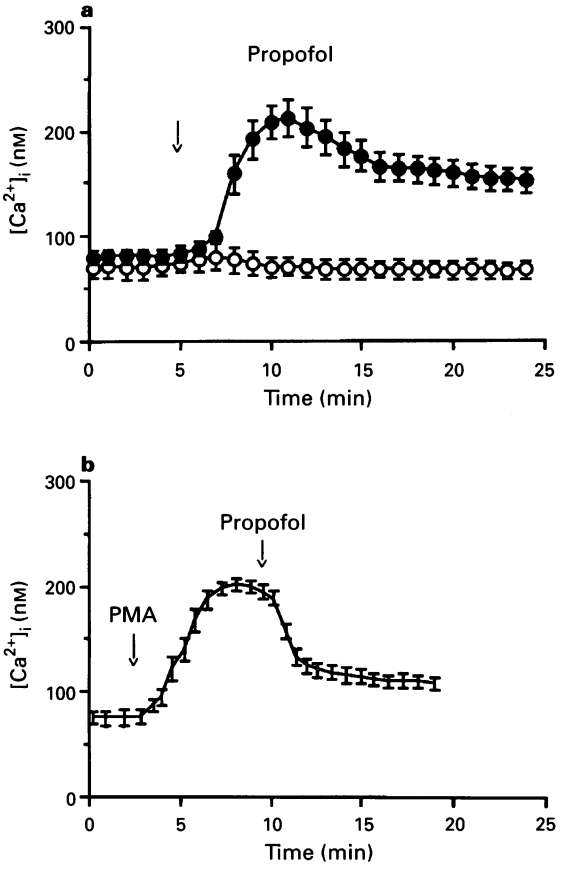


**Figure 4** Effects of propofol on $Ca^{2+}$ entry activated by phorbol esters in A10 cells. (a) Fura-2-loaded cells were superfused with HHBSS and stimulated by 500 nM 4$\beta$-PDD (●) and 500 nM 4$\alpha$-PDD (○) as indicated by the arrow. After 2 min, perfusion was restarted with the same buffer. Data are given as mean ± s.e.mean, $n=5$. (b) Cells were perfused with HHBSS and treated with 25 nM PMA as indicated by the arrow. At about 9 min when elevated $[Ca^{2+}]_i$ had reached a maximal level, the perfusion was quickly switched to the same buffer containing propofol (56 $\mu$M). Data are given as mean ± s.e.mean, $n=5$.

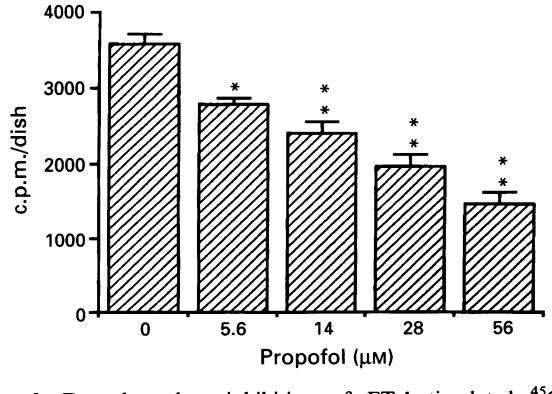


**Figure 3** Dose-dependent inhibition of ET-1-stimulated $^{45}Ca^{2+}$ uptake by propofol in A10 cells. Cells were incubated with HHBSS in the absence or presence of increasing concentrations of propofol (5.6–56 $\mu$M) for 5 min. $^{45}Ca^{2+}$ and 10 nM ET-1 were added and $^{45}Ca^{2+}$ accumulation was determined 5 min later. Basal uptake in the absence of ET-1 was determined and has been subtracted from values shown. Data are given as mean ± s.e.mean, $n=6$. Significantly different from control: $*P<0.05$; $**P<0.01$.

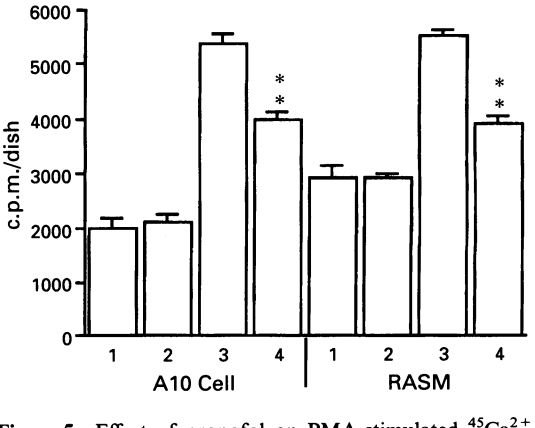


**Figure 5** Effect of propofol on PMA-stimulated $^{45}Ca^{2+}$ uptake in A10 and rat aortic smooth muscle (RASM) cells. Both A10 and RASM cells were incubated with HHBSS containing $^{45}Ca^{2+}$ (3 $\mu$Ci ml$^{-1}$) and were then treated with buffer alone (1), 56 $\mu$M propofol (2), 50 nM PMA (3) or propofol + PMA (4) as labelled in both types of cells. $^{45}Ca^{2+}$ uptake was determined as described in Figure 1b. Data are given mean ± s.e.mean, $n=4-6$. Significantly different from PMA alone. $**P<0.01$.

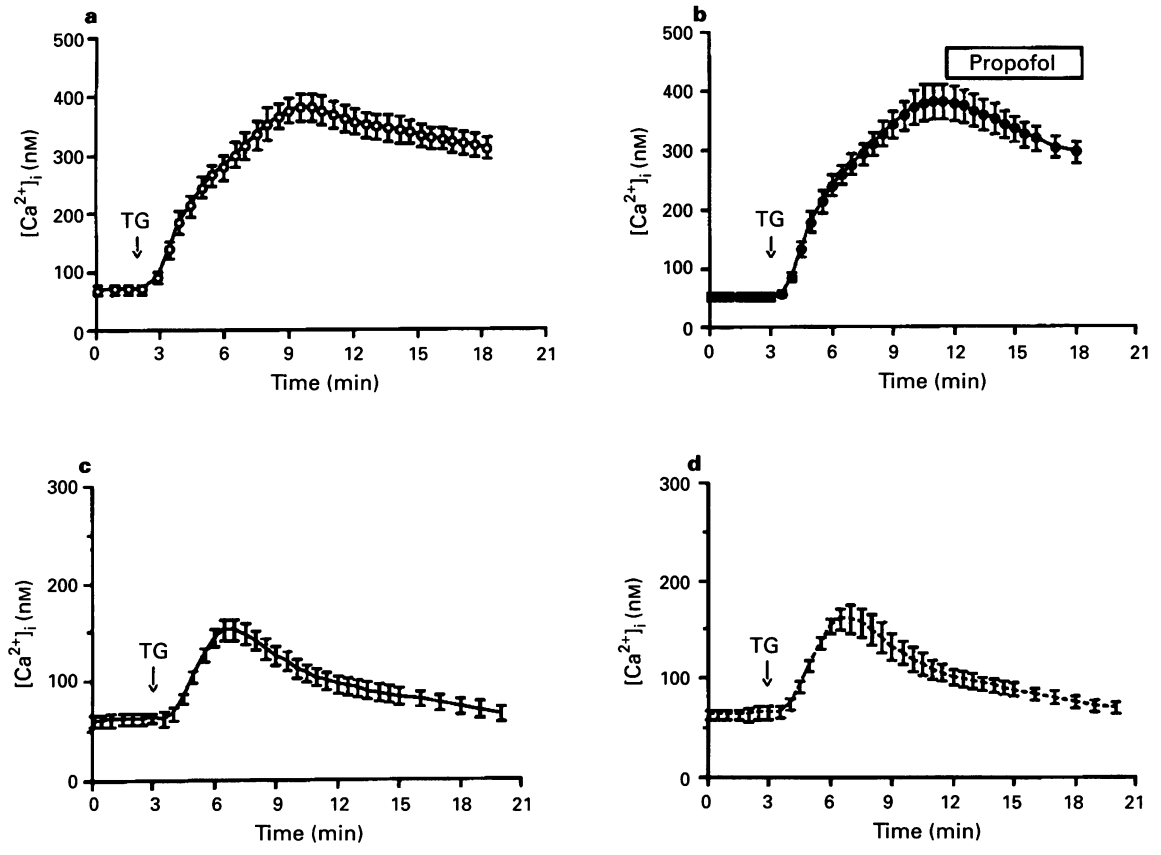


**Figure 6** Effects of propofol on thapsigargin (TG)-initiated $Ca^{2+}$ mobilization in A10 cells. Cells were superfused with HHBSS (a and b) and were then treated with 0.2 $\mu$M TG as indicated by the arrows. After 2 min exposure, perfusion was re-started with HHBSS. (b) During the sustained elevation of $[Ca^{2+}]_i$ induced by TG, propofol (56 $\mu$M) was included in the perfusion buffer (as indicated). Data are given as mean ± s.e.mean, $n=4$. (c and d) Effect of propofol on TG (0.2 $\mu$M)-initiated release of $Ca^{2+}$ from intracellular stores. Cells were perfused with $Ca^{2+}$-free HHBSS in the absence (c) or presence of 56 $\mu$M propofol (d). Cells were treated with 0.2 $\mu$M TG to mobilize intracellular $Ca^{2+}$. Data are given as mean ± s.e.mean, $n=4-5$.

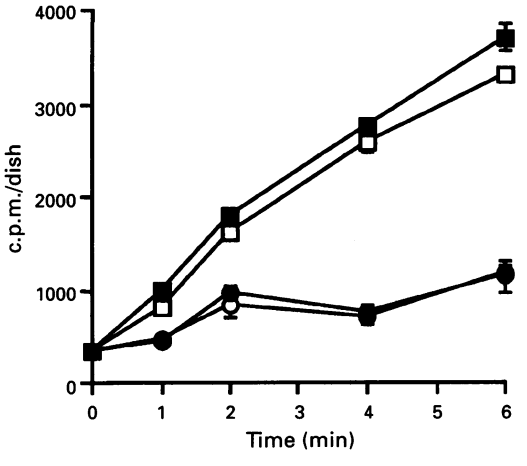


**Figure 7** Effect of propofol on thapsigargin (TG)-stimulated $^{45}Ca^{2+}$ influx in A10 cells. Cells were incubated with HHBSS and treated with buffer alone (○), 56 $\mu$M propofol (●), 0.2 $\mu$M TG (□) or TG + propofol (■). After 10 min, $^{45}Ca^{2+}$ was added to the cells to monitor the sustained $^{45}Ca^{2+}$ entry. $^{45}Ca^{2+}$ uptake was determined at various times as indicated. Data are given as mean ± s.e.mean, $n=4-6$.

## Discussion

In this study we have found that propofol reduces agonist-mediated production of inositol phosphates and selectively inhibits $Ca^{2+}$ entry through the nicardipine-sensitive L-type channel induced by ET-1 and phorbol esters. In contrast, this compound had no effect on $Ca^{2+}$ entry through the nicardipine-insensitive Ca channel initiated by thapsigargin. The data also demonstrate that ET-1 elevates $[Ca^{2+}]_i$ by activating a transient release of $Ca^{2+}$ from intracellular stores and a prolonged entry of extracellular $Ca^{2+}$ through mechanisms involving formation of $Ins(1,4,5)P_3$, production of diacylglycerol and activation of PKC (Xuan *et al.*, 1989; 1994; present study). The transient release of $Ca^{2+}$ from intracellular storage pools following ET-1 stimulation is probably regulated by $Ins(1,4,5)P_3$ because we had previously shown that it potently mobilizes $Ca^{2+}$ from the sarcoplasmic reticulum in saponin-permeabilized smooth muscle cells (Xuan *et al.*, 1991). Thus, propofol inhibition of ET-1- and AVP-stimulated formation of $Ins(1,4,5)P_3$ suggests that it may indirectly reduce release of $Ca^{2+}$ from the $Ins(1,4,5)P_3$-sensitive intracellular pool leading to a decreased $[Ca^{2+}]_i$, although a direct interaction of propofol with the $Ins(1,4,5)P_3$ receptors is not studied in this investigation.

The sustained elevation of $[Ca^{2+}]_i$ evoked by ET-1 was maintained by continued entry of $Ca^{2+}$ since it required extracellular $Ca^{2+}$ and was blocked by $Ni^{2+}$. $Ca^{2+}$ entry activated by ET-1 appears to involve the L-type channel. This is consistent with our previous findings (Xuan *et al.*, 1991; 1992). ET-1 activation of the dihydropyridine-sensitive channel was also demonstrated in vascular smooth muscle cells of porcine coronary artery (Goto *et al.*, 1989), human umbilical artery (Gardner *et al.*, 1992) and guinea-pig portal vein (Inoue *et al.*, 1990). This is also supported by findings that A10 cells express a high density of $ET_A$ receptors (Martin *et al.*, 1990) and L-type Ca channel activity (Friedman *et al.*, 1986). The present

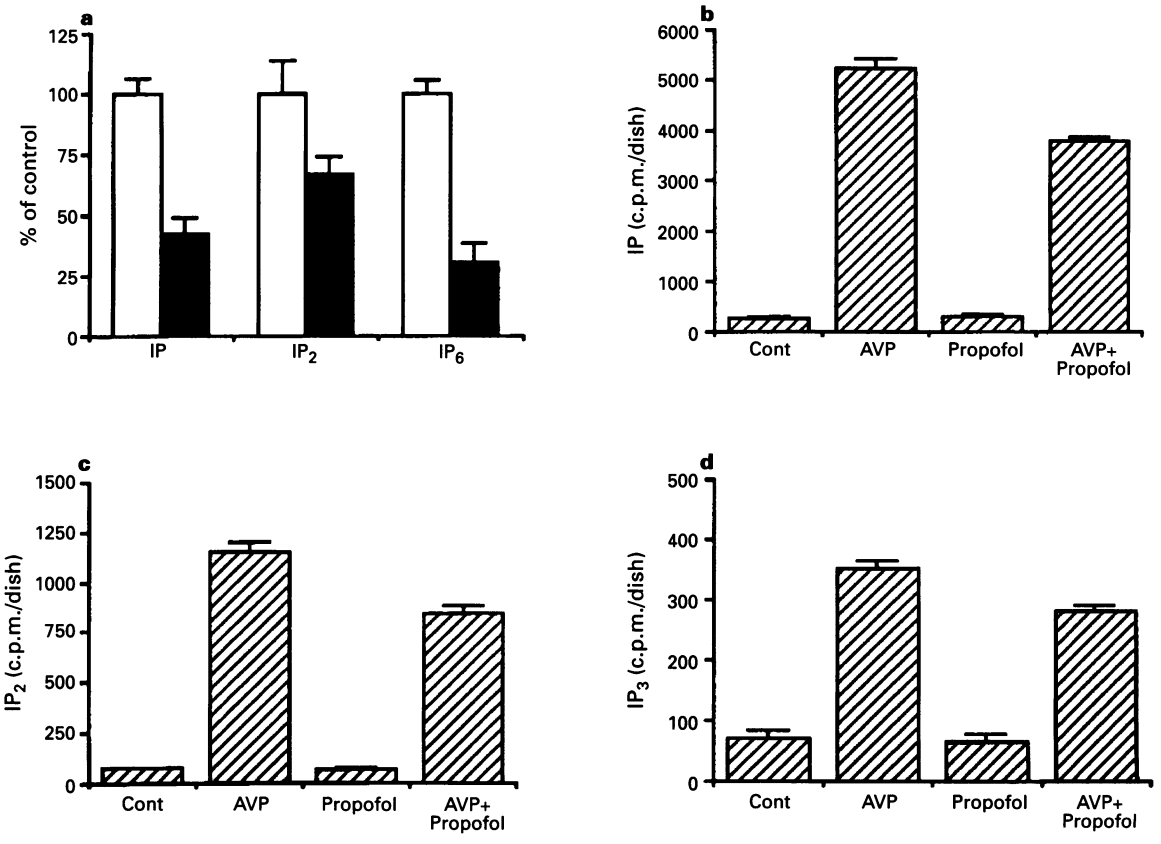


**Figure 8** Effects of propofol on production of inositol phosphates mediated by ET-1 and AVP in A10 cells. (a) [$^3$H]-inositol labelled cells were preincubated with HBSS containing 10 mM $Li^+$ in the absence (open columns) or presence (solid columns) of 56 $\mu$M propofol and were then treated with 10 nM ET-1 for 5 min. In (b), (c) and (d), labelled cells were treated with buffer alone as control (Cont), 56 $\mu$M propofol, 100 nM AVP or propofol + AVP as indicated. Inositol phosphates were extracted and separated by anion-exchange chromatography as described in the Methods. Data are given as mean ± s.e.mean, $n = 3$.

data clearly show that propofol regulates ET-1-mediated $Ca^{2+}$ entry through the L-type channel based on several lines of evidence. First, propofol reduces ET-1-stimulated $^{45}Ca^{2+}$ uptake in both a time- and dose-dependent manner. Second, when propofol is added to cells during the sustained elevation of $[Ca^{2+}]_i$ resulting from continued entry through the L-type channel (Xuan *et al.*, 1991; 1992), the entry is also blocked by this anaesthetic. In fact, propofol reduces contraction caused by adrenaline (Yamanoue *et al.*, 1994) which activates the L-type channel in smooth muscle (Nelson *et al.*, 1988). Finally, propofol inhibits $Ca^{2+}$ entry activated by phorbol esters which activate entry through a pharmacologically-similar nicardipine-sensitive L-type channel (Fish *et al.*, 1988; Xuan *et al.*, 1994).

The mechanisms underlying the inhibition of ET-1-mediated $Ca^{2+}$ entry through the L-type channel by propofol are not known. Since ET-1 appears to activate this channel through an indirect mechanism, presumably involving intracellular mediators (Inoue *et al.*, 1990; Naitoh *et al.*, 1990; Xuan *et al.*, 1991), we speculated that propofol may interfere with the ET-1-stimulated formation of intracellular mediators which regulate the activity of the Ca channels. Although propofol inhibits ET-1-mediated formation of inositol phosphates, which regulate $Ca^{2+}$ entry in other cell types (Vilven & Coronado, 1988; Berridge & Irvine, 1989), it is unknown whether they directly regulate $Ca^{2+}$ entry in smooth muscle cells. Diacylglycerol is another product derived from hydrolysis of phospholipids and activates PKC. Activation of PKC by phorbol esters causes a slowly-developing contraction (Rasmussen *et al.*, 1987), increases $Ca^{2+}$ currents (Lacerda *et al.*, 1988; Fish *et al.*, 1988) and phosphorylates myosin light chain (Rasmussen *et al.*, 1987). Furthermore, PKC inhibitors reduce ET-1-mediated contraction and elevation of $[Ca^{2+}]_i$ (Xuan *et al.*, 1994). Thus, a possible mechanism by which propofol regulates contraction and $Ca^{2+}$ entry may occur through interference with the activation of PKC in the ET-1-mediated signalling pathways. This appears likely because propofol inhibition of both ET-1-mediated phospholipase C activity (reduced production of diacylglycerol) and $Ca^{2+}$ response (lower intracellular free $Ca^{2+}$) would be expected to result in less translocation and therefore reduced activation of $Ca^{2+}$- and phospholipid-dependent PKC and its isoforms.

Other mechanisms are possible and require further investigation. For example, propofol inhibition of phorbol ester-stimulated $Ca^{2+}$ entry suggests that it may interact with the endogenous diacylglycerol in activating PKC or with the sites beyond PKC activation including a direct interaction with the L-type channel. Since propofol inhibits ET-1- and phorbol ester-stimulated $Ca^{2+}$ entry, but does not alter thapsigargin-initiated elevation of $[Ca^{2+}]_i$, it seems unlikely that this compound interferes with the activation of the plasma membrane $Ca^{2+}$-ATPase or $Na^+$-$Ca^{2+}$ exchanger which result from the elevated intracellular $Ca^{2+}$.

Thapsigargin has been found to elevate $[Ca^{2+}]_i$ contributed by both release of $Ca^{2+}$ from intracellular stores and entry of extracellular $Ca^{2+}$ through mechanisms involving selective inhibition of the sarcoplasmic reticulum $Ca^{2+}$-ATPase (Xuan *et al.*, 1992). Such inhibition prevents $Ca^{2+}$ sequestration and allows $[Ca^{2+}]_i$ to rise as $Ca^{2+}$ diffuses from the intracellular pool via a passive leak pathway. Interestingly, depletion of intracellular $Ca^{2+}$ pools by thapsigargin activates a $Ca^{2+}$ entry pathway. The thapsigargin-induced $Ca^{2+}$ influx appears to occur exclusively via a nicardipine-insensitive pathway and thus probably does not involve L-type channels (Xuan *et al.*, 1992). In fact, $Ca^{2+}$ entry initiated by thapsigargin in these A10 smooth muscle cells is indistinguishable from influx

through receptor-operated Ca channels in non-muscle cells (Dolor *et al.*, 1992). Our data clearly show that propofol does not alter thapsigargin-initiated $Ca^{2+}$ entry through the receptor-operated channel. Furthermore, we also find that $Ca^{2+}$ release from intracellular pools evoked by thapsigargin is unaffected by this compound. Additionally, we find that $Ca^{2+}$ entry through the receptor-operated Ca channel stimulated by bradykinin in bovine vascular endothelial cells is also not altered by propofol (data not shown).

In conclusion, the results from this study demonstrate that propofol regulates $Ca^{2+}$ entry through the nicardipine-sensitive L-type channel activated by phorbol esters and indirectly by ET-1. However, this anaesthetic does not alter the $Ca^{2+}$ entry and release initiated by thapsigargin. While the mechanisms by which propofol inhibits $Ca^{2+}$ entry through the L-type Ca channel remain unclear, our data suggest that it may interfere with ET-1-mediated activation of signalling transduction pathways and generation of intracellular messengers which regulate PKC activity and $Ca^{2+}$ mobilization.

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