SERCA2b Activity Is Regulated by Cyclophilins in Human Platelets

Juan A. Rosado, Jose A. Pariente, Gines M. Salido, Pedro C. Redondo

Objective—The role of cyclophils (chaperones that are widely expressed in different cell types, including human platelets) was explored in sarcoplasmic calcium (Ca\(^{2+}\)) adenosine triphosphatase (SERCA) activity.

Methods and Results—Cyclophilin inhibition by cyclosporin A (CsA) evoked a time- and concentration-dependent reduction of Ca\(^{2+}\) uptake by SERCA2b. However, other Ca\(^{2+}\)-adenosine triphosphatases expressed in platelets, such as SERCA3 and plasma membrane Ca\(^{2+}\) adenosine triphosphatase, remained unaltered after CsA treatment. Cypermethrin, a non-CsA-related calcineurin inhibitor, did not alter SERCA2b activity. Furthermore, SERCA2b was affected by other CSA analogues, which do not interfere with calcineurin, such as PKF-211 to PKF-811-NX5 (NIM811) and sanglifehrin A. Inhibition of the immunophilin family members using FK506 (tacrolimus) did not alter SERCA2b ability to sequester Ca\(^{2+}\) into the dense tubular system. Coimmunoprecipitation experiments confirmed that cyclophilin A associates with SERCA2b and STIM1 in resting platelets. This interaction is attenuated by the physiological agonist thrombin but enhanced by treatment with CsA or sanglifehrin A.

Conclusion—Cyclophilin A is a regulator of SERCA2b in human platelets. (Arterioscler Thromb Vasc Biol. 2010;30:419-425.)

Key Words: platelets ■ cyclophilin A ■ NIM811 ■ sanglifehrin A ■ SERCA2b

The immunophilin family groups proteins with rotenase or peptidylprolyl cis-transisomerase activity. These proteins have been classified according to their sensitivity to specific immunosuppressors drugs, cyclophilins (CyPs) are specific targets of cyclosporin A (CsA); immunophilins (FKBPs) are sensitive to FK506 (tacrolimus) and rapamycin (sirolimus), both structurally unrelated to CsA; and FCBPs are sensitive to CsA and FK506.

CyPs are involved in two major cellular events: (1) indirect and nonspecific regulation of serine-threonine kinase proteins, event mediated by the formation of stable CyP/CaNa complexes that interact with the regulatory center of calcineurin (CaN), PP2B, or calcium (Ca\(^{2+}\))/CaMK phosphatase, reducing its activity; and control of cell death by apoptosis through CyPD, which participates in the formation of the mitochondrial permeability transition pore and cytochrome c release in apoptotic cells. Furthermore, CyP B suppresses apoptosis evoked by oxidative, and endoplasmic reticulum, stress.

Immunophilins participate in Ca\(^{2+}\) homeostasis in several cell models. However, this role has been mainly attributed to FKBP5s that control the opening of Ca\(^{2+}\) channels located in the endoplasmic reticulum, such as ryanodine or inositol 1,4,5-trisphosphate receptors. In contrast, little is known about the role of immunophilins on Ca\(^{2+}\) uptake or extrusion mechanisms; and the current studies have reported contradictory results about the role of CyPs on the activity of sarcoplasmic Ca\(^{2+}\)-adenosine triphosphatase (ATPase) (SERCA) or the plasma membrane Ca\(^{2+}\)-ATPase (PMCA).

In the present study, we have investigated the role of CyPs in the regulation of Ca\(^{2+}\) reuptake by SERCA. We found that CsA reduces Ca\(^{2+}\) reuptake by SERCA2b in a concentration- and time-dependent manner, without having any effect on SERCA3 or the PMCA activities. Regulation of SERCA2b activity was specific for CyPs and does not involve immunophilins or calcineurin, with an important role of CyP A, which might regulate SERCA2b through protein-protein interaction.

Methods

Platelets obtained from human healthy donors, according to the Declaration of Helsinki and local ethics committee guidelines, were isolated using the methods published elsewhere. Platelets were electroporated and incubated with anti-SERCA3 antibody, loaded with fura-2; Ca\(^{2+}\)-adenosine triphosphatase (ATPase) (PMCA).

Results

CsA Increases Thrombin-Evoked Ca\(^{2+}\) Mobilization

In a Ca\(^{2+}\)-free medium (100 μmol/L of EGTA was added), treatment of platelets with thrombin (Thr; 0.1 U/mL) evokes
Figure 1. Effect of cyclosporin A (CsA) on cytosolic calcium mobilization induced by thrombin in human platelets. Human platelets were suspended in a calcium (Ca$^{2+}$)-free medium (100 μmol/L of EGTA was added) and preincubated for 5 minutes in the absence (black traces) or presence of 50 μmol/L of CsA (gray traces). Cells were then stimulated with thrombin (Thr), 0.1 U/mL. Changes in fura-2 fluorescence were monitored using the 340-nm/380-nm ratio and calibrated in terms of Ca$^{2+}$. *Inset, histogram represents Thr-evoked Ca$^{2+}$ mobilization, data are expressed as mean ± SEM of five separate experiments and presented as percentage of control. $P<0.005$.

CsA Reduces Ca$^{2+}$ Reuptake by SERCA2b in Human Platelets

Consistent with previous studies, increases in Ca$^{2+}$ release from the intracellular stores, the dense tubular system, and the acidic stores, followed by a Ca$^{2+}$ clearance through Ca$^{2+}$-ATPases. As shown in Figure 1, Ca$^{2+}$ mobilization evoked by Thr was significantly increased by 22.7±12.6% after incubation for 5 minutes at 37°C with 50 μmol/L of CsA (Figure 1, inset; $P<0.05$; n=5).

Effect of CsA and Interference With Mitochondria

To investigate a possible role of mitochondria on CsA-mediated effects on Ca$^{2+}$ mobilization, we tested the possible effects of CsA on PMCA. As shown in Supplemental Figure I (online Data Supplement; http://atvb.ahajournals.org), treatment of human platelets in a Ca$^{2+}$-free medium with 50 μmol/L of CsA for 5 minutes did not modify the rate of decay of Ca$^{2+}$ to basal levels after treatment with TG, 200 nmol/L, plus Iono, 100 nmol/L, which has been used as an indicator for PMCA activity in platelets; Ca$^{2+}$ extrusion by the sodium/Ca$^{2+}$ exchanger has a minor role in the range of concentrations reached in our experimental conditions. The decay constants in the presence and absence of CsA were 0.05288±0.00720 and 0.05132±0.00350, respectively ($P=0.82$; n=6).

Effect of CsA on Electrotransjection of SERCA2b, but not with SERCA3.

Later, we electrotransjected a mouse IgG of the same nature as the anti-SERCA3 antibody. In platelets electrotransjected with anti-SERCA3 antibody, Thr-evoked Ca$^{2+}$ elevation was reduced by 30.9±4.8% compared with control (IgG electrotransjected; $P<0.01$; n=4), which is a consequence of inhibition of SERCA3 and the subsequent attenuation of Ca$^{2+}$ reuptake. In the presence of anti-SERCA3 antibody, treatment with CsA significantly enhanced Thr-induced Ca$^{2+}$ release (Figure 2D, $P<0.05$, n=6), which is consistent with the results using TBHQ (Figure 2A).

CSA Does Not Alter SERCA3 Activity

We have further analyzed whether CsA alters SERCA3 activity by testing the effect of this inhibitor on Ca$^{2+}$ mobilization by Thr in combination with a low concentration, 10 nmol/L, of TG, which has been reported to selectively inhibit SERCA2b. As shown in Figure 3, we did not observe any significant differences in Thr plus TG-evoked Ca$^{2+}$ mobilization, post-stimulus Ca$^{2+}$, or the amount of Ca$^{2+}$ remaining in the stores in the absence or presence of 50 μmol/L of CsA. Taken together, these results indicate that CsA interferes with SERCA2b, but not with SERCA3.
electron transport chain disruptor, rotenone (10 μmol/L), and a mitochondrial hydrogen/ATPase inhibitor, oligomycin (10 μmol/L), to abolish ATP depletion as the result of mitochondrial hydrogen/ATPase working in reverse mode. As shown in Supplemental Figure IIA (online Data Supplement), we found that in the presence of rotenone and oligomycin incubation of platelets for 5 minutes with 50 μmol/L of CsA still modified Thr plus TBHQ evoked Ca\(^{2+}\) mobilization. We found that CsA increased Ca\(^{2+}\) mobilization by 15.5±1.7% (P<0.01, n=4–6), modified poststimulus Ca\(^{2+}\) by 2.2±2.6% (P>0.05, n=4–6); and significantly decreased the amount of Ca\(^{2+}\) remaining in the stores by 13.9%±5.7% (Supplemental Figure IIB, online Data Supplement; P<0.05, n=4–6).

**SERCA2b Activity Is Independent of Immunophilins**

We further tested whether immunophilins or FKBP5s also participate in the inhibition of SERCA2b activity previously reported. To investigate this issue, we incubated human platelets for 5 minutes at 37°C with 10 and 50 μmol/L of FK506. As shown in Figure 4, FK506 slightly increased the Ca\(^{2+}\) mobilization induced by Thr, 0.1 U/mL, plus TBHQ, 20 μmol/L, by 6% and 8% at 10 and 50 μmol/L, respectively (n=4–6). We did not detect any significant effect of FK506 on the rate of decay of Ca\(^{2+}\), or Ca\(^{2+}\) remaining in the stores.

**Reduction of SERCA2b Activity by CyP Inhibition Is Independent of the CsA-Calcineurin Complex**

It has been traditionally assumed that CsA is a specific inhibitor of CaN,26,27; however, general immunophilin inhibitors, such as CsA or FK506, are able to reduce CaN activity only after complexing with their CyP or immunophilin target proteins, respectively.26,27 Therefore, we needed to clarify whether CsA was affecting SERCA2b directly or through CaN.28 To test this possibility, we used the specific CyP inhibitor, which does not show immunosuppressant properties or interfere with CaN activity, PKF-211 to 811-NX-5, and the specific CaN inhibitor, cypermethrin.29 As shown in Figure 5, PKF211–811-NX-5 was able to modify Thr plus TBHQ–evoked Ca\(^{2+}\) mobilization, poststimulus Ca\(^{2+}\), or Ca\(^{2+}\) remaining in the stores.
Ca$^{2+}$/H$^{11001}$/ remaining in the stores. In contrast, cypermethrin was not able to reduce the amount of Ca$^{2+}$/H$^{11001}$/ sequestered by SERCA2b, thus suggesting that CaN is not involved in SERCA2b activity.

CyP A Complexes With SERCA2b and Regulates Its Function in Human Platelets

Human platelets express several types of CyPs, which have been shown to participate in platelet activation and platelet exocrine and paracrine functions.$^{30,31}$ To identify possible CyPs involved in SERCA2b regulation, we incubated human platelets for 30 minutes at 37°C with several concentrations, 10 to 500 nmol/L, of sanglifehrin A, a CsA analogue that specifically targets CyP A.$^{32}$ As shown in Figure 6A, treatment for 30 minutes at 37°C with 50 nmol/L of sanglifehrin A significantly increased Ca$^{2+}$/H$^{11001}$/ mobilization (43.6±12.1%, $P<0.001$, n=5), increased poststimulus Ca$^{2+}$/H$^{11001}$/ ($4.9±1.6%$, $P<0.01$, n=5), and reduced the amount of Ca$^{2+}$/H$^{11001}$/ remaining in the stores (21.4±11.1%, $P<0.05$, n=5). These findings suggest that Cyp A is involved in the regulation of SERCA2b activity in human platelets.

In addition, we investigated whether Cyp A interacts with SERCA2b in human platelets by coimmunoprecipitation. Human platelets, in a Ca$^{2+}$/H$^{11001}$/ -free medium, were incubated for 5 minutes in the absence or presence of CsA, 50 μmol/L, and then were stimulated for 1 minute with Thr, 0.1 U/mL, or a combination of Thr plus TG and lysed. Samples were immunoprecipitated with anti-SERCA2 (IID8) antibody followed by Western blotting with an anti-Cyp A antibody (anti-CyP A). In platelets, the anti-SERCA2 antibody recognizes SERCA2b, the only SERCA2 isoform identified. Our results revealed that CyP A associates with SERCA2b in resting cells (Figure 6B). This interaction was significantly decreased by 18% or 20% after treatment with Thr or Thr plus TG, respectively. The fact that no differences on CyP A/SERCA2b interaction are observed between samples treated with or without TG indicates that SERCA2b activity is not necessary for the association with CyP A. As shown in Figure 6B, the incubation of human platelets with CsA,
CsA increased by 10.1±18.6% the coupling between STIM1 and CyP A in resting platelets (\(P<0.05\), \(n=6\)). CsA was also able to enhance the STIM1–CyP A association by 32.7±10.9% in the presence of Thr (\(P<0.01\), \(n=6\)) and by 28.6±6.7% in the presence of Thr plus TG.

**Discussion**

Recent studies\(^{13–15}\) have reported that SERCA activity might be regulated by proteins targeted by CsA and other macrolide lactones, such as ivermectin or ascomycin, which mostly include several members of the immunophilin family. However, these studies\(^{13–15}\) have reported contradictory results depending on the cell type investigated. We found that CsA, a CyP inhibitor, modifies Thr-evoked Ca\(^{2+}\) mobilization without altering PMCA activity. This finding is different from previous results, but these discrepancies might be attributed to a different effect of CsA on distinct PMCA isoforms (PMCA1 and PMCA4), with PMCA4 being the isoform predominantly expressed in platelets, more likely insensitive to CsA.\(^{17,33,34}\)

To identify the SERCA isoform regulated by CsA, we stimulated platelets with Thr in the presence of specific inhibitors of the different SERCA isoforms in these cells (SERCA2b and SERCA3).\(^{18–21}\) When SERCA3 was inhibited by TBHQ, CsA was still able to alter Thr-induced Ca\(^{2+}\) mobilization, including the ability of platelets to accumulate Ca\(^{2+}\) into intracellular stores; however, when SERCA2b was inhibited by TG, CsA was unable to modify the Thr-evoked response, thus indicating that SERCA2b activity was inhibited in the presence of CsA. Meanwhile, SERCA3 activity seemed to be unaltered. SERCA2b inhibition, evoked by CsA, was confirmed by using cells electrotransfected with a specific anti–SERCA3 antibody.\(^{22}\)

Inhibition of SERCA1 isoform by CsA or ivermectin has been previously described by others;\(^{15}\) however, to our knowledge, this is the first time that a reduction of SERCA2b activity by CsA is reported, since previous studies reported that CsA either does not modify its activity\(^{15}\) or even that CsA enhances SERCA2b activity.\(^{16}\) A possible explanation for this controversy may be that previous studies did not consider the presence in their cell types of different rates of expression of SERCA isoatypes, including SERCA2b or SERCA3, which has been documented in HUVEC-derived EA.hy926 cells, as a possible explanation for the different responses to histamine and its effects on SERCA activity.\(^{35}\) In T-lymphocytes, addition of CsA, even at low concentrations, increased SERCA3a expression, while SERCA2b expression was diminished as observed by semiquantitative reverse transcriptase-PCR analysis.\(^{36}\) In human platelets the rate of protein expression has been shown to be almost null due to the residual mRNA contents derived from their megakaryocyte precursors, so the effect of CsA on SERCA2b observed cannot be attributed to a change in protein expression.

In addition to CyPs, CsA has also been found to interact with CaN activity.\(^{26–28}\) In order to ascertain what protein was responsible for the effects of CsA we performed additional tests with the CaN inhibitor cypermethrin, a CsA-unrelated agent that target CaN,\(^{29}\) which failed to reproduce the effects of CsA, thus suggesting that CaN was not involved in the
inhibition of SERCA2 activity induced by CsA. The effect of CsA was confirmed by using two nonimmunosuppressant CsA-derivates, PKF211–811-NX5 and sanglifehrin A, a specific inhibitor of CyP A. These findings further demonstrate that the effect of CsA in SERCA2b is exclusive of CyPs, including CyP A, and it is not the result of an indirect effect of CsA over other proteins like CaN.

We have found interaction between Cyp A and SERCA2b and also between STIM1 and CyP A by coimmunoprecipitation in resting cells. These interactions are decreased during platelet stimulation with Thr, which actively deplete the Ca^{2+} stores and initiate Ca^{2+} reuptake mechanisms. Since SERCA2b activity is enhanced after Ca^{2+} store depletion these findings suggest that CyP A might exert a negative role on SERCA2b so that disruption of the interaction between both proteins after Thr stimulation might be necessary for an increase in the activity of SERCA2b. The interaction between CyP A and SERCA2b was not affected by inhibition of SERCA2b by TG, which indicates that the activity of SERCA2b is not necessary for the formation of this protein complex. Similarly, STIM1/CyP A coupling found in resting platelets was reduced after intracellular Ca^{2+} stores were depleted, which might indicate that STIM1 is also involved in the regulation of SERCA2b function. In contrast, the activity of CyP A is necessary for the dissociation from SERCA2b after Thr stimulation, as demonstrated by using CsA and sanglifehrin A, which reduced the ability of Thr to dissociate the SERCA2b/CyP A interaction.

In conclusion, here we described a new regulatory role of CyP A on SERCA2b activity mediated by protein-protein interaction and modulated by physiological agonists like Thr. This regulatory mechanism might be a possible target for preventing disorders evoked by Ca^{2+} overload in human platelets and other cells.

Acknowledgments

We thank Isaac Jardín Polo and Mercedes Gómez Blázquez for their technical assistance.
Sources of Funding
This study was supported by MEC “Ramón y Cajal program” (RYC2007-00349) and MEC grant BFI2007-60104 (Dr Redondo). Sandoz-Novatis, Basel, Switzerland, generously provided the cyclophilin inhibitors PKF-211-811-NX5 and sanglifehirin A.

Disclosures
None.

References
9. Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, Parys JB, De Smedt H. Effects of the immunosuppressant FK506 on intracellular Ca2+ accumulation mech-
SERCA2b Activity Is Regulated by Cyclophilins in Human Platelets
Juan A. Rosado, Jose A. Pariente, Gines M. Salido and Pedro C. Redondo

Arterioscler Thromb Vasc Biol, published online February 5, 2010;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2010/02/05/ATVBAHA.109.194530.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2010/02/05/ATVBAHA.109.194530.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
MATERIAL AND METHODS

Materials

Fura-2 acetoxymethyl ester (fura-2/AM) was from Molecular Probes (Leiden, The Netherlands). Apyrase (grade VII), aspirin, bovine serum albumin (BSA), thrombin (Thr), thapsigargin (TG), oligomycin (OLI) and rotenone (ROT) were from Sigma (Madrid, Spain). Cyclosporin A (CsA) was from TOCRIS Bioscience (Bristol, U.K.). FK506 was from A.G. Scientific, Inc. (San Diego, U.S.A.). 2,5-di-(tert-butyl)-1,4-hydroquinone (TBHQ) was from Alexis (Nottingham, U.K.). Sanglifehrin A (Sang A) and PKF211-811-NX5 (NIM811) were generously provided by SANDOZ Novartis (Bassel, Switzerland). Cypermethrin (Cyp) was from MERCK Chemicals Ltd. (Nottingham, U.K.). Ionomycin (Iono) was from Calbiochem (Madrid, Spain). Anti-cyclophilin A antibody was from Abcam (Cambridge, U.K.). Monoclonal anti-SERCA2 (II8D) antibody and anti-SERCA3 (PL/IM430) antibody were from Santa Cruz Biothechnology (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody and horseradish peroxidase-conjugated sheep anti-mouse IgG antibody were from GE Healthcare (Bucks, UK). Anti-STIM1 antibody was from BD Transduction Laboratories (Franklin Lakes, NJ, U.S.A.). Protein A-agarose was from Upstate Biotechnology Inc. (Madrid, Spain). Immunoprecipitation Kit - Dynabeads® Protein G was from Invitrogen Corporation (Carlsbad, California, U.S.A.). Enhanced chemiluminescence detection reagents were from Pierce (Cheshire, U.K.). All other reagents were purchased from Panreac (Barcelona, Spain).
Platelet preparation and determination of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{c}\))

Blood was obtained from healthy volunteers and mixed with one-sixth volume of acid/citrate dextrose anticoagulant containing (in mM): 85 sodium citrate, 78 citric acid and 111 D-glucose. Platelet-rich plasma was then prepared by centrifugation for 5 min at 700 \(\times\) g and aspirin (100 \(\mu\)M) and apyrase (40 \(\mu\)g/ml) added. Platelet-rich plasma was incubated at 37 \(\degree\)C with 2 \(\mu\)M fura-2/AM for 45 min. Cells were then collected by centrifugation at 350 \(\times\) g for 20 min and resuspended in HEPES-buffered saline (HBS) containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 MgSO\(_4\), pH 7.45 and supplemented with 0.1% w/v bovine serum albumin and 40 \(\mu\)g/ml apyrase.

Fura-2 loaded platelets were alternatively excited at wavelengths of 340 and 380 nm and fluorescence emitted at 505 nm was recorded by using a Varian spectrofluorophotometer. Changes in [Ca\(^{2+}\)]\(_{c}\) were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz.\(^1\) In experiments were cells were treated with sanglifehrin A, Ca\(^{2+}\) determination is expressed as Fura-2 fluorescence (\(F_n/F_0\))\(^{-1}\) of 380 nm wavelength since this compound modified the Fura-2 fluorescence properties, so that fluorescence ratios were not suitable to be analyzed using the Grynkiewicz equation. Ca\(^{2+}\) mobilization stimulated by Thr, alone or in combination with TG or TBHQ (to estimate agonist-induced Ca\(^{2+}\) mobilization in the absence or presence of specific SERCA inhibitors) or TG + Iono (to calculate the amount of Ca\(^{2+}\) remaining in the stores) was estimated using the integral of the rise in [Ca\(^{2+}\)]\(_{c}\) or the fura-2 340/380 fluorescence ratio for 5 min after the addition of the stimulus and was expressed as nM.s, as previously described.\(^2\) Post-stimulus [Ca\(^{2+}\)]\(_{c}\) was estimated as the mean [Ca\(^{2+}\)]\(_{c}\) four and a half min after addition of the agonist, Thr, alone or in combination with SERCA inhibitors.

To compare the rate of decay of [Ca\(^{2+}\)]\(_{c}\) to basal values after platelet stimulation
with TG + Iono we used the constant of the exponential decay. Traces were fitted to the equation \( y = A(1 - e^{-K_1 T})e^{-K_2 T} \), where \( K_2 \) is the constant of the exponential decay.\(^3\)

**Immunoprecipitation**

Aliquots (250 µl) of platelet suspensions (4 x 10\(^8\) cell/ml), assessed by cellular count, were treated as described and 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 mM benzamidine. Immunoprecipitation of SERCA2b or STIM 1 were achieved by incubating samples either with 2 µg/ml of the anti-SERCA2 (IID8) antibody or 2 µg/ml of anti-STIM1 antibody and protein A-agarose overnight at 4°C. Immunoprecipitates were resolved by 15% SDS-PAGE and proteins were electrophoretically transferred, for 2 h at 0.8 mA/cm\(^2\), in a semi-dry bloter (Hoefer Scientific, Newcastle, Staffs., U.K.) onto nitrocellulose for subsequent probing.

**Western blotting**

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. Blocked membranes were then incubated for 2 h with the anti-cyclophilin A antibody (diluted 1:500 in TBST). The primary antibody was removed and blots washed six times for 5 min each with TBST. To detect the primary antibody, blots were incubated for 1 h with the appropriate secondary antibody, diluted 1:5000 in TBST, membranes were washed six times in TBST, and incubated with enhanced chemiluminescence reagents for 5 min. Blots were then exposed to photographic films and the optical density was estimated using scanning densitometry.
Platelets reversible electroporation procedure

The platelet suspension was transferred to an electroporation chamber containing antibodies at a highest final concentration of 10 µg/mL, and the antibodies were transjected according to published methods. Reversible electropermeabilization was performed at 4 kV/cm at a setting of 25-microfarad capacitance and was achieved by 4 pulses using a Bio-Rad Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA, U.S.A.). Following electroporation, cells were incubated with antibodies for an additional 60 min at 37 ºC then were centrifuged at 350 g for 20 min and resuspended in HBS containing 200 µM CaCl₂.

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’s test was used.

Reference section of supplementary data.


SUPPLEMENTAL FIGURE LEGENDS

FIGURE 1: Effect of CsA on PMCA in human platelets. Platelets suspended in a Ca\(^{2+}\)-free medium (100 µM of EGTA was added; arrow head), were preincubated for 5 min at 37 °C in the absence (black solid traces) or presence of CsA (50 µM; light grey dotted traces). Cells were then stimulated for 5 min with a combination of TG (200 nM) and ionomycin (100 nM). Changes in fura-2 fluorescence were monitored using the 340-nm/380-nm ratio and calibrated in terms of \([\text{Ca}^{2+}]_c\). Traces are representative of six independent experiments.

FIGURE 2: Effect of mitochondrial inhibitors on CsA-altered Ca\(^{2+}\) mobilization. (A) Platelets suspended in a Ca\(^{2+}\)-free medium (100 µM of EGTA was added; arrow head), were preincubated for 5 min at 37 °C in the absence or presence of CsA (50 µM) and then treated with rotenone (ROT; 10 µM) and oligomycin (OLI; 10 µM). Cells were stimulated with a combination of Thr (0.1 U/ml) + TBHQ (20 µM) and 4 min later cells were treated with TG (200 nM) + Iono (50 nM). (B) Histogram represents Ca\(^{2+}\) mobilization, post-stimulus \([\text{Ca}^{2+}]_c\) and the amount of Ca\(^{2+}\) remaining in the stores estimated as described in supplementary material. Values are mean ± SEM of four to six independent experiments. *, \(p < 0.05\) and **, \(p < 0.01\).
SUPPLEMENTAL FIGURE 1

![Graph showing changes in 
\([Ca^{2+}]_c\) (nM) over time (min) for different treatments: Control, CsA (50 µM), and TG+Iono. The graph illustrates a peak in 
\([Ca^{2+}]_c\) after the addition of TG+Iono and its subsequent decline.]