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Activation of microsomal cytochrome P450 mono-oxygenase by Ca^{2+} store depletion and its contribution to Ca^{2+} entry in porcine aortic endothelial cells

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1 We investigated how microsomal cytochrome P450 mono-oxygenase (Cyp450 MO) is regulated in cultured porcine aortic endothelial cells. The hypothesis that a Cyp450 MO-derived metabolite links Ca^{2+} store depletion and Ca^{2+} entry was studied further.

2 Microsomal Cyp450 MO was monitored fluorometrically by dealkylation of 1-ethoxypyrene-3,6,8-tris-(dimethyl-sulphonamide; EPSA) in saponin permeabilized cells or in subcellular compartments. Endothelial Ca^{2+} signalling was measured by a standard fura-2 technique, membrane potential was determined with the potential-sensitive fluorescence dye, bis-(1,3-dibutylbarbituric acid) pentamethine oxonol ($DiBAC_4(5)$) and tyrosine kinase was quantified by measuring the phosphorylation of a immobilized substrate with a horseradish peroxidase labelled phosphotyrosine specific antibody.

3 Depletion of cellular Ca^{2+} pools with inositol 1,4,5-trisphosphate (IP_3), thapsigargin or cyclopiazonic acid activated microsomal Cyp450 MO. Similar to direct Ca^{2+} store depletion, chelating of intra-microsomal Ca^{2+} with oxalate stimulated Cyp450 MO activity, while changing cytosolic free Ca^{2+} failed to influence Cyp450 MO activity. These data indicate that microsomal Cyp450 MO is activated by depletion of IP_3-sensitive stores.

4 Besides the common cytochrome P450 inhibitors, econazole, proadifen and miconazole, thiopentone sodium and methohexitone inhibited Cyp450 MO in a concentration-dependent manner. The physiological substrate of Cyp450 MO, arachidonic acid, inhibited EPSA dealkylation. In contrast to most other cytochrome P450 inhibitors used in this study, thiopentone sodium did not directly interfere with Ca^{2+} entry pathways, membrane hyperpolarization due to K^+ channel activation or tyrosine kinase activity.

5 Inhibition of Cyp450 MO by thiopentone sodium diminished Ca^{2+}/Mn^{2+} entry to Ca^{2+} store depletion by 43%, while it did not interfere with intracellular Ca^{2+} release by IP_3 or thapsigargin.

6 Cyp450 MO inhibition with thiopentone sodium diminished autacoid-induced membrane hyperpolarization.

7 Induction of Cyp450 MO with dexamethasone/clofibrate for 72 h yielded increases in thapsigargin-induced Cyp450 MO activity (by 35%), Ca^{2+}/Mn^{2+} entry (by 105%) and membrane hyperpolarization (by 40%).

8 The Cyp450 MO-derived compounds, 11,12 and 5,6-epoxyeicosatrienoic acids (EETs) yielded membrane hyperpolarization, insensitive to thiopentone sodium.

9 These data demonstrate that endothelial Cyp450 MO is activated by Ca^{2+} store depletion and Cyp450 MO produced compounds that hyperpolarize endothelial cells.

10 The data presented and our previous findings indicate that Cyp450 MO plays a crucial role in the regulation of store-operated Ca^{2+} influx. We propose that Cyp450 MO-derived EETs constitute a signal for Ca^{2+} entry activation and increase the driving force for Ca^{2+} entry by membrane hyperpolarization in porcine aortic endothelial cells.

Keywords: Bradykinin; $DiBAC_4(5)$; epoxy-eicosatrienoic acid; membrane hyperpolarization; ryanodine; tyrosine kinase; thapsigargin; thiopentone sodium

Introduction

In endothelial cells, Ca^{2+} entry is activated by autacoids, such as bradykinin which initially deplete intracellular Ca^{2+} pools (Schilling *et al*., 1992). This so called store-operated Ca^{2+} entry pathway (SOCP; Montero *et al*., 1993) is activated by a stimulating compound via the depletion of intracellular Ca^{2+} pools and was initially described by Putney (1990). Besides the physiological signal molecule for intracellular Ca^{2+} release, inositol 1,4,5-trisphosphate (IP_3), Ca^{2+} store depletion by the endoplasmic reticulum ATPase inhibitors thapsigargin and cyclopiazonic acid results in activation of this Ca^{2+} entry pathway (Schilling *et al*., 1992; Graier *et al*., 1995). The link between Ca^{2+} store depletion and Ca^{2+} entry is unclear, but possibly includes an enzymatically-produced second messenger (Parekh *et al*., 1993; Randriamampita & Tsien, 1993), a tyrosine kinase (Fleming *et al*., 1995), G proteins (Fasolato *et al*., 1993), protein-coupling (Berridge, 1995), vesicle transport and channel trafficking (Somasundaram *et al*., 1996). Recently, evidence was provided that in the signal cascade for SOCP activation in numerous non-excitable cells, like platelets (Sargeant *et al*., 1992), rat thymocytes (Alvarez *et al*., 1991) and endothelial cells (Graier *et al*., 1992) a cytochrome P450 enzyme might be involved. All of these studies are based on the inhibitory properties of imidazole antimycotics, such as clotrimazole, miconazole and econazole, on SOCP-mediated Ca^{2+} entry/influx in autacoid-stimulated cells. Since there is overwhelming evidence that these cytochrome P450 inhibitors have additional properties, like inhibition of K^+ channels (Alvarez *et al*., 1992), inhibition of L-type Ca^{2+} channels (Villalobos *et al*., 1992), inhibition of Ca^{2+} ATPases (Mason *et al*., 1993) and inhibition of tyrosine kinase activity (Sargeant *et*

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al., 1994), the suggestion of a cytochrome P450 involvement in SOCP activation based on these data is rather weak. Recently, we described another approach to investigate the involvement of cytochrome P450 in SOCP activation in endothelial cells and demonstrated that a common induction procedure for cytochrome P450 mono-oxygenases by β-naphtoflavone treatment enhanced histamine- and bradykinin-initiated Ca^{2+} entry in endothelial cells isolated from human umbilical veins and bovine aortae (Graier *et al*., 1995). Moreover, the endothelial Ca^{2+}/Mn^{2+} entry initiated by the cytochrome P450-derived compound, 5,6-epoxy-eicosatrienoic acid (5,6-EET) was indistinguishable from that activated by bradykinin. The formation of the cytochrome P450 epoxygenase-derived epoxides, 5,6-, 8,9-, 11,12- and 14,15-EET to autacoid stimulation of endothelial cells has been shown by Harder *et al*. (1995). Similar to our results in endothelial cells, EETs have been proposed to be involved in Ca^{2+} entry mechanism(s) in rat hepatocytes (Karara *et al*., 1991), pituitary cell (Snyder *et al*., 1986), parotid cells (Snowdowne *et al*., 1989) and epithelial cells (Madhun *et al*., 1991). As well as their proposed role in the Ca^{2+} signal cascade, EETs were shown to activate Ca^{2+}-activated K^+ channels in smooth muscle cells (Gebremedhin *et al*., 1992; Hu & Kim, 1993), and are thought to act as an endothelium-derived hyperpolarizing factor (EDHF) in blood vessels (Harder *et al*., 1995; Hecker *et al*., 1995).

Thus, there is strong evidence for the importance of endothelial cytochrome P450-derived epoxides in cellular Ca^{2+} homeostasis and vascular tone. Although it has been shown that EETs are produced by stimulating vascular endothelial cells with autacoids (Harder *et al*., 1995), the mechanism(s) of mono-oxygenase activation is still unclear and the role of EETs in endothelial Ca^{2+} homeostasis needs to be clarified. In this study, we investigated how cytochrome P450 mono-oxygenase is activated during autacoid stimulation and characterized this enzyme pharmacologically. In addition, the role of mono-oxygenase-derived EETs in endothelial Ca^{2+} homeostasis and membrane potential was determined.

Methods

Isolation and culture of endothelial cells

Endothelial cells from porcine or bovine aortae were isolated as described previously (Graier *et al*., 1995). Briefly, porcine aortae were collected at the local slaughterhouse and immediately transported to the department. Vessels were washed twice with PBS and incubated for 30 min at 37°C in serum-free Dulbecco's modified minimal essential medium (DMEM) supplemented with 200 u ml^{-1} collagenase type II (alternatively 2 u ml^{-1} dispase), 2 mg ml^{-1} bovine serum albumin, 0.5 mM Ca^{2+} and supplemented with dilutions (v/v) of 0.02 amino acids and 0.01 vitamins. Trypsin-inhibitor type II-S (1 mg ml^{-1}) was only present in the collagenase containing preparation medium. Cells were centrifuged and seeded in OPTI-MEM containing 3% foetal calf serum. Cells up to passage one (for Ca^{2+} measurement) or up to passage two (Cyp450 MO measurement) were used for experiments within 12 or 17 days after vessel preparation. Purity was controlled by typical cobblestone morphology under the microscope and the absence of immunofluorescence detection of smooth muscle α-actin. Cells cultured were > 99% pure.

Calibration of 1-ethoxypyrene-3,6,8-tris-(dimethylsulphonamide) (EPSA) dealkylation assay

A calibration curve for 1-hydroxypyrene-3,6,8-tris-(dimethylsulphonamide) (HPSA) in the range of 2 to 40 nM was constructed in the presence of 25 μM EPSA and 1 mg ml^{-1} saponin in an intracellular-like buffer (Capdevila *et al*., 1983) containing in mM: KCl 150, $MgCl_2$ 10 and Tris 50, pH adjusted. Increases in the fluorescence at excitation 495 nm and emission 550 nm were monitored and were described by: HPSA (nM) = $0.465 \times Fl - 2.152$ (Fl, fluorescence intensity at 495 nm excitation and 550 nm emission; $R = 0.996$).

Measurement of Cyp450 MO activity

Cultured endothelial cells were harvested by trypsin digestion (Graier *et al*., 1995). Cells were resuspended in intracellular-like buffer (see above) and transferred into a thermostatically controlled (37°C), stirred cuvette in a spectrofluorometer (Hitachi F-4500, Shimadzu RF5000PC). EPSA (25 μM) was added and cells were permeabilized with 1 mg ml^{-1} saponin, followed by the addition of a NADPH-regenerating system (isocitric dehydrogenase ($NADP^+$) EC 1.1.1.42, 25 iu ml^{-1}, D,L-isocitric acid, 8 mM; $NADP^+$, 1 mM; Capdevila *et al*., 1983). At 90 s, inhibitors of Cyp450 MO to be tested were added, followed by 20 μM EGTA at 180 s (i.e. approx. 0.14 μM free Ca^{2+}). Enzyme activity was stimulated by depletion of intracellular Ca^{2+} stores with compound indicated. The following increase in fluorescence by the conversion of EPSA to HPSA was monitored at excitation 495 nm and 550 nm emission.

Non Cyp450 MO-derived HPSA formation was determined by the same experimental protocol but without endothelial cells or cells heated for 15 min at 95°C. Non-specific HPSA formation could largely be attributed to a contamination in commercially available isocitric dehydrogenase type I (Crude, from porcine heart) and was substantially diminished in type IV preparations (Purified, from porcine heart). The values obtained for non Cyp450 MO-derived HPSA formation were subtracted from that measured with intact cells and Cyp450 MO activity was calculated by use of the standard calibration curve and is expressed as pmol min^{-1} per 10^6 cells or pmol min^{-1} mg^{-1} protein.

Preparation of subcellular organelles fractions

Cultured endothelial cells were suspended as described above and were sonicated six times for 10 s during 2 min on ice (Labsonic U, Braun, Freeport, Illinois, U.S.A.). Subcellular fractions were prepared at 4°C as described by Fleischer and Kervina (1974). Briefly, after sonication cells were subjected to subsequent centrifugation steps by 20 min centrifugation at 1000 *g* (2400 r.p.m. in Sorvall RT6000B with Sorvall H1000B rotor) to separate cells nuclei, plasma membranes, cell debris and unbroken cells. The supernatant was centrifuged at 34000 *g* for 30 min (24,000 r.p.m. in Beckman XL-70 centrifuge with Sorvall T1270 rotor) with the pellet containing peroxisomes, mitochondria and lysosomes (fraction #1). The supernatant was further centrifuged for 120 min at 105000 *g* (46,000 r.p.m. in the Beckman XL-70 centrifuge with Sorvall T1270 rotor) resulting in the microsomal pellet (fraction #3) and the cytosolic supernatant (fraction #2).

All pellets were resuspended in ice-cold intracellular-like buffer by means of short sonicating pulses. Small aliquots of all fractions were frozen at −70°C under nitrogen and quickly thawed just before the experiments. Before experiments, the protein content of each fraction was determined.

Ca^{2+} measurement

Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured by the fura-2 technique as described previously (Graier *et al*., 1995). Briefly, cells were incubated with 2.5 μM Fura-2/am at 37°C for 45 min in the dark. Intracellular Ca^{2+} was monitored by the ratio of 340 and 380 nm excitation and 510 nm emission. Due to the overall failure of the calibration techniques mentioned by many groups, intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in each experiment is expressed as the ratio 340/380 excitation at 510 nm emission (F_{340}/F_{380}). The maximal ratio response to 100 nM bradykinin or 1 μM thapsigargin was performed daily as a standard during the period of data collection. Thus, caution is necessary in comparing given ratio units between different figures.

For determination of SOCP activity, Mn^{2+} quench experiments were performed as previously described (Graier *et al*., 1995; Paltauf-Doburzynska & Graier, 1997). Briefly, in fura-2 loaded cells the entry of Mn^{2+}, a surrogate of endothelial SOCP, was monitored in the presence of 2 mM Mn^{2+} by the decrease of fura-2 fluorescence at the isosbestic (i.e. Ca^{2+}-insensitive) wavelength of fura-2 (360 nm excitation and 510 nm emission). The amount of Mn^{2+} entering the cells is expressed as percentage decrease in the initial fluorescence intensity.

Calibration of DiBAC$_4$(5) experiments for monitoring membrane potential

Measurements of membrane potential were performed as described previously (Graier *et al*., 1993). Briefly, cultured endothelial cells were suspended in a buffer containing (in mM): cholinchloride 145, KCl 5, HEPES free acid 10, $MgCl_2$ 1; adjusted with KOH to pH 7.4. The final cell concentration was approx. 9.3×10^6 cells ml^{-1}. $DiBAC_4(5)$ (1 μM) was added to the cell suspension under stirring just before the experiment. After the fluorescence at 590 nm excitation and 616 nm emission was constant, Gramicidin D (800 nM) was added. Cumulative portions of KCl solution were used to clamp the trans-membrane potential (E_m). Dilution effects of the KCl aliquots were considered in the calculation. The membrane potential corresponding to the extracellular KCl was calculated as described by Vieira *et al*. (1995). Changes in fluorescence were plotted against the calculated E_m values and fitted to a Boltzmann sigmoid curve following, E_m (mV) $= -2133 + (2715.3/(1 + e^{-1086}\text{-}F_{616}/388.8)$ (E_m, membrane potential; F_{616}, fluorescence intensity at 590 nm excitation and 616 nm emission; $R = 0.999$).

Fluorometric estimation of membrane potential

Cells were suspended in a cholinchloride buffer as mentioned above and incubated with $DiBAC_4(5)$ (1 μM) as described above. After the equilibration period, the compound to be tested was added and membrane potential was monitored at 590 nm excitation and 616 nm emission. Changes in membrane potential were calculated by use of a calibration curve and it was assumed that the trans-membrane resting potential against 5 mM KCl was approx. -34 mV (Graier *et al*., 1993; Groschner *et al*., 1994). Due to the known problems for estimation of absolute membrane potentials with the potential sensitive fluorescent probes (Krasznai *et al*., 1995) all E_m values obtained are expressed as differences compared to this resting potential in mV.

Determination of tyrosine kinase activity

Cultured endothelial cell were harvested by enzymatic digestion with trypsin (0.05%, type II) and cell lysates were obtained by sonication of the cell suspension on ice in a buffer containing 20 mM Tris, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 1 μg ml^{-1} pepstatin, 0.5 μg ml^{-1} leupeptin, 0.2 mM Na_3VO_4 and 5 mM mercaptoethanol, pH adjusted to 7.4. The extracts were centrifuged at 2000 *g* for 5 min and at 8000 *g* for 5 min and the supernatant was kept at -70°C for the experiments. Activity of protein tyrosine kinase was determined with a protein tyrosine kinase assay kit from Calbiochem-Novabiochem International. Briefly, cell extract aliquots were incubated in the presence of compounds to be tested with Mg^{2+} and ATP. Phosphorylation of an immobilized substrate was recognized by a horseradish peroxidase labelled phosphotyrosine specific antibody (PY20). The conversion of tetra-methylbenzidine as a substrate of horseradish peroxidase was monitored spectrophotometrically at 450 nm with a reference at 540 nm.

Materials

1 - Ethoxypyrene-3,6,8 - tris- (dimethylsulphonamide) (EPSA), 1 - hydroxypyrene-3,6,8 - tris- (dimethylsulphonamide) (HPSA) and fura-2 acetoxymethyl ester (Fura-2 AM) were obtained from Lambda Fluorescence Technologies (Graz, Austria). All tissue culture media, antibiotics and cell culture dishes were from Life Technologies (Vienna, Austria). Epoxyeicosatrienoic acids (EETs) were purchased from Cascade Ltd. (Reading, U.K.). Bis-(1,3-dibutylbarbituric acid) pentamethine oxonol ($DiBAC_4(5)$) was from Molecular Probes Inc. (Eugene, U.S.A.). Cyclopiazonic acid was purchased from Aldrich (Steinheim, Germany). Folin reagent was from Merck (Darmstadt, Germany). The protein tyrosine kinase assay kit was purchased from Calbiochem-Novabiochem International. All other chemicals used were obtained from Sigma (Vienna, Austria).

Statistics

K_m and V_{max} values were obtained by least square, non-linear fit of EPSA dealkylation activities in the presence of different EPSA concentrations to the Michaelis-Menten equation ($v = (V_{max} \times [S])/(K_m + [S])$) by use of Prism software (Version 2.01 for Windows95) from GraphPad (GraphPad Software Inc., San Diego, U.S.A.). Median inhibitory concentration (IC_{50}) values were extrapolated from collective concentration-response curves of each compound by use of non-linear curve fitting. All other results are expressed as mean values $\pm$s.e.mean. Statistical significance was evaluated with Student's unpaired *t* test. Level of significance was defined as $P < 0.05$ in all experiments.

Results

Distribution of cytochrome P450 mono-oxygenase (Cyp450 MO) in endothelial cells

Subcellular components of endothelial cells were isolated by differential centrifugation. Thereby, we separated three subcellular compartments, representing mitochondria, lysosomes, golgi complex and peroxisomes (fraction #1), cytosol (fraction #2) and microsoms, endoplasmic reticulum (fraction #3). Cyp450 MO activity was measured in each fraction (Figure 1). While there was no Cyp450 MO activity detectable in the cytosol (fraction #2; $n = 9$; NS vs solvent control), most Cyp450 MO activity was found in fraction #3 ($n = 9$; $P < 0.05$ vs solvent control). In addition to fraction #3, Cyp450 MO activity was detected to a smaller extent in fraction #1 ($n = 9$; $P < 0.05$ vs solvent control).

Cyp450 MO is activated by Ca^{2+} store depletion

In saponin (1 mg ml^{-1}) permeabilized cells, Ca^{2+} store depletion with the ATPase inhibitors, thapsigargin (TG; 1 μM) and cyclopiazonic acid (CPA; 10 μM) increased Cyp450 MO activity from 0.51 ± 0.12 (solvent control) to 1.93 ± 0.10 ($n = 26$; $P < 0.05$) and 2.36 ± 0.15 pmol min^{-1} per 10^6 cells ($n = 4$; $P < 0.05$), respectively (Figure 2). The apparent K_m and V_{max} values for thapsigargin-stimulated Cyp450 MO activity were 11.34 μM and 26 pmol mg^{-1} 10 min^{-1}, respectively, which is comparable to that described by Abraham *et al*. (1985) for benzo[a]pyrene hydroxylation. Chelating microsomal free Ca^{2+} with oxalate (3 mM; Gosh *et al*., 1989; Mullaney *et al*., 1987) increased Cyp450 MO activity from 0.51 ± 0.12 to 3.19 ± 0.17 ($n = 6$; $P < 0.05$; Figure 2). Thapsigargin-initiated Cyp450 MO activation in permeabilized cells remained unchanged when free Ca^{2+} concentration was clamped at 1 nM or 1 μM (data not shown).

In agreement with the results with the Ca^{2+} store-depleting/chelating compounds, the physiological agonist of endoplasmic Ca^{2+} release inositol-1,4,5 trisphosphate (10 μM; IP_3), yielded Cyp450 MO activation from 0.15 ± 0.15 to 0.67 ± 0.12 pmol min^{-1} per 10^6 cells ($n = 4$; $P < 0.05$; Figure 2).

In contrast to the compounds mentioned above, ryanodine in concentrations of 1 μM and 25 μM failed to increase Cyp450

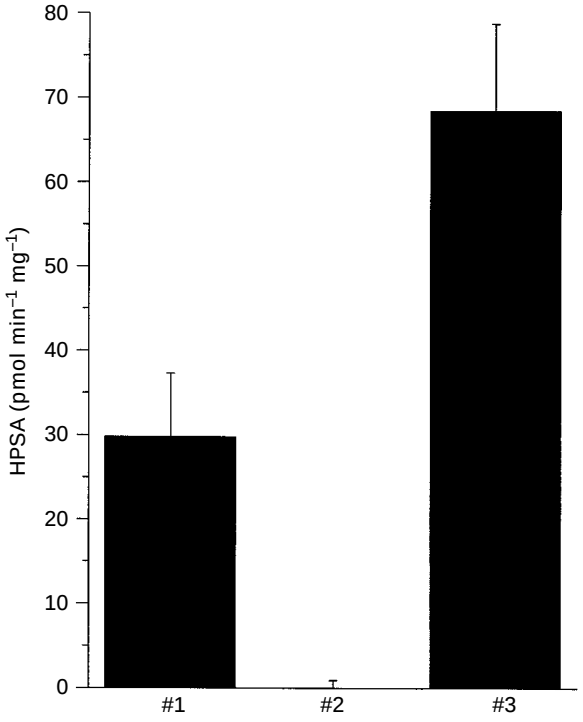


Figure 1 In porcine aortic endothelial cells, Cyp450 MO activity was mainly found in the microsomal fraction. Subcellular distribution of Cyp450 MO was investigated by separation of subcellular compartments with a centrifugation procedure as described in Methods. Each fraction (fraction #1: mitochondria, lysosomes, golgi complex and peroxisomes; fraction #2: cytosol and fraction #3: microsomes, endoplasmic reticulum) was dissolved to equal protein concentration in intracellular-like buffer containing a NADPH regenerating system (isocitric acid, isocitric dehydrogenase, $NADP^+$) and 25 μM EPSA. Each column represents the mean$\pm$s.e.mean ($n=9$).

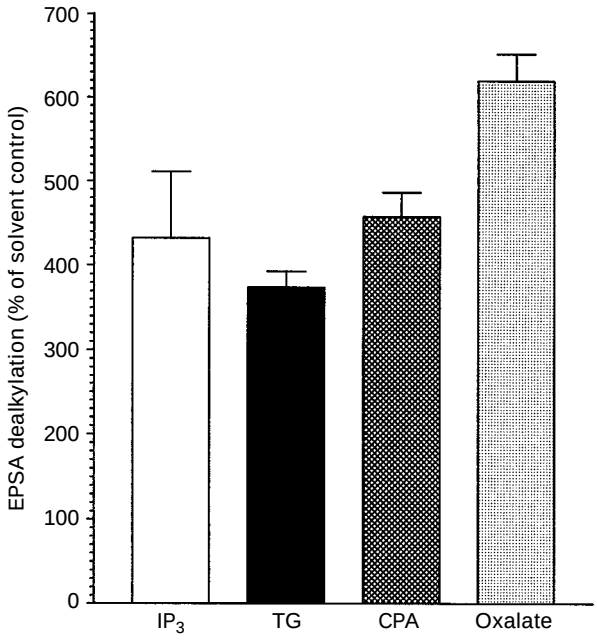


Figure 2 In endothelial cells, microsomal Cyp450 MO was activated by depletion of the intracellular Ca^{2+} pool by inositol 1,4,5-trisphosphate (IP_3), by inhibiting endoplasmic reticulum ATPase or by chelating microsomal Ca^{2+} with oxalate. Microsomal Cyp450 MO activity in saponin-permeabilized endothelial cells was monitored at 37°C in intracellular-like buffer in the presence of a NADPH regenerating system (isocitric acid, isocitric dehydrogenase, $NADP^+$) and 25 μM EPSA. Enzyme was activated by Ca^{2+} store depletion with IP_3 (10 μM), thapsigargin (1 μM; TG) and cyclopiazonic acid (10 μM; CPA) or by chelating intra-microsomal free Ca^{2+} with oxalate (3 mM). Due to different effects of the solvents used on Cyp450 MO activity, results are expressed as percentage increase of Cyp450 MO activity in the corresponding solvent control. Each column represents the mean$\pm$s.e.mean ($n=4-26$).

MO activity (data not shown). The effect of caffeine, another activator of Ca^{2+}-induced Ca^{2+} release mechanism, on Cyp450 MO activity could not be measured, due to interference of caffeine with the detection system (data not shown).

Pharmacological characterization of Cyp450 MO

The proposed physiological substrate of Cyp450 MO, arachidonic acid, inhibited Cyp450 MO-mediated HPSA formation with an IC_{50} value of 14.47 ± 2.75 μM (Figure 3a).

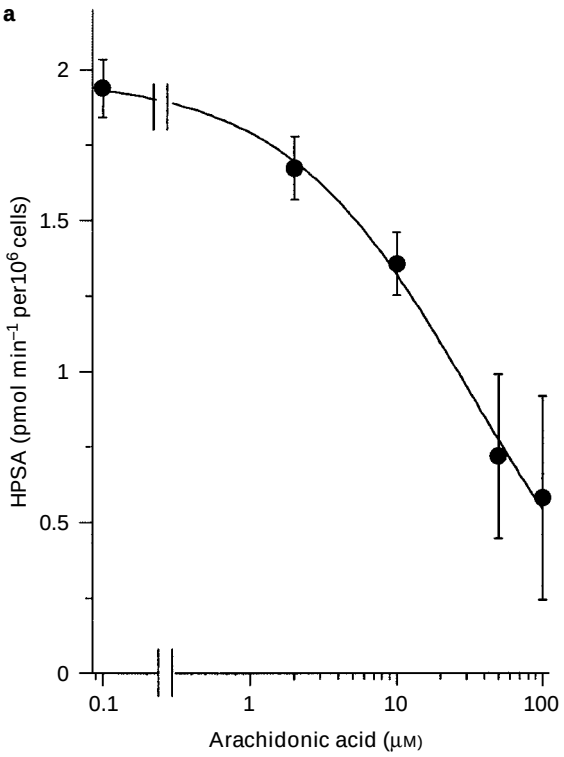
To characterize further endothelial Cyp450 MO, the inhibitory potency of well known cytochrome P450 inhibitors was established. The imidazol antimycotics, econazole and miconazole inhibited Cyp450 MO activity in a concentration-dependent manner with an IC_{50} of 41.08 ± 2.21 μM for econazole and 10.83 ± 4.88 μM for miconazole (Figure 3b). Proadifen, another common inhibitor of cytochrome P450 enzymes, decreased Cyp450 MO activity with an IC_{50} of 38.04 ± 3.14 μM (Figure 3b). Cyanide (250 μM), which attenuates cytochrome P450 enzyme activity, decreased Cyp450 MO activity by $56\pm3.3\%$ ($n=7$; $P<0.05$ vs in the absence of cyanide; data not shown).

In addition to these well known inhibitors of cytochrome P450 enzymes, methohexitone and thiopentone sodium which has been shown to inhibit hepatic Cyp450 MO (Lischke *et al.*, 1995) were investigated on their inhibitory potency on microsomal Cyp450 MO in endothelial cells. As shown in Figure 4, thiopentone sodium diminished Cyp450 MO activity in endothelial cells in a concentration-dependent manner with an IC_{50} of 84.46 ± 17.83 μM ($n=14$). In agreement with the results with thiopentone sodium, methohexitone (10 μM) attenuated endothelial Cyp450 MO activity by $84\pm0.6\%$ ($n=11$; $P<0.05$ vs in the absence of methohexitone; data not shown).

To test whether thiopentone sodium exhibits additional effects on endothelial Ca^{2+} signalling other than inhibition of Cyp450 MO activity, the effect of thiopentone sodium on Ca^{2+}-initiated membrane hyperpolarization and tyrosine kinase activity was tested in endothelial cells. Thiopentone sodium did not affect Ca^{2+}-induced membrane hyperpolarization of endothelial cells ($116.4\pm15.0\%$ of the Ca^{2+}-induced hyperpolarization in the absence of thiopentone sodium; $n=5$; NS), while tetrabutylammonium (10 mM) decreased the Ca^{2+}-initiated membrane hyperpolarization in endothelial cells ($23.1\pm6.0\%$ of the Ca^{2+}-induced hyperpolarization in the absence of tetrabutylammonium, $n=5$; $P<0.01$ vs control, $P<0.01$ vs in the presence of thiopentone sodium). In addition, thiopentone sodium up to 1 mM failed to affect tyrosine kinase activity (Inset Figure 4).

Contribution of Cyp450 MO to endothelial Ca^{2+} signalling

To investigate the role of Cyp450 MO on endothelial Ca^{2+} signalling activated by autacoids, thiopentone sodium was used to inhibit enzyme activity. In nominal free Ca^{2+} solution, 1 μM thapsigargin increased endothelial $[Ca^{2+}]_i$ in the absence of thiopentone sodium by 1.47 ratio units ($n=3$; $P<0.05$ vs basal; Figure 5a). In the presence of 300 μM thiopentone sodium, thapsigargin-induced Ca^{2+} release remained unchanged ($\Delta=1.42$ ratio units; $n=4$; NS vs control; Figure 5a). In contrast to thapsigargin-evoked Ca^{2+} release, Ca^{2+} entry on addition of 2.5 mM Ca^{2+} was significantly diminished in the presence of thiopentone sodium (Figure 5a). In the absence of thiopentone sodium, re-addition of extracellular Ca^{2+} to thapsigargin prestimulated cells yielded



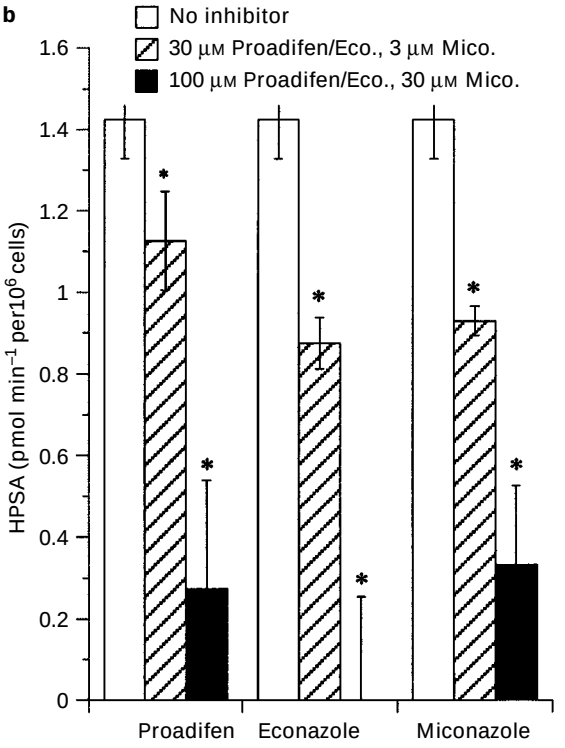


Figure 3 Microsomal Cyp450 MO-mediated EPSA dealkylation was inhibited by arachidonic acid (a), proadifen, econazole and miconazole in a concentration-dependent manner (b). (a) Cyp450 MO activity was measured in the presence of arachidonic acid in the concentrations as indicated. Each point represents the mean and vertical lines show s.e.mean ($n=8-12$). (b) Cyp450 MO activity was measured in the absence of any inhibitor and in the presence of 30 or 100 μM proadifen and econazole and 3 or 30 μM miconazole, respectively. Endothelial cells were permeabilized with 1 mg ml^{-1} saponin and enzyme activity was determined as described in Methods. Each column represents the mean$\pm$s.e.mean ($n=8-26$). *$P<0.05$ vs in the absence of the inhibitor.

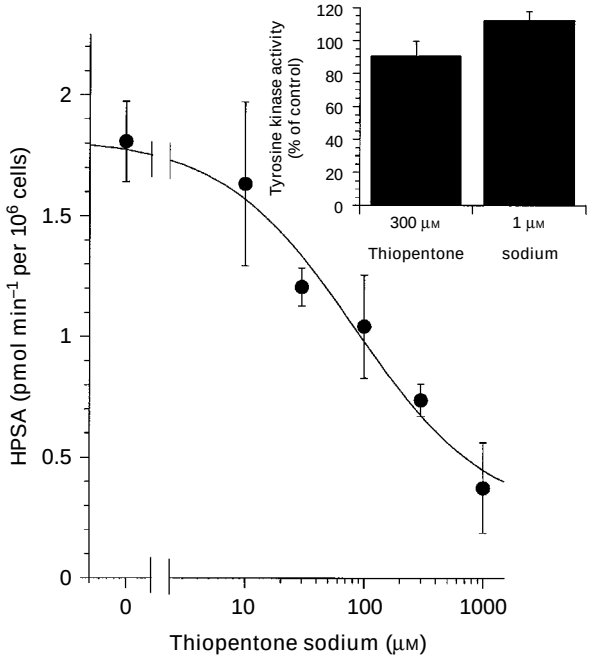


Figure 4 Thiopentone sodium inhibited Cyp450 MO-mediated HPSA formation in a concentration-dependent manner. In the presence of the thiopentone sodium concentrations indicated microsomal Cyp450 MO activity of saponin-permeabilized cells was measured by fluorometric determination of the conversion of EPSA to HPSA at 37°C in the presence of a NADPH re-generating system (isocitric acid, isocitric dehydrogenase, $NADP^+$). Each point represents the mean ($n=14$); vertical lines show s.e.mean. Inset: tyrosine kinase activity was measured in the absence and in the presence of 300 μM and 1 mM thiopentone sodium as described in Methods. Enzyme activity is expressed as percentage of activity in the absence of thiopentone sodium ($n=3$; NS vs in the absence of thiopentone sodium).

increases in $[Ca^{2+}]_i$ of 2.51 ratio units (Figure 5a), while in the presence of 300 μM thiopentone sodium, Ca^{2+} re-addition to thapsigargin prestimulated cells increased $[Ca^{2+}]_i$ by 1.52 ratio units ($n=4$; $P<0.05$ vs in the absence of thiopentone sodium; Figure 5a).

In the presence of 2.5 mM Ca^{2+}, thapsigargin (1 μM) increased $[Ca^{2+}]_i$ by 3.50 ratio units (Figure 5b). In the presence of 300 μM thiopentone sodium, thapsigargin-evoked increases in $[Ca^{2+}]_i$ were diminished to 1.16 ratio units ($n=4$; $P<0.05$ vs in the absence of thiopentone sodium; Figure 5b). After 6 min of thapsigargin stimulation, endothelial $[Ca^{2+}]_i$ in the presence of thiopentone sodium almost returned to that value measured in cells without extracellular Ca^{2+}, while in the absence of thiopentone sodium a plateau phase could be observed (Figure 5b).

Similar to thapsigargin, bradykinin-induced intracellular Ca^{2+} release remained unchanged in the presence of 300 μM thiopentone sodium (from 2.19 ± 0.03 to 2.60 ± 0.07 ratio units in the absence and from 2.20 ± 0.02 to 2.63 ± 0.07 ratio units in the presence of thiopentone sodium, $n=6$, NS). However, thiopentone sodium blunted the bradykinin-activated plateau phase in the presence of 2.5 mM Ca^{2+} (Figure 6a). Under control conditions, 100 nM bradykinin yielded an initial increase in $[Ca^{2+}]_i$ of 0.97 ratio units, followed by a plateau phase ($P<0.05$ vs basal; $n=5$; Figure 6a). In the presence of thiopentone sodium, the initial $[Ca^{2+}]_i$ increase due to bradykinin was reduced to 0.70 ratio units ($n=5$; $P<0.05$ vs in the absence of thiopentone sodium) and no plateau-phase was detectable after 3 min (Figure 6a). In agreement with our findings that thiopentone sodium inhibited SOCP stimulated by bradykinin, thiopentone sodium blunted bradykinin-induced Mn^{2+} entry, indicated by the lack of bradykinin-initi-

ated Mn^{2+} quench in the presence of thiopentone sodium (Figure 6b; $P<0.05$).

Addition of 300 μM thiopentone sodium to cells which were prestimulated by 100 nM bradykinin in the presence of 2.5 mM extracellular Ca^{2+}, yielded a slow decline of the bradykinin-evoked $[Ca^{2+}]_i$ plateau (data not shown). The thiopentone sodium-induced decline of $[Ca^{2+}]_i$ in stimulated cells was significantly slower (half declining time $=21.3\pm2.9$ s; $n=10$) than that observed with the K_{Ca} channel inhibitor tetrabutylammonium (10 mM; half-life of decline $=13.9\pm$ 2.1 s; $n=9$; $P<0.001$ vs thiopentone sodium).

In contrast to bradykinin and thapsigargin, ryanodine (5 μM) and caffeine (10 mM) failed to stimulate Ca^{2+} entry in endothelial cells, although both compounds yielded significant intracellular Ca^{2+} release (data not shown).

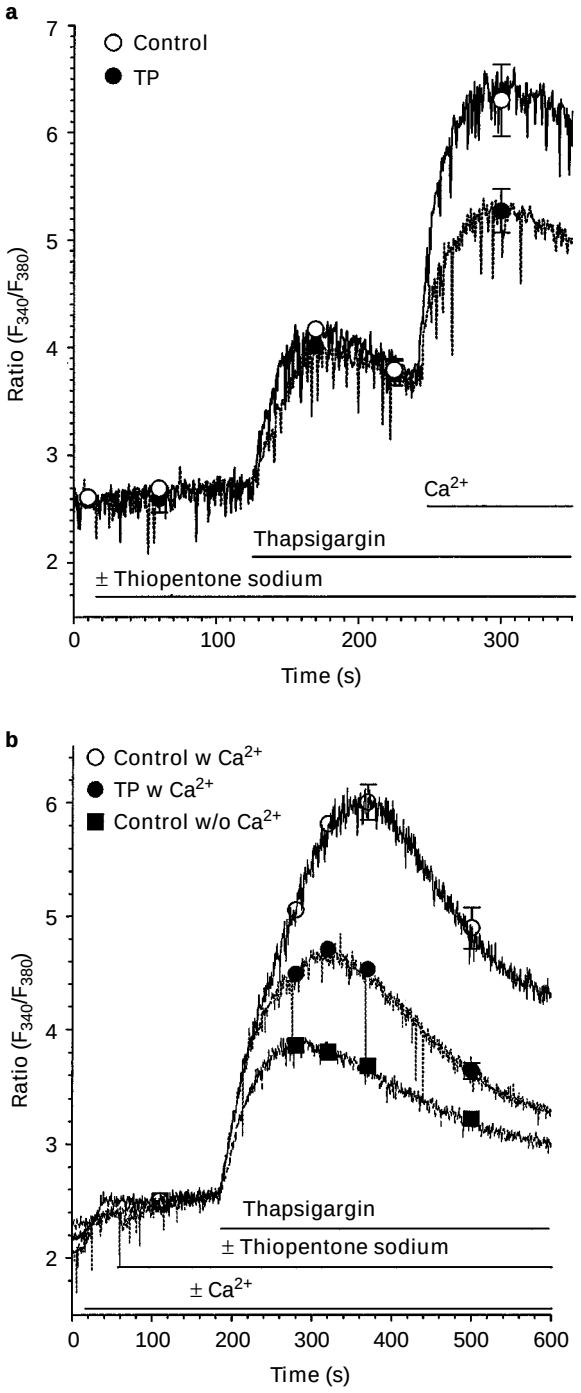


Figure 5 Thiopentone sodium did not affect thapsigargin-induced intracellular Ca^{2+} release, while it diminished Ca^{2+} entry after addition of 2.5 mM Ca^{2+} to thapsigargin prestimulated cells (a) and thapsigargin-initiated increases in the presence of 2.5 mM free Ca^{2+} (b). (a) Fura-2 loaded cells were stimulated in nominal Ca^{2+} free solution with 1 μM thapsigargin in the absence (Control) or presence of 300 μM thiopentone sodium (TP) followed by the addition of 2.5 mM extracellular Ca^{2+} as indicated. Tracings show representative experiments and points represent the mean$\pm$s.e.mean ($n=7$). (b) Endothelial cells were stimulated with 1 μM thapsigargin in 2.5 mM free Ca^{2+}-containing solution in the absence (Control w Ca^{2+}) or presence of 300 μM thiopentone sodium (TP w Ca^{2+}) or in the nominal absence of extracellular Ca^{2+} without thiopentone sodium (Control w/o Ca^{2+}). Each point represents the mean$\pm$s.e.mean ($n=4$).

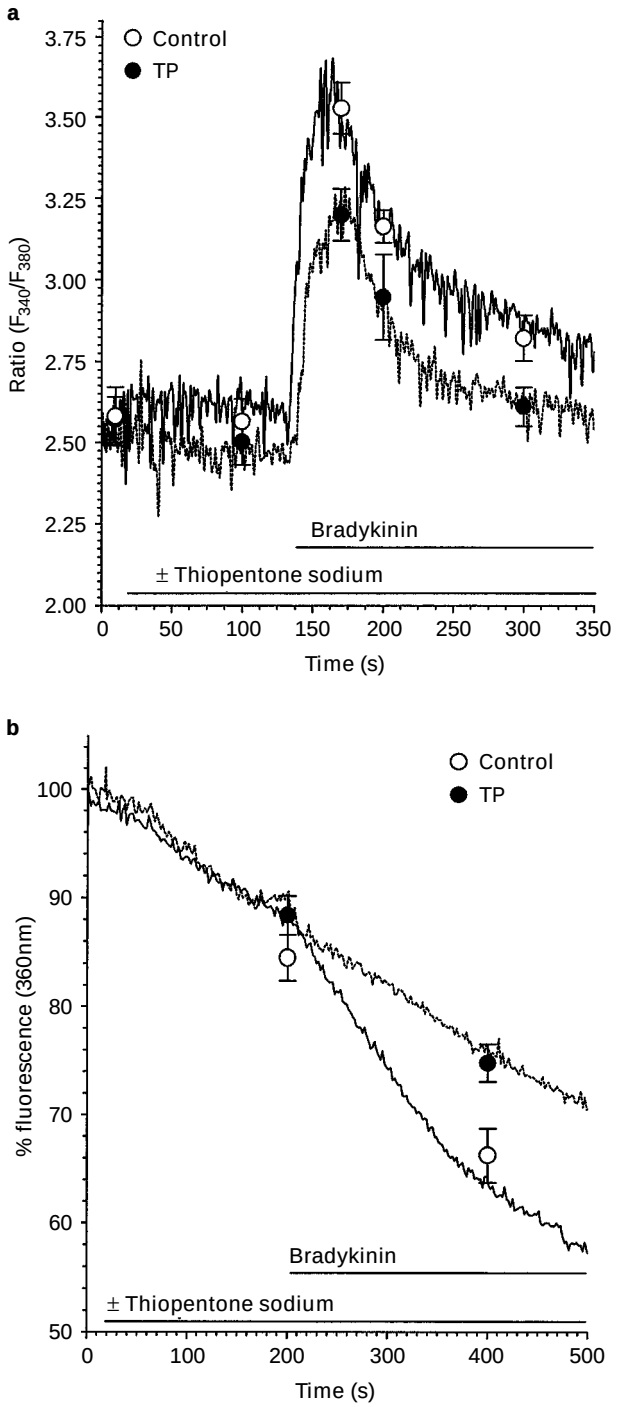


Figure 6 Thiopentone sodium blunted bradykinin-evoked Ca^{2+} (a) and Mn^{2+} (b) entry. (a) Cultured endothelial cells were stimulated with 100 nM bradykinin in 2.5 mM Ca^{2+}containing buffer in the absence (Control) and presence of 300 μM thiopentone sodium (TP). Points represent the mean $\pm$s.e.mean ($n=5$). (b) Fura-2 loaded cells were stimulated in the presence of 2 mM Mn^{2+} with 100 nM bradykinin in the absence (Control) and presence of 300 μM thiopentone sodium (TP). Each point represents the mean$\pm$s.e.mean ($n=6-8$).

Cyp450 MO contributes to membrane hyperpolarization following agonist stimulation

As described previously (Groschner, *et al*., 1992; Graier *et al*., 1993), stimulation of endothelial cells with bradykinin yielded membrane hyperpolarization (Figure 7). Addition of 100 nM bradykinin hyperpolarized endothelial cells by -20 ± 3 mV within 15 s ($n=8$), followed by a slow decline in membrane potential where membrane potential returned to basal values after about 450 s (Figure 7). In the presence of thiopentone sodium bradykinin-initiated membrane hyperpolarization was decreased to -11 ± 4 mV ($n=6$; $P<0.05$ vs in the absence of thiopentone sodium) and represents a transient phenomenon where membrane potential returned to basal value after 250 s ($P<0.05$ vs in the absence of thiopentone sodium; Figure 7).

Long-term incubation of endothelial cells with dexamethasone/clofibrate increased Cyp450 MO activity and altered stimuli-evoked Ca^{2+}/Mn^{2+} entry and membrane hyperpolarization

After 72 h of treatment with 3 μM dexamethasone and 5 μM clofibrate, endothelial Cyp450 MO activity in response to thapsigargin (1 μM) was increased by 35% in cells treated with dexamethasone/clofibrate ($n=12$; $P<0.05$; inset Figure 8a). Besides its effect on Cyp450 MO activity to thapsigargin, the 72 h pretreatment with dexamethasone/clofibrate augmented the $[Ca^{2+}]_i$ increase on addition of 2.5 mM extracellular Ca^{2+} following thapsigargin stimulation (1 μM) by 105% ($n=7-9$, $P<0.01$; Figure 8a). In contrast, pretreatment with dexamethasone/clofibrate did not affect intracellular Ca^{2+} release in response to thapsigargin (Figure 8a). Similar results were obtained with 100 nM bradykinin (data not shown). Convincingly, pretreatment with dexamethasone/clofibrate enhanced bradykinin (100 nM; Figure 8b) and thapsigargin (data not shown)–induced Mn^{2+} entry in endothelial cells, indicated by an increase in Mn^{2+} mediated quench by 105% ($n=4$, $P<0.05$; Figure 8b). Hence, pretreatment with dexametha-

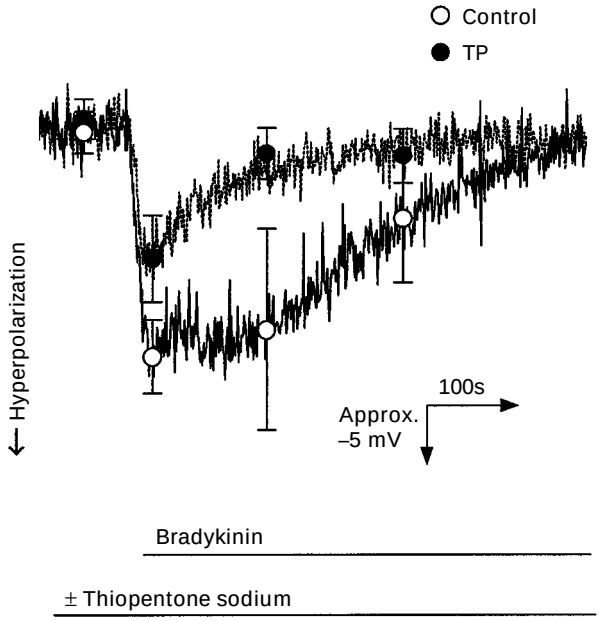


Figure 7 Bradykinin-evoked membrane hyperpolarization was diminished by thiopentone sodium. Porcine cultured endothelial cells were stimulated with 100 nM bradykinin in the absence (Control) or in the presence of 300 μM thiopentone sodium (TP). Changes in membrane potential were monitored with $DiBAC_4$ (5) as described in Methods. Each point represents the mean±s.e.mean ($n=6-8$).

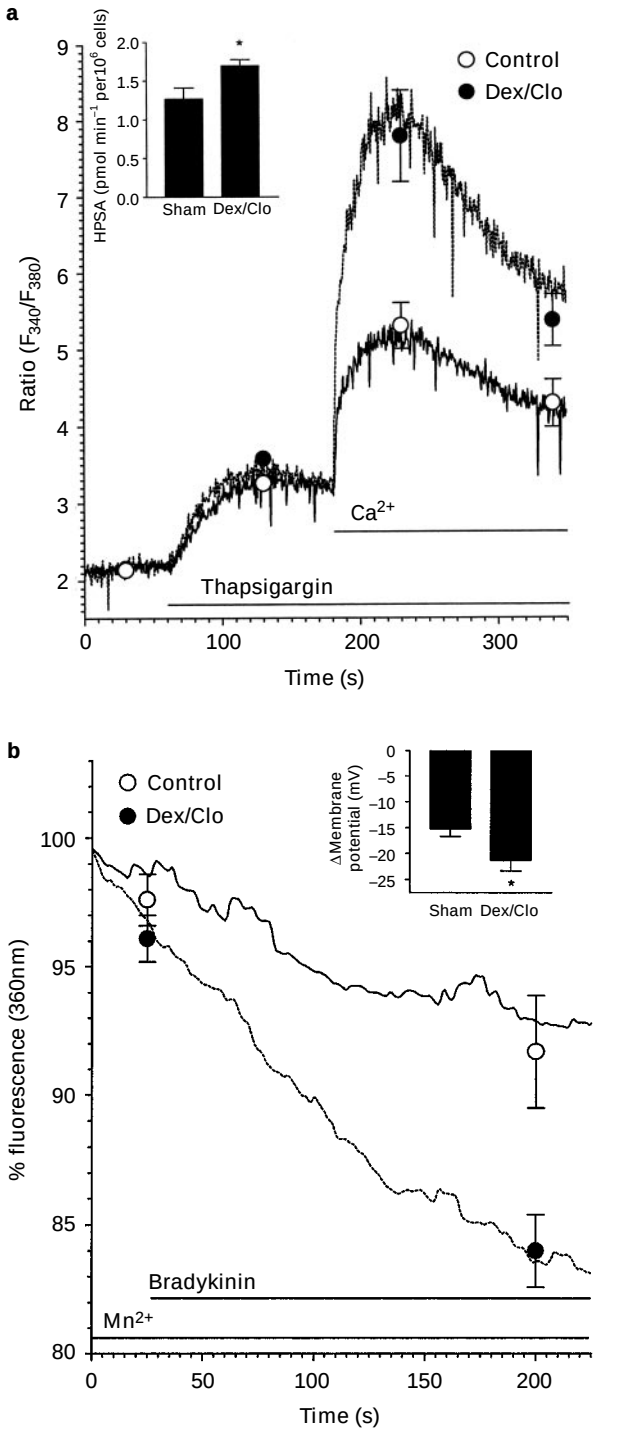


Figure 8 Pretreatment with dexamethasone/clofibrate increased stimuli-induced Cyp450 MO activity (inset in (a)), Ca^{2+} entry (a), Mn^{2+} quench (b) and membrane hyperpolarization (inset in (b)) in vascular endothelial cells. Porcine cultured endothelial cells were pretreated for 72 h with the solvent (DMSO 0.2%; Control) or 3 μM dexamethasone and 5 μM clofibrate (Dex/Clo, $n=7-9$). (a) Cells were loaded with fura-2 as described in Methods. In the nominal absence of extracellular Ca^{2+} cells were stimulated with 1 μM thapsigargin followed by the addition of 2.5 mM Ca^{2+} at times indicated. Inset in (a): Cyp450 MO activity was tested by measuring the EPSA dealkylation in thapsigargin-stimulated cells which were preincubated with or without dexamethasone/clofibrate ($n=12$). (b) In fura-2 loaded cells, Mn^{2+} entry on stimulation with 100 nM bradykinin was monitored by measuring the Mn^{2+}-initiated quench at the isosbestic wavelength of fura-2 (360 nm). Inset in (b): Thapsigargin-initiated membrane hyperpolarization (measured with $DiBAC_4$ (5) (1 μM)) of cells pretreated, in the absence or presence of dexamethasone/clofibrate as described in Methods. Each column represents the mean hyperpolarization in response to 1 μM thapsigargin ±s.e.mean ($n=4$) within 1000 s.

sone/clofibrate increased thapsigargin-induced membrane hyperpolarization by 40% ($n = 4$, $P<0.05$; inset Figure 9b).

The Cyp450 MO-derived compound, 11,12-EET yielded membrane hyperpolarization in endothelial cells which was resistant to thiopentone sodium

The Cyp450 MO-derived compound, 11,12-EET (19.4 μM) hyperpolarized endothelial cells by -12 ± 2 mV ($n=8$; $P<0.05$ vs basal; Figure 9a). In agreement with the inability of thiopentone sodium to prevent Ca^{2+}-initiated membrane hyperpolarization in endothelial cells as mentioned above, the 11,12-EET-evoked membrane hyperpolarization remained unchanged in the presence of 300 μM thiopentone sodium (Figure 9b). Similar to 11,12-EET, another arachidonic acid-derived epoxide, 5,6-EET also yielded membrane hyperpolarization in endothelial cells (data not shown).

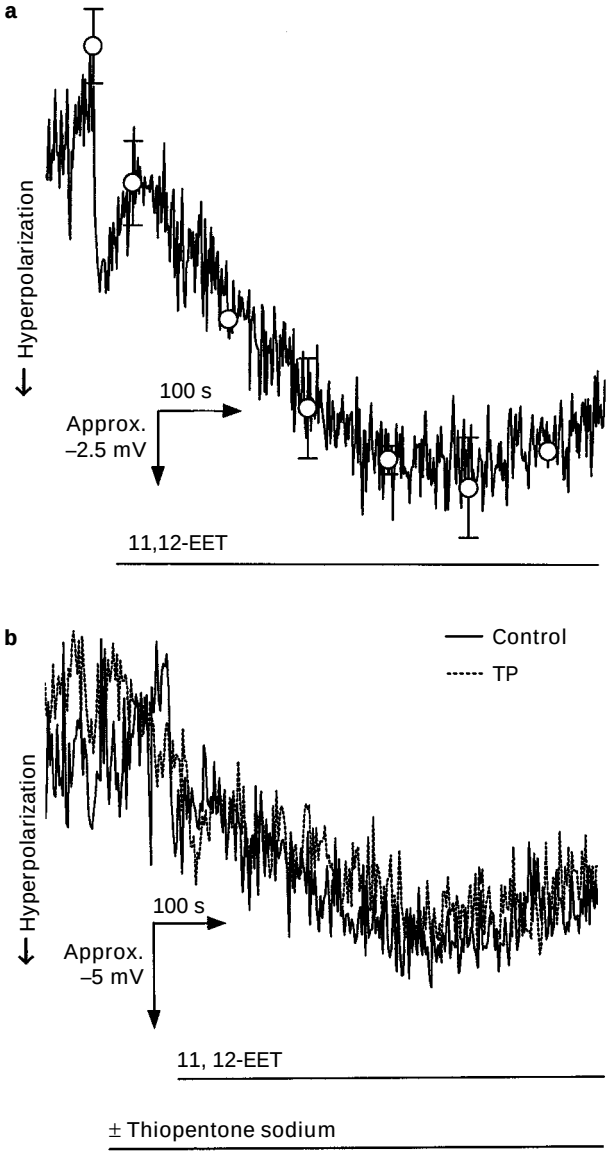


Figure 9 The Cyp450 MO-derived compound, 11,12-EET, hyperpolarized endothelial cells. This effect was not inhibited by thiopentone sodium. (a) Porcine cultured aortic endothelial cells were stimulated with 11,12-EET in nominal Ca^{2+} free solution. Changes in membrane potential were monitored with $DiBAC_4$ (5) as described in Methods. Points indicate the mean value $\pm$s.e.mean ($n=8$). (b) 11,12-EET-initiated membrane hyperpolarization was measured in nominal Ca^{2+}-free solution in the absence (continuous line) and presence (dotted plot) of 300 μM thiopentone sodium. Graph shows representative tracings ($n=6$).

Discussion

We investigated the mechanism of Cyp450 MO activation by Ca^{2+} store depletion to IP_3-generating autacoids and ATPase inhibitors. In addition, the importance of the Cyp450 MO-derived compounds, EETs, for Ca^{2+} entry via SOCP in endothelial cells was investigated. Our main finding was that Cyp450 MO was activated by lowering microsomal free Ca^{2+} due to intracellular Ca^{2+} release mediated by ATPase inhibition or IP_3 or by chelating intra-microsomal Ca^{2+}, while cytosolic free Ca^{2+} concentration was not important. While intracellular Ca^{2+} release remained unaffected by Cyp450 MO inhibition, autacoid-initiated SOCP activation and membrane hyperpolarization were diminished. The Cyp450 MO-derived epoxides, 5,6- and 11,12-EET yielded membrane hyperpolarization which was not affected by Cyp450 MO inhibition. Induction of endothelial Cyp450 MO by dexamethasone/clofibrate yielded increases in thapsigargin-initiated Cyp450 MO activity, SOCP activity and membrane hyperpolarization. Together with our previously published data (Graier *et al*., 1995), the present findings lend support to our hypothesis that Cyp450 MO-derived compounds may play a crucial role in the regulation of SOCP in vascular endothelial cells.

We and several other groups have failed to determine endothelial Cyp450 MO activity by measuring the dealkylation of ethoxyresorufine or 7-ethoxycoumarin (this study, Hecker *et al*., 1995). In contrast, benzo[a]pyrene was shown to be a suitable substrate for determination of endothelial Cyp450 MO activity (Abraham *et al*., 1985). In agreement with these findings, we found the cytochrome P450 substrate EPSA (Koller, 1994), a compound structurally similar to benzo[a]pyrene, to be a useful substrate for endothelial Cyp450 MO. In contrast to benzo[a]pyrene (Abraham *et al*., 1985), EPSA allows Cyp450 MO activity to be measured during the experiment. While this technique is very sensitive (detection limit of about 2 nM of HPSA in the presence of 25 μM EPSA) and reproducible one major drawback still remains, the permeabilization of the cells before the experiments.

The subcellular distribution of Cyp450 MO, which demonstrated Cyp450 MO to be located mainly in microsomes, lends support to our hypothesis that this enzyme might be located in/on the IP_3-sensitive Ca^{2+} pool. This hypothesis was further supported by our findings that IP_3-mediated Ca^{2+} release stimulated Cyp450 MO activity. Convincingly, thapsigargin and cyclopiazonic acid, which have been demonstrated to deplete IP_3-sensitive Ca^{2+} pools in endothelial cells (Schilling *et al*., 1992) and the intra-microsomal Ca^{2+} chelator oxalate (Mullaney *et al*., 1987), yielded Cyp450 MO activation. While Cyp450 MO stimulation strictly depended on a drop of intra-microsomal free Ca^{2+}, enzyme activity was not affected by changes in cytosolic free Ca^{2+} which excludes activation via cytosolic Ca^{2+}/calmodulin pathways.

Interestingly, ryanodine, an activator of Ca^{2+}-induced Ca^{2+} release, failed to activate endothelial Cyp450 MO, suggesting local/functional separation within the endoplasmic reticulum. Hence, ryanodine/caffeine have little or no ability to stimulate SOCP, although intracellular Ca^{2+} release has been found (Corda *et al*., 1995). Moreover, Corda *et al*. (1995) showed that caffeine-initiated SOCP activation only in thapsigargin prestimulated cells and suggested that thapsigargin-initiated depletion of IP_3-sensitive Ca^{2+} pools might generate a signal (Cyp450 MO activation?) essential for Ca^{2+} entry.

Endothelial Cyp450 MO was sensitive to econazole proadifen and miconazole with IC_{50} values of approximately 41, 11 and 38 μM, respectively. Thus, the concentrations necessary to prevent enzyme activity were rather high compared to their potency at inhibiting K^+ channels (Alvarez *et al*., 1992), ATPases (Mason *et al*., 1993), SOCP (Graier *et al*., 1995), tyrosine kinase (Sargeant *et al*., 1994) or L-type Ca^{2+} channels (Villalobos *et al*., 1992). Since we failed to detect any inhibitory effect of econazole on Cyp450 MO activity in concentrations less than 20 μM, the inability of econazole to prevent the cal-

cium influx factor (CIF) generation described by Randriamampita & Tsien (1993) was probably due to an insufficient concentration of econazole (10 μM) in these experiments.

As expected, the proposed physiological substrate of microsomal Cyp450 MO, arachidonic acid, prevented conversion of our fluorophore EPSA to HPSA, indicating that arachidonic acid, indeed, serves as a substrate for microsomal Cyp450 MO in endothelial cells. This is in agreement with findings of Harder *et al*. (1995), who showed arachidonic acid to be the main substrate for endothelial Cyp450 MO.

Since the cytochrome P450 inhibitors mentioned above were not useful for studying the contribution of Cyp450 MO on endothelial Ca^{2+} signalling, as they are not selective for Cyp450 MO, we studied two new inhibitors of Cyp450 MO, methohexitone and thiopentone (Lischke *et al*., 1995). In cells prestimulated with bradykinin in the presence of extracellular Ca^{2+}, addition of thiopentone sodium yielded a significantly slower decline of the $[Ca^{2+}]_i$ plateau than that obtained with the K_{Ca} channel inhibitor tetrabutylammonium (this study) or by inhibitors of the endothelial Ca^{2+} channel (Graier *et al*., 1995). Hence, hyperpolarization of endothelial cells to Ca^{2+} and to 5,6- and 11,12-EET remained unchanged in the presence of thiopentone sodium. These data suggest that thiopentone sodium does not interfere with endothelial K_{Ca} or Ca^{2+} channels directly. Moreover, thiopentone sodium, in contrast to imidazole antimycotics (Sargeant *et al*., 1994), did not affect endothelial tyrosine kinase activity, indicating that inhibition of tyrosine kinase does not account for the inhibitory effect of thiopentone sodium on endothelial SOCP. Thus, thiopentone sodium seems to be more selective at inhibiting Cyp450 MO activity than imidazole antimycotics or proadifen and may prove a suitable tool for investigation of the contribution of Cyp450 MO to the endothelial Ca^{2+} signalling.

When thiopentone sodium was applied before stimulation of endothelial cells with thapsigargin or bradykinin, SOCP-mediated Ca^{2+} entry was abolished, while intracellular Ca^{2+} release via IP_3 formation (bradykinin) or inhibition of endoplasmic reticulum ATPase (thapsigargin) remained unchanged in the presence of thiopentone sodium. These data indicate that inhibition of Cyp450 MO prevents stimulation and/or activity of SOCP to Ca^{2+} store depletion. The importance of endothelial Cyp450 MO for endothelial Ca^{2+} signalling was further supported by our findings that an induction procedure which yielded increases in thapsigargin-induced Cyp450 MO activity, enhanced thapsigargin-stimulated Ca^{2+}/Mn^{2+} entry and membrane hyperpolarization.

In general our present data together with our previous findings (Graier *et al*., 1995) suggest that Cyp450 MO-derived compounds to be involved in at least two crucial events of the endothelial Ca^{2+} signalling and stimulation of SOCP. Firstly, Cyp450 MO may generate an intracellular signal molecule for K^+ channel activation, resulting in hyperpolarization and thus, an increased driving force for Ca^{2+} to enter the endothelial cell by Ca^{2+} permeable channels. This hypothesis is supported by our findings that inhibition of Cyp450 MO by thiopentone sodium diminished membrane hyperpolarization induced by bradykinin in the absence of extracellular Ca^{2+}, where the inhibitory effect of thiopentone sodium on Ca^{2+} entry can be disregarded. The remaining hyperpolarization to bradykinin should be due to the release of Ca^{2+} and stimulation of Ca^{2+} activated K^+ channels (Groschner *et al*., 1992). Our findings that Cyp450 MO induction with dexamethasone/clofibrate did not affect thapsigargin-induced intracellular Ca^{2+} release, while thapsigargin-induced membrane hyperpolarization was increased, suggest that changes in intracellular Ca^{2+} concentrations for the activation of K_{Ca} channels can be excluded in the increased membrane hyperpolarization induced in dexamethasone/clofibrate-incubated cells. These data indicate that increased membrane hyperpolarization in response to thapsigargin is due to the elevation of Cyp450 MO activity. Convincingly, we demonstrated in this study that the Cyp450 MO-derived compounds, 5,6- and 11,12-EET hyperpolarize endothelial cells in concentrations known to be produced by endothelial cells (Rosolowsky & Cambell, 1993). Consistent with our hypothesis that the Cyp450 MO-derived EETs may serve as intracellular signal molecules for membrane hyperpolarization, thiopentone sodium had no effect on EET-initiated hyperpolarization. Secondly, Cyp450- MO-derived compounds may activate SOCP directly, as shown in rat hepatocytes (Karara *et al*., 1991), pituitary cells (Snyder *et al*., 1986), parotid cells (Snowdowne *et al*., 1989), epithelial cells (Madhun *et al*., 1991) and endothelial cells (Graier *et al*., 1995). However, in endothelial cells only 5,6-EET was shown to trigger SOCP directly (Graier *et al*., 1995). These data indicate, that all Cyp450 MO-derived compounds may hyperpolarize endothelial cells, while just one isomere, 5,6-EET triggers SOCP activation. The relative significance of these two different mechanisms for Cyp450 MO-derived compounds is unknown and additional studies are necessary to elucidate the function of microsomal Cyp450 MO in autacoid-initiated Ca^{2+} signalling in more detail.

In conclusion, our data suggest that microsomal Cyp450 MO plays a crucial role in autacoid-initiated Ca^{2+} signalling in endothelial cells. This microsomal enzyme becomes activated by IP_3-mediated depletion of intracellular Ca^{2+} pools and uses phospholipase-A_2-derived arachidonic acid to synthesize EETs. These compounds may hyperpolarize endothelial cells by stimulation of K^+ channels and, thus, increase the driving force for Ca^{2+} entry. In addition, one of these epoxides, 5,6-EET, or one of its metabolites, might be involved in the opening of Ca^{2+} permeable plasma membrane channels.

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References

ABRAHAM, N.G., PINTO, A., MULLANE, K.M., LEVERE, R.D. & SPOKAS, E. (1985). Presence of cytochrome P-450-dependent monooxygenase in intimal cells of hog aorta. *Hypertension*, **7,** 899 – 904.

ALVAREZ, J., MONTERO, M. & GARCÍA-SANCHO, J. (1991). Cytochrome P-450 may link intracellular Ca^{2+} stores with plasma membrane Ca^{2+} influx. *Biochem. J*., **274,** 193 – 197.

ALVAREZ, J., MONTERO, M. & GARCIA-SANCHO, J. (1992). High affinity inhibition of Ca^{2+}-dependent K^+ channels by cytochrome P-450 inhibitors. *J. Biol. Chem*., **267,** 11789 – 1193.

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

CAPDEVILA, J., CHACOS, N., FALCK, J.R., MANNA, S., NEGRO-VILLAR, A. & OJEDA, S.R. (1983). Novel hypothalmic arachidonic products stimulate somastotatin release from median eminence. *Endocrinology*, **113,** 421 – 423.

CORDA, S., SPURGEON, H.A., LAKATTA, E.G., CAPOGROSSI, M.C. & ZIEGELSTEIN, R. (1995). Endoplasmic reticulum Ca^{2+} depletion unmasks a caffeine-induced Ca^{2+} influx in human aortic endothelial cells. *Circ. Res*., **77,** 927 – 935.

FASOLATO, C., HOTH, M. & PENNER, R. (1993). A GTP-dependent step in the activation mechanism of capacitative calcium influx. *J. Biol. Chem*., **268,** 20737 – 20740.

FLEISCHER, S. & KERVINA, M. (1974). Subcellular fractionation of rat liver. *Methods Enzymol.*, **31,** 6–41.

FLEMING, I., FISSLTHALER, B. & BUSSE, R. (1995). Calcium signaling in endothelial cells involves activation of tyrosine kinase and leads to activation of mitogen-activated protein kinase. *Circ. Res.*, **76,** 522–529.

GEBREMEDHIN, D., MA, Y.-H., FALCK, J.R., ROMAN, R.J., VAN-ROLLINS, M. & HARDER, D.R. (1992). Mechanism of action of cerebral epoxyeicosatrienoic acids on cerebral arterial smooth muscle. *Am. J. Physiol.*, **263,** H519–H525.

GOSH, T.K., MULLANEY, J.M., TARAZI, F.I. & GILL, D.L. (1989). GTP-activated communication between distinct inositol 1,4,5-trisphosphate-sensitive and -insensitive calcium pools. *Nature*, **340,** 236–239.

GRAIER, W.F., GROSCHNER, K., SCHMIDT, K. & KUKOVETZ, W.R. (1992). SK&F 96365 inhibits histamine-induced formation of endothelium-derived relaxing factor in human endothelial cells. *Biochem. Biophys. Res. Commun.*, **186,** 1539–1545.

GRAIER, W.F., KUKOVETZ, W.R. & GROSCHNER, K. (1993). cAMP enhances agonist-induced Ca^{2+} entry into endothelial cells by activation of potassium channels and membrane hyperpolarization. *Biochem. J.*, **291,** 263–267.

GRAIER, W.F., SIMECEK, S. & STUREK, M. (1995). Cytochrome P450 mono-oxygenase-regulated signaling of endothelial Ca^{2+} entry. *J. Physiol.*, **482,** 259–274.

GROSCHNER, K., GRAIER, W.F. & KUKOVETZ, W.R. (1992). Activation of a small-conductance Ca^{2+}-dependent K^+ channel contributes to bradykinin-induced stimulation of nitric oxide synthesis in pig aortic endothelial cells. *Biochim. Biophys. Acta.*, **1137,** 162–170.

GROSCHNER, K., GRAIER, W.F. & KUKOVETZ, W.R. (1994). Histamine induces K^+, Ca^{2+} and Cl^- currents in human vascular endothelial cells–Role of ionic currents in stimulation of nitric oxide biosynthease. *Circ. Res.*, **75,** 304–314.

HARDER, D.R., CAMBELL, W.B. & ROMAN, R.J. (1995). Role of cytochrome P-450 enzymes and metabolites of arachidoic acid in the control of vascular tone. *J. Vasc. Res.*, **5,** 79–92.

HECKER, M., BARA, A.T., BAUERNSACHS, J. & BUSSE, R. (1995). Characterization of endothelium-derived hyperpolarizing factor as a cytochrome P450-derived arachidoic acid metabolite in mammals. *J. Physiol.*, **481,** 407–414.

HU, S. & KIM, H.S. (1993). Activation of K^+ channels in vascular smooth muscles by cytochrome P450 metabolites of arachidonic acids. *Eur. J. Pharmacol.*, **230,** 215–221.

KARARA, A., BREYER, M., FALCK, J.R. & CAPDEVILA, J. (1991). Epoxyeicosatrienoic acids (EETs) elevates cytosolic calcium in isolated rat hepatocytes. *FASEB J.*, **6,** A1053.

KOLLER, E. (1994). Fluoregenic substrates for enzyme analysis. *Am. Biotech. Lab.*, **12,** 14–15.

KRASZNAI, Z., MÁRIÁN, T., BALKAY, L., EMRI, M. & TRÓN, L. (1995). Flow cytometric determination of absolute membrane potential of cells. *J. Photochem. Photobiol. B: Biology*, **28,** 93–99.

LISCHKE, V., BUSSE, R. & HECKER, M. (1995). Selective inhibition by barbiturates of the synthesis of endothelium-derived hyperpolarizing factor in the rabbit carotid artery. *Br. J. Pharmacol.*, **115,** 969–974.

MADHUN, Z.T., GOLDTHWAIT, D.A., MCKAY, D., HOPFER, U. & DOUGLAS, J.G. (1991). An epoxygenase metabolite of arachidonic acid mediates angiotensin II-induced rises in cytosolic calcium in rabbit proximal tubule epithelial cells. *J. Clin. Invest.*, **88,** 456–461.

MASON, J.M., MAYER, B. & HYMEL, L.J. (1993). Inhibition of Ca^{2+} transport pathways in thymic lymphocytes by econazole, miconazole and SKF 96365. *Am. J. Physiol.*, **264,** C654–C662.

MONTERO, M., GARCIA-SANCHO, J. & ALVAREZ, J. (1993). Inhibition of the calcium store-operated calcium entry pathway by chemotactic peptide and by phorbol ester develops gradually and independently along differentiation of HL60 cells. *J. Biol. Chem.*, **268,** 26911–26919.

MULLANEY, J.M., CHUEH, S.H., GOSH, T.K. & GILL, D.L. (1987). Intracellular calcium uptake activated by GTP: evidence for a possible guanine nucleotide-induced transmembrane conveyance of intracellular calcium. *J. Biol. Chem.*, **262,** 13865–13872.

PALTAUF-DOBURZYNSKA, J. & GRAIER, W.F. (1997). Temperature dependence of agonist-stimulated Ca^{2+} signaling in cultured endothelial cells. *Cell Calcium*, **21,** 43–51.

PAREKH, A.B., TERLAU, H. & STÜHMER, W. (1993). 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. (1990). Capacitative calcium entry revisited. *Cell Calcium*, **11,** 611–624.

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.

ROSOLOWSKY, M. & CAMBELL, W.B. (1993). Role of PGI_2 and epoxyeicosatrienoic acids in relaxation of bovine coronary arteries to arachidonic acid. *Am. J. Physiol.*, **264,** H327–H335.

SARGEANT, P., CLARKSON, W.D., SAGE, S.O. & HEEMSKERK, J.W.M. (1992). Calcium influx evoked by Ca^{2+} store depletion in human platelets is more susceptible to cytochrome P-450 inhibitors than receptor-mediated calcium entry. *Cell Calcium*, **13,** 553–564.

SARGEANT, P., FARNDALE, R.W. & SAGE, S.O. (1994). The imidazole antimycotics econazole and miconazole reduce agonist-evoked protein-tyrosine phosphorylation and evoke membrane depolarisation in human platelets: cautions for their use in studying Ca^{2+} signalling pathways. *Cell Calcium*, **16,** 413–418.

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.

SNOWDOWNE, K.W., ROSENOER, L., YU, E. & CASHMAN, J.R. (1989). Eicosanoids evoke the release of amylase and increase cytoplasmic calcium in rat parotid cells. *Biochem. Biophys. Res. Commun.*, **161,** 379–384.

SNYDER, G., LATTANZIO, F., YADAGIRI, P., FALCK, J.R. & CAPDEVILA, J. (1986). 5,6-Epoxyeicosatrienoic acid mobilizes Ca^{2+} in anterior pituitary cells. *Biochem. Biophys. Res. Commun.*, **139,** 1188–1194.

SOMASUNDARAM, B., MAHAUT-SMITH, M.P. & FLOTOT, R.A. (1996). Temperature-dependent block of capacitative Ca^{2+} influx in the human leukaemic cell line KU-812J. *J. Biol. Chem.*, **271,** 26096–26104.

VIEIRA, L., SLOTKI, I. & CABANTCHIK, Z.I. (1995). Chloride conductive pathways which support electrogenic H^+ pumping by leishmania major promastigotes. *J. Biochem. Chem.*, **270,** 5299–5304.

VILLALOBOS, C., FONTERIZ, R., LOPEZ, M.G., GARCIA, A.G. & GARCIA-SANCHO, J. (1992). Inhibition of voltage-gated Ca^{2+} entry into GH3 and chromaffin cells by imidazole antimycotics and other P450 blockers. *FASEB J.*, **6,** 2742–2747.

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