A role for 5,6-epoxyeicosatrienoic acid in calcium entry by *de novo* conformational coupling in human platelets

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A major pathway for Ca^{2+} entry in non-excitable cells is activated following depletion of intracellular Ca^{2+} stores. A *de novo* conformational coupling between elements in the plasma membrane (PM) and Ca^{2+} stores has been proposed as the most likely mechanism to activate this capacitative Ca^{2+} entry (CCE) in several cell types, including platelets. Here we report that a cytochrome P450 metabolite, 5,6-EET, might be a component of the *de novo* conformational coupling in human platelets. In these cells, 5,6-EET induces divalent cation entry without having any detectable effect on Ca^{2+} store depletion. 5,6-EET-induced Ca^{2+} entry was sensitive to the CCE blockers 2-APB, lanthanum, SKF-96365 and nickel and impaired by incubation with anti-hTRPC1 antibody. Ca^{2+} entry stimulated by low concentrations of thapsigargin, which selectively depletes the dense tubular system and induces EET production, was impaired by the cytochrome P450 inhibitor 17-ODYA, which has no effect on CCE mediated by depletion of the acidic stores using 2,5-di-(tert-butyl)-1,4-hydroquinone. We have found that 5,6-EET-induced Ca^{2+} entry requires basal levels of H_2O_2, which might maintain a redox state favourable for this event. Finally, our results indicate that 5,6-EET induces the activation of tyrosine kinase proteins and the reorganization of the actin cytoskeleton, which might provide a support for the transport of portions of the Ca^{2+} store towards the PM to facilitate *de novo* coupling between IP_3R type II and hTRPC1 detected by coimmunoprecipitation. We propose that the involvement of 5,6-EET in TG-induced coupling between IP_3R type II and hTRPC1 and subsequently CCE is compatible with the *de novo* conformational coupling in human platelets.

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Capacitative Ca^{2+} entry (CCE) is regulated by the filling state of the intracellular Ca^{2+} stores (Putney, 1986), although the mechanism underlying this process is still not fully understood. A number of hypotheses have been proposed in different cell types to account for the communication between the intracellular Ca^{2+} stores and the plasma membrane (PM), which can be grouped into those that assume the generation of a diffusible molecule, a calcium influx factor (CIF), that gates capacitative Ca^{2+} channels in the PM and those that propose a constitutive physical interaction between Ca^{2+} channels in the PM and inositol 1,4,5-trisphosphate receptors (IP_3R) in the membrane of the intracellular Ca^{2+} stores, the conformational coupling hypothesis (Putney *et al.* 2001; Venkatachalam *et al.* 2002).

Recently, a modification of the classical conformational coupling hypothesis has been presented in several non-excitable cells. *De novo* conformational coupling is proposed to be based on a reversible trafficking of portions of the Ca^{2+} stores towards the PM to facilitate *de novo* coupling between the IP_3R in the endoplasmic reticulum (ER) and Ca^{2+} channels in the PM (Rosado *et al.* 2005). In human platelets, where it has been demonstrated, coupling occurs between the type II IP_3R and naturally expressed human canonical transient receptor potential 1 (hTRPC1) (Rosado *et al.* 2000*a*, 2004*a*; Rosado & Sage, 2000*a*, 2001*a*). In this process, formerly called 'secretion-like coupling', the actin cytoskeleton plays a dual role. Although actin polymerization is required for the activation of CCE, since cytoskeletal disruption impairs Ca^{2+} entry, the cortical actin network acts as a negative modulator of the interaction between the ER and PM (Rosado *et al.* 2000*a*).

The proposed alternative to the conformational coupling involves the action of a CIF. Suggested CIFs include cGMP (Pandol & Schoeffield-Payne, 1990), tyrosine kinases (Sargeant *et al.* 1993), small GTP-binding

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proteins (Bird & Putney, 1993), a still uncharacterized non-protein CIF (Randriamampita & Tsien, 1993), and a product of cytochrome P450. Cytochrome P450 metabolites have been proposed to act as CIFs based on the finding that cytochrome P450 inhibitors prevent CCE (Alonso-Torre *et al.* 1993). In particular, 5,6-epoxyeicosatrienoic acid (5,6-EET), a metabolite of cytochrome P450 epoxygenases, has been presented as a CIF (Graier *et al.* 1995; Xie *et al.* 2002), although other isomers, such as 11,12-EET (Mombouli *et al.* 1999) or 14,15-EET (Alvarez *et al.* 2004), have also been proposed as messengers involved in the activation of CCE. This hypothesis has recently received support from studies that suggest an important role for a Ca^{2+}-independent phospholipase A2 in the activation of CCE (Smani *et al.* 2003, 2004).

The *de novo* conformational coupling is a unique model that integrates some of the signalling molecules proposed as CIFs, such as tyrosine kinases or small GTP-binding proteins of the Ras family, with actin filament remodelling and conformational coupling between the IP_3R and hTRPC1 channels (Rosado & Sage, 2000*b*). Hence, we have investigated whether the cytochrome P450 epoxygenase metabolite 5,6-EET is involved in the activation of Ca^{2+} entry in human platelets, and, if this is the case, whether 5,6-EET might be a signalling molecule that participates in the *de novo* conformational coupling process in these cells.

Methods

Materials

Fura-2 acetoxymethyl ester (fura-2/AM), 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA), 2-(2,3-naphthalimino) ethyl trifluoromethanesulphonate (NT) and calcein-AM were from Molecular Probes (Leiden, the Netherlands). Apyrase (grade VII), aspirin, thapsigargin (TG), paraformaldehyde, Nonidet P-40, FITC-labelled phalloidin, β-naphthoflavone (BN), 17-octadecynoic acid (17-ODYA), methyl 2,5-dihydroxycinnamate (M-2,5-DHC), catalase, valinomycin and bovine serum albumin (BSA) were from Sigma (Madrid, Spain). Cytochalasin D (Cyt D), SKF 96365 and 2-aminoethoxydiphenyl borate (2-APB) were from Calbiochem (Nottingham, UK). 5,6-Epoxyeicosatrienoic acid (5,6-EET) and 2,5-di-(tert-butyl)-1,4-hydroquinone (TBHQ) were from Alexis (Nottingham, UK). Anti-phosphotyrosine monoclonal antibody (4G10) was from Upstate Biotechnology (Lake Placid, NY, USA). Horseradish peroxidase-conjugated ovine anti-mouse IgG antibody (NA931) was from Amersham (Buckinghamshire, UK). Anti-hTRPC1 polyclonal antibody was from Alomone Laboratories (Jerusalem, Israel). Anti-IP_3R type II polyclonal antibody (C-20), horseradish peroxidase-conjugated donkey anti-goat IgG antibody and horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents were of analytical grade.

Platelet preparation

Fura-2-loaded platelets were prepared as previously described (Rosado *et al.* 2000*a*) as approved by Local Ethical Committees and in accordance with the Declaration of Helsinki. Briefly, blood was obtained from healthy drug-free volunteers and mixed with one-sixth volume of acid/citrate dextrose anticoagulant containing (mM): 85 sodium citrate, 78 citric acid and 111 D-glucose. Platelet-rich plasma was then prepared by centrifugation for 5 min at 700 *g* and aspirin (100 μM) and apyrase (40 μg ml^{-1}) were added. Platelet-rich plasma was incubated at 37°C with 2 μM fura-2 acetoxymethyl ester for 45 min. Cells were then collected by centrifugation at 350 *g* for 20 min and resuspended in Hepes-buffered saline (HBS), pH 7.45, containing (mM): 145 NaCl, 10 Hepes, 10 D-glucose, 5 KCl, 1 $MgSO_4$, supplemented with 0.1% BSA and 40 μg ml^{-1} apyrase.

Cell viability

Cell viability was assessed using calcein and trypan blue. For calcein loading, cells were incubated for 30 min with 5 μM calcein-AM at 37°C and centrifuged, and the pellet was resuspended in fresh HBS. Fluorescence was recorded from 2 ml aliquots using a Cary Eclipse Spectrophotometer (Varian Ltd, Madrid, Spain). Samples were excited at 494 nm and the resulting fluorescence was measured at 535 nm. The results obtained with calcein were confirmed using the trypan blue exclusion technique. Ninety-five per cent of cells were viable in our platelet preparations, at least during the performance of the experiments.

Measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$)

Fluorescence was recorded from 2 ml aliquots of magnetically stirred platelet suspension (2×10^8 cells ml^{-1}) at 37°C using a fluorescence spectrophotometer with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in $[Ca^{2+}]_i$ were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz *et al.* (1985). Mn^{2+} influx was monitored as a quenching of fura-2 fluorescence at the isoemissive wavelength of 360 nm, which is presented on an arbitrary linear scale (Sage *et al.* 1989).

Ca^{2+} entry was estimated using the integral of the rise in $[Ca^{2+}]_i$ for 45 s after addition of $CaCl_2$ (Rosado *et al.* 2000*a*). Control experiments were performed for all experimental procedures in order to correct Ca^{2+} entry by subtraction of the $[Ca^{2+}]_i$ elevation due to leakage

of the indicator. To calculate the initial rate of Ca^{2+} elevation after the addition of Ca^{2+} to the medium, the traces were fitted to the equation $y = A \times (1 - e^{-Kx})$, and to estimate the initial rate of fura-2 fluorescence quenching after the addition of Mn^{2+} to the medium, the traces were fitted to the equation $y = S \times e^{-Kx} + A$, where K is the slope, S is the span and A is the plateau. Ca^{2+} release was estimated using the integral of the rise in $[Ca^{2+}]_i$ for 3 min after the addition of 5,6-EET, BN or TG. Both Ca^{2+} entry and release are expressed as nanomolar taking a sample every second (nM · s), as previously described (Heemskerk *et al.* 1997; Rosado & Sage, 2000*c*).

Immunoprecipitation and Western blotting

The immunoprecipitation and Western blotting were performed as previously described (Rosado & Sage, 2000*a*). Briefly, 500 μl aliquots of platelet suspension (2×10^9 cell ml^{-1}) were lysed with an equal volume of lysis buffer, pH 7.2, containing 316 mM NaCl, 20 mM Tris, 2 mM EGTA, 0.2% SDS, 2% sodium deoxycholate, 2% Triton X-100, 2 mM Na_3VO_4, 2 mM phenylmethylsulphonyl fluoride, 100 μg ml^{-1} leupeptin and 10 mM benzamidine. Aliquots of platelet lysates (1 ml) were immunoprecipitated by incubation with 2 μg of anti-hTRPC1 polyclonal antibody and 25 μl of protein A–agarose overnight at 4°C on a rocking platform. The immunoprecipitates were resolved by 8% SDS-PAGE and separated proteins were electrophoretically transferred onto nitrocellulose membranes for subsequent probing. Blots were incubated overnight with 10% (w/v) BSA in Tris-buffered saline with 0.1% Tween 20 (TBST) to block residual protein binding sites. Immunodetection of IP_3R type II and hTRPC1 was achieved using the anti-IP_3RII polyclonal antibody diluted 1 : 500 in TBST for 3 h or the anti-hTRPC1 antibody diluted 1 : 200 in TBST. The primary antibody was removed and blots were washed six times for 5 min each with TBST. To detect the primary antibody, blots were incubated with horseradish peroxidase-conjugated donkey anti-goat IgG antibody or horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody diluted 1 : 10 000 in TBST and then exposed to enhanced chemiluminescence reagents for 1 min. Blots were then exposed to photographic films. The density of bands on the film was measured using a scanning densitometry.

Protein tyrosine phosphorylation

Protein tyrosine phosphorylation was detected by gel electrophoresis and Western blotting (Rosado & Sage, 2000*a*). Platelets stimulation was terminated by mixing with an equal volume of 2 × Laemmli's buffer (Laemmli, 1970) with 10% dithiothreitol followed by heating for 5 min at 95°C. One-dimensional SDS-electrophoresis was performed with 10% polyacrylamide minigels, and separate proteins were electrophoretically transferred, for 2 h at 0.8 mA cm^{-2}, in a semidry blotter (Hoefer Scientific, Newcastle-under-Lyne, Staffordshire, UK) onto nitrocellulose for subsequent probing. Blots were incubated overnight with 10% (w/v) BSA in Tris-buffered saline with 0.1% Tween 20 (TBST) to block residual protein binding sites. Immunodetection of tyrosine phosphorylation was achieved using the anti-phosphotyrosine antibody 4G10 diluted 1 : 2500 in TBST for 1 h. The primary antibody was removed and blots washed six times for 5 min each with TBST. To detect the primary antibody, blots were incubated with horseradish peroxidase-conjugated ovine anti-mouse IgG antibody diluted 1 : 10000 in TBST, washed six times in TBST, and exposed to enhanced chemiluminescence reagents for 5 min. Blots were then exposed to photographic films and the optical density of the entire lane was estimated using scanning densitometry.

Measurement of F-actin content

The F-actin content of resting and activated platelets was determined according to a previously published procedure (Rosado & Sage, 2000*c*). Briefly, washed platelets (2×10^8 cells ml^{-1}) were activated in HBS. Samples of platelet suspension (200 μl) were transferred to 200 μl ice-cold 3% (w/v) formaldehyde in phosphate-buffered saline (PBS) for 10 min. Fixed platelets were permeabilized by incubation for 10 min with 0.025% (v/v) Nonidet P-40 detergent dissolved in PBS. Platelets were then incubated for 30 min with FITC-labelled phalloidin (1 μM) in PBS supplemented with 0.5% (w/v) bovine serum albumin. After incubation the platelets were collected by centrifugation for 90 s at 3000 *g* and resuspended in PBS. Staining of 2×10^7 cells ml^{-1} was measured using a fluorescence spectrophotometer. Samples were excited at 496 nm and emission was at 516 nm.

Intracellular ROS production through the oxidation of CM-H_2DCFDA

CM-H_2DCFDA is a ROS-sensitive probe that can be used to detect ROS production in living cells. It passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases, releasing the corresponding dichlorodihydrofluorescein derivative. CM-H_2DCFDA oxidation yields a fluorescent adduct, dichlorofluorescein (DCF) that is trapped inside the cell (Zhang *et al.* 2003). Cells were incubated at 37°C with 10 μM CM-H_2DCFDA acetyl ester for 30 min, then centrifuged and the pellet was resuspended in fresh HBS. Fluorescence was recorded from 2 ml aliquots using a fluorescence spectrophotometer. Samples were excited at 488 nm and the resulting fluorescence was measured at 530 nm. ROS levels were

quantified as the integral of the rise in DCF fluorescence for 5 min after platelet treatment.

Determination of EET production

EET production was determined following a method based on a previously published procedure (Nithipatikom *et al.* 2000). Briefly, platelets (2×10^8 cells ml^{-1}) were preincubated in the absence or presence of 10 μM 17-ODYA. Cells were stimulated in with 10 nM TG or 20 μM TBHQ for 5 min at 37°C or left untreated and then fixed with ice-cold 3% (w/v) formaldehyde in PBS for 10 min, as described above, and sonicated. Freshly prepared NT (1 mM) in anhydrous acetonitrile was added to the samples and vortexed lightly for 2 s as previously described (Yue *et al.* 2004). The reaction tubes were placed in the dark at room temperature for 15 min and fluorescence was determined using a fluorescence spectrophotometer. Samples were excited at 259 nm and emission was at 394 nm.

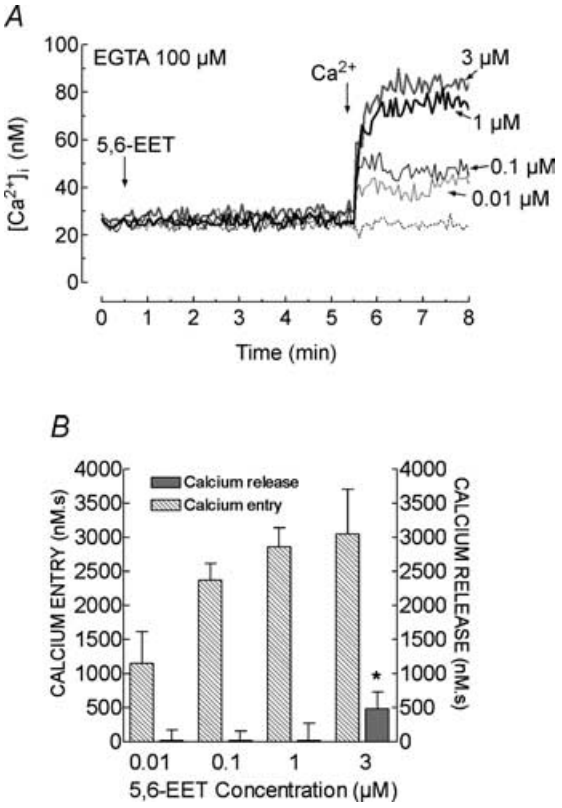

Figure 1. Effect of 5,6-EET on Ca^{2+} release and entry in human platelets
A, fura-2-loaded human platelets were treated in a Ca^{2+}-free medium (100 μM EGTA was added) with various concentrations of 5,6-EET (0.01–3 μM) or left untreated (dotted trace) followed by addition of $CaCl_2$ (final concentration 300 μM) to initiate Ca^{2+} entry. Modifications in $[Ca^{2+}]_i$ were monitored using the 340/380 nm ratio and traces were calibrated in terms of $[Ca^{2+}]_i$. *B*, bars indicate the amount of Ca^{2+} entry and release in the presence of different concentrations of 5,6-EET. Ca^{2+} entry and release were estimated using the integral of the rise in $[Ca^{2+}]_i$ for 3 min after addition of $CaCl_2$ or 5,6-EET, respectively, and expressed as nanomolar seconds (nM s) as described in Methods. Values are expressed as the mean ± S.E.M. from six independent experiments. *$P < 0.05$ compared to the resting level.

Statistical analysis

Analysis of statistical significance was performed using Student's *t* test. $P < 0.05$ was considered to be significant for a difference.

Results

Treatment of platelets with 5,6-EET induces divalent cation entry

In the absence of extracellular Ca^{2+}, treatment of fura-2-loaded human platelets in stirred cuvettes at 37°C with different concentrations of 5,6-EET has a negligible effect on Ca^{2+} release from the intracellular stores (Fig. 1). Only when platelets were stimulated with 3 μM 5,6-EET was a significant Ca^{2+} release from the intracellular stores detected (Fig. 1*B*; $P < 0.05$). Interestingly, subsequent addition of Ca^{2+} (300 μM) to the suspension of 5,6-EET-treated platelets resulted a concentration-dependent increase in $[Ca^{2+}]_i$ indicative of Ca^{2+} entry (Fig. 1). A significant Ca^{2+} entry was detected at 0.01 μM 5,6-EET (the integral of the rise in $[Ca^{2+}]_i$ above basal for 45 s after addition of Ca^{2+} taking data every second was 350 ± 86 nM · s) and reached a maximum after treatment of platelets with 3 μM 5,6-EET (the integral of the rise in $[Ca^{2+}]_i$ above basal was 1008 ± 157 nM · s; $n = 6$). The initial rate of Ca^{2+} elevation after the addition of Ca^{2+} to the external medium was similar for all the concentrations of 5,6-EET tested (the initial slope was 0.0589 ± 0.0057, 0.0571 ± 0.0052, 0.0575 ± 0.0055 and 0.0561 ± 0.0048 for 3, 1, 0.1 and 0.01 μM 5,6-EET). As observed in Fig. 1*A*, dashed trace, in the absence of stimulus Ca^{2+} entry in platelets is negligible, which indicates that the elevation in $[Ca^{2+}]_i$ above basal after addition of $CaCl_2$ to EET-treated cells was not due to leakage of the indicator.

Mn^{2+} was used to evaluate the effect of 5,6-EET on divalent cation influx. This cation can be used as a surrogate for Ca^{2+} entry given its quenching effect on fura-2 (Sage *et al.* 1989). Fura-2 was excited at the isoemissive wavelength, 360 nm, to allow monitoring of quenching of fluorescence by Mn^{2+}. Addition of Mn^{2+} (300 μM) to platelets treated for 5 min with 5,6-EET resulted in a sustained quenching of fura-2 fluorescence (Fig. 2, traces *a–d*) compared with non-stimulated cells (Fig. 2, trace *e*). The initial slope for the rate of fura-2 fluorescence quenching after the addition of Mn^{2+} were 0.0509 ± 0.0049, 0.0471 ± 0.0055, 0.0325 ± 0.0035 and 0.0191 ± 0.0028 for 3, 1, 0.1 and 0.01 μM 5,6-EET These

results indicate that 5,6-EET in the range of 0.01–3 μM induces divalent cation entry in a concentration-dependent manner.

5,6-EET induces Ca^{2+} entry that shows characteristics of CCE in human platelets

In order to investigate the nature of the cation entry induced by platelet treatment with 5,6-EET we have examined the effect of 2-APB and SKF 96365, two CCE blockers in human platelets (Jenner & Sage, 2000; Diver *et al.* 2001) and other cell types (Enfissi *et al.* 2004; Gratschev *et al.* 2004; Ng *et al.* 2005), on Ca^{2+} entry induced by 1 μM 5,6-EET, the highest concentration used that induces Ca^{2+} entry without any detectable Ca^{2+} release (see Fig. 1). As shown in Fig. 3*A*, *B* and *F*, in the presence of 100 μM 2-APB or 10 μM SKF 96365, 1 μM 5,6-EET was unable to induce Ca^{2+} entry ($P < 0.001$; $n = 5$–9), suggesting that this process exhibits pharmacological properties characteristics of CCE. To confirm this possibility we explored the effect of 5,6-EET on Ca^{2+} entry in the presence of La^{3+} or Ni^{2+}, two well described blockers of CCE (Wang *et al.* 2004). As depicted in Figs 3*C*, *D* and *F*, both cations abolished Ca^{2+} entry induced by 1 μM 5,6-EET ($P < 0.001$; $n = 5$). These findings suggest that 5,6-EET-induced Ca^{2+} entry, in the range of 0.01–1 μM, shows characteristics of CCE in human platelets without any detectable Ca^{2+} release from the internal stores.

hTRPC1 has been shown to form Ca^{2+} permeable cation channels (Sinkins *et al.* 1998; Xu & Beech, 2001; Beech *et al.* 2004). The transfection of different cell lines with hTRPC1 has been reported to increase CCE (Liu *et al.* 2000; Wu *et al.* 2000) indicating that hTRPC1, unlike some other TRP isoforms is regulated by the filling state of the intracellular Ca^{2+} stores. In addition, we have shown that hTRPC1 might conduct CCE in human platelets activated by store depletion (Rosado & Sage, 2000*a*; Rosado *et al.* 2002). In order to investigate whether Ca^{2+} entry stimulated by 5,6-EET is conducted by hTRPC1 channels we have performed a number of experiments using the anti-hTRPC1 antibody, which blocks hTRPC1 channel function by binding to the extracellular sequence 557–571 located in the pore region (Rosado *et al.* 2002). As shown in Fig. 3*E* and *F*, incubation of human platelets with anti-hTRP1 antibody for 10 min abolished Ca^{2+} entry stimulated by 1 μM 5,6-EET ($n = 5$).

Role of cytochrome P450 enzymes in CCE in human platelets

We have recently identified two mechanisms for CCE in human platelets activated by depletion of two independent Ca^{2+} pools (Rosado *et al.* 2004*b*). The major store, presumably the dense tubular system (DTS), is sensitive to low concentrations of TG, while the acidic store is sensitive to TBHQ and high concentrations of TG (Lopez *et al.* 2005*a*). We have now investigated whether depletion of these stores is able to induce EET production determined using the fluorescent indicator NT, which reacts with EETs and forms highly fluorescent derivatives (Maier *et al.* 2000; Nithipatikom *et al.* 2000). Treatment of platelets with 10 nM TG to deplete the DTS (Lopez *et al.* 2005*a*) for 5 min significantly increased NT fluorescence to 121.3 ± 6.8% of control ($P < 0.05$; $n = 12$). In contrast TBHQ (20 μM), used to deplete the acidic stores, did not significantly modify NT fluorescence (NT fluorescence after treatment with TBHQ was 93.2 ± 4.7% of control; $n = 12$). To examine whether the increase in NT fluorescence induced by TG was due to EET production we repeated the experiments in the presence of 17-ODYA, a substrate inhibitor that selectively and irreversibly inhibits cytochrome P450 epoxygenases and ω-hydrolases (Dong *et al.* 1997). Preincubation of platelets for 10 min at 37°C with 10 μM 17-ODYA prevented the TG-evoked increase in NT fluorescence (in the presence of 17-ODYA, NT fluorescence in TG-treated cells was 100.6 ± 5.9% of control; $n = 12$) while having no significant effects on NT fluorescence in resting or TBHQ-treated platelets (NT fluorescence was 100.7 ± 9.6 and 95.5 ± 3.3% of control, in non-stimulated and TBHQ-treated platelets pretreated with 17-ODYA, respectively; $n = 12$). These findings suggest that the increase in NT fluorescence observed in platelets stimulated with TG is due to EET production by cytochrome P450 epoxygenases.

Hence, we have further investigated the role of these enzymes on CCE induced by low concentrations of

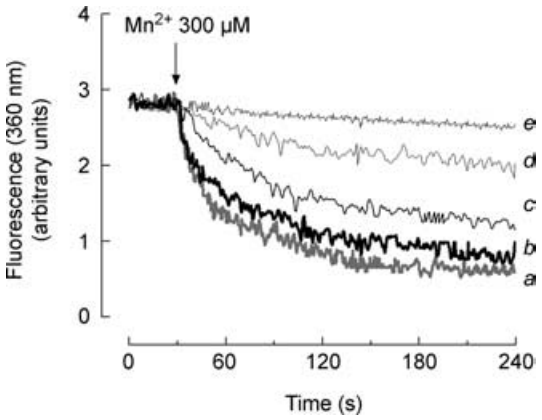

Figure 2. Effect of 5,6-EET on extracellular Mn^{2+} entry
Human platelets were loaded with fura-2 and resuspended in a Ca^{2+}-free medium as described in Methods. Fura-2-fluorescence was measured with an excitation wavelength of 360 nm, the isoemissive wavelength. Platelets were stimulated with different concentrations of 5,6-EET (0.01–3 μM, traces *a*–*d*) 5 min before addition of $MnCl_2$ (final concentration 300 μM). Mn^{2+} was added to untreated control cells (trace *e*) or cells treated with 5,6-EET. Traces are representative of seven separate experiments.

TG in platelets. In the absence of extracellular Ca^{2+}, addition of TG (10 nM) to fura-2-loaded human platelets in stirred cuvettes at 37°C evoked a prolonged elevation in $[Ca^{2+}]_i$ due to release of Ca^{2+} from internal stores. Subsequent addition of Ca^{2+} (300 μM) to the external medium induced a sustained increase in $[Ca^{2+}]_i$ indicative of CCE (Fig. 4*A*). Pretreatment of human platelets with 17-ODYA (10 μM), significantly reduced CCE induced by 10 μM TG by 42% (Fig. 4*B* and *F*; $P < 0.05$; $n = 9$). The initial slope for the rise in $[Ca^{2+}]_i$ after the addition of Ca^{2+} was significantly reduced from 0.0682 ± 0.0060 to 0.0422 ± 0.0042 in control and 17-ODYA-treated cells, respectively ($P < 0.05$). Treatment with 17-ODYA had no significant effects on Ca^{2+} release from the intracellular store, indicating that accumulation of Ca^{2+} in the DTS was unaffected by inhibition of cytochrome P450 epoxygenases (Fig. 4*B* and *E*; $n = 9$).

It is well known that depolarization of the membrane potential decreases the driving force for CCE. To check whether the inhibitory effect of 17-ODYA could be attributable to changes in membrane potential, we studied its effect on Ca^{2+} influx in the presence of the K^+ ionophore valinomycin, which stabilizes the platelet membrane potential close to the K^+ equilibrium potential (Mahaut-Smith *et al.* 1990). 17-ODYA inhibited TG-induced CCE to the same extent in the presence and in the absence of 3 μM valinomycin (17-ODYA reduced CCE by 42 and 45% in the absence and presence of valinomycin). This finding indicates that the effects of the 17-ODYA are not due to a decrease in the membrane potential.

To confirm that the inhibitory effect of 17-ODYA on CCE was mediated by inhibition of the cytochrome P450 epoxygenases we performed a series of experiments adding 5,6-EET (1 μM) at the same time as TG. As shown in Fig. 4*C*, *E* and *F*, treatment of platelets with a combination of TG plus 5,6-EET induced a similar Ca^{2+} mobilization as TG alone (Fig. 4*A*). In addition, platelet stimulation with TG + 5,6-EET overcame the effect of 17-ODYA on TG-induced CCE (Fig. 4*D* and *F*; $n = 9$). These findings indicate that the effect of 17-ODYA is mediated by inhibition of cytochrome P450 epoxygenases. Cytochrome P450 arachidonic acid epoxygenases catalyse the metabolism of endogenous arachidonic acid to

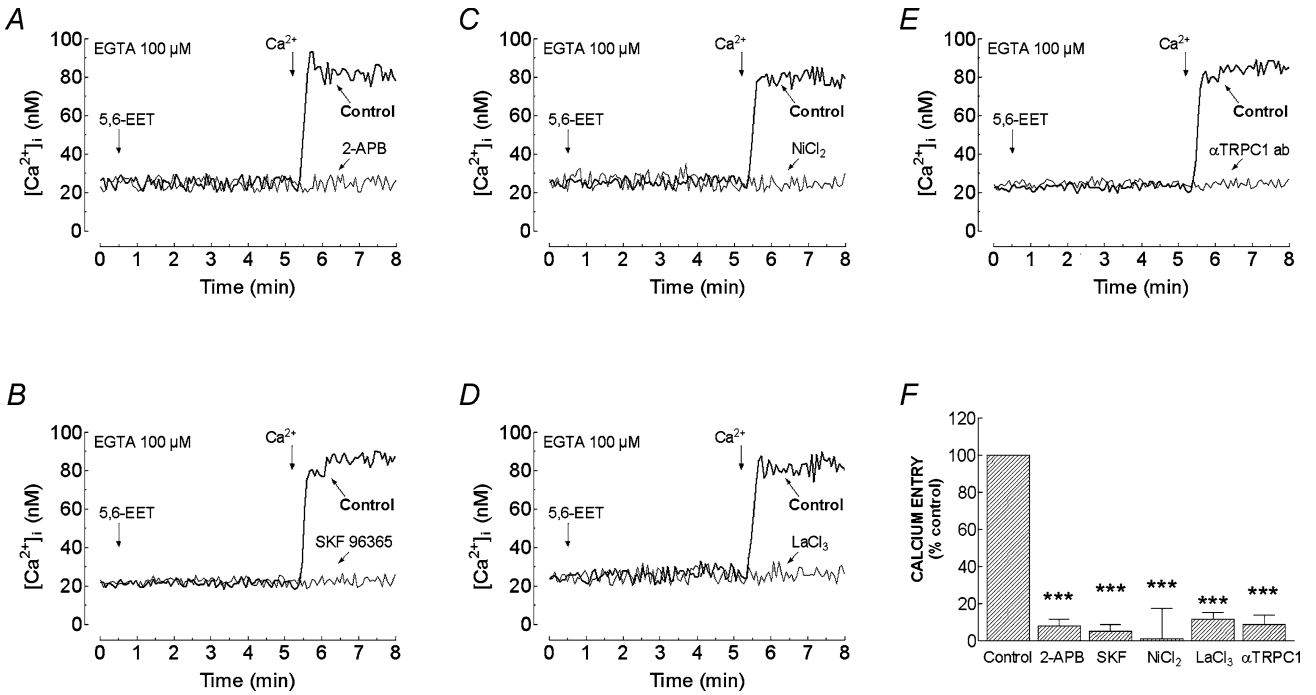

Figure 3. 5,6-EET-induced Ca^{2+} entry is inhibited by 2-APB, SKF 96365, $LaCl_3$, $NiCl_2$ and the anti-hTRPC1 antibody

Fura-2-loaded human platelets were preincubated at 37°C with 100 μM 2-APB (*A*) or 10 μM SKF 96365 for 10 min (*B*), or with 10 μM $NiCl_2$ (*C*) or 100 μM $LaCl_3$ for 1 min (*D*), or with 15 μg ml^{-1} anti-hTRPC1 antibody (αTRPC1 ab) for 10 min (*E*) or the vehicles as Control (bold traces) and then treated in a Ca^{2+}-free medium (100 μM EGTA was added) for 5 min with 1 μM 5,6-EET followed by addition of $CaCl_2$ (300 μM) to initiate Ca^{2+} entry. *F*, bars indicate the percentage of Ca^{2+} entry under the different experimental conditions relative to their respective control. Ca^{2+} entry was determined as described in Methods and values are expressed as the mean ± S.E.M. from five to nine independent experiments. ***$P < 0.001$ compared to control.

5,6-EET, 8,9-EET, 10,11-EET and 14,15-EET (Roman, 2002). In addition, these observations indicate that the product of cytochrome P450 epoxygenases, specifically 5,6-EET, is required for the activation of CCE by low concentrations of TG in human platelets.

We have further investigated the role of cytochrome P450 epoxygenases and 5,6-EET in CCE evoked by TBHQ. As shown in Fig. 5*A*, treatment of platelets with 20 μM TBHQ in the absence of extracellular Ca^{2+} resulted in a small rise in $[Ca^{2+}]_i$ as the acidic Ca^{2+} stores depleted and the subsequent addition of $CaCl_2$ (300 μM) resulted in a rise in $[Ca^{2+}]_i$ indicative of CCE ($n = 11$). Preincubation of platelets for 10 min with 10 μM 17-ODYA did not significantly modify TBHQ-induced Ca^{2+} release or entry, which indicates that the products of cytochrome P450 epoxygenases are not required either for Ca^{2+} release or for the activation of CCE induced by depletion of the acidic stores in human platelets (Fig. 5*B*; $n = 11$). These findings are consistent with the lack of EET production by TBHQ reported above. In addition, these findings suggest that 17-ODYA is not a Ca^{2+} chelators or Ca^{2+} channel blocker.

Effect of cytochalasin D on 5,6-EET-induced Ca^{2+} entry in human platelets

Both pathways for the activation of CCE in human platelets are differentially modulated by the actin cytoskeleton, so

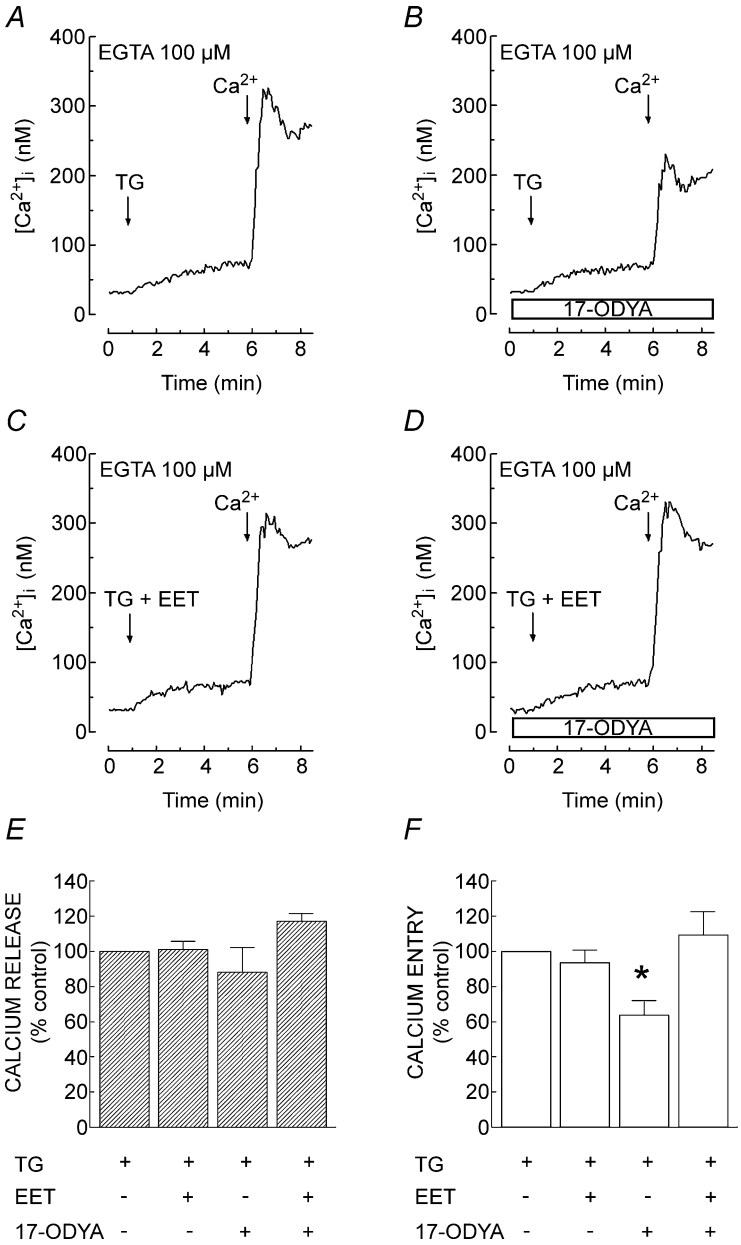

Figure 4. Role of cytochrome P450 enzymes on CCE induced by low concentrations of TG
Fura-2-loaded human platelets were preincubated for 10 min at 37°C with 10 μM 17-ODYA (*B* and *D*) or the vehicle as Control (*A* and *C*) and then treated in a Ca^{2+}-free medium (100 μM EGTA was added) for 5 min with TG (10 nM) in the absence or presence of 1 μM 5,6-EET, as indicated, followed by addition of $CaCl_2$ (300 μM) to initiate Ca^{2+} entry. *E* and *F*, bars indicate the percentage of Ca^{2+} release (*E*) and entry (*F*) under the different experimental conditions relative to their control (vehicle was added). Ca^{2+} release and entry were determined as described in Methods and values are expressed as the mean ± S.E.M. from nine independent experiments. *$P < 0.05$ compared to control.

that actin disassembly by Cyt D impairs CCE mediated by depletion of the DTS by low concentrations of TG while facilitates Ca^{2+} entry stimulated by depletion of the acidic stores using TBHQ (Rosado *et al.* 2004*b*; Lopez *et al.* 2005*b*). To further investigate whether 5,6-EET is required solely for CCE induced by low TG concentrations we examine the effect of Cyt D on Ca^{2+} entry evoked by 5,6-EET.

As shown in Fig. 6*A*, pretreatment of platelets for 40 min with 10 μM Cyt D abolished Ca^{2+} entry induced by 5,6-EET (1 μM; $P < 0.001$; $n = 6$). A similar result was observed when 5,6-EET was endogenously generated by using the cytochrome P450 inducer BN (Graier *et al.* 1995; Xie *et al.* 2002). Induction of cytochrome P450 by BN (3 μM) led to a significant Ca^{2+} entry in human platelets, similar to that found with (1 μM) 5,6-EET, whereas intracellular Ca^{2+} release remained unchanged. In Cyt D-pretreated cells, BN was unable to induce Ca^{2+} entry (Fig. 6*B*; $P < 0.001$; $n = 6$). These findings indicate that inhibition of actin polymerization prevents Ca^{2+} entry mediated by 5,6-EET.

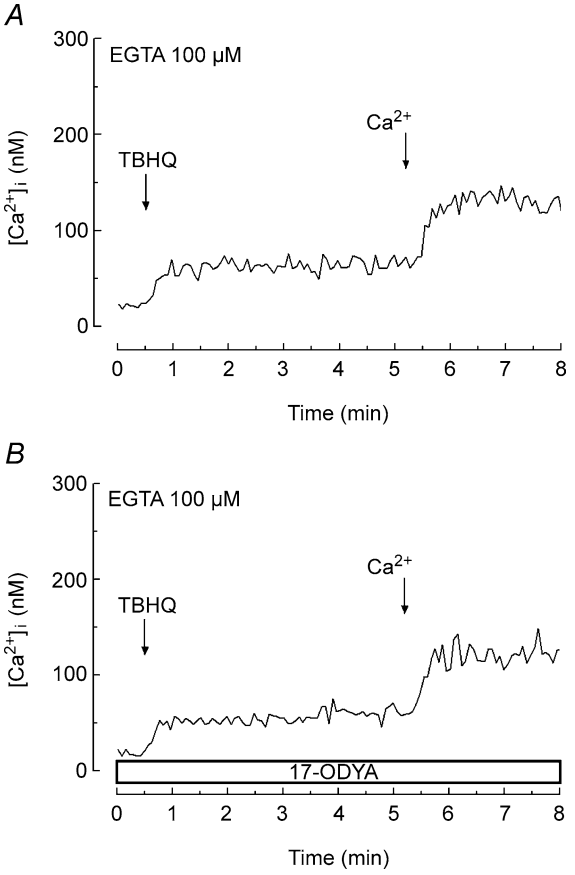

Figure 5. Role of cytochrome P450 enzymes on CCE induced by treatment with TBHQ
Fura-2-loaded human platelets were preincubated for 10 min at 37°C with 10 μM 17-ODYA (*B*) or the vehicle as Control (*A*) and then treated in a Ca^{2+}-free medium (100 μM EGTA was added) for 5 min with TBHQ (20 μM) followed by addition of $CaCl_2$ (300 μM) to initiate Ca^{2+} entry. Changes in $[Ca^{2+}]_i$ were monitored using the 340/380 nm ratio and traces were calibrated in terms of $[Ca^{2+}]_i$. Traces are representative of 11 separate experiments.

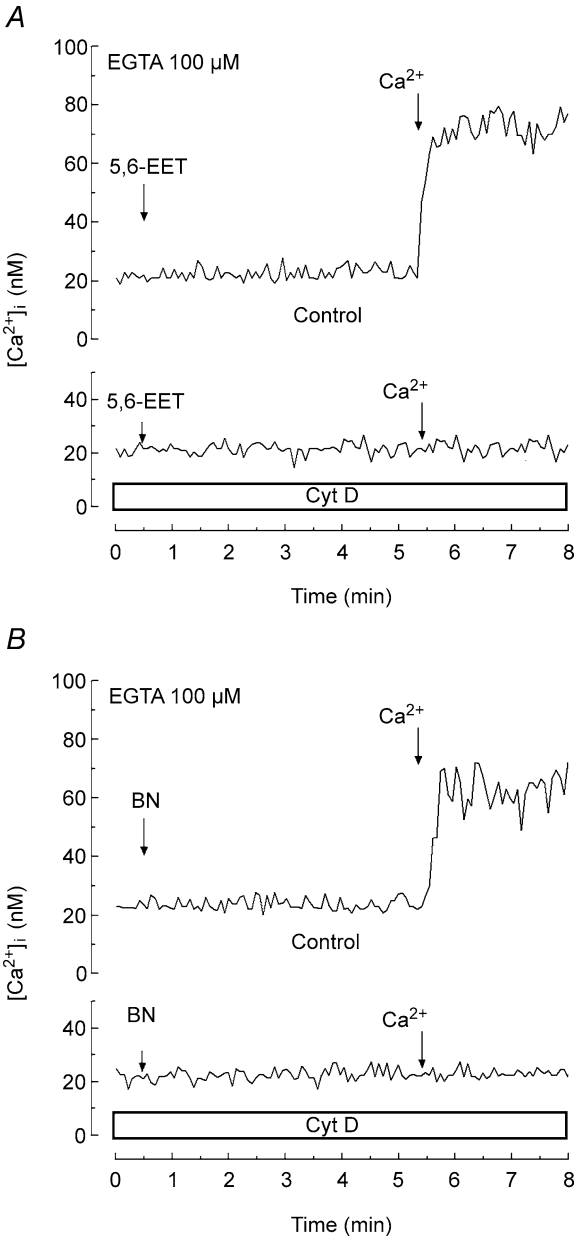

Figure 6. Effect of Cytochalasin D on 5,6-EET-induced Ca^{2+} entry
Fura-2-loaded human platelets were preincubated for 40 min at 37°C with 10 μM Cyt D or the vehicle as Control and then treated in a Ca^{2+}-free medium (100 μM EGTA was added) for 5 min with 1 μM 5,6-EET (*A*) or 3 μM BN (*B*) followed by addition of $CaCl_2$ (300 μM) to initiate Ca^{2+} entry. Changes in $[Ca^{2+}]_i$ were monitored using the 340/380 nm ratio and traces were calibrated in terms of $[Ca^{2+}]_i$. Traces are representative of six separate experiments.

5,6-EET is a component of the *de novo* conformational coupling mechanism for the activation of CCE in human platelets

In human platelets, depletion of the DTS mediates the activation of CCE by a *de novo* conformational coupling between the IP_3R type II and hTRPC1 (Rosado *et al.* 2005). A number of cellular events have been shown to be involved in the activation of this process, including H_2O_2 generation, activation of tyrosine kinases and actin filament reorganization (Rosado & Sage, 2000*d*; Xie *et al.* 2002; Rosado *et al.* 2004*a*, 2005). We have now investigated whether the involvement of 5,6-EET in Ca^{2+} entry in platelets might be consistent with the *de novo* conformational coupling.

We have described above that Cyt D impairs 5,6-EET-evoked Ca^{2+} entry, which indicates that actin filament reorganization is required for this mechanism. In addition, we have now further investigated the involvement of 5,6-EET in the *de novo* conformational coupling model by exploring the role of 5,6-EET on actin filament reorganization, a process required for the activation of CCE by *de novo* conformational coupling in platelets. Treatment of human platelets with 1 μM 5,6-EET induced a rapid increase in the actin filament content, which was maximal 10 s after 5,6-EET stimulation and then decreased (Fig. 7).

Furthermore, we have investigated whether H_2O_2 is required for the activation of 5,6-EET-mediated Ca^{2+} entry by exploring the ability of 5,6-EET to produce H_2O_2 and the effect of catalase, an enzyme that activates the decomposition of H_2O_2 into water and oxygen (Luo *et al.* 1998). The amount of intracellular H_2O_2 was estimated using CM-H_2DCFDA, which is sensitive to H_2O_2 generation (Zhang *et al.* 2003). As shown in Fig. 8*A*, treatment of platelets with 1 μM 5,6-EET did not induce any detectable change in the DCF fluorescence, suggesting that 5,6-EET is unable to stimulate H_2O_2 production in human platelets. Therefore, we have investigated whether basal levels of H_2O_2 are required to maintain a redox state that favours 5,6-EET-mediated Ca^{2+} entry by using catalase. Our results indicate that treatment of human platelets with 300 U ml^{-1} catalase abolished 10 μM H_2O_2-induced Ca^{2+} entry (Fig. 8*B*), as well as the increase in DCF fluorescence induced by addition of exogenous H_2O_2 (10 μM; data not shown). Treatment of platelets with catalase significantly reduced Ca^{2+} entry stimulated by 5,6-EET (1 μM) by $75 \pm 8\%$ (Fig. 8*C*; $P < 0.001$; $n = 10$). The initial slope for the rise in $[Ca^{2+}]_i$ after the addition of Ca^{2+} was significantly reduced from 0.0582 ± 0.0054 to 0.0382 ± 0.0038 in control and catalase-treated cells, respectively ($P < 0.05$). Since we have previously shown that catalase is not a Ca^{2+} channel blocker in platelets (Rosado *et al.* 2004*a*), our results suggest that basal levels of H_2O_2 are required for the activation of Ca^{2+} entry mediated by 5,6-EET. To further investigate the involvement of H_2O_2 on 5,6-EET-mediated Ca^{2+} entry we induced cytochrome P450 using BN. As shown in Fig. 8*D*, preincubation of platelets with catalase (300 U ml^{-1}) abolished BN-induced Ca^{2+} entry ($P < 0.001$; $n = 10$).

A role for protein-tyrosine phosphorylation in the regulation of CCE has been reported on the basis of the correlation between an increase in the phosphotyrosine levels of cellular proteins and the filling state of the intracellular Ca^{2+} stores (Vostal *et al.* 1991; Sargeant *et al.* 1993; Sargeant *et al.* 1994). These observations have been confirmed by the effects of different tyrosine kinase inhibitors on agonist- and TG-evoked CCE (Yule *et al.* 1994; Camello *et al.* 1999). Tyrosine kinases have been shown to be involved in the *de novo* conformational coupling (Rosado *et al.* 2000*b*). To further explore whether 5,6-EET belongs to the same model for the activation of Ca^{2+} entry in platelets we have investigated the effect of 5,6-EET on protein tyrosine phosphorylation. 5,6-EET-induced protein-tyrosine phosphorylation was assessed by gel electrophoresis and Western blotting with a specific anti-phosphotyrosine antibody. Platelets were stimulated with different concentrations of 5,6-EET and samples for protein phosphorylation analysis were taken from the spectrophotometer cuvette 5 s before and 5 min after platelet stimulation. As shown in Fig. 9*A* and *B*, treatment of platelets with 5,6-EET induced a biphasic and concentration-dependent increase in protein-tyrosine phosphorylation relative to its control ($n = 4$). 5,6-EET caused a detectable increase at 0.01 μM and a maximal effect at 1 μM, decreasing the ability of 5,6-EET to induce protein tyrosine phosphorylation at higher concentrations (Fig. 9*A* and *B*). We have further investigated whether

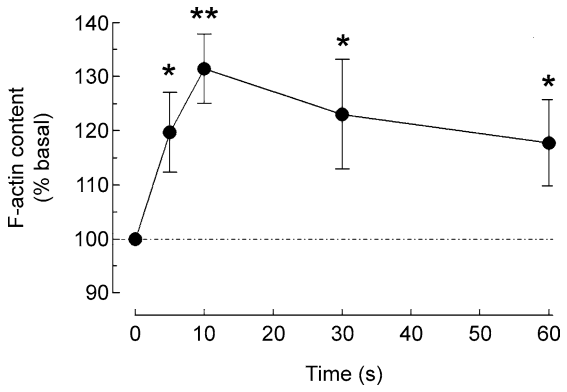

Figure 7. Effect of 5,6-EET on actin polymerization in platelets
Human platelets were treated with 1 μM 5,6-EET in a Ca^{2+}-free medium (100 μM EGTA was added). Samples were removed 5 s before and 5, 10, 30 and 60 s after the addition of 5,6-EET and the actin filament content was determined as described in Methods. Values given are 5,6-EET-evoked actin filament formation as a percentage of control and results are expressed as mean ± S.E.M. of nine separate determinations. *$P < 0.05$, **$P < 0.01$ compared to control (resting cells).

tyrosine kinases are required for 5,6-EET-induced Ca^{2+} entry by using M-2,5-DHC, which effectively inhibits protein tyrosine phosphorylation in platelets (Rosado *et al.* 2000*b*). As shown in Fig. 9*C*, pretreatment of human platelets for 30 min at 37°C with 1 μg ml^{-1} M-2,5-DHC decreased Ca^{2+} entry by 60%. The initial slope for the rise in $[Ca^{2+}]_i$ after the addition of Ca^{2+} was significantly reduced from 0.0571 ± 0.0050 to 0.0392 ± 0.0035 in control and M-2,5-DHC-treated cells, respectively ($P < 0.05$).

Finally, we have tested the role of 5,6-EET on the coupling between the IP_3R type II and hTRPC1 by coimmunoprecipitation using a commercial antibody that specifically recognizes the sequence hTRPC1$^{557–571}$, which is present in hTRPC1 and not in other hTRP proteins (Wang *et al.* 1999). We have recently tested the specificity of the commercial antibody with the anti-hTRP1 antibody T1E3 (Rosado *et al.* 2002), which has been shown to be a specific and powerful tool in the study of mammalian TRP1 proteins (Xu & Beech, 2001; Bergdhal *et al.* 2003). As shown in Fig. 10, after immunoprecipitation with anti-hTRPC1 antibody, Western blotting revealed the presence of IP_3R type II in samples from 5,6-EET-treated cells but not in resting platelets ($n = 4$). Western blotting with anti-hTRPC1 confirmed a similar content of this protein in all lanes. The efficacy of 5,6-EET-induced coupling between hTRPC1 and IP_3R type II was smaller than that observed with TG (10 nM; about 50% of the

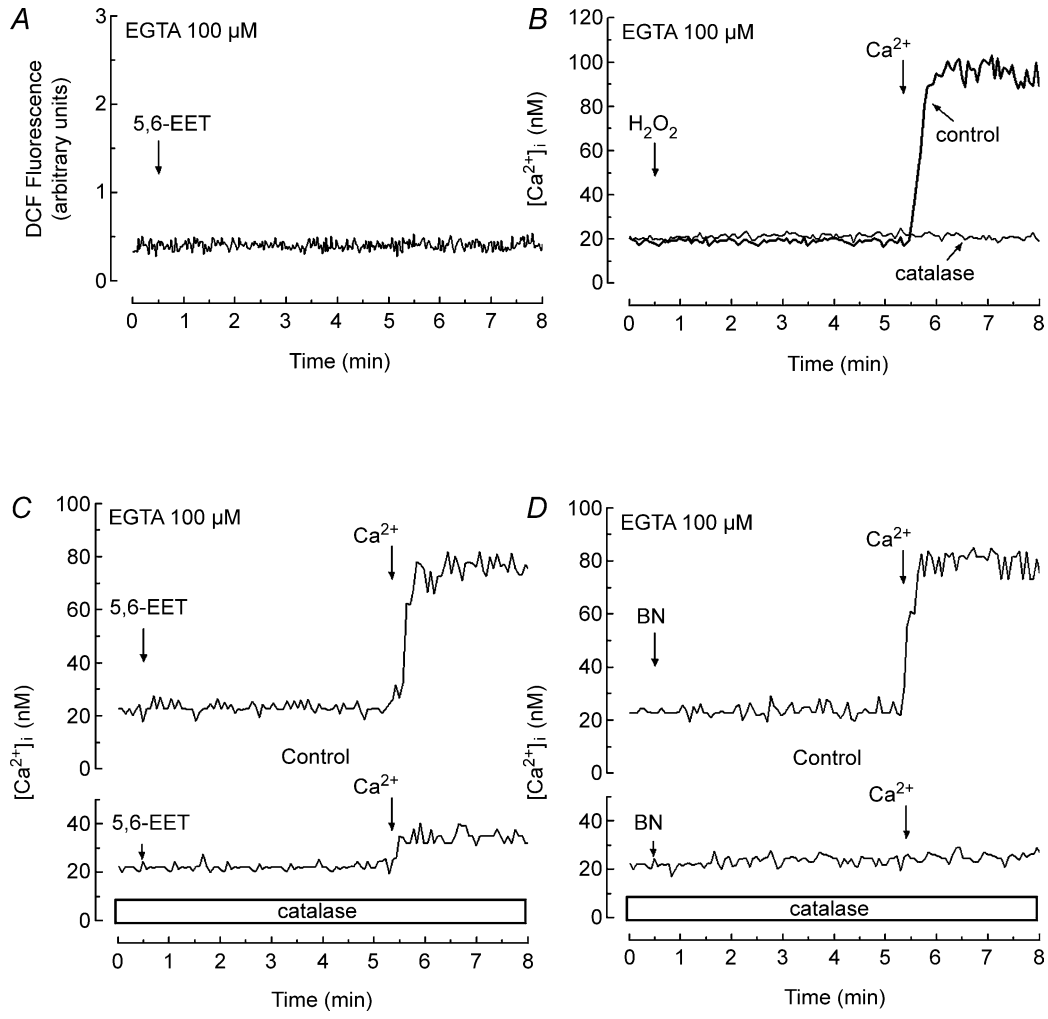

Figure 8. Effect of catalase on 5,6-EET-induced Ca^{2+} entry
A, human platelets loaded with CM-H_2DCFDA were stimulated with 1 μM 5,6-EET in a Ca^{2+}-free medium (100 μM EGTA was added). Traces are representative of eight independent experiments. *B–D*, fura-2-loaded human platelets were preincubated for 10 min at 37°C with 300 U ml^{-1} catalase or the vehicle as Control and then treated in a Ca^{2+}-free medium (100 μM EGTA was added) for 5 min with 10 μM H_2O_2 (*B*), 1 μM 5,6-EET (*C*) or 1 μM BN (*D*) followed by addition of $CaCl_2$ (300 μM) to initiate Ca^{2+} entry. Changes in $[Ca^{2+}]_i$ were monitored using the 340/380 nm ratio and traces were calibrated in terms of $[Ca^{2+}]_i$. Traces are representative of 10 separate experiments.

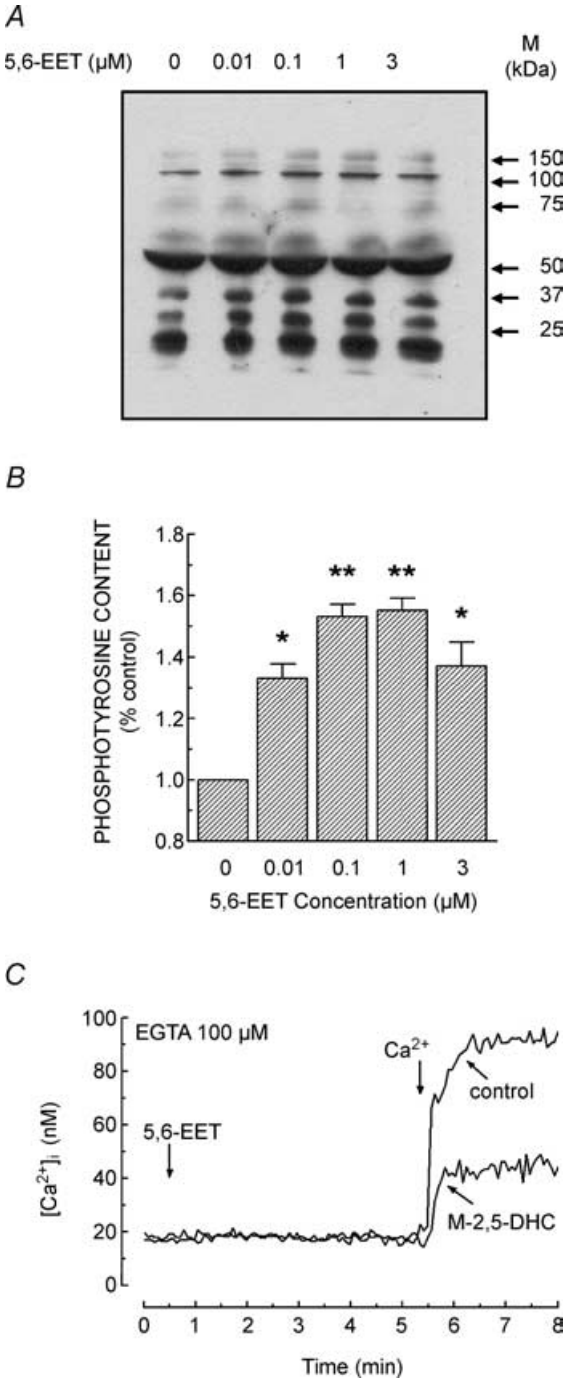

Figure 9. Role for protein tyrosine phosphorylation on 5,6-EET-induced Ca^{2+} entry in platelets

A, human platelets were treated with different concentrations of 5,6-EET (0.01–3 μM) in a Ca^{2+}-free medium (100 μM EGTA was added). Samples were taken from the platelet suspension 5 s before and 5 min after the addition of different concentrations of 5,6-EET. Platelet proteins were analysed by 10% SDS-PAGE and subsequent Western blotting with a specific anti-phosphotyrosine antibody, and the presence of phosphotyrosine residues quantified by densitometry in Western blots as described. Molecular masses (M) indicated on the right were determined using molecular-mass markers run in the same gel. *B*, bars represent the integrated optical density for entire lanes under each condition. Results are expressed as fold-increases (mean ± S.E.M. of four separate experiments) over the integrated optical density of resting platelet. $^*P < 0.05$, $^{**}P < 0.01$ compared to control (resting cells). *C*, fura-2-loaded human platelets were incubated at 37°C with 1 μg ml^{-1} M-2,5-DHC or the vehicle for 30 min. At the time of the experiment 100 μM EGTA was added. Cells were then stimulated with 5,6-EET (1 μM) and, 5 min later, $CaCl_2$ (final concentration 300 μM) was added to the medium to initiate Ca^{2+} entry. Elevations in $[Ca^{2+}]_i$ were monitored using the 340/380 nm ratio as described in Methods. Traces shown are representative of four others.

TG-evoked response). This effect is also observed by using the inhibitor of cytochrome P450 epoxygenases, 17-ODYA, which reduced TG-induced coupling between hTRPC1 and IP_3R type II by 50% ($P < 0.05$; $n = 4$).

Discussion

A number of hypotheses have been presented to account for the activation of CCE, and these can be divided into two classes: those suggesting a diffusible messenger (the CIF hypothesis) and those suggesting a physical coupling between elements in the membrane of the Ca^{2+} store and PM (the conformational coupling hypothesis) (Parekh & Putney, 2005). In platelets, depletion of the intracellular Ca^{2+} stores leads to the activation of CCE by a *de novo* conformational coupling based on the reversible trafficking and coupling between the type II IP_3R in the Ca^{2+} store and endogenously expressed hTRPC1 in the PM (Rosado & Sage, 2000*a*; Rosado *et al.* 2005). A number of molecules presented as candidates for the CIF hypothesis have also been reported to be involved in the *de novo* conformational coupling hypothesis, such as tyrosine kinases (Sargeant *et al.* 1994; Rosado *et al.* 2000*b*; Redondo *et al.* 2003; Vazquez *et al.* 2004), GTP-binding proteins of the Ras family (Bird *et al.* 1993; Fasolato *et al.* 1993; Rosado & Sage, 2000*c*) or protein kinase C (Bode & Goke, 1994; Ma *et al.* 2001; Rosado *et al.* 2004*b*).

Cytochrome P450 metabolites, such as EET isomers (EETs), have been presented as components of the CIF pathway (Alonso-Torre *et al.* 1993). Numerous physiological roles have been suggested for the EETs and, collectively, EETs appear to have clear effects on ion channels (Campbell *et al.* 1996). The 5,6-EET has been reported as a diffusible messenger involved in the activation of CCE in different cell types, including astrocytes (Rzigalinski *et al.* 1999) and endothelial cells (Graier *et al.* 1995; Xie *et al.* 2002). Consistent with this, we have now found that 5,6-EET is likely to be a CCE activator in human platelets. In these cells, 5,6-EET, as well as the cytochrome P450 inducer BN, induces Ca^{2+} entry through cation channels, also permeable to Mn^{2+}, similar to those conducting CCE. Ca^{2+} entry by 5,6-EET was found to be inhibited by 2-APB, SKF 96365 and the divalent and trivalent cations Ni^{2+} and La^{3+}, which

indicates that this process shows properties characteristic of CCE. Furthermore, 5,6-EET-stimulated Ca^{2+} entry was blocked by incubation with the anti-hTRPC1 antibody, which we have previously reported to impair CCE in human platelets (Rosado *et al.* 2002), providing evidence for a role of hTRPC1 in the conduction of Ca^{2+} entry by 5,6-EET.

It has recently been reported that there are two functionally separated Ca^{2+} stores in human platelets, an IP_3-sensitive major store with high affinity to TG, corresponding to the DTS, and an acidic store with low affinity to TG but sensitive to TBHQ and NAADP (Cavallini *et al.* 1995; Kovacs *et al.* 1997; Lopez *et al.* 2005*a*,*b*). We now report that depletion of the DTS induces EET production, and using the substrate inhibitor of cytochrome P450 epoxygenases, 17-ODYA, we have found that 5,6-EET is involved in CCE induced by depletion of the DTS but not in CCE induced by discharge of the acidic stores. Cytochrome P450 enzymes produce a number of EET isomers; however, our results indicate that among them, 5,6-EET is involved in Ca^{2+} entry in human platelets since its addition overcame the effect of 17-ODYA on TG-induced CCE.

These results are confirmed by the effect of Cyt D on Ca^{2+} entry induced by 5,6-EET. We have previously shown that CCE induced by depletion of the DTS by TG and the acidic stores by TBHQ are differentially regulated by the actin cytoskeleton, which plays a dual role in the activation of CCE by TG: a positive role as a support for the transport of portions of the Ca^{2+} store to the proximity of the PM to allow coupling to occur, which is impaired by Cyt D, and a negative effect provided by the membrane-associated cytoskeleton to prevent constitutive activation of Ca^{2+} entry (Rosado *et al.* 2004*b*). In contrast, TBHQ-induced CCE is regulated by the membrane cytoskeleton, which acts only as a physical actin barrier to prevent coupling between the Ca^{2+} stores and PM under resting conditions and therefore is facilitated by Cyt D (Rosado *et al.* 2004*b*). If the 5,6-EET is involved in TG-induced CCE one would postulate that Cyt D would inhibit 5,6-EET-stimulated Ca^{2+} entry. In contrast, an increase in Ca^{2+} entry activated by 5,6-EET would be expected if 5,6-EET were a component of the TBHQ-activated pathway. The inhibitory effect of Cyt D on Ca^{2+} entry induced by exogenous or endogenously generated 5,6-EET clearly confirms that 5,6-EET is a component of the mechanism involved in the activation of CCE upon depletion of the DTS by low concentrations of TG, the so called *de novo* conformational coupling.

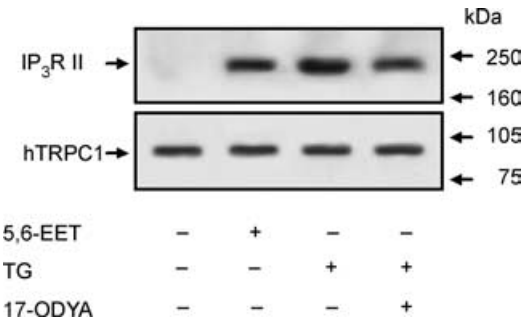

Figure 10. Role of 5,6-EET on TG-induced coupling between IP_3R II and hTRPC1 in human platelets
Platelets were preincubated for 10 min at 37°C in the presence (lane 4) or absence (lanes 1–3) of 10 μM 17-ODYA. Cells were then stimulated with either 5,6-EET (1 μM) or TG (10 nM). Samples were taken 5 s before and 3 min after the addition of 5,6-EET or TG and lysed. Whole cell lysates were immunoprecipitated with anti-hTRPC1 antibody. Immunoprecipitates were analysed by Western blotting using either anti-IP_3R type II polyclonal antibody (upper panel) or anti-hTRPC1 antibody (lower panel) as described in Methods. These results are representative of four independent experiments.

We have found that 5,6-EET-mediated Ca^{2+} entry, as well as the *de novo* conformational coupling (Rosado *et al.* 2004*a*), requires a favourable redox state, where H_2O_2, a powerful oxidizing compound (Törnquist *et al.* 2000), plays an important role. Although speculative, H_2O_2 might maintain an oxidative redox state that delays 5,6-EET degradation. These findings might provide an explanation for the requirement of H_2O_2 for the activation of Ca^{2+} entry in human platelets (Rosado *et al.* 2004*a*).

To further support the involvement of 5,6-EET in the *de novo* conformational coupling, we provide evidence supporting a role for 5,6-EET in the activation of protein tyrosine kinases and actin filament polymerization. A role for tyrosine kinases in CCE has been suggested in several cell types (Yule *et al.* 1994; Camello *et al.* 1999), including platelets (Vostal *et al.* 1991; Sargeant *et al.* 1993, 1994), where we have recently reported the involvement of tyrosine kinases in the *de novo* conformational coupling mediated by the reorganization of the actin cytoskeleton (Rosado *et al.* 2000*b*). We show that 5,6-EET induces a concentration-dependent increase in the phosphotyrosine content of platelet proteins, which together with the effect of the tyrosine kinase inhibitor, M-2,5-DHC, supports that tyrosine kinases are involved in 5,6-EET-evoked Ca^{2+} entry. We have previously reported that the *de novo* conformational coupling requires a mechanical support provided by the cytosolic actin filament network (Rosado *et al.* 2000*a*). Inhibition of actin reorganization by Cyt D prevented EET-induced Ca^{2+} entry, which supports that the actin polymerizing mechanism activated by 5,6-EET is likely to be involved in 5,6-EET-induced Ca^{2+} entry.

The most clear evidence of the involvement of 5,6-EET in the activation of Ca^{2+} entry by a *de novo* conformational coupling in platelets comes from the findings that demonstrate that 5,6-EET activates the coupling between the IP_3R type II and hTRPC1 at the concentration that induce Ca^{2+} entry, which is impaired by 17-ODYA. The effect of 5,6-EET on the coupling between the IP_3R type II and hTRPC1 was smaller than that induced by TG suggesting that an independent pathway must be

involved in this process. We have recently demonstrated in platelets that TG-induced coupling between the IP_3R type II and hTRPC1, and subsequently CCE, is mediated by both cytoskeleton-dependent and -independent pathways (Rosado & Sage, 2001*b*). Since inhibition of actin polymerization by Cyt D resulted in complete inhibition of 5,6-EET-induced Ca^{2+} entry, this messenger might only be a component of the cytoskeleton-dependent branch of the cellular machinery for the activation of coupling between the IP_3R type II and hTRPC1 and CCE by TG.

In summary, we have shown that 5,6-EET induces Ca^{2+} entry without having any effect on Ca^{2+} store depletion in human platelets. 5,6-EET is likely to be a messenger molecule involved in Ca^{2+} entry mediated by depletion of the DTS, compatible with the *de novo* conformational coupling, where store depletion might stimulate the synthesis of 5,6-EET, which, in turns, induces the activation of tyrosine kinase proteins and the reorganization of the actin cytoskeleton. Actin filament remodelling might provide a support for the transport of portions of the Ca^{2+} store towards the PM to allow the *de novo* coupling of IP_3R type II to hTRPC1, which we have suggested may underlie the activation of CCE in human platelets (Rosado *et al.* 2000*a*, 2002, 2004*a*, 2005; Rosado & Sage, 2000*a*, 2001*a*).

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