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Store-operated Ca^{2+} Entry-associated Regulatory factor (SARAF) Plays an Important Role in the Regulation of Arachidonate-regulated Ca^{2+} (ARC) Channels*

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The store-operated Ca^{2+} entry-associated regulatory factor (SARAF) has recently been identified as a STIM1 regulatory protein that facilitates slow Ca^{2+}-dependent inactivation of store-operated Ca^{2+} entry (SOCE). Both the store-operated channels and the store-independent arachidonate-regulated Ca^{2+} (ARC) channels are regulated by STIM1. In the present study, we show that, in addition to its location in the endoplasmic reticulum, SARAF is constitutively expressed in the plasma membrane, where it can interact with plasma membrane (PM)-resident ARC forming subunits in the neuroblastoma cell line SH-SY5Y. Using siRNA-based and overexpression approaches we report that SARAF negatively regulates store-independent Ca^{2+} entry via the ARC channels. Arachidonic acid (AA) increases the association of PM-resident SARAF with Orai1. Finally, our results indicate that SARAF modulates the ability of AA to promote cell survival in neuroblastoma cells. In addition to revealing new insight into the biology of ARC channels in neuroblastoma cells, these findings provide evidence for an unprecedented location of SARAF in the plasma membrane.

Cellular agonists stimulate Ca^{2+} influx via different signaling pathways and plasma membrane (PM)[4] channels, ranging from receptor-operated channels, directly gated by agonist-receptor binding, to second messenger-gated channels and store-operated channels. Arachidonic acid (AA) is a second messenger generated from the membrane phospholipids by the enzymatic activity of phospholipase A2 or the combined actions of phospholipase C and diacylglerol lipase, that mediates a number of cellular functions, including Ca^{2+} entry (1). Arachidonate-regulated Ca^{2+} (ARC) channels were identified in 1996 as receptor-operated, store-independent, channels in cells from the exocrine avian nasal gland (2). Since then, the biophysical properties of this ubiquitous route for Ca^{2+} entry have been characterized (3) and the ARC channel has been reported to consist of a heteropentameric complex of three Orai1 subunits and two Orai3 subunits (4). Further evidence reported that ARC channels are dependent on the minor pool of STIM1 that constitutively resides in the PM (4–5). Although the role of STIM1 in the regulation of ARC channels has not been completely characterized a recent report has suggested that STIM1 might be necessary to maintain the interaction of Orai1/Orai3 with an unknown soluble factor (6). More recently, it has been reported that protein kinase A (PKA)-mediated phosphorylation of Thr-389 of STIM1 is essential for the activation of the ARC channels (7).

STIM1 is an essential component of the activation of both CRAC and ARC channels. SARAF, a novel regulator of STIM1 function, has been recently identified as a modulator of Ca^{2+} homeostasis. SARAF is a 339 amino acid endoplasmic reticulum (ER)-resident protein that associates with STIM to facilitate slow Ca^{2+}-dependent inactivation of store-operated Ca^{2+} entry (SOCE), prevent STIM1 spontaneous activation and promote STIM1 deoligomerization after Ca^{2+} store refilling. Furthermore, SARAF has been reported to modulate cytosolic and ER Ca^{2+} concentration (8). The activation of SARAF requires the intraluminal (N-terminal) region, while the interaction with SARAF involves the cytosolic region, which interacts with the C-terminal inhibitory domain of STIM1 (downstream the STIM1 Orai1 activation region (SOAR)) to regulate the STIM1/Orai1 interaction (9). SARAF is associated with STIM1 under resting conditions and translocates to ER-PM regions in a STIM1-dependent manner (8).

In the present study we have investigated the role of SARAF in the modulation of ARC channel function. We show for the first time the expression of a pool of SARAF in the plasma membrane that interacts with Orai proteins and that SARAF plays a regulatory role in the activation of ARC channels in neuroblastoma cells.

Experimental Procedures

Materials—Fura-2 acetoxymethyl ester (fura-2/AM) and jasplakinolide were from Molecular Probes (Leiden, The Netherlands). Thapsigargin (TG), arachidonic acid (AA), rabbit anti-Orai1 antibody (directed toward amino acids 288–301 of human Orai1), all-*trans* RA (ATRA), rabbit anti-Orai3 antibody, and bovine serum albumin (BSA) were from Sigma (Madrid, Spain). Rabbit anti-SARAF antibody, siOrai3 and

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[4] The abbreviations used are: PM, plasma membrane; AA, arachidonid acid; ARC channels, arachidonate-regulated Ca^{2+} channels; ATRA, all-*trans* retinoic acid; $[Ca^{2+}]_c$, cytosolic Ca^{2+} concentration; ER, endoplasmic reticulum; RA, retinoic acid; SARAF, store-operated Ca^{2+} entry-associated regulatory factor; SOC channels, store-operated Ca^{2+} channels; SOCE, store-mediated calcium entry; TG, thapsigargin.

Turbofect transfection reagent were from Thermo Fisher (Madrid, Spain). Horseradish peroxidase-conjugated anti-rabbit IgG antibody was from Abcam (Madrid, Spain). Protein A-agarose was from Upstate Biotechnology Inc. (Madrid, Spain). Complete EDTA-free protease inhibitor tablets were from Roche (Madrid, Spain). Enhanced chemiluminescence detection reagents were from Pierce (Cheshire, UK). All other reagents were of analytical grade.

Plasmid Construction—Plasmids were based on the previously published SARAF sequences (GenBank™: JQ348891.1). The DNA of the complete cds was isolated from NG115–401L cells using specific primers (Forward: 5′-AAAAAACCCGGGATGGCCGCAGCCTGCGGGCC-3′; and reverse: 5′-AAAAAAGAATTCTTATCGTCTCCTGGTACCACCATAT-3′). Final cDNA was purified and cloned into the EcoRV site previously inserted in the pIRES2-eGFP-RV expression vector. Nucleotide sequence of this construct was verified by sequencing.

To knockdown expression of SARAF, a pLKO.1-puro plasmid-based shRNA targeting the sequence: CGGACTTAGATATTGCATACA (clone ID: TRCN0000146643; Sigma-Aldrich) was used (SARAF-shRNA). In addition, a non-targeting shRNA plasmid (NT-shRNA) that targets no known human sequence was used as a control. A primer containing the target sequence along with a stem loop followed by the reverse target sequence was annealed to a complimentary primer and inserted into the EcoRI and AgeI sites of the pLKO.1-puro plasmid (Addgene; number 10878). The resulting hairpin consisted of the following sequence: 5′-CCGGCGGACTTAGATATTGCATACACTCGAGTGTATGCAATATCTA AGTCCGTTTTTTG-3′. The correct insertion of the hairpin into pLKO.1 plasmid was finally checked by sequencing.

Cell Culture and Transfection—SH-SY5Y and NG115–401L cell lines were obtained from ATCC (Manassas, VA) and cultured at 37 °C with a 5% CO_2 in RPMI or DMEM, respectively, supplemented with 10% (*v*/*v*) fetal bovine serum, 2 mM L-glutamine, and 100 units/ml penicillin and streptomycin.

Cells were transfected with expression plasmids for pIRES2-EGFP-SARAF as well as with the siSARAF or scramble plasmid as described previously (10–11) using Turbofect transfection reagent.

Measurement of Changes in Cytosolic Free-Ca^{2+} Concentration ($[Ca^{2+}]_c$)—Cells were loaded with fura-2 by incubation with 2 μM fura 2/AM for 30 min at room temperature. Coverslips with cultured cells were mounted on a perfusion chamber and placed on the stage of an epifluorescence inverted microscope (Nikon Diaphot T200, Melville, NY) with image acquisition and analysis system for videomicroscopy (Hamamatsu Photonics, Hamamatsu, Japan). Cells were continuously superfused with HBS and were alternatively excited with light from a xenon lamp passed through a high-speed monochromator (Polychrome IV, Photonics, Hamamatsu, Japan) at 340/380 nm. Fluorescence emission at 505 nm was detected using a cooled digital CCD camera (Hisca CCD C-6790, Hamamatsu, Japan) and recorded using Aquacosmos 2.5 software (Hamamatsu Photonics, Hamamatsu, Japan). Fluorescence ratio (F_{340}/F_{380}) was calculated pixel by pixel and data are presented as $\Delta F_{340}/F_{380}$. AA-evoked Ca^{2+} influx was measured as the integral of $\Delta F_{340}/F_{380}$ above basal for 3 min after the addition of the agonist in the presence of external Ca^{2+}.

Immunoprecipitation and Western Blotting—The immunoprecipitation and Western blotting were performed as described previously (12). Briefly, 500-μl aliquots of cell suspension (4×10^6 cell/ml) were lysed with an equal volume of 2× Nonidet P-40 buffer, pH 8, containing 274 mM NaCl, 40 mM Tris, 4 mM EDTA, 20% glycerol, 2% Nonidet P-40, 2 mM Na_3VO_4, and complete EDTA-free protease inhibitor tablets. Aliquots of cell lysates (1 ml) were immunoprecipitated by incubation with 2 μg of anti-SARAF and 25 μl of protein A-agarose overnight at 4 °C on a rocking platform. The immunoprecipitates were resolved by 10% 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 Orai1and SARAF was achieved by incubation for 2 h with anti-Orai1 or anti-Orai3 antibody diluted 1:200 in TBST or the anti-SARAF antibody diluted 1:1000 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 for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody diluted 1:10,000 in TBST and then exposed to enhanced chemiluminiscence reagents for 4 min. The density of bands was measured using C-DiGit Chemiluminescent Western blot Scanner. Data were normalized to the amount of protein recovered by the antibody used for the immunoprecipitation.

Biotinylation Protocol—Cells were washed three times with HBS (without added serum or protein). Söerscen's buffer (3 ml) (16 mM Na_2HPO_4 and 114 mM NaH_2PO_4, pH 7.0) containing 2.5 mg EZ-Link sulfo-NHS-LC-biotin was added, and cells were incubated at 4 °C for 1 h as previously described (13). The biotinylation reaction was terminated by addition of Tris-base to a final concentration of 33 mM. Following biotinylation, cells were washed twice in Söerscen's buffer, disrupted using Nonidet P40 buffer and sonicated. Cells were harvested by centrifugation (16,000 × *g* for 5 min at 4 °C). Samples were incubated with 25 μl of streptavidin beads overnight at 4 °C, centrifuged, and resuspended in Laemmli´s buffer for subsequent analysis by Western blotting.

Determination of Apoptosis—Apoptosis was assessed using the Direct *In situ* DNA Fragmentation Assay Kit (Abcam, Cambridge, UK) as previously described (14). Briefly, cells were fixed by adding 5 ml of paraformaldehyde (1% w/v in PBS) and placed in ice for 15 min. Cells were then washed and suspended in 70% (*v*/*v*) ethanol for 30 min at −20 °C and incubated with the staining solution for 60 min at 37 °C. Staining solution consists of (each ~51 μl): TdT reaction buffer (10 μl), TdT enzyme (0.75 μl), FITC-dUTP (8 μl), and ddH20 (32.25 μl). Cells were washed twice and the final pellet was incubated for 30 min in 0.5 ml of propidium iodide/RNase A solution. The percentage of apoptotic cells was evaluated by flow cytometry. The number of apoptotic cells is expressed as percentage of the total cell number.

Statistical Analysis—Analysis of statistical significance was performed using one-way analysis of variance. For comparison

between two groups Student's *t* test was used. $p < 0.05$ was considered to be significant for a difference.

Results

SARAF Modulates Ca^{2+} Entry Evoked by Arachidonic Acid—SARAF has been reported to modulate STIM1 function, including the activation of SOCE (8). Since STIM1 is required for the activation of AA-regulated, store-independent, Ca^{2+} entry via the ARC channels, we have explored the possible regulation of Ca^{2+} entry through the ARC channels by SARAF. As depicted in Fig. 1, *A* and *C*, treatment of SH-SY5Y cells, which have been previously reported to display ARC channel currents (15), with AA (8 μM) in a medium containing 1.2 mM Ca^{2+} resulted in a sustained rise in $[Ca^{2+}]_c$ due to Ca^{2+} entry via ARC channels (Fig. 1*A*, *top panel*; $n = 12$). AA was unable to induce Ca^{2+} release from intracellular stores in the absence of extracellular Ca^{2+} (Fig. 1*A*, *bottom panel*; $n = 6$). AA-evoked Ca^{2+} entry was significantly inhibited by 46 ± 7% in cells overexpressing SARAF ($p < 0.001$; $n = 9$). By contrast, the response to AA was significantly enhanced by 29 ± 6% in cells where endogenous SARAF levels were reduced by siRNA (Fig. 1, *B* and *C*; $p < 0.05$; $n = 8$). As reported in Fig. 1, *B* and *C*, the effect of SARAF down-regulation on AA-induced Ca^{2+} entry was successfully rescued by ectopic expression of GFP-SARAF ($n = 5$). These findings indicate that SARAF plays a regulatory role on ARC channel function. Fig. 1*D* shows the expression of SARAF in cells overexpressing SARAF or treated with siRNA SARAF or empty vectors ($n = 5$).

To further assess whether the Ca^{2+} signal evoked by AA was mediated by the activation of ARC channels SH-SY5Y cells were transfected with si Orai3 or scramble plasmid. As depicted in Fig. 1*E*, attenuation of Orai3 expression by siRNA reduced AA-induced Ca^{2+} entry by 83 ± 5% as compared with cells treated with scramble plasmid. Interestingly, in cells treated with siRNA Orai3, silencing or overexpression of SARAF were without effect (Fig. 1*F*), thus suggesting that the effect of SARAF is mostly mediated by regulation of ARC channels. Fig. 1*F* shows that the expression of Orai3 in cells treated with siRNA Orai3 was reduced by 80% as compared with that of cells transfected with empty vectors ($n = 5$). These findings indicate that the regulation of AA-induced Ca^{2+} entry by SARAF is likely mediated by modulation of the ARC channels.

The possible involvement of SARAF in SOCE and AA-evoked Ca^{2+} influx in SH-SY5Y cells was further explored by successive activation of both Ca^{2+} entry pathways. Treatment of SH-SY5Y cells with TG in a Ca^{2+}-free medium resulted in a transient increase in $[Ca^{2+}]_c$ due to discharge of the intracellular Ca^{2+} stores. Subsequent addition of 1.2 mM Ca^{2+} to the medium resulted in an elevation in $[Ca^{2+}]_c$, indicative of SOCE (Fig. 2*A*). The addition of AA (8 μM) after SOCE had been initiated by the addition of Ca^{2+} to store-depleted cells resulted in further Ca^{2+} entry (Fig. 2*A*). Since addition of AA does not evoke further release of Ca^{2+} from the stores (Fig. 1*A*, *bottom panel*), the AA-evoked Ca^{2+} entry is indicative of store-independent ARC channel activation. Overexpression of SARAF in SH-SY5Y cells clearly attenuated both SOCE and AA-stimulated Ca^{2+} entry, without altering the ability of cells to accumulate Ca^{2+} into the stores. These findings support the hypothesis that SARAF modulates cytosolic Ca^{2+} signals through SOCE (8–9), and extends its role to another important Ca^{2+} influx pathway, the AA-induced Ca^{2+} signal, a role that is likely to be relevant in protecting cells from Ca^{2+} overload.

In an attempt to confirm the essential role of STIM1 in store-operated Ca^{2+} (SOC) and ARC channel activation we repeated the maneuver depicted in Fig. 2*A* in NG115–401L cells, a neuroblastoma cell line that expresses a negligible amount of endogenous STIM1 (13, 16). As depicted in Fig. 2*B*, treatment of NG115–401L cells with TG in a Ca^{2+}-free medium resulted in a transient increase in $[Ca^{2+}]_c$ due to Ca^{2+} release from the internal stores. However, neither the subsequent addition of 1,2 mM Ca^{2+} to the extracellular medium nor the addition of AA (8 μM) in the presence of extracellular Ca^{2+} were able to induce any significant increase in $[Ca^{2+}]_c$, which reveals the relevant role of STIM1 in the activation of both SOCE and AA-evoked Ca^{2+} influx.

SARAF Is Constitutively Expressed in the Plasma Membrane and Interacts with Orai1—Since SARAF was found to play a role in the activation of Ca^{2+} influx by AA we explored the interaction of SARAF with the ARC channels. SARAF has been presented as an ER single pass membrane protein (17); however, the product of *TMEM66*, the former name of the gene encoding SARAF, has been suggested to be located in the PM (18). Since ARC channels consist of a heteropentameric complex of three Orai1 and two Orai3 subunits with the participation of PM-resident STIM1 (4–5) we investigated whether there is a pool of SARAF constitutively expressed in the PM and whether SARAF interacts with ARC channels. The location of SARAF in the PM was assessed by biotinylation of plasma membrane proteins and collection with streptavidin-coated agarose beads. SDS-PAGE and Western blotting were used to identify SARAF. As shown in Fig. 3*A*, analysis of biotinylated proteins shows that SARAF is present in the PM in resting cells. Therefore, the pool of SARAF present in the PM might be responsible for the regulation of ARC channels as the ER-resident SARAF has been reported to modulate SOCE (8–9).

To investigate whether SARAF in the PM associates with the ARC channels we have designed a maneuver based on the use of jasplakinolide, a cell-permeant peptide isolated from *Jaspis johnstoni* that induces polymerization and stabilization of actin filaments exclusively at the cell periphery, near the PM (19). Jasplakinolide induces the formation of a cortical actin filament barrier that prevents the interaction of intracellular organelles with the PM (19–21). As represented schematically in Fig. 3*C*, a model based on the interaction between ER-resident SARAF and ARC channel subunits in the PM might be expected to be impaired by jasplakinolide but an interaction between PM-located SARAF and ARC channel subunits should be resistant to treatment with jasplakinolide. We therefore tested for the interaction between SARAF and Orai1 by looking for co-immunoprecipitation from SH-SY5Y cell lysates. Immunoprecipitation and subsequent SDS-PAGE and Western blotting were conducted using control cells and cells stimulated with AA (8 μM) in the presence of extracellular Ca^{2+} (1.2 mM). Cells were preincubated for 30 min at 37 °C with 10 μM jasplakinolide (19). After immunoprecipitation with the anti-SARAF antibody, Western blotting revealed the presence of Orai1 in samples

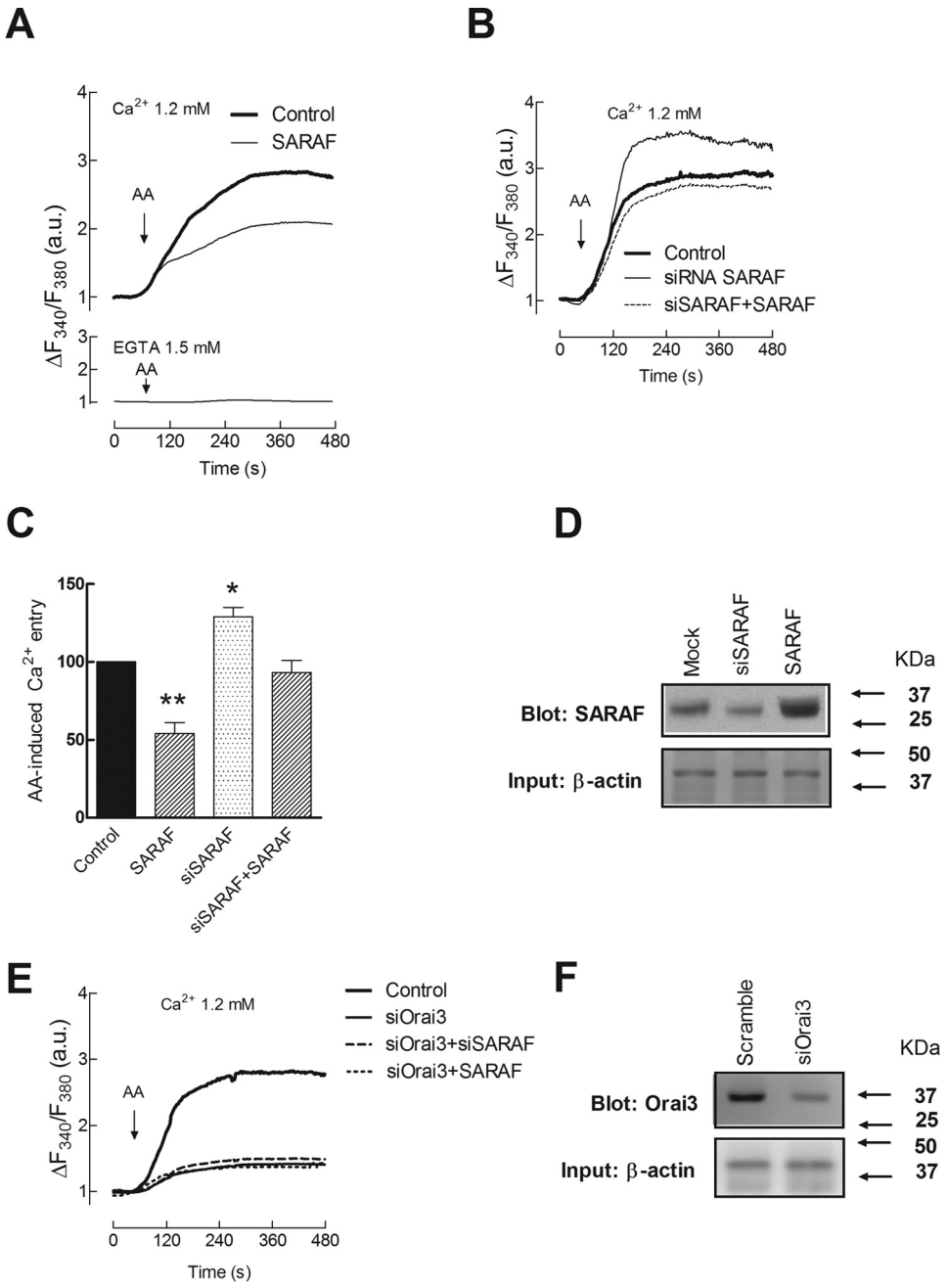

FIGURE 1. **SARAF modulates arachidonic acid-evoked Ca^{2+} entry in neuroblastoma SH-SY5Y cells.** SH-SY5Y cells were loaded with fura-2 and resuspended in a medium containing 1.2 mM Ca^{2+} or in a Ca^{2+}-free medium (1.5 mM EGTA added) as described under "Experimental Procedures." Elevations in $[Ca^{2+}]_i$ were monitored by using the 340/380 nm fluorescence ratio and presented as $\Delta F_{340}/F_{380}$. *A*, SH-SY5Y cells overexpressing SARAF (SARAF) and mock-treated cells (Control and *bottom panel*) were treated with AA (8 μM) in the presence of 1.2 mM extracellular Ca^{2+} or in a Ca^{2+}-free medium as indicated. *B*, SH-SY5Y cells transfected with si SARAF, alone or in combination with SARAF overexpression plasmid, or scramble plasmids, as indicated, were stimulated with AA (8 μM) in the presence of 1.2 mM extracellular Ca^{2+}. *C*, bar graph indicates the amount of Ca^{2+} entry after cell stimulation with AA in the presence of extracellular Ca^{2+}. Data are expressed as mean ± S.E. * and **, $p < 0.05$ and $p < 0.01$, respectively. *D*, cells overexpressing SARAF or transfected with si SARAF and their respective controls were lysed, and whole cell lysates were subjected to 10% SDS-PAGE and subsequent Western blotting with a specific anti-SARAF antibody. Membranes were reprobed with anti-β-actin antibody for protein loading control. The panels show results from one experiment representative of 4 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. *E*, SH-SY5Y cells transfected with si Orai3, alone or in combination with SARAF overexpression plasmid or siRNA SARAF, or scramble plasmid, as indicated, were stimulated with AA (8 μM) in the presence of 1.2 mM extracellular Ca^{2+}. *F*, cells transfected with si Orai3 or scramble plasmid were lysed, and whole cell lysates were subjected to 10% SDS-PAGE and subsequent Western blotting with a specific anti-Orai3 antibody. Membranes were reprobed with anti-β-actin antibody for protein loading control. The panels show results from one experiment representative of 4 others. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel.

from unstimulated cells (Fig. 3*B*, *top panel*; $n = 6$). Cell stimulation with AA significantly enhanced the interaction between SARAF and Orai 1 to 170% of control (Fig. 3*B*, *top panel*; $p < 0.05$; $n = 6$). Western blotting with anti-SARAF confirmed a similar content of this protein in all lanes (Fig. 3*B*, *bottom panel*). These findings indicate that SARAF located in the PM is able to interact constitutively with Orai1 channels and that this interaction is enhanced by AA.

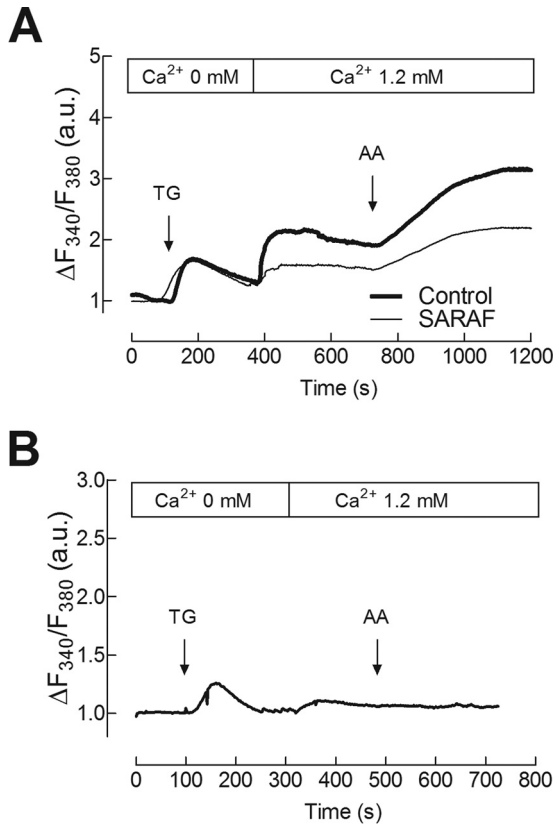

FIGURE 2. **SOCE and ARC-mediated Ca^{2+} entry require STIM1 and are modulated by SARAF.** *A*, SH-SY5Y cells overexpressing SARAF (SARAF) and mock-treated cells (Control) were treated in a Ca^{2+}-free medium (1.5 mM EGTA added) with 1 μM TG followed by addition Ca^{2+} to the medium (final concentration 1.2 mM) to initiate Ca^{2+} entry. Cells were then stimulated with AA (8 μM) to evoke store-independent Ca^{2+} entry via ARC channels. *B*, NG115-401L cells were treated in a Ca^{2+}-free medium (1.5 mM EGTA added) with 1 μM TG followed by addition of Ca^{2+} (final concentration 1.2 mM). Cells were then stimulated with AA (8 μM). The traces shown are representative of ten to twelve independent experiments.

SARAF Modulates the Ability of AA to Promote Cell Survival—Recent studies have revealed that retinoic acid induces cell survival in SH-SY5Y neuroblastoma cells thorugh the generation of AA (22). Bell *et al.* have reported that inhibition of phospholipase A2 (PLA2) in SH-SY5Y cells treated with all-trans RA (ATRA; 2–15 μM) results in an increase in the percentage of apoptotic cells, thus supporting a role of AA in cell survival (22). Therefore, we have further explored the functional role of SARAF in AA-induced cellular effects. SH-SY5Y cells were stimulated with increasing concentrations of ATRA (5, 10, and 15 μM) and the percentage of apoptotic cells was determined 24 h latter. As shown in Fig. 4, treatment with ATRA alone does not significantly increase the percentage of apoptotic cells (a slight increase was detected after treatment with 15 μM ATRA). As previously reported (22), inhibition of PLA2 using 10 μM AACOCF3, a concentration that has been previously used to inhibit AA release in neuroblastoma cell lines (23), significantly enhance the percentage of apoptotic cells at all the ATRA concentrations tested (Fig. 4). The percentage of apoptotic cells was also increased in cells overexpressing SARAF (Fig. 4), which impairs AA-induces Ca^{2+} response (see Fig. 1*A*). To investigate whether the effect of SARAF is mediated through the modulation of AA-induced Ca^{2+} signals cells overexpressing SARAF were treated with AACOCF3. As depicted in Fig. 4, the percentage of apoptotic cells upon treatment with AACOCF3 alone or in combination with SARAF overexpression was not significantly different, thus suggesting that the effect of SARAF in cell survival occurs downstream the PLA2/AA pathway.

Discussion

Here we report that the STIM1 regulatory protein SARAF modulates AA-evoked Ca^{2+} entry. The store-independent, AA-induced, Ca^{2+} influx is mediated via activation of ARC channels, which consists of a combination of Orai1 and Orai3 subunits, with the cooperation of PM-resident STIM1 (4–5). Our results indicate that overexpression of SARAF results in attenuation of AA-induced Ca^{2+} response, while transfection of siRNA SARAF enhances AA-stimulated Ca^{2+} influx, thus suggesting that SARAF is a negative regulator of AA-induced Ca^{2+} signals. The effect of SARAF on AA-evoked Ca^{2+} entry was found to be mediated by regulation of the ARC channels, since in cells transfected with siRNA Orai3, the changes in SARAF expression were without effect.

Since SARAF has been presented as an STIM1 regulator and the ARC channels involved the participation of the PM pool of STIM1, we have further explored for the presence of SARAF in the PM. Using biotinylation analysis we detected SARAF expressed in the PM, which is consistent with the suggested location of the product of *TMEM66* in prostate cancer cells (18). Furthermore, we have found that SARAF in the PM is able to associate with Orai1 and this interaction is enhanced by stimulation with AA.

Despite the interaction of SARAF with Orai1 in the PM might be associated to SOCE as well, and we cannot rule out this possibility, the finding that AA, which is unable to discharge the Ca^{2+} stores, stimulates the association between both proteins strongly suggests that SARAF associates to ARC channel-forming Orai1. The coimmunoprecipitation of SARAF with Orai1 might occur by direct SARAF-Orai1 interaction or through the interaction of SARAF with PM-resident STIM1, which, in turn, associates with the Orai1/Orai3 channel complex.

A study by Putney's group reported a mutual antagonism between SOCE and AA-induced Ca^{2+} entry in HEK-293 cells where both pathways coexists (24). These observations might suggest an alternative explanation to the positive effect of AA on the interaction between SARAF and Orai1 in neuroblastoma cells. Since SARAF is a negative modulator of Ca^{2+} entry via SOCE (8) or ARC channels (Fig. 1) AA might antagonize SOCE by promoting the interaction between SARAF and SOC channel-forming Orai1, as a mechanism to protect the cell from deleterious Ca^{2+} overload. However, in neuroblastoma cells we have not detected antagonism between SOCE and AA-evoked Ca^{2+} influx, as shown in Fig. 2*A*, where the extent of the change in the fura-2 340/380 fluorescence ratio evoked by AA after the activation of SOCE was comparable with that observed in cells not treated with TG (Figs. 1*A* *versus* 2*A*).

We have further explored the functional significance of the interaction of SARAF with ARC channels. In cancer cells, RA

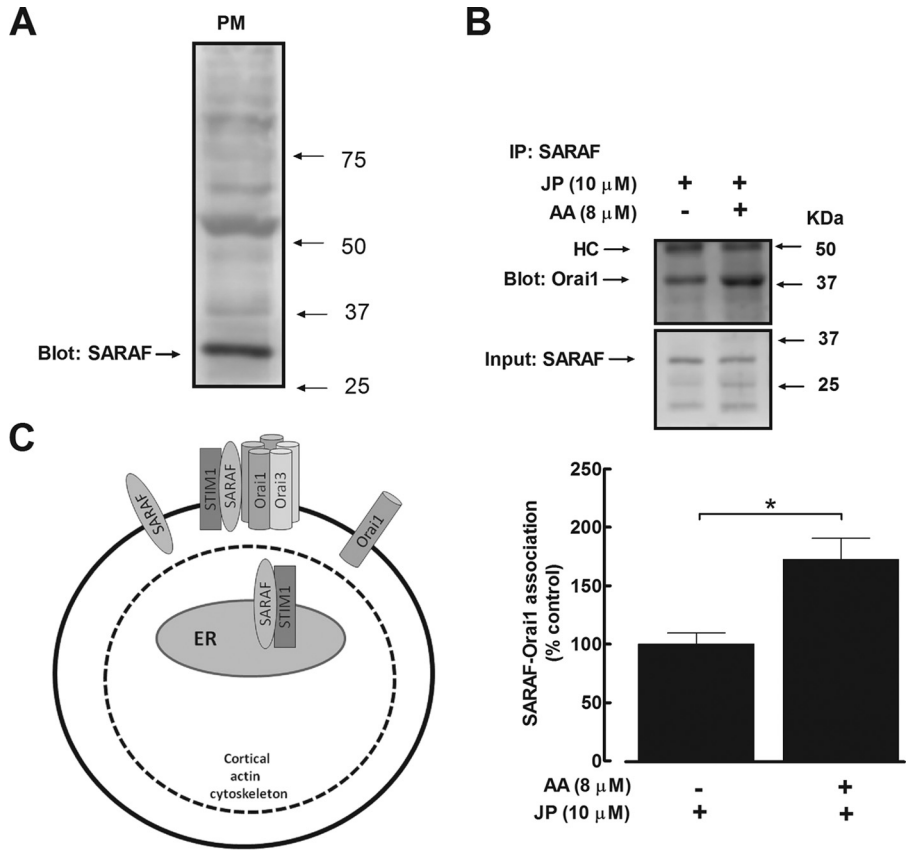

FIGURE 3. **SARAF is constitutively expressed in the plasma membrane and interacts with Orai1.** *A*, SH-SY5Y cells were mixed with ice-cold Söerscen's buffer containing 2.5 mg EZ-Link sulfo-NHS-LC-biotin, and cell surface proteins were labeled by biotinylation, as described under "Experimental Procedures." Labeled proteins were extracted with streptavidin-coated agarose beads and analyzed by SDS-PAGE and Western blotting using the anti-SARAF antibody. These results are representative of four separate experiments. *B*, SH-SY5Y cells were pretreated for 30 min with 10 μM jasplakinolide and then were stimulated in the presence of 1.2 mM extracellular Ca^{2+} with AA (8 μM) and lysed. Whole cell lysates were immunoprecipitated with anti-SARAF antibody. Immunoprecipitates were analyzed by Western blotting (WB) using anti-Orai1 antibody (*top*) and reprobed with anti-SARAF antibody (*bottom*) for protein loading control. Positions of molecular mass markers are shown on the right. *HC*, heavy chain of the Ig used for immunoprecipitation. Histograms indicate the quantification of SARAF-Orai1 interaction presented as percentage of control (cells not stimulated with AA). Data are expressed as mean ± S.E. of six independent experiments. *C*, schematic presentation of the effect of jasplakinolide on actin filament reorganization. Actin polymerization induced by jasplakinolide forms a tight cortical actin barrier, displacing the ER and preventing physical interaction with the plasma membrane.

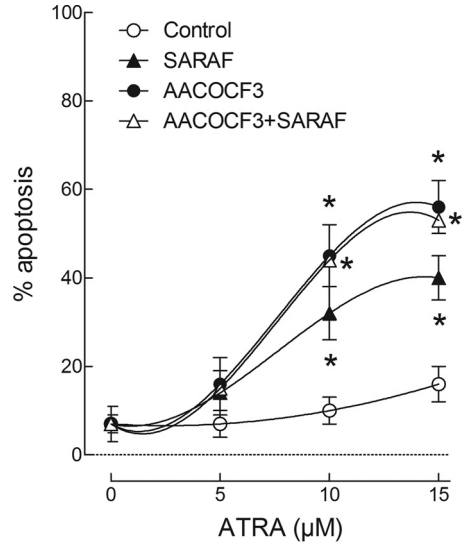

FIGURE 4. **SARAF modulates the ability of AA to promote cell survival.** SH-SY5Y cells overexpressing SARAF (SARAF) and mock-treated cells were treated for 24h with ATRA (5–15 μM) alone or in combination with the PLA2 inhibitor AACOCF3 (10 μM). Apoptosis was determined as described under "Experimental Procedures." Data are expressed as mean ± S.E. of four independent experiments. *, $p < 0.05$ as compared with Control.

has been reported to exert either cell death or cell survival (25–26). In SH-SY5Y neurblastoma cells, RA induces cell survival through the activation of the PLA2/AA pathway since inhibition of PLA2 results in an increase in the percentage of apoptotic cells (22). Here we report that SARAF overexpression attenuates cell survival of cells stimulated with ATRA thus suggesting that impairment of AA-evoked Ca^{2+} signals by SARAF attenuates its role in cell survival. The percentage of apoptotic cells was increased in cells treated with ATRA in the presence of the PLA2 inhibitor, AACOCF3, and this percentage was not modified in cells overexpressing SARAF, which strongly suggest that the effect of SARAF is most likely mediated through the PLA2/AA pathway.

Our findings shed new light on the mechanisms involved in the regulation of ARC channels. We have shown for the first time that SARAF modulates Ca^{2+} entry stimulated by AA, as well as the PM expression of SARAF, which constitutively associates to the pore-forming subunit Orai1. The interaction between SARAF and Orai1 that happens in the PM is enhanced by AA. The regulatory mechanism of SARAF might modulate the ability of AA to promote survival of SH-SY5Y neuroblastoma cells.

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Store-operated Ca^{2+} Entry-associated Regulatory factor (SARAF) Plays an Important Role in the Regulation of Arachidonate-regulated Ca^{2+} (ARC) Channels

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