Endogenously Expressed Trp1 Is Involved in Store-mediated Ca\(^{2+}\) Entry by Conformational Coupling in Human Platelets*

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Physical interaction between transient receptor potential (Trp) channels and inositol 1,4,5-trisphosphate receptors (IP\(_3\)Rs) has been presented as a candidate mechanism for the activation of store-mediated Ca\(^{2+}\) entry. The role of a human homologue of Drosophila transient receptor potential channel, hTrp1, in the conduction of store-mediated Ca\(^{2+}\) entry was examined in human platelets. Incubation of platelets with a specific antibody, which recognizes the extracellular amino acid sequence 557–571 of hTrp1, inhibited both store depletion-induced Ca\(^{2+}\) and Mn\(^{2+}\) entry in a concentration-dependent manner. Stimulation of platelets with the physiological agonist thrombin activated coupling between the IP\(_3\) receptor type II and endogenously expressed hTrp1. This event was reversed by refilling of the internal Ca\(^{2+}\) stores but maintained after removal of the agonist if the stores were not allowed to refill. Inhibition of IP\(_3\) recycling using Li\(^+\) or inhibition of IP\(_3\)Rs with xestospongin C or treatment with jasplakinolide, to stabilize the cortical actin filament network, abolished thrombin-induced coupling between hTrp1 and IP\(_3\)R type II. Incubation with the anti-hTrp1 antibody inhibited thrombin-evoked Ca\(^{2+}\) entry without affecting Ca\(^{2+}\) release from intracellular stores. These results provide evidence for the involvement of hTrp1 in the activation of store-mediated Ca\(^{2+}\) entry by coupling to IP\(_3\)R type II in normal human cells.

The molecular basis of the activation and maintenance of SMCE is not fully understood, and two main questions still remain: elucidation of the mechanism of activation of SMCE after depletion of the internal Ca\(^{2+}\) stores and identification of the channel that mediates extracellular Ca\(^{2+}\) entry. Several hypotheses have been proposed to account for the activation of SMCE, which fall into two main categories: those suggesting a diffusible messenger that gates plasma membrane channels and those suggesting a conformational coupling between elements in the endoplasmic reticulum (possibly the IP\(_3\)R receptors) and the plasma membrane (3, 6). Recently, a modification of the conformational coupling model has been proposed to operate in several cell types. This “secretion-like coupling model” is based on the trafficking and coupling of portions of the endoplasmic reticulum with the plasma membrane, where the actin cytoskeleton plays an important role (7–9).

Studies aiming to identify the channels involved in the conduction of SMCE have focused attention on human homologues of Drosophila transient receptor potential (Trp) channels (see Ref. 6). Recent studies have provided evidence that relates Trp proteins to SMCE channels. Functional expression of Trp proteins enhances SMCE in several mammalian cells, including COS cells (10) and human salivary gland cells (11). A second argument for Trp channels mediating SMCE is provided by antisense studies showing interference with the expression of trp sequences affects the activation of SMCE (10).

Human platelets have been shown to express mRNA for Trp1 and its splice variant Trp1A (12). In addition, Trp1 proteins have been detected in platelets using an anti-hTrp1 antibody specific for the amino acid residues 557–571 (13). Both Trp1 and Trp1A have been shown to form non-selective cation channels regulated by store depletion in a number of expression systems (14, 15). In agreement with the conformational coupling hypothesis, functional IP\(_3\)R receptors (IP\(_3\)Rs) have been shown to be required for the activation of SMCE (13, 16), and physical interaction between IP\(_3\)Rs and Trp channels has been reported in several transfected cell lines (17–20) and in platelets endogenously expressing Trp1 (13). In human platelets, this coupling is activated by depletion of the intracellular Ca\(^{2+}\) stores (13). We report here that physiological agonists also stimulate coupling of IP\(_3\)R receptors to hTrp1 in human platelets and that incubation with anti-Trp1 antibody resulted in a dramatic decrease in agonist- or store depletion-evoked Ca\(^{2+}\) entry, providing strong evidence for the involvement of Trp1 in the SMCE in normal human cells.

EXPERIMENTAL PROCEDURES

Materials—Fura-2 acetoxyethyl ester (fura-2AM) was from Texas Fluorescence (Austin, TX). Apgarase (grade VII), aspirin, bovine serum albumin, paraformaldehyde, Nonidet P-40, thrombin, HISTOPAQUE-1119, HISTOPAQUE-1077, and thapsigargin (TG) were from Sigma. Xestospongin C (Xest C), ionomycin (Iono), and D-Pho-Pro-Arg-chloromethylketone dihydrochloride (PPACK) were from Calbiochem. Dimethyl bis(o-aminophenoxo)-ethane-N,N,N’,N’-tetra-acetic acid (BAPTA, bis(o-aminophenoxo)-ethane-N,N,N’,N’-tetra-acetic acid

Cytoplasmic Ca\(^{2+}\) is a ubiquitous modulator of a large number of cellular processes such as contraction, secretion, cell growth, and cell death (for example, Ref. 1). Elevations in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) can be mediated by release of Ca\(^{2+}\) from the intracellular Ca\(^{2+}\) stores and entry of Ca\(^{2+}\) across the plasma membrane (2, 3). In nonexcitable cells, stores and entry of Ca\(^{2+}\) in a concentration-dependent manner. Stimulation of platelets with the physiological agonist thrombin activated coupling between the IP\(_3\) receptor type II and endogenously expressed hTrp1. This event was reversed by refilling of the internal Ca\(^{2+}\) stores but maintained after removal of the agonist if the stores were not allowed to refill. Inhibition of IP\(_3\) recycling using Li\(^+\) or inhibition of IP\(_3\)Rs with xestospongin C or treatment with jasplakinolide, to stabilize the cortical actin filament network, abolished thrombin-induced coupling between hTrp1 and IP\(_3\)R type II. Incubation with the anti-hTrp1 antibody inhibited thrombin-evoked Ca\(^{2+}\) entry without affecting Ca\(^{2+}\) release from intracellular stores. These results provide evidence for the involvement of hTrp1 in the activation of store-mediated Ca\(^{2+}\) entry by coupling to IP\(_3\)R type II in normal human cells.

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The abbreviations used are: SMCE, store-mediated calcium entry; Trp, transient receptor potential; hTrp, human Trp; IP\(_3\)R, inositol 1,4,5-trisphosphate receptor; PPACK, D-Phe-Pro-Arg-chloromethylketone dihydrochloride; TG, thapsigargin; Iono, ionomycin; Xest C, xestospongin C; JP, jasplakinolide; HBS, HEPES-buffered saline; PM, plasma membrane; TBST, Tris-buffered saline/0.1% Tween 20; CAP, control antigen peptide; BAPTA, bis(o-aminophenoxo)-ethane-N,N,N’,N’-tetra-acetic acid; FITC, fluorescein isothiocyanate.

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Trp1 and Store-mediated Ca\(^{2+}\) Entry in Platelets

(BAPTA), acetylcholine ester, and jasplakinolide (JP) were from Molecular Probes (Leiden, The Netherlands). Anti-h-Trp polyclonal antibody and control antigen peptide were obtained from Alomone Laboratories (Jerusalem, Israel). TIE3 antibody was kindly provided by Dr. David J. Beech (School of Biomedical Sciences, University of Leeds, UK). FITC-conjugated donkey anti-rabbit IgG antibody, anti-IP,R type II polyclonal antibody (C-20), and horseradish peroxidase-conjugated donkey anti-goat IgG antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody and anti-mouse IgG antibody were from Amersham Biosciences. All other reagents were of analytical grade.

**Platelet Preparation and Leukocyte Separation**—Experiments were carried out on human blood platelets or peripheral blood leukocytes obtained from healthy drug-free volunteers with local ethical committee approval. Platelet-rich plasma was prepared by centrifugation for 5 min at 700 × g and aspirin (100 μM) and apyrase (40 μg/ml) added as described previously (21). Peripheral blood leukocytes were obtained by gradient centrifugation using Histopaque-1119 and 1077 Medium (22). Fura-2-loaded platelets, co-loaded with dimethyl BAPTA when required, were prepared as described previously (13) and resuspended in HEPES-buffered saline (HBS) containing 145 mM NaCl, 10 mM HEPES, 10 mM glucose, 1 mM MgSO\(_4\), pH 7.45, and supplemented with 0.1% w/v bovine serum albumin and 40 μg/ml apyrase.

**Measurement of [Ca\(^{2+}\)] and Determination of Ca\(^{2+}\) Entry**—Fluorescence was recorded from 200-μl aliquots of platelet suspension (10⁶ cells/ml) at 37°C using a Cairn Research Spectrophotometer (Cairn Research Ltd., Sittingbourne, Kent, UK) with excitation wavelengths of 340 and 380 nm and emission at 500 nm. Changes in [Ca\(^{2+}\)]\(_i\) were measured as the integral of the rise in [Ca\(^{2+}\)]\(_i\), above basal for 150 s after addition of thrombin in the presence of external Ca\(^{2+}\), corrected by subtraction of the integral over the same period of stimulation in the absence of external Ca\(^{2+}\) (with 100 μM EGTA). Fura-2 fluorescence was stimulated after depletion using TG. Ca\(^{2+}\) entry was estimated using the integral of the rise in [Ca\(^{2+}\)]\(_i\), for 150 s after addition of Ca\(_{Cl}\)\(_2\)

**Measurement of [Sr\(^{2+}\)] and Sr\(^{2+}\) Entry**—Sr\(^{2+}\) was used to monitor non-capacitative cation entry to avoid complications arising from stimulation of the platelet plasma membrane Ca\(^{2+}\) ATPase by thrombin (25) since Sr\(_{Cl}\) is carried with lower affinity than Ca\(^{2+}\) by the platelet plasma membrane Ca\(^{2+}\) ATPase (26). Sr\(^{2+}\) entry was determined in Ca\(^{2+}\)-free HBS containing EGTA (100 μM) to minimize the effects of contaminating Ca\(^{2+}\). Cytosolic Sr\(^{2+}\) was monitored using the fura-2 340-nm/380-nm fluorescence ratio.

**Immunochemistry**—Samples of platelet suspension (200 μl; 2 × 10⁹ cells/ml) were transferred to 200-μl vials of ice-cold 3% (w/v) formaldehyde in phosphate-buffered saline for 10 min and then incubated for 1 h with 1 μg/ml anti-h-Trp1 antibody. The platelets were then collected by centrifugation and washed twice in phosphate-buffered saline containing 137 mM NaCl, 2.7 mM KCl, 5.62 mM NaH\(_2\)PO\(_4\), 1.09 mM NaH\(_2\)PO\(_4\), 1.47 mM KH\(_2\)PO\(_4\), pH 7.2, and supplemented with 0.5% (w/v) bovine serum albumin. To detect the primary antibody, samples were incubated with 0.02 μg/ml FITC-conjugated donkey anti-rabbit IgG antibody for 1 h and washed twice in phosphate-buffered saline. Fluorescence was measured using a fluorescence spectrophotometer (PerkinElmer Life Sciences). Samples were excited at 495 nm, and emission was at 516 nm. When permeabilized platelets were required, the cells were incubated for 10 min with 0.02% (w/v) Nonident P-40 detergent.

**Immunoprecipitation and Western Blotting**—500-μl aliquots of platelet suspension (2 × 10⁹ cells/ml) 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\(_3\)VO\(_4\), 2 mM phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin, and 10 μg/ml aprotinin. Aliquots (1 ml) were then immunoprecipitated by incubation with 2 μg of anti-h-Trp polyclonal antibody (Alomone), 2 μg of TIE3 polyclonal antibody, or 3 μg of anti-IP,R type II polyclonal antibody and protein A-agarose overnight at 4°C. Immunoprecipitates were resolved by 8% SDS-PAGE, and separated proteins were transferred onto nitrocellulose membrane. Immunodetection of hTrp1 or IP,R type II was achieved using the anti-h-Trp1 or TIE3 antibodies diluted 1:200 and 1:600 in TBST, respectively, or with anti-IP,R RI1 diluted 1:500 in TBST for 3 h. To detect the primary antibody, blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody or horseradish peroxidase-conjugated donkey anti-goat IgG antibody diluted 1:10,000 in TBST, washed six times in TBST, and exposed to enhanced chemiluminescence reagents for 1 min. Blots were then exposed to preflashed photographic film. For reprobing, membranes were incubated for 30 min at 50°C in stripping buffer containing 100 mM 2-mercaptoethanol, 65.5 mM Tris, and 2% SDS, pH 6.7. Membranes were then washed and Western blotting was performed as described previously.

**Statistical Analysis**—Analysis of statistical significance was performed using Student’s t test. For multiple comparisons, one-way analysis of variance combined with the Dunnett test was used.

**RESULTS**

**Thrombin Stimulates Coupling between hTrp1 and IP,R Type II**—Recent studies have reported expression of hTrp1 mRNA in human platelets and megakaryocytes (12). Using a commercial antibody that specifically recognizes the sequence hTrp\(^{557-571}\), which is present only in hTrp1 and not in other reported hTrp proteins (27), a single protein band of ~100 kDa displayed reactivity toward the antibody in human platelets (Fig. 1A, lanes 1 and 2) (13). The specificity of the commercial antibody was tested with the anti-Trp1 antibody TIE3, which has been shown to be a specific and powerful tool in the investigation of mammalian Trp1 proteins (28). As shown in Fig. 1A, after immunoprecipitation with the commercial antibody, TIE3 displayed reactivity toward exactly the same protein band, suggesting that both antibodies specifically recognize Trp1 in human platelets. Both antibodies recognized comparable amounts of Trp1 in either resting or store-depleted platelets (Fig. 1A; n = 4). To further investigate whether these antibodies specifically recognized the same protein, both antibodies were used on the same samples. As shown in Fig. 1A, after immunoprecipitation with the commercial antibody, probing with the same antibody revealed reactivity against a protein band of ~100 kDa (Fig. 1B). Reprobing with the TIE3 antibody revealed reactivity toward the same protein (Fig. 1C). Proteins in this or other molecular mass ranges were not detected in human peripheral blood leukocytes (Fig. 1D, lanes 2 and 3), where kTrp1 mRNA expression has not been detected (29).

We have recently reported that depletion of the intracellular Ca\(^{2+}\) stores using thapsigargin stimulates coupling between endogenously expressed hTrp1 and the IP,R type II in human platelets independently of rises in [Ca\(^{2+}\)]\(_i\) (13). As shown in Fig. 1A (lanes 5 and 6), immunoprecipitation with anti-IP,R type II followed by SDS-PAGE and Western blotting with TIE3 confirmed de novo coupling between hTrp1 and IP,R type II when the intracellular Ca\(^{2+}\) stores were depleted using TG. To investigate the physiological significance of this coupling, the effect of thrombin was investigated. Normal platelets or platelets loaded with the intracellular Ca\(^{2+}\) chelator dimethyl BAPTA were stimulated with 0.1 units/ml thrombin in the absence of extracellular Ca\(^{2+}\)(100 μM EGTA was added) and lysed 3 min later. Control platelets were compared with cells heavily loaded with the Ca\(^{2+}\) chelator dimethyl BAPTA so as to differentiate between Ca\(^{2+}\)- and store-depletion-dependent responses (30). We have recently demonstrated that dimethyl BAPTA loading prevents thrombin-evoked [Ca\(^{2+}\)]\(_i\) elevations (9). After immunoprecipitation with anti-h-Trp1 antibody, Western blotting revealed the presence of IP,R type II in samples from thrombin-treated but not resting platelets (Fig. 2A, upper panel; n = 4). Thrombin-induced coupling between hTrp1 and IP,R type II was independent of elevations in [Ca\(^{2+}\)]\(_i\), since it was also observed in dimethyl BAPTA-loaded platelets. Western blotting with anti-h-Trp1 confirmed a similar content of this protein in all lanes (Fig. 2A, lower panel).

We also conducted converse experiments, immunoprecipitating platelet lysates with the anti-IP,R type II antibody and detecting for the presence of hTrp1. Consistent with the above results, after immunoprecipitation with anti-IP,R type II,
type II antibody. As shown in A, hTrp1 was detected in samples from thrombin-treated but not resting cells (Fig. 2B, upper panel; n = 4). Again, thrombin-evoked coupling in both control and dimethyl BAPTA-loaded platelets.

To investigate whether the coupling between IP₃RII and hTrp1 is disassembled by removal of thrombin, we have performed a series of experiments in which, after platelet stimulation with thrombin, the agonist was washed away. In HBS containing 1 mM Ca²⁺, thrombin (0.1 units/ml) evoked a rise in [Ca²⁺], in platelets (Fig. 3A, part i) due to release of Ca²⁺ from the stores and Ca²⁺ entry. However, after removal of thrombin and incubation of the cells in HBS (1 mM Ca²⁺ added to allow refilling of the stores), Ca²⁺ entry was inactivated (Fig. 3A, part ii). Consistent with this, we found that IP₃RII and hTrp1 couple in response to thrombin stimulation, whereas removal of the agonist under conditions that allow refilling of the Ca²⁺ stores clearly reversed the extent of coupling (Fig. 3A, part iii).

If, after stimulation with thrombin, the agonist was washed away and, in the absence of added Ca²⁺, the thrombin antagonist PPACK (1 μM, sufficient to completely block any rise in [Ca²⁺], evoked by 0.1 units/ml thrombin) was added to inactivate residual traces of the agonist, the coupling was maintained (Fig. 3A, part iii).

We have shown previously that store depletion-induced Ca²⁺ entry and coupling requires both IP₃ and activation of IP₃Rs and is blocked by stabilization of the cortical actin filaments (9, 13, 31). To examine whether the mechanism of activation of the coupling between IP₃RII and hTrp1 by thrombin has the same requirements, we investigated the role of IP₃ and IP₃R activation by using Li⁺ or the inhibitor of IP₃R function Xest C. As reported previously (13), treatment of human platelets for 30 min with 20 μM Xest C or for 2 h with 10 mM Li⁺ abolished thrombin-evoked release of Ca²⁺ from the intracellular stores. As shown in Fig. 3B, incubation of human platelets with Xest C...
or Li⁺ completely blocked the coupling between IP₃RII and hTrp1. These findings confirm that IP₃ and functional IP₃RII are required for the coupling with the hTrp1 protein.

To assess the role of the cortical actin filaments in thrombin-induced coupling between IP₃RII and hTrp1, we investigated the effect of JP. We have reported previously that JP reduced thrombin-induced Ca²⁺ entry without affecting Ca²⁺ release from the stores (32). Consistent with this, treatment of human platelets with 10 μM JP for 30 min abolished the coupling between IP₃RII and hTrp1 induced by thrombin (Fig. 3C).

**Fig. 4.** Extracellular location of the epitope recognized by the anti-hTrp1 antibody. Fixed human platelets were incubated with either 0.02 μg/ml FITC-conjugated donkey anti-rabbit IgG antibody for 1 h (column a) or for 1 h with 1 μg/ml anti-hTrp1 antibody (Alomone, incubated previously in the absence (column b) or presence (column c) of CAP) followed by incubation with the FITC-conjugated anti-rabbit IgG for a further 1 h. In column d, cells were stimulated with TG (1 μM) in a Ca²⁺-free HBS (100 μM EGTA was added) and fixed 3 min later. Platelets were then incubated for 1 h with 1 μg/ml anti-hTrp1 antibody (Alomone) followed by incubation with the FITC-conjugated anti-rabbit IgG. In column e, platelets were fixed and then permeabilized. The suspension was then incubated for 1 h with 1 μg/ml anti-hTrp1 antibody (Alomone) followed by incubation with the FITC-conjugated anti-rabbit IgG. Histograms indicate the percentage of immunofluorescence under different experimental conditions relative to their control (non-permeabilized resting cells). Values are mean ± S.E. of four independent experiments.

**Location of the Epitope Recognized by the Anti-hTrp1 Antibody**—The commercial antibody used to perform the majority of our studies specifically recognizes the peptide sequence QLYDKGYTSKEQKDC, which corresponds to the amino acid residues 557–571 of the human hTrp1 protein. This antibody has been shown to be effective in Western blotting, immunoprecipitation, and immunolocalization studies (13, 27, 33, 34).

The sequence 557–571 has been shown to be located between the transmembrane domains 5 and 6; thus, it would be expected to be located at the external face of the PM (29, 35). To further investigate the location of the epitope recognized by the anti-hTrp1 antibody, we performed a series of immunofluorescence experiments. As shown in Fig. 4, incubation of fixed, non-permeabilized platelets in suspension with 1 μg/ml anti-hTrp1 antibody followed by detection using a FITC-conjugated secondary antibody revealed the presence of hTrp1 proteins in the platelet membranes (Fig. 4, column b; n = 4), indicating that the epitope is located extracellularly. The fluorescence observed was not due to nonspecific binding of the secondary antibody as demonstrated by the lower fluorescence detected in samples incubated with this antibody alone (Fig. 4, column a; n = 4). The increase in fluorescence detected after incubation with the anti-hTrp1 antibody was almost completely prevented when the antibody was incubated previously for 1 h in the presence of the control antigen peptide (CAP) (1 μg/ml; Fig. 4, column c; n = 4), confirming the antibody specificity. To investigate whether store depletion induced increased expression of the hTrp1 protein in the PM, we repeated the experimental protocol using TG-treated cells. Treatment of human platelets with 200 nM TG for 3 min in a Ca²⁺-free medium slightly increased the detection of hTrp1 in the membrane; however, this rise was not significant (Fig. 4, column c; p = 0.39; n = 4). Similar results were obtained when the studies were performed in cells permeabilized with the detergent Nonidet P-40, con-
TG-induced Ca$^{2+}$ protocol used for the anti-hTrp1 antibody was unable to block medium induced a sustained increase in [Ca$^{2+}$] when the antibody was incubated previously for 1 hour in the presence of CAP. Ca$^{2+}$ stores. Subsequent addition of Ca$^{2+}$ to control (vehicle was added) in the absence or presence of the CAP. Ca$^{2+}$ entry was estimated as described under “Experimental Procedures.” Values are mean ± S.E. Significance values indicate differences compared with TG-treated cells in the absence of the antibody. **, p < 0.01; ***, p < 0.001.

As shown in D, fura-2-fluorescence was measured at the isoemissive excitation wavelength, 360 nm. Mn$^{2+}$ was also added to untreated unstimulated control cells (trace f). Traces are representative of four separate experiments.

Fig. 5. Concentration dependence of inhibition of TG-evoked store-regulated cation entry by anti-hTrp1 antibody (ab). Fura-2-loaded human platelets were incubated for 10 min with 1.5, 5, 10, or 15 μg/ml anti-hTrp1 antibody (Alomone, traces b, c, d, e, respectively) or the vehicle (trace a) and then stimulated with TG (200 nM) in a Ca$^{2+}$-free medium, and 3 min later, CaCl$_2$ (A) or MnCl$_2$ (D), final concentration 300 μM, was added to the medium. In C, fura-2-loaded human platelets were incubated for 10 min with 15 μg/ml anti-mouse IgG antibody (Alomone) in the absence or presence of CAP (traces b and c, respectively) or the vehicle (trace a) and then stimulated with TG (200 nM) in a Ca$^{2+}$-free medium, and 3 min later, CaCl$_2$ (final concentration, 300 μM) was added to the medium. As shown in A and C, elevations in [Ca$^{2+}$] were monitored using the 340-nm/380-nm ratio and calibrated in terms of [Ca$^{2+}$]$.i$. Traces are representative of three to four independent experiments. In B, histograms indicate the percentage of Ca$^{2+}$ entry in the presence of different concentrations of the anti-hTrp1 antibody relative to their control (vehicle was added) in the absence or presence of the CAP. Ca$^{2+}$ entry was estimated as described under “Experimental Procedures.” Values are mean ± S.E. Significance values indicate differences compared with TG-treated cells in the absence of the antibody. **, p < 0.01; ***, p < 0.001.

Incubation with Anti-hTrp1 Antibody Inhibits TG-induced Store-mediated Ca$^{2+}$ and Mn$^{2+}$ Entry—Since the sequence 557–571 is located in the pore-forming region between the fifth transmembrane domain and region VII of hTrp1 (29, 30), we investigated whether the antibody to this sequence could block channel function. To assess this, human platelet suspensions were incubated for 10 min with increasing concentrations of the anti-hTrp1 antibody (1.5–15 μg/ml) followed by depletion of the intracellular Ca$^{2+}$ stores using TG to activate SMCE. In a Ca$^{2+}$-free medium, TG evoked a prolonged elevation of [Ca$^{2+}$]$_i$ in platelets due to leakage of Ca$^{2+}$ from intracellular stores. Subsequent addition of Ca$^{2+}$ (300 μM) to the external medium induced a sustained increase in [Ca$^{2+}$]$_i$, indicative of SMCE (Fig. 5A). Incubation with the anti-hTrp1 antibody for 10 min significantly reduced SMCE by 34 ± 3, 46 ± 3, 60 ± 2, and 76 ± 2% (n = 4) at concentrations of 1.5, 5, 10, and 15 μg/ml, respectively (Fig. 5B; p < 0.01). The effect of the highest concentration used in this study (15 μg/ml) was prevented when the antibody was incubated previously for 1 h in the presence of the CAP (15 μg/ml). To investigate the specificity of this assay, the effect of incubation with an antibody directed to a protein not related to hTrp proteins or any other platelet protein was tested. Incubation of platelets for 10 min in the presence of 15 μg/ml anti-mouse IgG antibody following the protocol used for the anti-hTrp1 antibody was unable to block TG-induced Ca$^{2+}$ entry either in the absence or presence of the CAP (15 μg/ml; Fig. 5C; n = 3).

SMCE has been widely demonstrated to occur through non-selective cation channels (see Ref. 24). Thus, we have used Mn$^{2+}$ to evaluate the effect of incubation with anti-hTrp1 antibody on TG-evoked divalent cation entry. This cation can be used as a surrogate for Ca$^{2+}$ entry given its quenching effect on fura-2 fluorescence at the isoemissive wavelength, 360 nm (24). Addition of Mn$^{2+}$ (300 μM) to platelets with TG-depleted intracellular Ca$^{2+}$ stores resulted in a sustained quenching of fluorescence (Fig. 5D, trace a) as compared with undepleted cells (Fig. 5D, trace f). When platelets were incubated for 10 min with anti-hTrp1 antibody, Mn$^{2+}$ entry was attenuated. The initial rate of Mn$^{2+}$-evoked fluorescence quenching in platelets incubated with the antibody and then treated with TG was significantly decreased to 85 ± 4, 67 ± 4, 54 ± 4, and 33 ± 7% (n = 4) of the initial rate observed in cells treated with TG alone, at concentrations of 1.5, 5, 10, and 15 μg/ml, respectively (Fig. 5D; p < 0.01). These results indicate that the anti-hTrp1 antibody blocked divalent cation entry in human platelets in a concentration-dependent manner.

Anti-hTrp1 Antibody Inhibits Thrombin-evoked Ca$^{2+}$ Entry—As shown in Fig. 6, A and B, incubation of fura-2-loaded human platelets for 10 min with 15 μg/ml anti-hTrp1 antibody resulted in a substantial inhibition of the elevation in [Ca$^{2+}$]$_i$, evoked by the physiological agonist thrombin (0.05 units/ml) in medium containing 1 mM Ca$^{2+}$ (n = 4; p < 0.001). In contrast, incubation with 15 μg/ml anti-mouse IgG antibody did not interfere with thrombin-induced Ca$^{2+}$ entry (Fig. 6C; n = 3). Since we have recently demonstrated that thrombin is able to activate non-capacitative cation entry in platelets (32), we used a low concentration of the agonist (0.05 units/ml) to reduce this...
Fig. 6. Effect of incubation with anti-hTrp1 antibody on thrombin-evoked elevations in [Ca\(^{2+}\)], in the presence and absence of external Ca\(^{2+}\) and non-capacitative cation entry. As shown in A–F, human platelets were loaded with fura-2 and resuspended in a Ca\(^{2+}\)-free medium. Elevations in [Ca\(^{2+}\)], were monitored using the 340-nm/380-nm ratio and calibrated in terms of Ca\(^{2+}\). Platelets were incubated for 10 min in the presence of either 15 μg/ml anti-hTrp1 antibody (Alomone, B and E) or 15 μg/ml anti-mouse IgG antibody (C and F) or the vehicle (A and D). At the time of experiment, either 1 mM CaCl\(_2\) (A–C) or 100 μM EGTA (D–F) was added. Human platelets were stimulated with thrombin (0.05 units/ml) at the time indicated. As shown in G, cells were incubated for 10 min in the absence (a and b) or presence (Alomone, c) of 15 μg/ml anti-hTrp1 antibody. At the time of experiment, 100 μM EGTA was added. Cells were then treated with TG (1 μM) and Iono (50 nM), and 3 min later, SrCl\(_2\) (final concentration, 300 μM) was added followed by thrombin (10 units/ml (a and c) or 0.05 units/ml (b)). The traces shown are representative of three to four separate experiments.

**DISCUSSION**

The mechanism that activates SMCE after depletion of the intracellular Ca\(^{2+}\) stores is not fully understood, and identification of the channels involved in this process would assist in the advancement of knowledge of this signaling mechanism. Recent studies have presented compelling evidence for the involvement of mammalian homologues of the *Drosophila* Trp channels in SMCE in certain cells (9, 15, 36). We have reported previously that hTrp1 couples with the IP\(_3\)R type II in platelets after depletion of the internal Ca\(^{2+}\) stores using TG (13). The anti-hTrp1 antibody used in these studies specifically recognizes the sequence hTrp1\(^{567-571}\), which is present in hTrp1 and no other proteins (27). Using this antibody, hTrp1 proteins were detected in human platelets, where hTrp1 mRNA expression has been demonstrated previously (12), but not in human leukocytes lacking hTrp1 mRNA expression (29). In the present study, we report that thrombin was able to induce coupling between the IP\(_3\)R type II and naturally expressed hTrp1 proteins in human platelets. This coupling was reversed by removal of the agonist and, as for store depletion-induced coupling between IP\(_3\)R type II and hTrp1 proteins, requires IP\(_3\) and functional IP\(_3\)R and some degree of reorganization of the cortical actin barrier. To our knowledge, this is the first time
that agonist-stimulated coupling between hTrp proteins and IP$_3$Rs has been reported, suggesting that direct coupling between hTrp1 and IP$_3$R type II is a physiological mechanism by which SMCE could be activated in platelets.

Reports that store depletion results in coupling between hTrp1 and IP$_3$R type II in platelets provide only circumstantial evidence for the involvement of hTrp1 in SMCE. Thus, using an antibody that specifically recognizes the amino acid sequence 557–571 of hTrp1, we have further investigated the involvement of hTrp1 in the mediation of SMCE in platelets. Since the sequence 557–571 is located in the pore region of the hTrp1 channel (29), one might expect that binding of this antibody to the hTrp1 channel could interfere with its channel function. Our results showing that incubation of platelets with the anti-
hTrp1 antibody inhibits Ca$^{2+}$ entry provide strong evidence for the involvement of hTrp1 proteins in the normal response of platelets to stimuli that increase IP$_3$ levels and activate store-depletion-activated cation entry channels, which participate in the normal response of platelets to physiological stimuli. In agreement with previous studies in several cell types (19), including platelets (13), activation of this channel might require coupling with the IP$_3$ receptor located in the membrane of the endoplasmic reticulum, which might be the sensor and transducer of the Ca$^{2+}$ content in the intracellular stores (38).

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