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# Cytochrome P450 mono-oxygenase-regulated signalling of $Ca^{2+}$ entry in human and bovine endothelial cells

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1. We tested the hypothesis that agonist-stimulated $Ca^{2+}$ entry, and thus formation of endothelium-derived nitric oxide (EDNO) in vascular endothelial cells, is related to activation of microsomal P450 mono-oxygenase (P450 MO) and the biosynthesis of 5,6-epoxyeicosatrienoic acid (5,6-EET).
2. Several P450 inhibitors diminished the sustained $[Ca^{2+}]_i$ plateau response to agonist or intracellular $Ca^{2+}$ store depletion with ATPase inhibitors by 31–69% (fura-2 technique). $Mn^{2+}$ influx stimulated by agonists or ATPase inhibitors was prevented by P450 inhibitors.
3. Histamine- or ATPase inhibitor-stimulated formation of EDNO was strongly attenuated (50–83%) by P450 inhibitors, without any effect on EDNO formation by the $Ca^{2+}$ ionophore A23187, indicating that decreased EDNO synthesis is due specifically to the inhibition of $Ca^{2+}$ entry by these compounds.
4. Induction of P450 MO by $\beta$-naphthoflavone potentiated agonist-induced $Ca^{2+}$ and $Mn^{2+}$ influx by 60 and 53%, respectively. Intracellular $Ca^{2+}$ release remained unchanged.
5. The P450 MO product, 5,6-EET (< 156 nmol $l^{-1}$), activated $Ca^{2+}/Mn^{2+}$ entry without any depletion of intracellular $Ca^{2+}$ stores. The 5,6-EET-stimulated $Ca^{2+}/Mn^{2+}$ entry was not affected by P450 inhibitors.
6. As with the bradykinin-stimulated $Ca^{2+}$entry pathway, the 5,6-EET-activated $Ca^{2+}$ entry pathway was permeable to $Mn^{2+}$ and $Ba^{2+}$, sensitive to $Ni^{2+}$, $La^{3+}$ and membrane depolarization, and insensitive to the removal of extracellular $Na^{+}$ or the organic $Ca^{2+}$ antagonist, nitrendipine.
7. In the presence of 5,6-EET, stimulation with bradykinin only transiently increased $[Ca^{2+}]_i$. *Vice versa*, 5,6-EET failed to increase $[Ca^{2+}]_i$ further in bradykinin-stimulated cells. The sustained $[Ca^{2+}]_i$ plateau phase induced by a co-stimulation with bradykinin and 5,6-EET was identical to that observed with bradykinin or 5,6-EET alone.
8. These results demonstrate that $Ca^{2+}$ entry induced by the P450 MO product, 5,6-EET, is indistinguishable to that observed by stimulation with bradykinin.
9. All data support our hypothesis that depletion of endothelial $Ca^{2+}$ stores activates microsomal P450 MO which in turn synthesizes 5,6-EET. We propose that the arachidonic acid metabolite 5,6-EET or one of its metabolites is a second messenger for activation of endothelial $Ca^{2+}$ entry.

It is now well established that many endothelial vascular functions are associated with autacoid-induced increases in endothelial free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$; for review see Graier, Sturek & Kukovetz, 1994*b*). Intracellular $Ca^{2+}$ release is due to G protein-mediated activation of phospholipase C followed by inositol 1,4,5-trisphosphate ($IP_3$) formation which, in turn, releases intracellularly stored $Ca^{2+}$ from $IP_3$-sensitive $Ca^{2+}$ pools (Graier *et al.* 1994*b*). In contrast to the second messenger cascade of agonist-induced $Ca^{2+}$ mobilization in endothelial cells, the exact second messenger(s) for agonist-induced $Ca^{2+}$ entry is (are) still obscure.

There are several main hypotheses for endothelial $Ca^{2+}$-influx mechanisms, including an involvement of G protein(s) and/or inositol 1,3,4,5-tetrakisphosphate (for

review see Graier *et al.* 1994*b*). Recently, it has been suggested that depletion of the $IP_3$-sensitive pools is involved in the mechanism(s) of endothelial $Ca^{2+}$ entry regulation (Schilling,Cabello & Rajan, 1992). Schilling *et al.* (1992) clearly demonstrated that depletion of $IP_3$-sensitive stores using the $Ca^{2+}$-ATPase-pump inhibitors, 2,5-di-(tert-butyl)-hydroquinone (BHQ), thapsigargin (TG) and cyclopiazonic acid (CPA), stimulates $Ca^{2+}$ entry into vascular endothelial cells which is indistinguishable from that activated by bradykinin. Such 'store depletion-activated $Ca^{2+}$ entry' was also found in various non-excitable cells, like platelets (Sargeant, Clarkson, Sage & Heemskerk, 1992), thymocytes (Alvarez, Montero & García-Sancho, 1991) and neutrophils (Montero, Alvarez & García-Sancho, 1992). Collectively, these data provide strong evidence for $Ca^{2+}$ store depletion-activated $Ca^{2+}$ entry. The question remaining is: Which second messengers link store depletion with $Ca^{2+}$ entry?

There is evidence that cytochrome P450 may be the link between depletion of intracellular $Ca^{2+}$ stores and plasmalemmal $Ca^{2+}$ channels in platelets (Sargeant *et al.* 1992), rat thymocytes (Alvarez *et al.* 1991) and neutrophils (Montero *et al.* 1992). This is due to the observation that imidazole antimycotics and P450 inhibitors like econazole potently inhibit $Ca^{2+}$ entry. We have previously reported that SK&F 96365, a drug very similar to econazole, abolished $Ca^{2+}$ entry and endothelium-derived relaxing factor formation in cultured human endothelial cells (Graier, Groschner, Schmidt & Kukovetz, 1992). Montero *et al.* (1992) provided evidence for the existence of a short-lived mediator in human neutrophils. They proposed that the formation of this labile compound may be linked to store depletion and result in the opening of plasmalemmal ion channels. Consistent with the hypothesis of a labile second messenger between store depletion and $Ca^{2+}$ entry, Parekh, Terlau & Stühmer (1993) described a labile compound (half-life, a few seconds) that was synthesized in *Xenopus* oocytes after store depletion and activated a non-selective cation current. Thus, there is strong evidence for the existence of another second messenger which is synthesized after $IP_3$-mediated depletion of $Ca^{2+}$ stores and activates $Ca^{2+}$ entry into non-excitable cells. Neither the nature nor pharmacological profile of this messenger is known.

We determined whether agonist-stimulated $Ca^{2+}$ entry in endothelial cells is due to the biosynthesis of a diffusible second messenger that is dependent on the activity of endothelial microsomal P450 mono-oxygenase (P450 MO). The role of the P450 MO-derived arachidonic acid metabolite, 5,6-epoxyeicosatrienoic acid (5,6-EET), as a second messenger mediating endothelial $Ca^{2+}$ influx was investigated.

## METHODS

### Materials

Fura-2 acetoxymethyl ester (fura-2 AM) was obtained from Lambda Fluorescence Technology (Graz, Austria) or from Molecular Probes (Eugene, OR, USA). Tissue culture media and drugs were purchased from Gibco/BRL (Eggenstein, Germany) or Boehringer–Mannheim (Germany). Cyclopiazonic acid was from Aldrich (Steinheim, Germany) and ionomycin was purchased from Calbiochem (La Jolla, CA, USA). Epoxyeicosatrienoic acid isomers were obtained from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA). All other chemicals were purchased from Sigma (St Louis, MO, USA). Cell culture Petri dishes were from Tissue Culture Products (Houslow, UK or Costar, Cambridge, MA, USA).

### Isolation and culture of endothelial cells

Endothelial cells from bovine coronary arteries and human umbilical cords were isolated as described previously (Graier, Kukovetz & Groschner, 1993; Sturek, Smith & Stehno-Bittle, 1994*c*). Briefly, bovine blood vessels were prepared in the slaughterhouse and put in a chilled buffer with a composition similar to Eagle's minimal essential medium including Hepes (EH), containing (mmol $l^{-1}$): 135 NaCl, 1 $MgCl_2$, 5 KCl, 0·44 $KH_2PO_4$, 0·34 $NaH_2PO_4$, 2·6 $NaHCO_3$, 20 Hepes, 10 D-glucose; plus dilutions (v/v): 0·02 amino acids, 0·01 vitamins, 0·002 Phenol Red, 0·01 penicillin–streptomycin, 2% serum, adjusted with NaOH at pH 7·4. Human umbilical cords were from healthy, women of normal body weight who volunteered to give their umbilical cords for experimental procedures. All donors were properly informed of the nature of the study. The study was performed according to the Austrian law for using human tissue. Immediately after birth, human umbilical cords were put into chilled Dulbecco's modified minimal essential medium (DMEM). In the laboratory, vessels were washed twice and incubated in serum-free, low $Ca^{2+}$ (0·5 mmol $l^{-1}$) EH with 294 U $ml^{-1}$ collagenase plus (mg $ml^{-1}$): 2 bovine serum albumin, 1 trypsin inhibitor and 0·4 DNAase I. After 30–90 min at 37 °C, cell suspensions were directly incubated with 2·5 $\mu$M fura-2 AM for fluorometric measurement or centrifuged and resuspended in Opti-MEM containing 3% fetal calf serum for culture. All experiments were performed with freshly dispersed endothelial cells within 3 days after vessel isolation or with cultured cells from primary culture and one passage within 10 days after isolation. Purity was indicated by the typical cobblestone morphology and the lack of immunofluorescence detection of contaminating smooth muscle cells ($\alpha$-actin). Human umbilical vein endothelial cells were also detected by immunofluorescence staining with factor VIII antibody. Cell culture was > 99% pure endothelial cells.

### $Ca^{2+}$ measurement

Intracellular free $Ca^{2+}$ concentration was measured using the fura-2 technique (Sturek, Caldwell, Humphrey & Wagner-Mann, 1991*a*; Sturek, Smith & Stehno-Bittle, 1991*b*; Sturek, Stehno-Bittle & Obye, 1991*c*; Graier *et al.* 1993). Briefly, suspended cells were loaded at 37 °C for 30 min with 2·5 $\mu$mol $l^{-1}$ fura-2 AM and equilibrated for 30 min in EH.

**$Ca^{2+}$ measurement in suspended cells.** Cells were centrifuged, washed and resuspended to a concentration of ~1·25 × $10^6$ cells $ml^{-1}$ in 2 mmol $l^{-1}$ $Ca^{2+}$–$Na^+$ buffer containing (mmol $l^{-1}$): 135 NaCl,

1 $MgCl_2$, 5 KCl, 2 $CaCl_2$, 10 Hepes, 10 D-glucose, adjusted with NaOH to pH 7·4. Cell suspensions (each 2 ml) were placed in a thermometrically controlled (37 °C) cuvette and experiments were performed after a 5 min equilibration. Intracellular free $Ca^{2+}$ concentration was monitored using the ratio technique with microcomputer-controlled spectrofluorometers (Shimadzu Rf 5000, Shimadzu Europe Corp., Vienna or a modified Hitachi F-2000; Hitachi, Vienna, Austria). Excitation wavelengths were changed every 2 s between 340 and 380 nm and emission light was detected at 500 nm at both excitation wavelengths. Autofluorescence measurements were performed after each experiment using 10 $\mu$mol $l^{-1}$ ionomycin followed by the addition of 5 mmol $l^{-1}$ $MnCl_2$.

**$Ca^{2+}$ measurement in single cells.** Single cell $Ca^{2+}$ recording was performed with a microfluorometric system established by Sturek *et al.* (1991*a,b,c*). Briefly, cells were centrifuged and resuspended in 2 mmol $l^{-1}$ $Ca^{2+}$–$Na^+$ buffer. One drop of this cell suspension was put on a superfusion chamber. Cells were allowed to settle down and thereafter superfused at a constant flow of 1–2 ml $min^{-1}$. Single cells or cells in small sheets were excited alternately every 50 ms with 360 and 380 nm light and emission light was detected at 510 nm using the photon counting technique. The counts were converted to analog from the optical processing circuitry (Sturek *et al.* 1994*b*) and then two separated emission intensities at two different excitation wavelengths were registered by a microcomputer running data acquisition programs, written by Dr M. Sturek based on AxoBASIC® 1·0 (Axon Instruments, Foster City, CA, USA).

**Data acquisition.** Due to the uncertainties of the $[Ca^{2+}]_i$ calibration in our microfluorometric experiments (Sturek *et al.* 1991*a,c*), intracellular free $Ca^{2+}$ ($[Ca^{2+}]_i$) is expressed as a ratio: 340/380 and 360/380 nm emission for cell suspension and single cell recordings, respectively. Endothelial $[Ca^{2+}]_i$ values are given as ratio units. Due to minor instrument drift and the resulting difference in maximum ratio values over several months of data collection, the maximum ratio response to bradykinin was used and shown daily as a standard during experiments.

### $Mn^{2+}$ quench experiments

Fura-2 AM loaded cells were equilibrated for 5 min in $Ca^{2+}$-free solution containing 2 mmol $l^{-1}$ $Mn^{2+}$, a surrogate of endothelial agonist-activated $Ca^{2+}$ entry. Activation of $Mn^{2+}$ entry was detected as a decrease in the fura-2 fluorescence at the isosbestic ($Ca^{2+}$-insensitive) wavelength of fura-2 (360 nm excitation and 510 nm emission). The amount of $Mn^{2+}$-induced decreases at 360 nm excitation and 510 nm emission (expressed as the percentage decrease in the initial fluorescence intensity) shows the effect of agonists on the permeability of the endothelial $Ca^{2+}$ entry pathway.

### Endothelium-derived relaxing factor measurement

Formation of endothelium-derived relaxing factor (i.e. endothelium-derived nitric oxide) was monitored by observing increases in intracellular cGMP levels as previously described (Graier *et al.* 1992). Briefly, confluent endothelial cells were washed and pre-incubated at 37 °C for 15 min with Hepes buffer containing 1 mmol $l^{-1}$ 3-isobutyl-1-methyl-xanthine (IBMX). After 11 min, P450 inhibitors or vehicle was added and the incubation was started after 15 min by the addition of the compound to be tested. The experiment was stopped after 4 min by removal of the incubation buffer and the addition of 1 ml HCl (0·01 mol $l^{-1}$). Endothelial cGMP levels were measured in the supernatant by radioimmunoassay. All increases in endothelial cGMP level by compounds tested were abolished in the presence of 100 $\mu$mol $l^{-1}$ $^{\omega}$*N*-nitro-L-arginine, an inhibitor of the nitric oxide (NO) synthase, indicating that increases in intracellular [cGMP] by these compounds were related to stimulation of endothelial NO synthase.

### P450 induction

P450 MO has been found in the endothelium (Abraham, Pinto, Mullane, Levere & Spokas, 1985), while Kutsky, Falck, Weiss, Manna, Chacos & Capdevila (1983) failed to detect mono-oxygenase in vascular smooth muscle cells. Like other P450 enzymes, endothelial P450 MO protein expression and enzyme activity can be induced by certain compounds. In the present study, primary cultured endothelial cells from human umbilical veins were seeded out in culture media (Opti-MEM with 3% fetal calf serum) containing 1 $\mu$mol $l^{-1}$ $\beta$-naphthoflavone, which induces endothelial P450 MO (Pinto, Abraham & Mullane, 1986; Nakai, Ward, Gannon & Rifkind, 1992). After 3 days, when cells reached confluency, the culture medium was removed, the cells were washed twice with DMEM and prepared for $Ca^{2+}$ measurements as described above. There were no differences in growth or morphology of the endothelial cells after this treatment compared with cells which were sham treated with the vehicle (0·1% dimethylsulphoxide).

### Statistics

All experiments were performed with three different batches, at least, of endothelial cell preparations. When shown as group data the results are expressed as mean values ± S.E.M. Statistical significance was evaluated with a one- or two-way analysis of variance including Scheffe's *post hoc* analysis. Significance was defined as $P < 0·05$ in all experiments.

## RESULTS

### P450 inhibitors diminish endothelial $Ca^{2+}$ entry

Addition of 100 nmol $l^{-1}$ bradykinin to single endothelial cells freshly isolated from bovine coronary artery results in a twofold increase in the fura-2 ratio ($n = 29$; Fig. 1*A*). In all figures, drug exposures and solution changes are indicated by the horizontal lines. Addition of the P450 inhibitor, econazole (10 $\mu$mol $l^{-1}$), decreased the bradykinin-induced $[Ca^{2+}]_i$ plateau by 63% ($n = 29$; $P < 0·05$ *vs.* plateau). At this time decreased extracellular $Na^+$ concentration ($[Na^+]_o = 19$ mmol $l^{-1}$) increased $[Ca^{2+}]_i$ threefold in the presence of econazole ($n = 5$; $P < 0·05$ *vs.* $[Na^+]_o = 139$ nmol $l^{-1}$; Fig. 1*A*). Neither the econazole-insensitive $[Ca^{2+}]_i$ plateau nor the $[Na^+]_o$-induced $[Ca^{2+}]_i$ increase were sensitive to 300 $\mu$mol $l^{-1}$ $Ni^{2+}$, a concentration which has already been shown to block agonist-activated $Ca^{2+}$ entry in endothelial cells (Graier *et al.* 1994*b*). In the presence of 10 $\mu$mol $l^{-1}$ econazole, bradykinin-induced $Mn^{2+}$ quenching was abolished (Fig. 1*B*).

In $Ca^{2+}$-free solution, stimulation with 100 nmol $l^{-1}$ bradykinin results in a transient increase of endothelial $[Ca^{2+}]_i$ (Fig. 2). Neither the duration of the transient $Ca^{2+}$

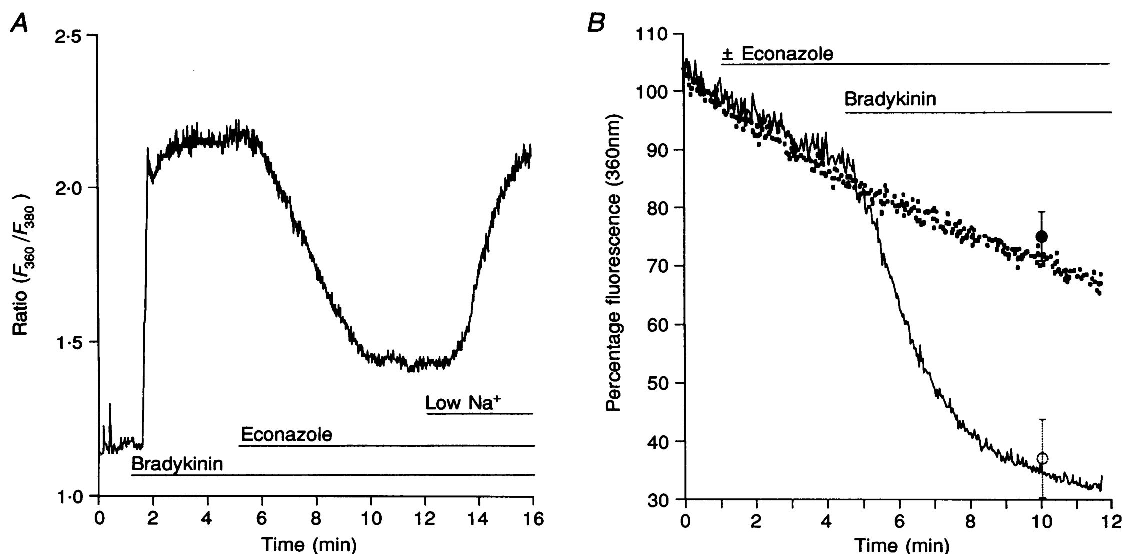


**Figure 1. Effect of econazole on bradykinin-stimulated endothelial cells freshly isolated from bovine coronary artery**

*A*, bradykinin (100 nmol $l^{-1}$)-induced changes in $[Ca^{2+}]_i$ ($n = 29$). Econazole (10 $\mu$mol $l^{-1}$) was added and extracellular free $Na^+$ concentration was decreased from 143 to 19 mmol $l^{-1}$ ($n = 5$). *B*, bradykinin (100 nmol $l^{-1}$)-induced $Mn^{2+}$ quenching in the absence (continuous line, ○) or presence (dotted plot, ●) of 10 $\mu$mol $l^{-1}$ econazole.

response nor the slope of the decline in $[Ca^{2+}]_i$ were altered by 5 $\mu$mol $l^{-1}$ econazole. Change of the superfusion solution to 2 mmol $l^{-1}$ $CaCl_2$-containing buffer after 3 min of bradykinin-stimulation in nominal $Ca^{2+}$-free solution produced a large increase in endothelial $[Ca^{2+}]_i$ in control conditions (from 1·38 ± 0·03 to 4·45 ± 0·24 ratio units; $n = 26$; Fig. 2). In the presence of 5 $\mu$mol $l^{-1}$ econazole, this increase upon re-addition of extracellular $Ca^{2+}$ was abolished (from 1·23 ± 0·04 to 1·39 ± 0·04 ratio units; $n = 9$; $P < 0.05$ *vs.* control). The small increases observed upon re-addition of extracellular $Ca^{2+}$ in the presence of econazole were similar to those observed upon re-addition of extracellular $Ca^{2+}$ without prior addition with bradykinin.

As with freshly isolated endothelial cells from bovine coronary artery, the agonist-induced $[Ca^{2+}]_i$ plateau in cultured human umbilical vein endothelial cells was sensitive to P450 inhibition (Table 1). To avoid $[Ca^{2+}]_i$ oscillations supramaximal concentrations of histamine

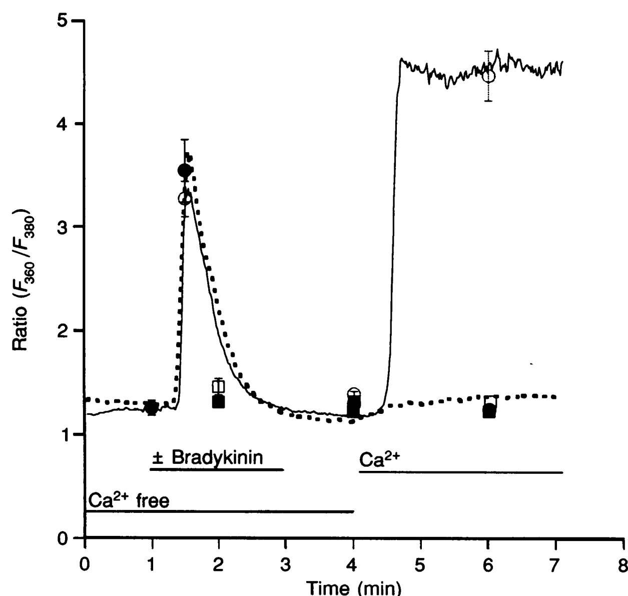


**Figure 2. Effect of econazole on bradykinin-induced $Ca^{2+}$ entry in cells freshly isolated from bovine coronary artery**

In nominal $Ca^{2+}$-free solution (no $Ca^{2+}$ added plus $10^{-5}$ mol $l^{-1}$ EGTA) cells were stimulated with 100 nmol $l^{-1}$ bradykinin in the absence (○, continuous line) or presence (●, dotted plot) of 5 $\mu$mol $l^{-1}$ econazole. Extracellular $Ca^{2+}$ (2 mmol $l^{-1}$) was added to the superfusion solution in the absence of bradykinin. Control values without a stimulation with bradykinin are shown as □ in the absence and ■ in the presence of 5 $\mu$mol $l^{-1}$ econazole ($n$ = 9–26).

**Table 1. Effects of the P450 inhibitors, econazole, miconazole, SK&F 96365, SK&F 525A and cyanide, on increases in human umbilical vein endothelial free-Ca$^{2+}$ concentration upon exposure to histamine, thapsigargin, 2,5-di-(tert-butyl)-hydroquinone (BHQ) or cyclopiazonic acid (CPA)**

| | Histamine (100 μmol l$^{-1}$) | Thapsigargin (1 μmol l$^{-1}$) | BHQ (15 μmol l$^{-1}$) | CPA (10 μmol l$^{-1}$) |
|---|---|---|---|---|
| Control | 2·57 ± 0·07 (57) | 3·2 ± 0·11 (16) | 3·10 ± 0·18 (5) | 3·17 ± 0·11 (14) |
| Econazole (5 μmol l$^{-1}$) | 1·79 ± 0·08* (13) | 2·26 ± 0·34* (6) | 2·05 ± 0·37* (4) | 2·04 ± 0·16* (5) |
| Miconazole (5 μmol l$^{-1}$) | 2·03 ± 0·15* (6) | 2·13 ± 0·16* (4) | 2·15 ± 0·23* (4) | 2·17 ± 0·15* (3) |
| SK&F 96365 (20 μmol l$^{-1}$) | 1·86 ± 0·09* (7) | 2·22 ± 0·21* (3) | 2·25 ± 0·07* (4) | 2·17 ± 0·11* (4) |
| SK&F 525A (50 μmol l$^{-1}$) | 1·75 ± 0·15* (7) | 2·15 ± 0·25* (5) | 2·17 ± 0·14* (4) | 2·31 ± 0·21* (4) |
| Cyanide (250 μmol l$^{-1}$) | 2·04 ± 0·18* (6) | 2·37 ± 0·11* (4) | 2·57 ± 0·09* (7) | 2·62 ± 0·32 (6) |

Cultured human umbilical vein endothelial cells were loaded with fura-2 AM as described under Methods. After equilibration, suspended cells were stimulated with histamine or the ATPase inhibitor in the concentration indicated. P450 inhibitors were added during the sustained plateau in elevated $[Ca^{+}]_i$. Endothelial $[Ca^{2+}]_i$ is expressed as a ratio of 340/380 nm excitation and 500 nm emission. Values represent means ± S.E.M. in ratio units from the number of experiments as indicated. The basal $[Ca^{2+}]_i$ value in all experiments was 1·41 ± 0·02 ratio units ($n = 144$). *$P < 0·05$ *vs.* the effect in the absence of the P450 inhibitors. Numbers in parentheses, $n$.

(i.e. 100 μmol l$^{-1}$) were used (Jacob, Merrit, Hallam & Rink, 1988). Convincingly, all P450 inhibitors tested strongly diminished histamine-induced $Mn^{2+}$ quench in human umbilical vein endothelial cells (inhibition: 98% by econazole, miconazole and SKF 525A, 89% by SK&F 96365 and 84% by cyanide; results not shown).

In agreement with these results the $[Ca^{2+}]_i$ plateau induced by the ATPase inhibitors TG, BHQ or CPA was diminished by an addition of 5 μmol l$^{-1}$ econazole (Table 1). Pretreatment with 5 μmol l$^{-1}$ econazole completely prevented TG-induced $Mn^{2+}$ entry by 97 ± 4·6% ($n = 6$, $P < 0·05$ *vs.* TG without econazole).

To test whether the inhibitory properties of P450 inhibitors on endothelial $Ca^{2+}$ entry were due to inhibition of $K^{+}$ channels (prevention of membrane hyperpolarization and, thus, minimizing the driving force for $Ca^{2+}$ entry), we investigated the effect of econazole on ionomycin (4 μmol l$^{-1}$)-induced $Ca^{2+}$ entry. Membrane depolarization by 80 mmol l$^{-1}$ $K^{+}$ diminished ionomycin-induced $[Ca^{2+}]_i$ plateau by 64% from 5·07 ± 0·23 to 2·75 ± 0·12 ratio units ($n = 4$, $P < 0·05$), while 10 μmol l$^{-1}$ econazole failed to affect ionomycin-induced increases of $[Ca^{2+}]_i$ (5·13 ± 0·19 ratio units, $n = 4$).

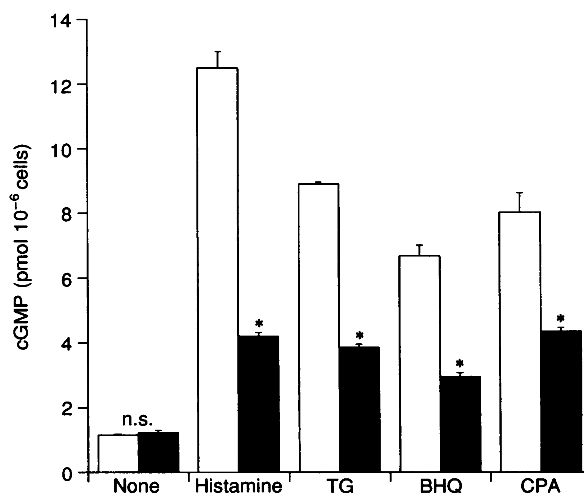


**Figure 3. Effect of econazole on the formation of nitric oxide in cultured human umbilical vein endothelial cells stimulated by histamine or the ATPase inhibitors, thapsigargin (TG), 2,5-di-(tert-butyl)-hydroquinone (BHQ) or cyclopiazonic acid (CPA)**

Confluent endothelial cells grown in 24 plastic plates, were washed and pre-incubated with 1 mmol l$^{-1}$ IBMX at 37 °C for measurement of cGMP as an assay for NO. After 15 min cells were stimulated for 4 min with 100 μmol l$^{-1}$ histamine, 1 μmol l$^{-1}$ TG, 15 μmol l$^{-1}$ BHQ or 10 μmol l$^{-1}$ CPA without (□) or with (■) a pretreatment for 2 min with 5 μmol l$^{-1}$ econazole ($n = 9$). *$P < 0·05$ *vs.* the effect in the absence of econazole. n.s., not significant.

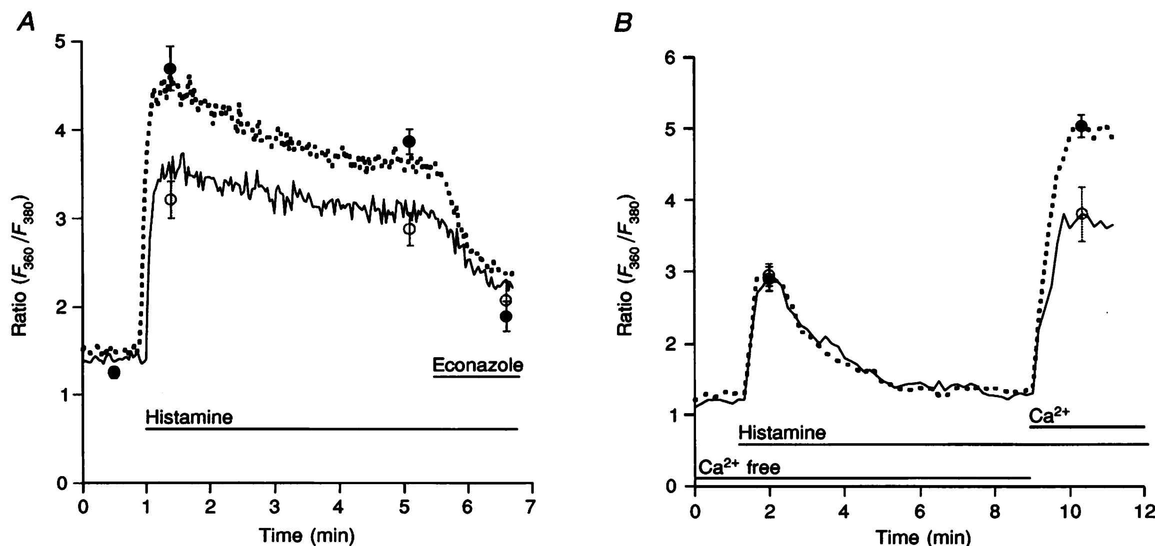


**Figure 4. Effect of an induction of P450 enzyme on histamine-induced $[Ca^{2+}]_i$ response in cultured human umbilical vein endothelial cells**

Cells were treated with 1 $\mu$mol $l^{-1}$ $\beta$-naphthoflavone (dotted plots, ●) or vehicle (continuous line, ○) during 3 days of culture. *A*, cells were stimulated in $Ca^{2+}$-containing solution with 100 $\mu$mol $l^{-1}$ histamine, then 10 $\mu$mol $l^{-1}$ econazole was added. *B*, cells were stimulated in $Ca^{2+}$-free solution with 100 $\mu$mol $l^{-1}$ histamine, then 2 mmol $l^{-1}$ $Ca^{2+}$ was added ($n = 11-17$).

### Inhibition of P450 enzyme(s) strongly attenuates $[Ca^{2+}]_i$-dependent stimulation of nitric oxide synthase by histamine and ATPase inhibitors

Stimulation of endothelial constitutive NO synthase by agonists is dependent upon the $[Ca^{2+}]_i$-increasing properties of the stimulating agent. Therefore, it was of interest to find out whether inhibition of $Ca^{2+}$ entry by P450 inhibitors affects agonist-stimulated NO formation. Formation of EDNO was monitored by observing increases in the intracellular level of cGMP. Figure 3 shows that econazole strongly decreased the effect of histamine (100 nmol $l^{-1}$), TG (100 nmol $l^{-1}$), BHQ (10 $\mu$mol $l^{-1}$) and CPA (10 $\mu$mol $l^{-1}$) on cGMP production in human umbilical vein endothelial cells. Basal cGMP levels remained unaffected by econazole. In contrast, the effects of 1 $\mu$mol $l^{-1}$ A23187, which stimulates NO synthase due to its $Ca^{2+}$ ionophore properties, and 1 mmol $l^{-1}$ sodium nitroprusside, which activates endothelial soluble guanylyl cyclase directly, were not altered by econazole up to a concentration of 10 $\mu$mol $l^{-1}$ (A23187: control 9·2 ± 1·0 *vs.* econazole 8·3 ± 0·9 pmol cGMP $(10^6 \text{ cells})^{-1}$; $n = 9$, n.s. and sodium nitroprusside: control 14·2 ± 0·5 *vs.* econazole 13·7 + 0·7 pmol cGMP $(10^6 \text{ cells})^{-1}$; $n = 9$, n.s., respectively).

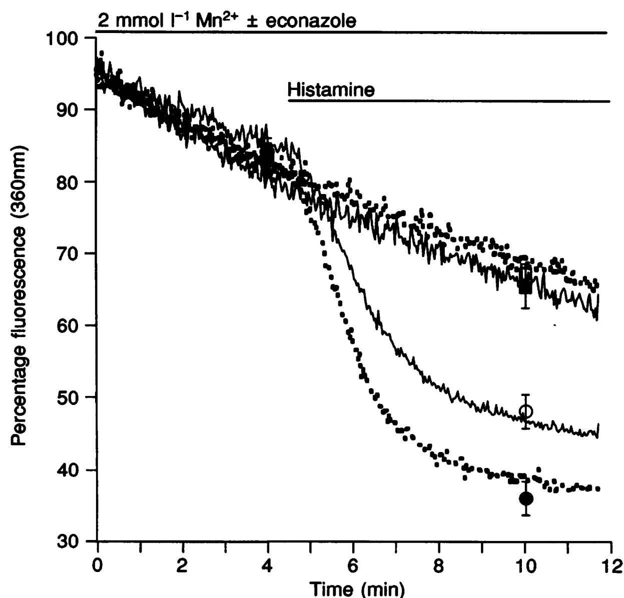


**Figure 5. Effect of an induction of P450 enzyme(s) on histamine-induced $Mn^{2+}$ entry in cultured human umbilical vein endothelial cells and its inhibition by econazole**

Cells treated for 3 days with 1 $\mu$mol $l^{-1}$ $\beta$-naphthoflavone (dotted plots, ●, ■) or vehicle (continuous lines, ○, □) were stimulated in the absence (●, ○) or in the presence (■, ●) of 10 $\mu$mol $l^{-1}$ econazole ($n = 4-7$).

### Induction of P450 enzyme(s) potentiates agonist-stimulated $[Ca^{2+}]_i$ influx

Cultured endothelial cells from human umbilical veins were treated during 3 days of culture with $\beta$-naphthoflavone, an inducer of endothelial P450 MO (Pinto *et al.* 1986). In sham-treated human umbilical vein endothelial cells (0·1% dimethylsulphoxide), stimulation with 100 $\mu$mol $l^{-1}$ histamine resulted in an increase of 2·6 ratio units ($n = 15$, $P < 0.05$ *vs.* basal), followed by a plateau phase at 2·87 ± 0·18 ratio units ($n = 15$, $P < 0.05$ *vs.* basal; Fig. 4*A*). In cells pretreated with 1 $\mu$mol $l^{-1}$ $\beta$-naphthoflavone, histamine (100 $\mu$mol $l^{-1}$) increased $[Ca^{2+}]_i$ from 1·27 ± 0·05 to 4·69 ± 0·25 ratio units ($n = 19$; $P < 0.05$ *vs.* basal; $P < 0.05$ *vs.* control), followed by a sustained plateau at 3·86 ± 0·14 ratio units ($n = 19$; $P < 0.05$ *vs.* basal; $P < 0.05$ *vs.* control; Fig. 4*A*). Endothelial basal $[Ca^{2+}]_i$ levels remained unchanged by pretreatment with 1 $\mu$mol $l^{-1}$ $\beta$-naphthoflavone. Addition of 10 $\mu$mol $l^{-1}$ econazole decreased the histamine-induced $[Ca^{2+}]_i$ plateau in cells treated with $\beta$-naphthoflavone to 1·89 ± 0·17 ($n = 4$; $P < 0.05$ *vs.* $[Ca^{2+}]_i$ plateau; Fig. 4*A*). In sham-treated cells, econazole (10 $\mu$mol $l^{-1}$) decreased the histamine-induced $[Ca^{2+}]_i$ plateau to 2·07 ± 0·21 ($n = 6$; $P < 0.05$ *vs.* $[Ca^{2+}]_i$ plateau; Fig. 4*A*). In sham-treated cells, econazole (10 $\mu$mol $l^{-1}$) decreased the histamine-induced $[Ca^{2+}]_i$ plateau to 2·07 ± 0·21 ($n = 6$; $P = 0.05$ *vs.* $[Ca^{2+}]_i$ plateau; Fig. 4*A*).

In $Ca^{2+}$-free solution, histamine (100 $\mu$mol $l^{-1}$) transiently increased $[Ca^{2+}]_i$ in sham-treated cells from 1·15 ± 0·09 to 2·95 ± 0·15 ratio units ($n = 11$; $P < 0.05$ *vs.* basal; Fig. 4*B*). In cells pretreated for 3 days with 1 $\mu$mol $l^{-1}$ $\beta$-naphthoflavone, histamine-induced $Ca^{2+}$ release was unchanged from 1·17 ± 0·03 to 2·90 ± 0·17 ratio units ($n = 17$, $P < 0.05$ *vs.* basal, n.s. *vs.* sham-treated cells). In contrast to $Ca^{2+}$ release, $Ca^{2+}$ entry upon the addition of 2·5 mmol $l^{-1}$ extracellular $Ca^{2+}$ to histamine-prestimulated cells was significantly enhanced by the P450 MO induction procedure (Fig. 4*B*; $\beta$-naphthoflavone-treated cells: 5·03 ± 0·16 ratio units, $n = 17$; sham-treated cells: 3·80 ± 0·38 ratio units, $n = 8$, $P < 0.05$).

In agreement with these results, histamine-induced $Mn^{2+}$ quench was significantly enhanced in $\beta$-naphthoflavone-pretreated cells (53%; $n = 4$) compared with the effect of histamine in sham-treated cells ($n = 7$, $P < 0.05$; Fig. 5). Pre-incubation with 10 $\mu$mol $l^{-1}$ econazole prevented histamine-induced $Mn^{2+}$ quench in $\beta$-naphthoflavone-pretreated cells ($n = 5$) and in the control group ($n = 4$; Fig. 5).

### Endothelial, P450 mono-oxygenase-derived compound stimulates $Ca^{2+}$ entry

P450 MO in blood vessels is mainly located in vascular endothelium (Abraham *et al.* 1985). This cytochrome P450-containing enzyme was shown to synthesize four isomers of epoxyeicosatrienoic acid (EET) by using arachidonic acid in the presence of NADPH (for review see Zimniak & Waxman, 1993). In this study we investigated the effect of two epoxyeicosatrienoic acid products, 5,6-EET and 8,9-epoxyeicosatrienoic acid (8,9-EET) on endothelial $[Ca^{2+}]_i$. Due to the labile chemical nature of these compounds (the reported half-life of 5,6-EET in aqueous solutions is less than 1 min; Proctor, Falck & Capdevila, 1987), the concentration indicated is the initial concentration in the superfusion buffer.

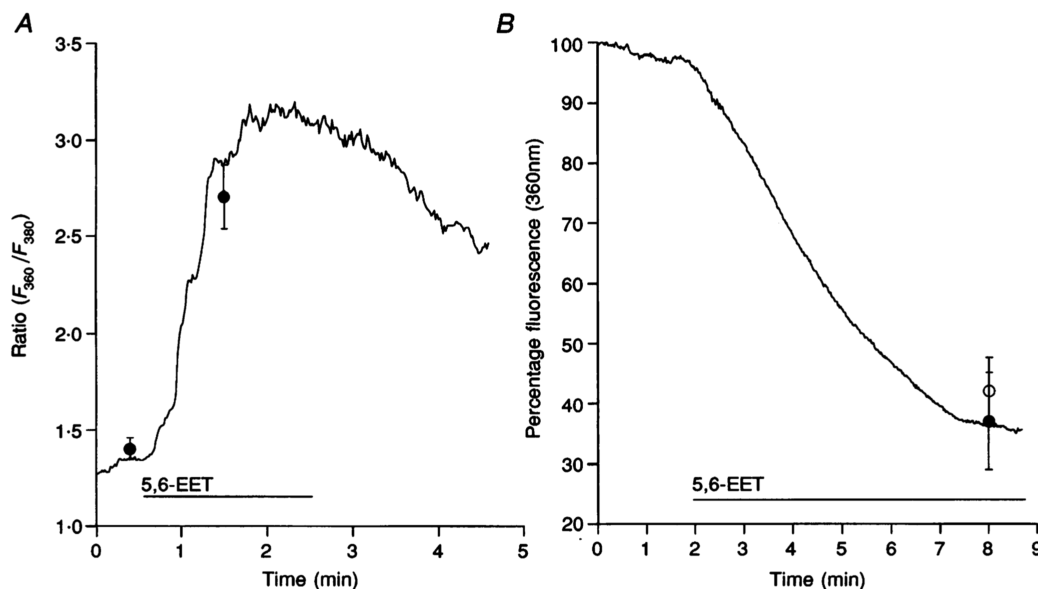


**Figure 6. Effect of the P450 mono-oxygenase product, 5,6-epoxyeicosatrienoic acid (5,6-EET; <156 nmol $l^{-1}$), in cells freshly isolated from bovine coronary artery in 2 mmol $l^{-1}$ $Ca^{2+}$-containing solution**

Due to the labile nature of this compound concentration given is the initial concentration in the superfusion buffer. *A*, $[Ca^{2+}]_i$ response. *B*, $Mn^{2+}$ quenching in the absence (○) or presence (●) of 10 $\mu$mol $l^{-1}$ econazole ($n = 6$–17).

Figure 6*A* shows the effect of 5,6-EET on $[Ca^{2+}]_i$ in cells freshly isolated from bovine coronary artery. Addition of 5,6-EET (< 156 nmol $l^{-1}$) resulted in a rapid increase from $1{\cdot}40 \pm 0{\cdot}05$ to $2{\cdot}70 \pm 0{\cdot}16$ ratio units ($n = 17$; $P < 0{\cdot}05$; Fig. 6*A*), similar to bradykinin in this series of experiments (from $1{\cdot}13 \pm 0{\cdot}02$ to $2{\cdot}12 \pm 0{\cdot}13$ ratio units, $n = 28$; $P < 0{\cdot}05$). Incubation with the vehicle (0·1 % ethanol) had no effect on endothelial $[Ca^{2+}]_i$. The effects of 5,6-EET were not modulated in the presence of 100 $\mu$mol $l^{-1}$ aspirin or by lowering extracellular $Na^+$ concentration (19 mmol $l^{-1}$), but strictly depended on the presence of extracellular $Ca^{2+}$, as indicated by the lack of an increase in $[Ca^{2+}]_i$ to 5,6-EET in nominal $Ca^{2+}$-free solution (up to 780 nmol $l^{-1}$ 5,6-EET; from $1{\cdot}17 \pm 0{\cdot}14$ to $1{\cdot}20 \pm 0{\cdot}12$, $n = 5$, n.s.). In contrast to the stimulatory properties of 5,6-EET, another epoxyeicosatrienoic acid isomer, 8,9-EET, failed to affect $[Ca^{2+}]_i$ in cells freshly isolated from bovine coronary artery (up to 780 nmol $l^{-1}$; $n = 11$).

### Characteristics of 5,6-EET-induced increases in $[Ca^{2+}]_i$

To characterize further the mechanism of 5,6-EET-induced increases of endothelial $[Ca^{2+}]_i$, $Mn^{2+}$ quench experiments were performed. As shown in Fig. 6*B*, stimulation with <156 nmol $l^{-1}$ EET yielded an influx of $Mn^{2+}$ ($n = 7$). The amount of quenching in response to 5,6-EET was almost identical to that obtained by activation with bradykinin (Fig. 1*B*). In the presence of 10 $\mu$mol $l^{-1}$ econazole, a concentration which had already been shown to prevent agonist-induced $Mn^{2+}$ influx, 5,6-EET-activated $Mn^{2+}$ entry remained unchanged ($n = 6$; Fig. 6*B*). In four of sixty-seven experiments, stimulation of 5,6-EET (<156 $\mu$mol $l^{-1}$) resulted in an oscillation in $[Ca^{2+}]_i$ with a frequency of about 1 spike $min^{-1}$ and a range from 1·45 to 2·7 spike $min^{-1}$ ($n = 36$), while resting $[Ca^{2+}]_i$ during the spikes was slightly increased (from $1{\cdot}13 \pm 0{\cdot}13$ to $1{\cdot}67 \pm 0{\cdot}16$ ratio units; $n = 4$, $P < 0{\cdot}05$).

### Comparison of 5,6-EET- and bradykinin-induced $Ca^{2+}$ entry

To compare 5,6-EET- and bradykinin-induced $Ca^{2+}$ entry the effect of a short exposure to <156 nmol $l^{-1}$ 5,6-EET on $[Ca^{2+}]_i$ was investigated. In contrast to a short stimulation with bradykinin, which results in a long lasting $Ca^{2+}$ entry period even when bradykinin has been washed out (Fig. 2; Graier *et al.* 1994*b*), the $[Ca^{2+}]_i$ increase in response to 5,6-EET strictly depends on the presence of this compound and decreased to basal level within $1{\cdot}87 \pm 0{\cdot}33$ min ($n = 7$) after removal of 5,6-EET.

Co-stimulation of endothelial cells with 5,6-EET (< 156 nmol $l^{-1}$) and bradykinin (100 nmol $l^{-1}$) transiently increased $[Ca^{2+}]_i$ from $1{\cdot}05 \pm 0{\cdot}02$ to $2{\cdot}02 \pm 0{\cdot}06$ ratio units ($n = 5$, $P < 0{\cdot}05$), followed by a sustained $[Ca^{2+}]_i$ plateau at $1{\cdot}68 \pm 0{\cdot}08$ ratio units. There was no detectable difference in the sustained $[Ca^{2+}]_i$ plateau compared with the sustained $[Ca^{2+}]_i$ plateau induced by 5,6-EET or bradykinin alone ($1{\cdot}63 \pm 0{\cdot}04$, $n = 4$ and $1{\cdot}70 \pm 0{\cdot}04$ ratio units, $n = 3$, respectively; Fig. 7*A*). The bradykinin-induced peak area under the curve (AUC) in the presence of 5,6-EET

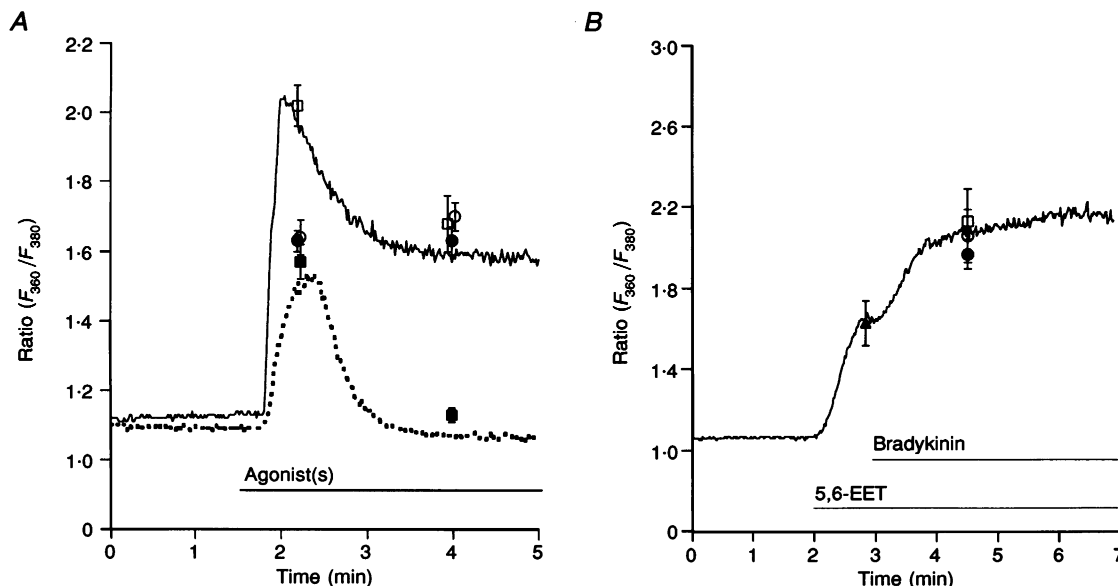


**Figure 7. Interaction between 5,6-EET- and bradykinin-induced $[Ca^{2+}]_i$ increases in cells from bovine coronary artery**

*A*, co-stimulation (continuous line, □) with maximally effective concentrations of 5,6-EET (< 156 nmol $l^{-1}$) and bradykinin (100 nmol $l^{-1}$) was compared with the effects of 5,6-EET (●) or bradykinin (○) alone, and intracellular $Ca^{2+}$ release by 100 nmol $l^{-1}$ bradykinin in nominal $Ca^{2+}$-free solution (dotted plot, ■). *B*, cells were prestimulated with sub-maximal effective concentration of 5,6-EET (< 52 nmol $l^{-1}$; ▲). Bradykinin (100 nmol $l^{-1}$) was added (continuous line, □). The effects of <156 nmol $l^{-1}$ 5,6-EET (●) and 100 nmol $l^{-1}$ bradykinin (○) alone are presented ($n = 4$–8).

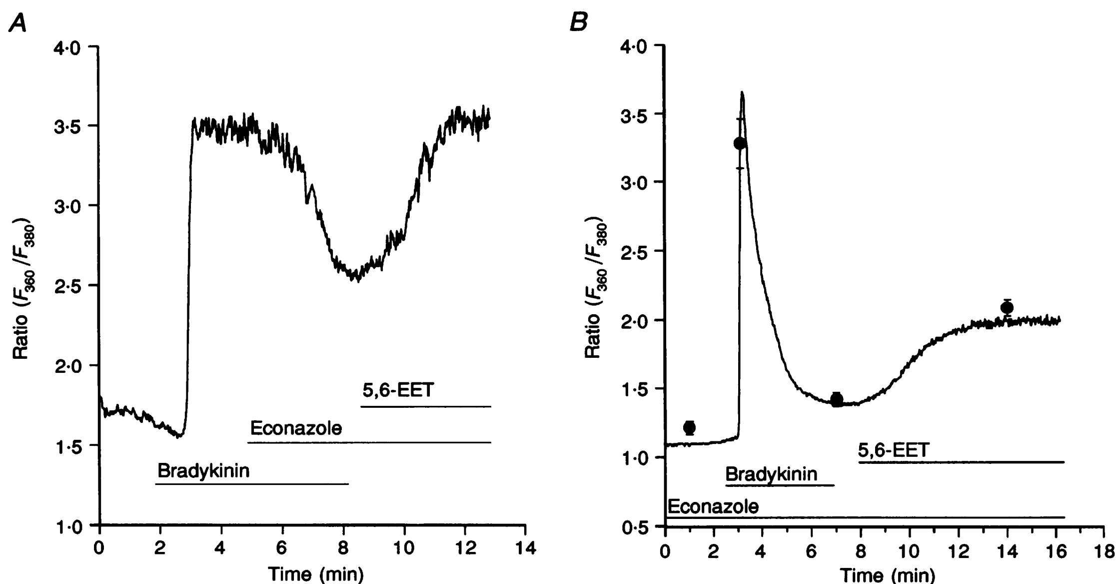


**Figure 8. Econazole failed to modulate the effect of 5,6-EET on $[Ca^{2+}]_i$ in endothelial cells freshly isolated from bovine coronary artery**

*A*, cells were stimulated with 100 nmol $l^{-1}$ bradykinin. After reaching a constant plateau, bradykinin-induced $Ca^{2+}$ entry was blocked by the inhibition of P450-enzyme(s) with 10 $\mu$mol $l^{-1}$ econazole. In the presence of econazole, <156 nmol $l^{-1}$ 5,6-EET was added. Tracing shows a typical experiment. *B*, cells were pre-incubated for 2 min with 10 $\mu$mol $l^{-1}$ econazole and stimulated with 100 nmol $l^{-1}$ bradykinin followed by <78 nmol $l^{-1}$ 5,6-EET ($n = 8$).

(AUC = 0·065 ± 0·009 $R_{min}$, where $R$ represents ratio, $n = 3$) was not different to $Ca^{2+}$ release induced by bradykinin in nominal $Ca^{2+}$-free solution (AUC = 0·071 ± 0·007 $R_{min}$, $n = 5$, Fig. 7*A*). In agreement with these results, $Mn^{2+}$ quench induced by a co-stimulation with 100 nmol $l^{-1}$ bradykinin and <156 nmol $l^{-1}$ 5,6-EET (50 ± 7·2% after 2 min) was similar to that observed with bradykinin or 5,6-EET alone (54 ± 4·1 and 48 ± 6·9%, respectively). In bradykinin-prestimulated cells, a further addition of <156 nmol $l^{-1}$ 5,6-EET failed to change the bradykinin-induced sustained $[Ca^{2+}]_i$ plateau (from 1·67 ± 0·07 to 1·65 ± 0·06 ratio units, $n = 5$, n.s.) and $Mn^{2+}$ entry (data not shown). In cells which were stimulated with a sub-maximal concentration of 5,6-EET (< 52 nmol $l^{-1}$), a further stimulation with 100 nmol $l^{-1}$ bradykinin increased the $[Ca^{2+}]_i$ plateau to exactly the same value as that

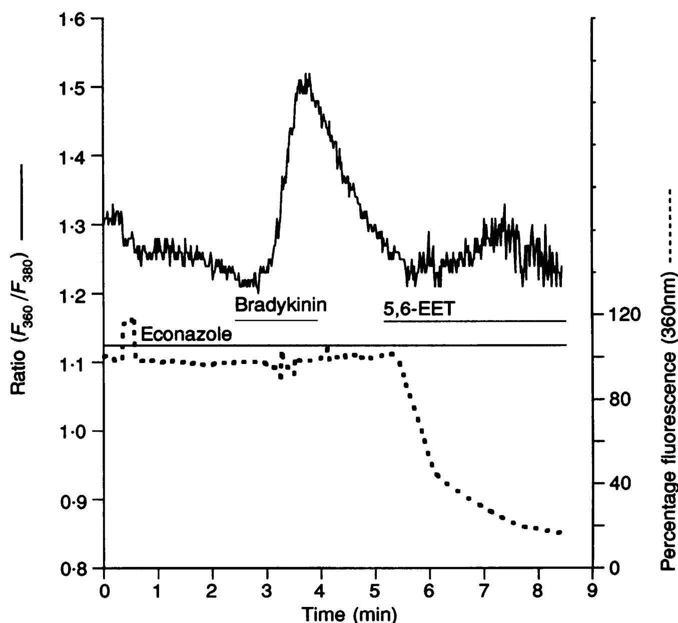


**Figure 9. Econazole prevented bradykinin-induced $Mn^{2+}$ quenching, while addition of <156 nmol $l^{-1}$ 5,6-EET results in unchanged $Mn^{2+}$, even in the presence of econazole (given at time 0)**

While econazole prevented $Ca^{2+}/Mn^{2+}$ influx induced by intracellular $Ca^{2+}$ store depletion with bradykinin, econazole failed to modulate 5,6-EET-induced $Ca^{2+}/Mn^{2+}$ entry.

obtained with bradykinin or <156 nmol $l^{-1}$ 5,6-EET alone (Fig. 7*B*), indicating that both compounds may stimulate the same $Ca^{2+}$ entry pathway. Thus, sustained $[Ca^{2+}]_i$ plateau induced by either 100 nmol $l^{-1}$ bradykinin or <156 nmol $l^{-1}$ 5,6-EET may reflect a maximal stimulation of the same $Ca^{2+}$ entry pathway. A further stimulation with 5,6-EET or bradykinin is without effect.

To test whether prestimulation with bradykinin may modulate the effect of 5,6-EET, cells were prestimulated with 100 nmol $l^{-1}$ bradykinin. Addition of 10 $\mu$mol $l^{-1}$ econazole decreased the bradykinin-induced $[Ca^{2+}]_i$ plateau as already shown in Fig. 1. Further addition of 5,6-EET (< 156 nmol $l^{-1}$) in the presence of econazole increased $[Ca^{2+}]_i$ from 2·41 ± 0·26 to 3·87 ± 0·34 ratio units ($n = 4$, $P < 0.05$, Fig. 8*A*). When pre-incubated with 10 $\mu$mol $l^{-1}$ econazole, cells only transiently responded to 100 nmol $l^{-1}$ bradykinin, while a further addition of 5,6-EET (< 78 nmol $l^{-1}$) to these cells yielded an unchanged increase from 1·42 ± 0·05 to 2·09 ± 0·06 ratio units ($n = 8$, $P < 0.05$; Fig. 8*B*). Convincingly, stimulation of econazole pretreated cells (2 min) with 100 nmol $l^{-1}$ bradykinin in the presence of 2 mmol $l^{-1}$ $Mn^{2+}$ transiently increased $[Ca^{2+}]_i$ due to $Ca^{2+}$ release from the intracellular stores but failed to activate $Mn^{2+}$ entry (Fig. 9). However, addition of <156 nmol $l^{-1}$ 5,6-EET to those cells resulted in a large $Mn^{2+}$ entry (Fig. 9), indicating that the stimulatory effect of 5,6-EET does not depend on P450 activity. Thus, when activation of P450 MO is prevented by econazole, prestimulation with bradykinin does not modulate the effect of 5,6-EET.

As with bradykinin (for review see Schilling & Elliott, 1992; Graier *et al.* 1994*b*) 5,6-EET-induced $Ca^{2+}$ entry was sensitive to high $K^+$ buffer. As shown in Fig. 10, superfusion with 80 mmol $l^{-1}$ $K^+$-containing solution abolished the effect of 5,6-EET (from 2·23 ± 0·26 to 1·14 ± 0·11; $n = 4$, $P < 0.05$). Previous studies have shown that bradykinin-induced $Ca^{2+}$ influx is not related to activation of $Na^+$–$Ca^{2+}$ exchange (Colden-Stanfield, Schilling, Ritchie, Eskin, Navarro & Kunze, 1987). In agreement, neither bradykinin- nor 5,6-EET-induced $Ca^{2+}$ entry were altered by reduction of extracellular $Na^+$ to 19 mmol $l^{-1}$ (data not shown).

The inorganic blocker, $Ni^{2+}$ (300 $\mu$mol $l^{-1}$), diminished 5,6-EET-induced $Ca^{2+}/Mn^{2+}$ entry (46%), a reduction similar to that after stimulation with 100 nmol $l^{-1}$ bradykinin (50%). Addition of 100 $\mu$mol $l^{-1}$ $La^{3+}$ completely prevents 5,6-EET- and bradykinin-induced sustained $Mn^{2+}$ quenching (data not shown). In contrast to the inhibitory properties of these inorganic blockers, the organic $Ca^{2+}$ channel antagonist nitrendipine (1 $\mu$mol $l^{-1}$) failed to affect 5,6-EET-induced $Ca^{2+}/Mn^{2+}$ entry (data not shown).

Finally the permeability to $Ba^{2+}$ was tested. Increases in intracellular $Ba^{2+}$ concentration can be detected by the fura-2 technique (for review see Schilling & Elliott, 1992). In the presence of 2 mmol $l^{-1}$ extracellular $Ba^{2+}$ (no $Ca^{2+}$ added), stimulation with <156 nmol $l^{-1}$ 5,6-EET resulted in $Ba^{2+}$ influx, indicated by an increase in the ratio from 1·03 ± 0·01 to 1·19 ± 0·01 within 4 min ($n = 4$, $P < 0.05$ *vs.* basal). Bradykinin (100 nmol $l^{-1}$) yielded an almost identical $Ba^{2+}$ influx to that obtained with 5,6-EET. Addition of 2 mmol $l^{-1}$ extracellular $Ba^{2+}$ to cells prestimulated in $Ca^{2+}$-free solution with bradykinin increased the ratio from 1·04 ± 0·01 to 1·27 ± 0·02 within 4 min ($n = 5$, $P < 0.05$ *vs.* basal, n.s. *vs.* 5,6-EET). However, the detected $Ba^{2+}$ influx in cells stimulated with both compounds was very small. This might be due to the inhibitory properties of $Ba^{2+}$ on endothelial $Ca^{2+}$-activated $K^+$ channels, resulting in prevention of membrane hyperpolarization and, thus, decreased driving force for the $Ba^{2+}$ entry.

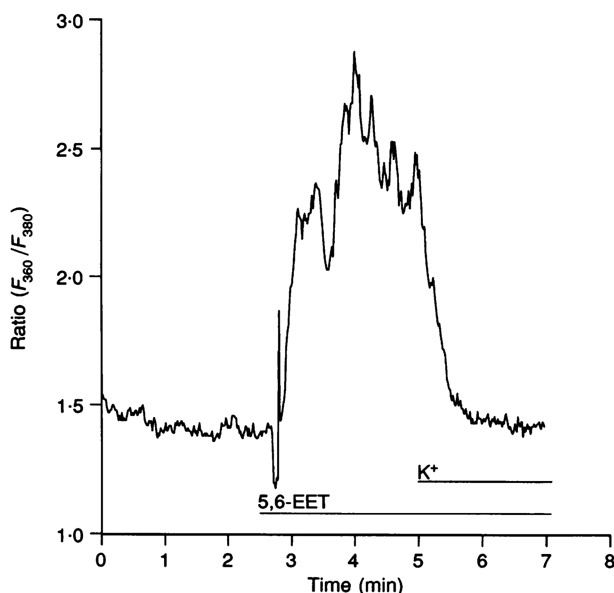


**Figure 10. Membrane depolarization with high extracellular $K^+$ prevents the effect of 5,6-EET on $[Ca^{2+}]_i$ in cells freshly isolated from bovine coronary artery**

Cells were stimulated in the presence of 2 mmol $l^{-1}$ extracellular $Ca^{2+}$ with <156 nmol $l^{-1}$ 5,6-EET and depolarized in the presence of 5,6-EET with 80 mmol $l^{-1}$ $K^+$ (equimolar substitution for $Na^+$) in the superfusion solution as lines indicate.

## DISCUSSION

We investigated the role of microsomal P450 MO in the regulation of endothelial $Ca^{2+}$ entry. The main finding was that this cytochrome P450 enzyme leads to the formation of the arachidonic acid metabolite, 5,6-EET, as the final second messenger that is essential for both agonist-induced and $Ca^{2+}$ store depletion-induced $Ca^{2+}$ entry. Various chemically distinct P450 inhibitors attenuated $Ca^{2+}$ entry, induction of P450 MO augmented $Ca^{2+}/Mn^{2+}$ entry, and 5,6-EET elicited P450-independent $Ca^{2+}/Mn^{2+}$ entry indistinguishable from that activated by bradykinin. All these data support the mechanism schematically illustrated in Fig. 11.

Several groups have reported evidence for the involvement of P450-related enzyme(s) in $Ca^{2+}$ entry in various non-excitable cells, such as platelets (Sargeant *et al.* 1992), rat thymocytes (Alvarez *et al.* 1991) and neutrophils (Montero, Alvarez & García-Sancho, 1991; Montero *et al.* 1992). Since there is evidence that the P450 inhibitors, based on imidazole antimycotics (e.g. econazole, miconazole) have additional properties, like inhibition of $Ca^{2+}$ ATPases (Mason, Mayer & Hymel, 1993), inhibition of L-type $Ca^{2+}$ channels (Merritt *et al.* 1990; Villalobos, Fonteriz, Lopez, García & García-Sancho, 1992) and inhibition of $K^+$ channels (Alvarez, Montero & García-Sancho, 1992), we first investigated the effect of various P450 inhibitors with different chemical properties on endothelial $Ca^{2+}$ entry. We used the imidazole-related P450 inhibitors, econazole, miconazole, and SK&F 96365, the phenylpropylbenzene derivative, SK&F 525A, and cyanide, which did not change cellular ATP content during the 4 min incubation (P. Dittrich, unpublished observation). All P450 inhibitors tested diminished the $[Ca^{2+}]_i$ plateau induced by agonists or store depletion via ATPase inhibitors in cultured human umbilical vein and freshly isolated bovine coronary artery endothelial cells. In agreement with these data, P450 inhibitors prevented agonist-induced $Mn^{2+}$ quench of fura-2 by agonists or ATPase inhibitors.

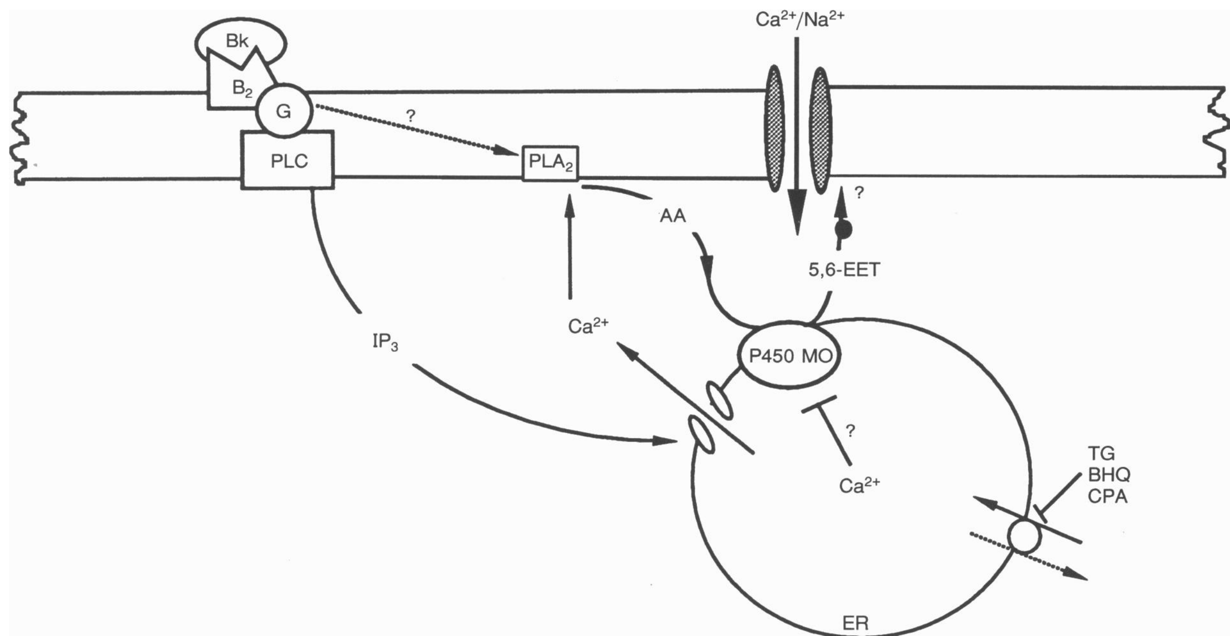


**Figure 11. Scheme of the proposed mechanisms involved in the regulation of agonist-induced $Ca^{2+}$ entry into vascular endothelial cells**

Agonists, like bradykinin (Bk) interact with their endothelial surface receptors ($B_2$) resulting in a G protein (G) -mediated stimulation of phospholipase C (PLC) and perhaps in a direct stimulation of phospholipase $A_2$ ($PLA_2$). PLC synthesizes inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol (not shown). $IP_3$ releases $Ca^{2+}$ from certain parts of microsomal $Ca^{2+}$ stores (i.e. endoplasmatic reticulum; ER). $Ca^{2+}$ release stimulates $PLA_2$, resulting in arachidonic acid (AA) release. Intracellular $Ca^{2+}$-store depletion activates, via an as yet unknown mechanism (calmodulin? tyrosine kinase?), microsomal P450 mono-oxygenase (P450 MO), which synthesizes epoxyeicosatrienoic acid(s) from $PLA_2$ released arachidonic acid in an NADPH-dependent pathway. One of these compounds, 5,6-epoxyeicosatrienoic acid (5,6-EET) or a metabolite of 5,6-EET ($^{2+}$) activates directly, or via some intermediary step, $Ca^{2+}$-permeable membrane channels. Besides these channels, EET(s) may also stimulate endothelial (and smooth muscle) $K^+$ channels and may also influence $Na^+$–$K^+$ pump activity (not shown).

The finding that the increase in $[Ca^{2+}]_i$ upon re-addition of extracellular $Ca^{2+}$ to bradykinin- or TG-stimulated cells was abolished in the presence of econazole indicates that inhibition of P450 enzyme(s) completely prevents depletion-induced $Ca^{2+}$ entry. Econazole did not interfere with $IP_3$-induced $Ca^{2+}$ release, as indicated by identical $[Ca^{2+}]_i$ transients in $Ca^{2+}$-free solution in both the presence and absence of econazole.

In contrast to pretreatment with P450 inhibitors (Fig. 2), acute addition of P450 inhibitors to stimulated cells failed to decrease the $[Ca^{2+}]_i$ plateau to basal level (Fig. 1*A*). Also, decreasing $[Na^+]_o$ enhanced the $[Ca^{2+}]_i$ plateau, suggesting $Na^+$–$Ca^{2+}$ exchange is responsible for the remaining $[Ca^{2+}]_i$ plateau in the presence of econazole. Endothelial $Ca^{2+}$-permeable channels are not highly selective for $Ca^{2+}$ and, thus, significant influx of $Na^+$ through these channels is expected during agonist stimulation (Colden-Stanfield *et al.* 1987). Under these conditions the intracellular concentrations of both ions ($Na^+$ and $Ca^{2+}$) may increase, resulting in only small changes of the equilibrium potential of the $Na^+$–$Ca^{2+}$ exchange. This is indicated by the lack of a contribution of the $Na^+$–$Ca^{2+}$ exchange to agonist-stimulated increases in $[Ca^{2+}]_i$ in the absence of P450 inhibitors (Colden-Stanfield *et al.* 1987; this study). In contrast, during channel inhibition by P450 inhibitors in cells prestimulated with bradykinin, $[Ca^{2+}]_i$ may drop rapidly via plasmalemmal $Ca^{2+}$ pump activity (Colden-Stanfield *et al.* 1987), while intracellular $Na^+$ remains high. Thus, the reverse mode of $Na^+$–$Ca^{2+}$ exchange (i.e. 3 $Na^+$ out, 1 $Ca^{2+}$ in) would be favoured, resulting in $Ca^{2+}$ influx via the $Na^+$–$Ca^{2+}$ exchanger and, in turn, an increase of $[Ca^{2+}]_i$ (remaining plateau phase under econazole in Fig. 1*A*). In such '$Na^+$-loaded cells' decreased $[Na^{2+}]_o$ may amplify the driving force for $Na^+$ efflux and $Ca^{2+}$ influx through the $Na^+$–$Ca^{2+}$ exchanger, resulting in an enhanced $[Ca^{2+}]_i$ (Fig. 1*A*). Since low $[Na^{2+}]_o$ failed to increase $[Ca^{2+}]_i$ in agonist-stimulated cells pretreated with econazole, an activation of $Na^+$ entry other than through the P450 inhibitor-sensitive, non-selective pathway seems unlikely. In cells stimulated with bradykinin in $Na^+$-free solution, econazole (in $Na^+$-containing solution) diminished the $[Ca^{2+}]_i$ plateau to about 10% above basal. The remaining, very small $[Ca^{2+}]_i$ plateau was insensitive to removal of extracellular $Ca^{2+}$ (data not shown). This may reflect the modest inhibition of $Ca^{2+}$-ATPases by P450 inhibitors (Mason *et al.* 1993).

Since $Ca^{2+}$ entry into endothelial cells depends not only on channel opening, but also on the driving force for $Ca^{2+}$ influx, i.e. hyperpolarization via $Ca^{2+}$-activated $K^+$ currents (for review see Graier *et al.* 1994*b*), one may speculate that inhibition of endothelial $K^+$ channels is an underlying mechanism of econazole-induced blockade of $Ca^{2+}$ entry, as shown in smooth muscle (Alvarez *et al.* 1992). However, the results on ionomycin-induced $Ca^{2+}$ entry, which was highly sensitive to membrane depolarization and insensitive to econazole, clearly exclude inhibition of endothelial $K^+$ channels as the main reason for the $Ca^{2+}$ entry-blocking effect of P450 inhibitors in the endothelial cell preparations we studied.

Several groups have described the formation of NO as dependent on $Ca^{2+}$ entry and enhanced by $K^+$ channel-mediated membrane hyperpolarization (Graier *et al.* 1994*b*). Our results in this study indicate that inhibition of P450 enzyme(s) strongly diminished histamine-, TG-, BHQ-, and CPA-induced increases in endothelial cGMP levels, which reflect formation of endothelium-derived nitric oxide (EDNO; Graier *et al.* 1992). At the concentration used, cytochrome P450 inhibitors did not interfere with the stimulatory effects of either A23187, which increases cGMP by its $Ca^{2+}$ ionophore properties leading to constitutive NO synthase stimulation, or sodium nitroprusside, which stimulates soluble guanylyl cyclase directly. Purified brain constitutive NO synthase was unchanged by econazole up to 30 $\mu$mol $l^{-1}$ (P. Klatt, unpublished observation). Thus, even though constitutive NO synthase and soluble guanylyl cyclase have been shown to constitute P450-containing proteins, the P450 inhibitors in the concentration used failed to influence constitutive NO synthase and/or soluble guanylyl cyclase activity, indicating that the inhibitory effect of P450 inhibitors on histamine-, TG-, BHQ- or CPA-induced EDNO increases are due to inhibition of endothelial $Ca^{2+}$ entry. Finally, the absence of an effect of P450 inhibitors on $Ca^{2+}$ ionophore-induced cGMP formation parallels the $[Ca^{2+}]_i$ response, thus providing further evidence against a non-specific action of P450 inhibitors on $K^+$ channels.

Pinto *et al.* (1986) showed that induction of endothelial P450 MO, which is mainly located on endothelial microsomal membranes (Abraham *et al.* 1985; Capdevila *et al.* 1992), potentiated endothelium-dependent relaxations to arachidonic acid. Using a method similar to their protocol for P450 MO induction in vascular endothelium, we treated cultured endothelial cells from human umbilical veins and porcine aortae with $\beta$-naphthoflavone, a well-known P450 MO inducer in the vascular endothelium (Pinto *et al.* 1986; Moffat, Ward, Bend, Mock, Farhangkhoee & Karmazyn, 1993). Induction of P450 MO with $\beta$-naphthoflavone significantly potentiated the agonist-induced $[Ca^{2+}]_i$ response (Fig. 4*A*). As shown in Fig. 4*B*, P450 induction did not change agonist-induced $Ca^{2+}$ release, while $Ca^{2+}$/$Mn^{2+}$ entry was enhanced. These findings rule out changes in agonist-sensitive $Ca^{2+}$ store size and/or degree of discharge upon agonist and provide further evidence for the role of P450 MO in agonist-induced $Ca^{2+}$ entry. In contrast to $\beta$-naphthoflavone, phenobarbitone failed to affect the agonist-induced $[Ca^{2+}]_i$ increase. This finding is in agreement with the data obtained from other groups who reported that vascular

cytochrome P450 enzymes are not inducible with phenobarbitone (Pinto *et al.* 1986).

We do not know how microsomal P450 MO might be regulated. Alvarez *et al.* (1991) described a possible role of microsomal calmodulin for the regulation of a microsomal P450 enzyme which may link $Ca^{2+}$ store depletion and $Ca^{2+}$ entry in thymocytes. Thus, $Ca^{2+}$–calmodulin in the lumen of the endoplasmic reticulum is thought to block P450 activity. Depletion of $Ca^{2+}$ stores may liberate calmodulin from its $Ca^{2+}$–calmodulin complexes, resulting in a lack of P450 inhibition (Alvarez *et al.* 1991). On the other hand, there is evidence that tyrosine kinase-dependent phosphorylation may regulate the formation of the second messenger for $Ca^{2+}$ entry stimulation in platelets (Vostal, Jackson & Shulman, 1991). Convincingly, the phosphatase inhibitor okadaic acid was shown to enhance $Ca^{2+}$ entry in *Xenopus* oocytes (Parekh *et al.* 1993) and endothelial cells (Graier *et al.* 1993). Further studies are necessary to find out whether P450 MO is directly regulated by $Ca^{2+}$–calmodulin and/or by a tyrosine kinase-mediated phosphorylation.

Endothelial microsomal P450 MO has been shown to produce some chiral epoxyeicosatrienoic acids (5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET acid) isomers from arachidonic acid in a NADPH-dependent pathway (Fritzpatrik & Murphy, 1989). In this study we used 5,6-EET and 8,9-EET. In contrast to 8,9-EET, which failed to increase endothelial $[Ca^{2+}]_i$, 5,6-EET increased $[Ca^{2+}]_i$ to an extent similar to that induced by bradykinin or TG. The effect of 5,6-EET was unaffected by aspirin which supported the idea that cyclo-oxygenase is not involved in this phenomenon. The finding that 5,6-EET failed to increase endothelial $[Ca^{2+}]_i$ in $Ca^{2+}$ free solution but increased $Mn^{2+}$ quenching in the presence of 2 mmol $l^{-1}$ $Mn^{2+}$, clearly demonstrates that 5,6-EET activates endothelial $Ca^{2+}$ entry without prior depletion of intracellular $Ca^{2+}$ stores.

As expected, $Ca^{2+}/Mn^{2+}$ entry stimulated by 5,6-EET was not sensitive to P450 inhibitors. This suggests that prevention of 'store-depletion-activated $Ca^{2+}$ entry' by the P450 inhibitors might reflect the lack of biosynthesis of 5,6-EET by microsomal P450 MO rather then a direct blockade of endothelial $Ca^{2+}$ channels. These findings further confirm our suggestion that P450 inhibitors did not block agonist-induced $Ca^{2+}$ entry via inhibition of membrane hyperpolarization, because under such circumstances 5,6-EET would not be able to stimulate $Ca^{2+}$ entry.

Table 2 summarizes the pharmacological differences between the effects of 5,6-EET and bradykinin on endothelial $[Ca^{2+}]_i$. The three major differences are: (1) bradykinin-stimulated $Ca^{2+}$ entry was due to intracellular $Ca^{2+}$ release, while 5,6-EET (< 780 $\mu$mol $l^{-1}$) activates $Ca^{2+}$ entry without any $Ca^{2+}$ store depletion; (2) inhibition of microsomal P450 MO prevented bradykinin-induced $Ca^{2+}$ entry, while the effect of 5,6-EET remained unchanged, and (3) while a short stimulation with bradykinin results in a long-lasting activation of $Ca^{2+}$ entry even when bradykinin has been removed. $Ca^{2+}$ entry induced by 5,6-EET, on the other hand, strictly depends on the presence of this compound and is terminated by the removal of 5,6-EET. These findings are consistent with our idea that 5,6-EET, or one of its metabolites, might be the second messenger for bradykinin-induced $Ca^{2+}$ entry in endothelial cells. This hypothesis is further supported by the fact that $Ca^{2+}$ entry induced by 5,6-EET and bradykinin is indistinguishable. We found that both compounds activate a $Ca^{2+}$ entry pathway which is $Mn^{2+}$ and $Ba^{2+}$ permeable, sensitive to the inorganic $Ca^{2+}$ channel blockers $Ni^{2+}$ and $La^{3+}$, but not sensitive to the organic $Ca^{2+}$ channel blocker nitrendipine. Furthermore, $Ca^{2+}$ entry stimulated by 5,6-EET or bradykinin (for review see Graier *et al.* 1994*b*) is sensitive to membrane depolarization but remained unchanged in low

**Table 2. Comparison of the effects of bradykinin and 5,6-EET on endothelial $[Ca^{2+}]_i$**

| | Bradykinin | 5,6-EET |
|---|---|---|
| Concentration | 100 nmol $l^{-1}$ | < 156 nmol $l^{-1}$ |
| Interaction with | $B_2$ receptor | ? |
| $Ca^{2+}$ release | + (via $IP_3$) | – |
| $Ca^{2+}$ entry | + | + |
| permeable to $Mn^{2+}/Ba^{2+}$ | + | + |
| sensitive to $Ni^{2+}/La^{3+}$ | + | + |
| insensitive to nitrendipine | + | + |
| sensitive to membrane depolarization | + | + |
| unchanged in low extracellular $Na^+$ | + | + |
| sensitive to P450 mono-oxygenase inhibition | + | – |
| requires $Ca^{2+}$ store depletion | + | – |
| terminated by removal of the activator | – | + |

extracellular $Na^+$, indicating the lack of involvement of $Na^+-Ca^{2+}$ exchange activity in the $[Ca^{2+}]_i$ increasing effects of bradykinin and 5,6-EET. Additional evidence for a common $Ca^{2+}$ entry pathway stimulated by 5,6-EET and bradykinin is provided by the lack of additivity of $Ca^{2+}$ entry induced by these compounds, as indicated by the findings that: (1) 5,6-EET failed to stimulate $Ca^{2+}$ entry in bradykinin prestimulated cells, (2) bradykinin only transiently increased $[Ca^{2+}]_i$ in cells co-stimulated with the maximal concentration of 5,6-EET which reflects intracellular $Ca^{2+}$ release (Fig. 7*A*) and (3) that in cells prestimulated with sub-maximal concentrations of 5,6-EET, maximal concentration of bradykinin increased sustained $[Ca^{2+}]_i$ plateau to the same value as obtained by maximal concentrations of bradykinin or 5,6-EET alone (Fig. 7*B*). Interestingly, in cells prestimulated for 1 min with sub-maximal concentrations of 5,6-EET, bradykinin failed to elicit a transient spike as evidence for discharge from $Ca^{2+}$ stores (Fig. 7*B*). This might partly reflect depletion of the bradykinin-sensitive $Ca^{2+}$ pool by $Ca^{2+}$-induced $Ca^{2+}$ release. We have shown that in endothelial cells the bradykinin-releaseable $Ca^{2+}$ pool can be depleted by $Ca^{2+}$-induced $Ca^{2+}$ release (Graier, Simecek, Bowles & Sturek, 1994*a*). Thus, although 5,6-EET does not directly deplete intracellular $Ca^{2+}$ stores, elevation of $[Ca^{2+}]_i$ by the 5,6-EET-induced $Ca^{2+}$ entry may deplete intracellular $Ca^{2+}$ stores in vascular endothelial cells as a secondary action.

It has been proposed that this epoxyeicosatrienoic acid is involved in the agonist-induced $[Ca^{2+}]_i$ response in a large number of non-excitable cells. In isolated rat hepatocytes (Karara, Breyer, Falck & Capdevila, 1991), pituitary cells (Snyder, Lattanzio, Yadagiri, Falck & Capdevila, 1986), parotid cells (Snowdowne, Rosenoer, Yu & Cashman, 1989) and epithelial cells (Madhun, Goldthwait, McKay, Hopfer & Douglas, 1991), 5,6-EET increases $[Ca^{2+}]_i$ due to the direct stimulation of $Ca^{2+}$ entry. Additional data provided evidence for a cyclo-oxygenase-related $Ca^{2+}$ release with higher concentrations of 5,6-EET (Kuno, Kawawaki, Shibata & Gotani, 1993). Our findings that 5,6-EET failed to increase $[Ca^{2+}]_i$ in $Ca^{2+}$-free solution and that cyclo-oxygenase inhibition did not affect 5,6-EET-induced $Ca^{2+}$ entry strongly suggest a direct stimulation of endothelial $Ca^{2+}$ entry by this P450 MO product (Fig. 11). Snowdowne *et al.* (1989) have also described results similar to ours in epithelial cells, including the inhibition of agonist-stimulated $Ca^{2+}$ entry by P450 inhibitors and 5,6-EET-induced increases in $[Ca^{2+}]_i$, but little activity of other isomers. Our data suggest that, in endothelial cells, long-lasting $Ca^{2+}$ entry induced by autacoids or $Ca^{2+}$ store depletion is regulated by the P450 MO product, 5,6-EET, or a metabolite of 5,6-EET. Thus, $Ca^{2+}$ release induced by $IP_3$ or inhibition of microsomal ATPase may stimulate phospholipase $A_2$, as described by Buckley, Barchowsky, Dolor & Whorton (1991). The role of direct G protein-linked, $Ca^{2+}$ increase-independent phospholipase $A_2$ activation (Buckley *et al.* 1991) in the agonist-induced $[Ca^{2+}]_i$ response in endothelial cells remains unclear, but may explain the endothelial $Ca^{2+}$ entry induced by direct G protein-activating compounds (Graier *et al.* 1994*b*). In endothelial cells, Goligorsky, Menton, Laszlo & Lum (1989) provided strong evidence for the involvement of phospholipase $A_2$ activation in thrombin-induced sustained $Ca^{2+}$ entry. A key role of phospholipase $A_2$ stimulation for agonist-stimulated $Ca^{2+}$ entry was also described in mast cells (Kuno *et al.* 1993). Accordingly, all authors proposed a $Ca^{2+}$ release- or direct G protein- (without increased $[Ca^{2+}]_i$) mediated stimulation of phospholipase $A_2$, resulting in a non-cyclo-oxygenase-related arachidonic acid metabolite which opened $Ca^{2+}$-permeable channels.

Very recently, EETs were also proposed to be endothelium-derived hyperpolarizing factors (Hu & Kim, 1993), thought to act by diffusing to smooth muscle cells and stimulating smooth muscle $K^+$ channels. Although hyperpolarization alone does not increase $[Ca^{2+}]_i$ in non-stimulated endothelial cells (Graier *et al.* 1993; Graier *et al.* 1994*b*), we cannot rule out that, besides $Ca^{2+}$ entry, 5,6-EET stimulates endothelial $K^+$ channels, not only by its $[Ca^{2+}]_i$-increasing property, but also directly, as described for smooth muscle (Hu & Kim, 1993). In agreement with these reported effects of 5,6-EET, the intracellular second messenger which is proposed to be responsible for $Ca^{2+}$ entry stimulation in *Xenopus* oocytes was also shown to stimulate $K^+$ channels (Parekh *et al.* 1993).

Thus, 5,6-EET fulfills all the criteria of the reported nature and properties of the intracellular messenger of 'store depletion-activated $Ca^{2+}$ entry' in non-excitable cells: it is a low molecular weight, labile compound (Montero *et al.* 1992; Parekh *et al.* 1993; this study); it is synthesized by a P450-containing enzyme (P450 MO) at the microsomal membrane (for review see Alvarez *et al.* 1991; this study); it depends on phospholipase $A_2$ activity (Goligorsky *et al.* 1989; Kuno *et al.* 1993); it is diffusible (Parekh *et al.* 1993); its stability and half-life may be influenced by intracellular pH (Thuringer, Diarra & Sauvé, 1991); as an epoxycompound it is sensitive to oxidant stress (Schilling & Elliott, 1992); and, most importantly, 5,6-EET activates $Ca^{2+}$ entry.

In conclusion, our data present $Ca^{2+}$ entry into endothelial cells as a complex phenomenon (Fig. 11). Agonists like bradykinin may deplete $Ca^{2+}$ stores via an $IP_3$-dependent mechanism. Due to the decreased microsomal $Ca^{2+}$ concentration, microsomal P450 MO is activated. This enzyme uses arachidonic acid, which is liberated by $Ca^{2+}$ release- and/or G protein-stimulated phospholipase $A_2$ and synthesizes epoxyeicosatrienoic acids. One of these metabolites, 5,6-EET, or a metabolite of 5,6-EET, may directly, or via an intermediary step, open endothelial $Ca^{2+}$-

permeable channels and, perhaps, $K^+$ channels. These findings provide a new perspective on second messenger regulation of $Ca^{2+}$-permeable ion channels in non-excitable cells.

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