Cell Calcium 65 (2017) 91–101

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Orai3 channel is the 2-APB-induced endoplasmic reticulum calcium leak

Daniel Leon-Aparicio [a], Jonathan Pacheco [b], Jesus Chavez-Reyes [a], Jose M. Galindo [a], Jesus Valdes [a], Luis Vaca [b], Agustin Guerrero-Hernandez [b,*]

[a] Department of Biochemistry, Cinvestav, Mexico City, 07000, Mexico
[b] Department of Cell Biology and Development, Instituto de Fisiología Celular, UNAM, Mexico City, 04510, Mexico

ARTICLE INFO

Article history:
Received 11 August 2016
Received in revised form 17 January 2017
Accepted 20 January 2017
Available online 23 January 2017

Keywords:
Orai3 channel
Endoplasmic reticulum calcium leak
2-APB
Luminal calcium concentration
SERCA pump

ABSTRACT

We have studied in HeLa cells the molecular nature of the 2-APB induced ER Ca^{2+} leak using synthetic Ca^{2+} indicators that report changes in both the cytoplasmic ($[Ca^{2+}]_i$) and the luminal ER ($[Ca^{2+}]_{ER}$) Ca^{2+} concentrations. We have tested the hypothesis that Orai channels participate in the 2-APB-induced ER Ca^{2+} leak that was characterized in the companion paper. The expression of the dominant negative Orai1 E106A mutant, which has been reported to block the activity of all three types of Orai channels, inhibited the effect of 2-APB on the $[Ca^{2+}]_{ER}$ but did not decrease the ER Ca^{2+} leak after thapsigargin (TG). Orai3 channel, but neither Orai1 nor Orai2, colocalizes with expressed IP_3R and only Orai3 channel supported the 2-APB-induced ER Ca^{2+} leak, while Orai1 and Orai2 inhibited this type of ER Ca^{2+} leak. Decreasing the expression of Orai3 inhibited the 2-APB-induced ER Ca^{2+} leak but did not modify the ER Ca^{2+} leak revealed by inhibition of SERCA pumps with TG. However, reducing the expression of Orai3 channel resulted in larger $[Ca^{2+}]_i$ response after TG but only when the ER store had been overloaded with Ca^{2+} by eliminating the acidic internal Ca^{2+} store with bafilomycin. These data suggest that Orai3 channel does not participate in the TG-revealed ER Ca^{2+} leak but forms an ER Ca^{2+} leak channel that is limiting the overloading with Ca^{2+} of the ER store.

1. Introduction

Calcium influx stimulated by depletion of the endoplasmic reticulum (ER) Ca^{2+} store or SOCE involves the coupling of STIM in the ER membrane and Orai channels at the plasma membrane [1,2]. There are three types of Orai channels, all of them can support SOCE [3] or ICRAC currents [4], but Orai1 is by far the main channel involved in generating this type of Ca^{2+} influx [5,6]. In this sense the location in the cell of Orai1 channel correlates with this function, since Orai1 is present at the plasma membrane [7,8]. However, the location of Orai2 and Orai3 appears to be different, in many instances, these two channels are located to a large extent in intracellular compartments, the ER among others [9–11]. It has been reported that Orai3 needs Orai1 to reach the plasma membrane due to the absence of the tripeptide sequence EFA in the carboxy terminus of Orai3 channel [12]. The question is then whether these channels in the ER are functional or just channels that are going in transit to the plasma membrane.

There are some reports that Orai channels can have what appears to be a leak activity, which does not require the presence of STIM1 to be observed. The first report we are aware of is a work carried out in yeast vesicles expressing functional Orai1 channels and resulted in a reduced luminal $[Ca^{2+}]$ when compared with those vesicles expressing no functional Orai1 mutant channels, such as Orai1R91W and Orai1E106Q [13]. Similarly, the expression of Orai1 E106A in PC12 cells, a channel that cannot transport Ca^{2+} [9] and that inhibits the activity of all three different types of Orai channels [4], showed a higher luminal ER $[Ca^{2+}]$ (above 300 μM) than those cells not expressing this mutant channel, with luminal $[Ca^{2+}]_{ER}$ below 300 μM [9]. Another work trying to determine the molecular nature of ER Ca^{2+} leak channels found that Orai2 channel regulates the luminal $[Ca^{2+}]_{ER}$ since this concentration was larger when silencing Orai2 channel and smaller than control when overexpressing Orai2 channels in HEK293T cells [14]. Moreover, it has been shown that Orai channels localization in HeLa cells is not the same for each type. Orai1 is mainly in the plasma membrane, while Orai2 is located in intracellular compartments, but not necessar-

Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; Ca^{2+}, calcium; $[Ca^{2+}]_{ER}$, Endoplasmic Reticulum Ca^{2+} concentration; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; IP_3, Inositol 1,4,5- trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; SERCA, sarco/endoplasmic reticulum calcium ATPase; TG, thapsigargin; AUC, area under the curve.

* Corresponding author at: Departamento de Bioquímica, Cinvestav, Mexico City, 07000, Mexico.
E-mail address: aguerrero@cinvestav.mx (A. Guerrero-Hernandez).

http://dx.doi.org/10.1016/j.ceca.2017.01.012
0143-4160/

ily in the ER, and Orai3 shows a large colocalization with a BAP31, a protein marker of the ER [10]. However, in this work there was no data showing the role of Orai3 channel in the regulation of the $[Ca^{2+}]_{ER}$ in HeLa cells [10]. All these data point to the idea that Orai channels can have ER Ca^{2+} leak activity.

Initially, the chemical 2-APB was introduced as a cell-permeable inhibitor of IP_3Rs [15]. However, it turned out to be nonspecific modulator of ion channels; in the case of Orai channels this chemical is rather interesting because it has different effects on each one of the Orai channels. Orai 1 is activated by this compound by a mechanism that does not require STIM [16] and also involves an increase in the size of the channel pore [17]; at low concentrations (<10 μM) this activation is sustained, while at higher concentrations (>30 μM), this activation is rapidly followed by a sustained inhibition [16]. Orai2 is relatively refractory to this compound [4,16] and Orai3 is strongly activated [4,16] to the extent that switches the channel from Ca^{2+} selective to a Ca^{2+}-permeable but the channel pore is now so wide that becomes a nonselective cation channel, at least when expressed in the plasma membrane [18]. Importantly, these effects of 2-APB on Orai3 channel do not appear to require the participation of STIM proteins [16,18].

We have observed that 2-APB produces an ER Ca^{2+} leak in HeLa cells that is inhibited with partially depleted ER Ca^{2+} stores and is not involved in the ER Ca^{2+} leak after TG [19]. Our data shown here suggest that 2-APB-induced ER Ca^{2+} leak in HeLa cells is mediated by activation of Orai3 channels that are not involved in the TG-induced ER Ca^{2+} leak but more likely function as a safety mechanism to avoid Ca^{2+} overloading of the ER store.

2. Materials and methods

2.1. Materials

We have used the cell permeable forms of Fura-2/AM (Invitrogen) and Mag-Fluo-4/AM (Invitrogen) to load HeLa cells with fura-2 in the cytoplasm and Mag-fluo-4 in the lumen of the ER. These dyes were dissolved in dehydrated dimethyl sulfoxide (DMSO, Sigma) as well as 2-APB (Sigma), thapsigargin (Invitrogen) and bafilomycin (Sigma) to make a 1000-fold concentrated stock solution. Therefore the DMSO concentration was 0.1% v/v on the indicated additions.

2.2. Cell culture and plasmid transfections

HeLa cells (ATCC) were grown in 60-mm dishes (Corning) with high glucose (25 mM) Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 mg/mL streptomycin and 100 units/mL penicillin (all from Invitrogen) and maintained at 37 °C in 5% CO_2 humidified atmosphere. The transfection of HeLa cells with Orai1-E106A was carried out using nucleofector (Amaxa, Germany) according to the protocol of the manufacturer. In brief, 2 μg of the plasmid was transfected to one million cells, and then these cells were plated on 60-mm dishes for 24 h. Transfections of Orai1-CFP, Orai2-CFP, Orai3-CFP and pcDNA3 (as mock) were carried out using lipofectamine 2000 in Opti-MEM (Invitrogen) following manufacturerís protocol. Transfection protocol for confocal imaging experiments of Orai channels and IP_3R was calcium precipitation using 1 μg of each plasmid, cells were grown on 25 mm circular coverslips for 48 h before carrying out imaging acquisition as described below.

2.3. Simultaneous recording of the $[Ca^{2+}]_i$ and changes in the luminal $[Ca^{2+}]_{ER}$ in HeLa cell suspensions

Each experiment represents simultaneous responses of both the $[Ca^{2+}]_i$ and the $[Ca^{2+}]_{ER}$ of 0.5×10^6 transfected cells using a Xenon arc lamp-based fluorescence spectrophotometer (PTI) that has a built-in device for continuous stirring of cell samples. Each experiment involved the following steps; HeLa cells were harvested with 0.05% trypsin in PBS, viability was checked with trypan blue exclusion, loading with 1 μM Fura-2/AM and 1 μM Mag-Fluo-4/AM (both from Molecular Probes, Life Technologies) for a period of 2 h at room temperature and protected from light, next cells were washed twice in saline solution and finally resuspended in the same saline solution composed by (in mM) 121 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 1.8 $CaCl_2$, 25 HEPES, 6 $NaHCO_3$ and 5.5 Glucose (pH 7.3) or in the same saline solution but lacking added $CaCl_2$ and supplemented with 0.1 mM EGTA. Changes in the $[Ca^{2+}]_i$ and the free luminal ER Ca^{2+} level ($[Ca^{2+}]_{ER}$) were recorded after allowing for an equilibration period of 10 min (basal level). Fura-2 and Mag-Fluo-4 fluorescence signals were obtained by alternating excitation (2.7 Hz at 100 ms integration time) at 340, 360 and 380 nm for Fura-2 and 485 nm for Mag-Fluo-4, emission was fixed at 515 nm. Fura-2 and Mag-Fluo-4 signals were smoothed, after background subtraction, with a 7-point window using the Savitzky Golay's algorithm contained in the analysis software FeliX32 (PTI). To transform Fura-2 fluorescence signal in $[Ca^{2+}]_i$, the maximum fluorescence ratio (R_{max}) was obtained by adding digitonin. Minimum fluorescence ratio (R_{min}) was determined by adding EGTA. Fluorescence background of the cell suspension was determined by Fura-2 quenching with Mn^{2+} addition. $[Ca^{2+}]_i$ was calculated using the Grynkewiczís equation [20] where $[Ca^{2+}]_i = Kd^*\beta\ (R - R_{min})/(R_{max} - R)$, where Kd is the apparent Ca^{2+} dissociation constant for Fura-2 (200 nM), R is the ratio of fluorescence value at 340 nm over the fluorescence at 380 nm after background subtraction and the viscosity correction procedure described by Poenie [21]. Mag-Fluo-4 is a low-affinity Ca^{2+} indicator with a reported Kd of 22 μM, that is widely used to report changes in the $[Ca^{2+}]_{ER}$ [22–28]. Mag-Fluo-4 fluorescence signal was not transformed in $[Ca^{2+}]$ but it was normalized using the resting level (F/Fo) calculated during the initial 10 min period of equilibration (basal level). For clarity this ratio was multiplied by 100 and expressed as percentage of change.

2.4. Confocal microscopy and imaging of transfected cells

Confocal images were performed with an upright TCS-SP5 LEICA confocal microscope using an APO 63X water immersion objective. HeLa cells were imaged at room temperature on Krebs solution containing (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 1.8 $CaCl_2$, 20 HEPES, 1 NaH_2PO_4 and 11 glucose (pH 7.4). Images were obtained from live cells co-expressing YFP-IP3R and one of the Orai channels, either Orai1-CFP, Orai2-CFP or Orai3-CFP. CFP and YFP were excited with 458 and 514 nm laser lines, respectively. Fluorescence was collected from 465 to 509 nm for CFP and from 525 to 600 nm for YFP. Colocalization analysis was performed using Mander's coefficient routine in ImageJ (NIH).

2.5. Reducing expression of channels with small interfering RNA

HeLa cells were grown in 35 mm dishes and transfected with 1 μg of Mission® esiRNA for Orai3 (EHU131741, Sigma-Aldrich Química, S.L, Toluca, Mexico) or siControl with Lipofectamine 2000 in Opti-MEM. The medium was replaced after 24 h with DMEM without siRNA for another 24 h. After this time, cells were harvested for simultaneous recording of changes in the $[Ca^{2+}]_i$ and the $[Ca^{2+}]_{ER}$ as described above.

2.6. Statistical analysis

Data shown are the mean ± SEM, where n, indicates the number of different experiments (implies different cell passage). Statistical analysis of data was carried out using unpaired Student's *t*-test for comparison between two groups, and ANOVA with a post hoc Dun-

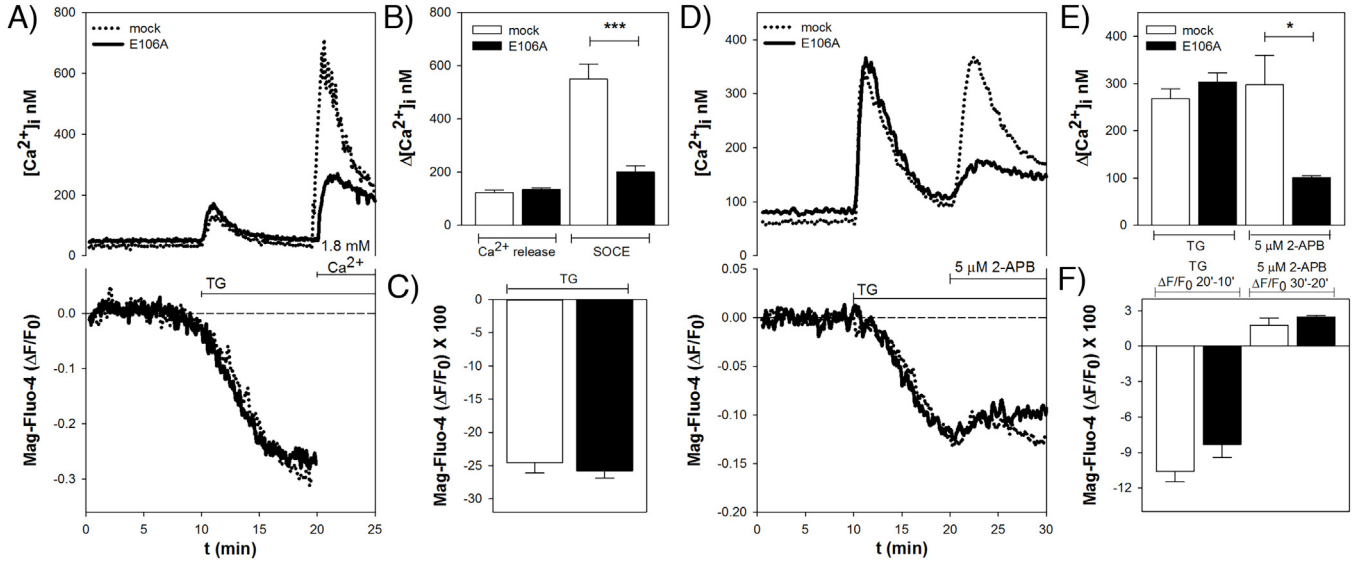

Fig. 1. Expression of dominant negative Orai1 E106A channel inhibits SOCE but does not affect the TG-revealed ER Ca^{2+} leak. A) HeLa cells in the absence of external [Ca^{2+}] (0.1 mM EGTA) were transfected with either an empty plasmid (dotted line) or with a plasmid containing a dominant negative Orai1 E106A mutant (solid line) and TG followed by 1.8 mM Ca^{2+} were added at the times indicated. B) This mutant channel did not modify the [Ca^{2+}] response to TG, but clearly inhibited SOCE. C) The extent of the ER depletion promoted by TG was not affected by the expression of this ion channel mutant. D) Same experiment as in A) except the external [Ca^{2+}] was 1.8 mM. E) $[Ca^{2+}]_i$ response due to the addition of TG was no modified by Orai1 E106A, but reactivation of SOCE with 5 μM 2-APB was strongly inhibited. F) The extent of reduction in the $[Ca^{2+}]_{ER}$ was not altered by the expression of this mutant channel.

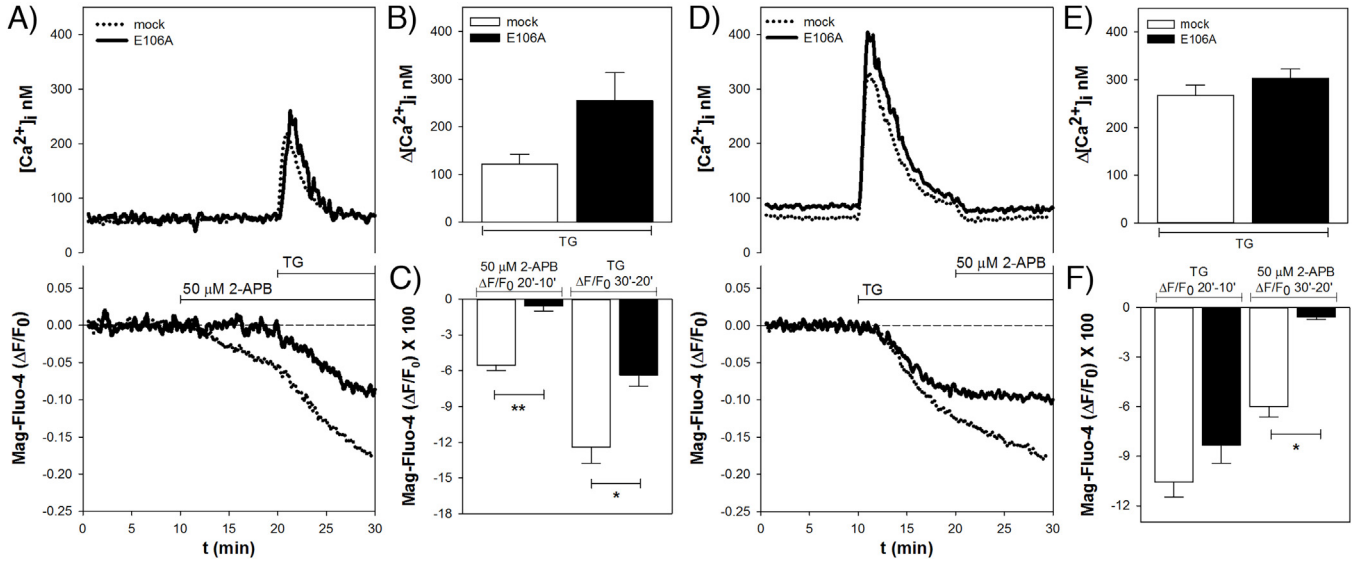

Fig. 2. Expression of Orai1 E106A inhibits the 2-APB-induced ER Ca^{2+} leak. A) HeLa cells transfected with either an empty plasmid (dotted line) or the mutant Orai1 E106A (solid line) were recorded in the presence of 1.8 mM external [Ca^{2+}] and exposed to 2-APB followed by TG at the time indicated. B) The $[Ca^{2+}]_i$ response after TG was if anything increased by Orai1 E106A. C) Orai1 E106A inhibited both the ER Ca^{2+} leak induced by 2-APB and the one observed after TG. D) Same experiment as in A) except that TG was added before 2-APB as indicated. E) $[Ca^{2+}]_i$ responses to TG were not affected by the expression of Orai1 E106A. F) $[Ca^{2+}]_{ER}$ reduction after TG was not affected by Orai E106A but the effect of 2-APB was clearly inhibitory on the $[Ca^{2+}]_{ER}$ in the presence of Orai1 E106A (right solid bar).

nett's test for comparison among multiple groups. The difference between means was considered significant with $p < 0.05$.

3. Results

3.1. Dominant negative Orai1 E106A mutant did not affect the ER Ca^{2+} leak but inhibited the 2-APB-induced ER Ca^{2+} leak

It has been described that 2-APB strongly activates Orai3 channels while to a more limited extent does the same with Orai1 channels; Orai2 channel is rather refractory to this chemical [17]. To determine whether Orai channels are involved in the effect of 2-APB, as a first approach we have used the dominant negative mutant Orai1 E106A which inhibits the activity of all Orai channels [4]. This mutant modified neither the effect of TG on the $[Ca^{2+}]_i$ response nor the reduction in the luminal $[Ca^{2+}]_{ER}$ (Fig. 1A, 1B, 1C) but strongly inhibited SOCE, that is the $[Ca^{2+}]_i$ response resulting from the addition of 1.8 mM Ca^{2+} (Fig. 1B, solid bars). These data suggest that Orai channels were not participating in the ER Ca^{2+} leak in HeLa cells. Since the effect of 2-APB on the ER Ca^{2+} leak depends on a Ca^{2+}-filled ER store [19], we decided to carry out this experiment in 1.8 mM external [Ca^{2+}]. In this case again, the overexpression of the mutant Orai1 E106A, did not modify Ca^{2+} responses after TG (Fig. 1D, 1E, 1F) and it is clear then that Orai channels

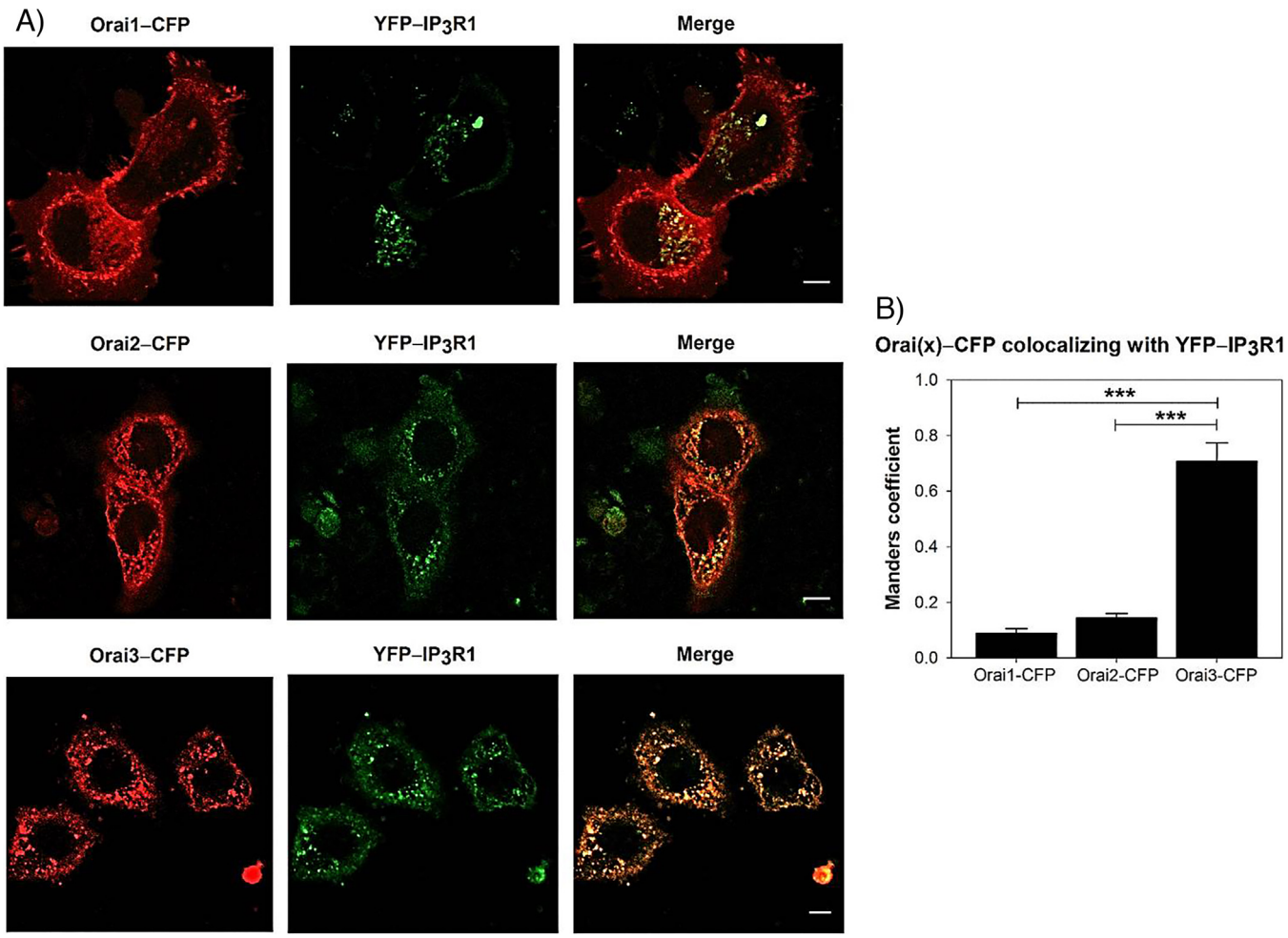

Fig. 3. Orai3 colocalizes with IP_3Rs. A) Transfection of Orai-CFP channels together with YFP-IP_3R1 showed different degree of colocalization depending on the type of channel expressed. Orai1 is shown in the upper panel. Orai2 in the middle while Orai3 in the lower panel. B) Manders' colocalization coefficient showed that Orai3 and IP_3Rs colocalizes while this was not the case for Orai1 or Orai2.

did not contribute significantly to the $[Ca^{2+}]_i$ response after blocking SERCA pumps with TG, in the presence of external $[Ca^{2+}]$; the absence of this effect is most likely due to the rapid inactivation of Orai1 channels induced by Ca^{2+} [29,30]. To verify that Orai1 E106A had inhibited SOCE, we have reactivated Orai1 channels with 5 μM 2-APB, in this case, there was a large and transient increase in the $[Ca^{2+}]_i$ that was totally abolished by the expression of the mutant Orai1 E106A (Fig. 1D, 1E). Since the reduction in the $[Ca^{2+}]_{ER}$ triggered by TG was not affected by Orai1 E106A further supports the contention that Orai channels were not involved in the ER Ca^{2+} leak. However, the 2-APB-induced reduction in the luminal $[Ca^{2+}]_{ER}$ was totally abolished by overexpressing Orai1 E106A (Fig. 2A, C), arguing for the participation of Orai channels in this effect of 2-APB. Intriguingly, the TG-revealed ER Ca^{2+} leak judged by the reduction in the $[Ca^{2+}]_{ER}$ was clearly decreased by the expression of the mutant Orai1 channel (Fig. 2C). One way to reconcile data shown in Fig. 1 and those shown here is to consider that 2-APB is blocking the late phase of TG revealed Ca^{2+} leak as we have described this inhibitory effect of 2-APB on TG-induced ER Ca^{2+} leak [19]. To verify that this was the case, we have applied 2-APB after TG and 2-APB fully inhibited the TG-revealed ER Ca^{2+} leak, but only in those cells that were expressing the Orai1 mutant (Fig. 2D, F). Collectively, these data suggest that Orai channels decrease luminal $[Ca^{2+}]_{ER}$ in response to 2-APB but they do not seem to participate in the TG-revealed ER Ca^{2+} leak.

3.2. Orai3 channel colocalized with IP_3R in HeLa cells

It has been shown in HeLa cells that Orai3 localizes in the endoplasmic reticulum [10], to verify that our cells show that distribution as well, we have co-expressed Orai channels together with IP_3R1 and we have determined the extent of colocalization with Manders' coefficient (Fig. 3). Data show that Orai1 distribution was primarily in the cell periphery (Fig. S1), with very little inside the cell. Orai2 channel localization appears to be more intracellular (Fig. S1); however it did not colocalize with IP_3R to a large extent (Fig. 3B). However, the picture was completely different with Orai3 channel since this protein was located intracellularly with a very similar pattern to the one observed for IP_3R1 (Fig. 3A, B). These data say that, to variable extent, all three Orai channels were present in intracellular compartments, but only Orai3 channels showed a large Manders' colocalization coefficient with IP_3Rs in HeLa cells (Fig. 3B). Additionally, we have observed that Orai3 channel had an intracellular distribution in HEK293 cells that clearly resembles the ER (Fig. S1). These data say that Orai3 location was mainly in the ER both in HeLa and HEK293 cells.

3.3. Only Orai3 channel supports the 2-APB-induced ER Ca^{2+} leak

It has been known that Orai channels can form heteromultimers [4,31] and this is the explanation as to why Orai1 E106A is able to block the activity of all types of Orai channels. Accordingly, to obtain

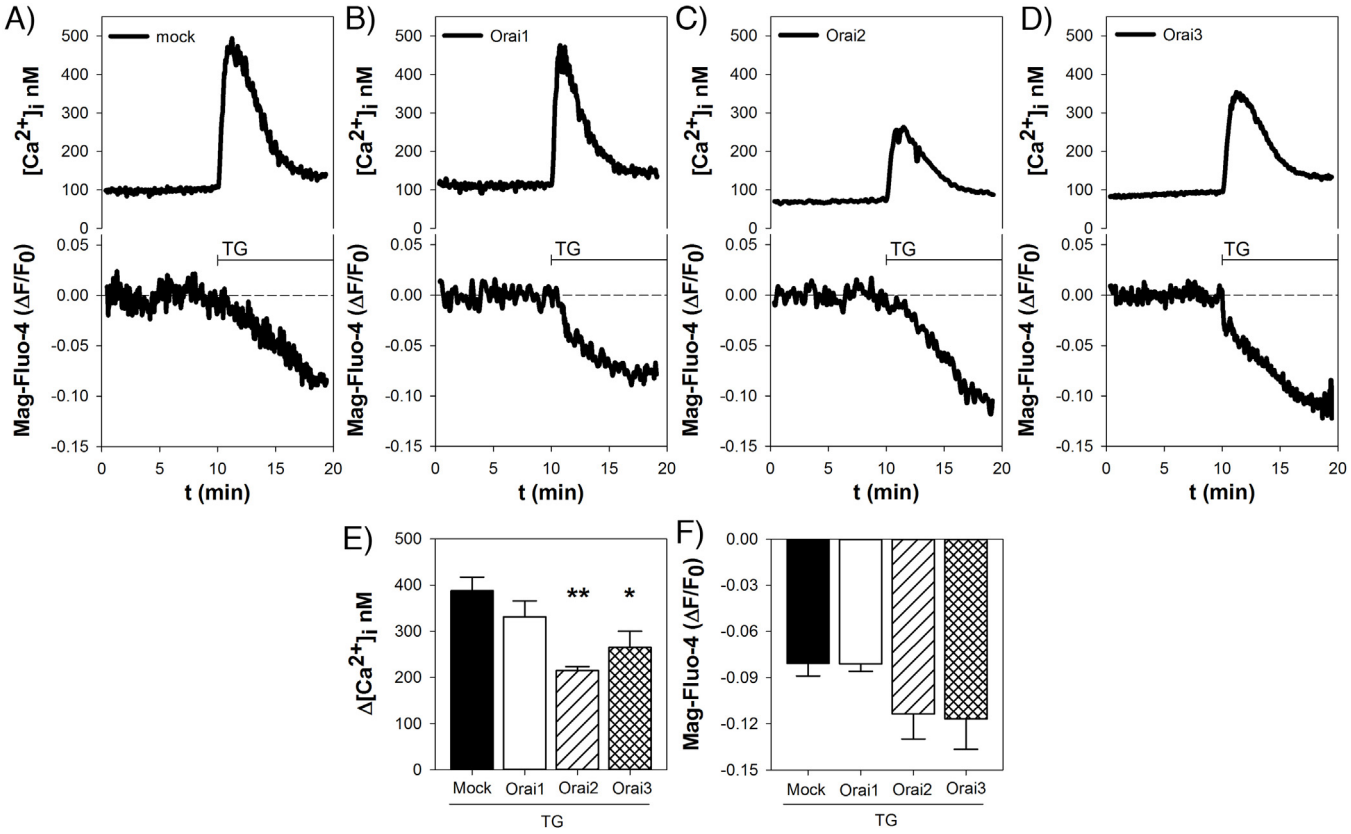

Fig. 4. The effect of Orai channels overexpression on the TG-induced ER Ca^{2+} leak. HeLa cells that were transfected with A) an empty vector, B) Orai1, C) Orai2 and D) Orai3 were placed in the presence of 1.8 mM external [Ca^{2+}] and simultaneously recorded the $[Ca^{2+}]_i$ (upper panel) and $[Ca^{2+}]_{ER}$ (lower panel) responses when exposed to TG at the times indicated. E) Overexpression of Orai2 or Orai3 clearly decreased the $[Ca^{2+}]_i$ response to TG. F) The ER Ca^{2+} leak after TG was not modified by the expression of any Orai channels.

a hint of which Orai channel is responding to 2-APB in the ER, we have overexpressed each one of the three Orai channels and determined the effect on 2-APB-induced and TG-revealed ER Ca^{2+} leaks in the presence of external [Ca^{2+}]. As an initial approach we have determined the effect of Orai channels on the TG-revealed ER Ca^{2+} leak (Fig. 4). The overexpression of Orai1 did not change the $[Ca^{2+}]_i$ response (Fig. 4B, E). However, the overexpression of either Orai2 or Orai3 resulted in a smaller TG-induced $[Ca^{2+}]_i$ response (Fig. 4C, D and E), suggesting that these two channels can behave as an ER Ca^{2+} leak. Importantly, the overexpression of any Orai channel did not change the reduction in the $[Ca^{2+}]_{ER}$ in response to TG. If anything, Orai2 and Orai3 displayed a slightly larger reduction in the luminal $[Ca^{2+}]_{ER}$ but this difference did not reach significance (Fig. 4F). The same experiment, but in this case cells were exposed to 50 μM 2-APB for 10 min before the application of TG showed that mock cells had the typical reduction in the $[Ca^{2+}]_{ER}$ induced by 2-APB and further reduction in the $[Ca^{2+}]_{ER}$ after blocking SERCA pumps with TG, although presented a smaller $[Ca^{2+}]_i$ response (compare Fig. 4E with Fig. 5E). The overexpression of wild type Orai1 channel increased the $[Ca^{2+}]_i$ responses to 2-APB and to TG (Fig. 5B, E, open bars). However, it strongly inhibited the 2-APB- and the TG-revealed ER Ca^{2+} leaks (Fig. 5B, F, open bars). In the case of Orai2 overexpression, this channel did not produce any increase in the $[Ca^{2+}]_i$ in response to 2-APB and did not modify the $[Ca^{2+}]_i$ response after TG (Fig. 5C, E crossed bars). However, it produced a complete inhibition of the 2-APB-induced ER Ca^{2+} leak and also a strong reduction of the ER Ca^{2+} leak after TG (Fig. 5C, F, crossed bars). The overexpression of Orai3 produced a complete different set of responses on the $[Ca^{2+}]_{ER}$ (Fig. 5D). There was no significant effect on the $[Ca^{2+}]_i$ response after TG, if anything was slightly increased (Fig. 5E, crosshatched bar) and the 2-APB-induced reduction of the $[Ca^{2+}]_{ER}$ was also slightly increased (Fig. 5F, left crosshatched bar). The ER Ca^{2+} leak after TG was not modified by Orai3 channel (Fig. 5F, right crosshatched bar). In conclusion, the overexpression of Orai1 or Orai2 strongly inhibited the 2-APB-induced reduction of the $[Ca^{2+}]_{ER}$ (Fig. 5F) and also the ER Ca^{2+} leak after TG but only when this has been preceded by the addition of 2-APB. These data can be explained if it is assumed that Orai channels form heteromultimers but only the homomultimers of Orai3 channels are responding to 2-APB. We can argue that the channel activated by 2-APB helps in the reduction of the $[Ca^{2+}]_{ER}$ after TG and the most likely candidate for this role is Orai3, while Orai2 and Orai1 would have inhibitory roles in the response to 2-APB by having a "dilution effect" of pure Orai3 channels. Collectively these data suggest that Orai3 channel, more than Orai2 or Orai1, is responsible for the 2-APB-induced reduction in the $[Ca^{2+}]_{ER}$.

3.4. *Reducing the expression of Orai3 inhibited the 2-APB-induced ER Ca^{2+} leak*

We have used siRNA against Orai3 to reduce the expression of this channel and to test the idea that this is the channel responding to 2-APB in the ER. This particular set of siRNAs against Orai3 channel specifically decreased Orai3 mRNA because the mRNAs of the two other channels were not decreased (Fig. S2). The expression of Orai3 channel at the protein level was also clearly decreased after 48 h (Fig. S2). The reduction in the expression of Orai3 channel greatly decreased the effect of 2-APB on the $[Ca^{2+}]_{ER}$ (Fig. 6A solid line, 6C closed bar) when compared to the effect of 2-APB on cells transfected with a siRNA control (Fig. 6A dotted line, Fig. 6C open

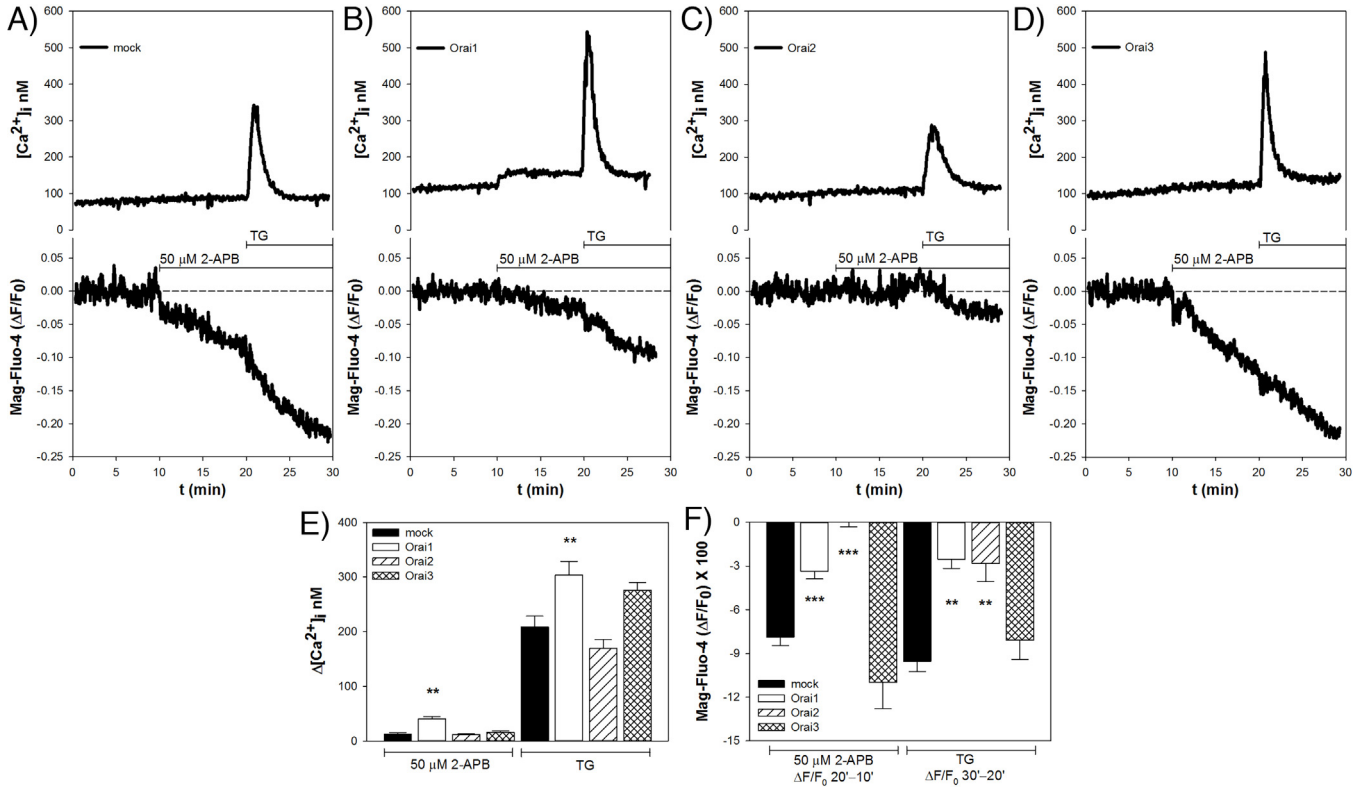

Fig. 5. Expression of only Orai3 channel supported the 2-APB-induced ER Ca^{2+} leak. HeLa cells that were transfected with A) an empty vector, B) Orai1, C) Orai2 and D) Orai3 were in the presence of 1.8 mM external $[Ca^{2+}]$ and simultaneously recorded the $[Ca^{2+}]_i$ (upper panel) and $[Ca^{2+}]_{ER}$ (lower panel) responses when exposed to 2-APB followed by TG at the times indicated. E) Overexpression of Orai1 increased the $[Ca^{2+}]_i$ response to 2-APB addition (left open bar) and also potentiated the $[Ca^{2+}]_i$ after TG (right open bar). F) The 2-APB-induced ER Ca^{2+} leak (left closed bar) was partially inhibited by Orai1 (left open bar), strongly inhibited by Orai2 (left dashed bar), and slightly increased by Orai3 (left hatched bar). The ER Ca^{2+} leak after TG was also inhibited by either Orai1 (right open bar) or Orai2 (right dashed bar) and not affected by Orai3 (right hatched bar).

bar). However, the $[Ca^{2+}]_i$ response after TG was not affected by SiRNA for Orai3 (Fig. 6A, B). Collectively, these data say that Orai3 is the channel responsible for the reduction in the $[Ca^{2+}]_{ER}$ induced by 2-APB but it seems this channel does not participate in the ER Ca^{2+} leak after TG. Additionally, these data are in agreement with data shown in the companion paper that the channel activated by 2-APB is not involved in the ER Ca^{2+} leak after TG [19].

3.5. Reducing the expression of Orai3 modified the ER Ca^{2+} leak after TG

To verify that Orai3 is not participating in the ER Ca^{2+} leak after TG, we have knocked down Orai3 and we have assessed ER Ca^{2+} leak by adding TG in the absence of external $[Ca^{2+}]$. The reduction in the expression of Orai3 channel modified neither the $[Ca^{2+}]$i response after TG (Fig. 7A, B) nor its associated reduction in the $[Ca^{2+}]_{ER}$ (Fig. 7A, 7C). The addition of 1.8 mM $[Ca^{2+}]$ showed that Orai3 channel did not participate in SOCE in HeLa cells (Fig. 7A, B). Unexpectedly, the reduction in the expression of Orai3 channel resulted in a prompt reduction in the $[Ca^{2+}]_{ER}$ after TG, since the lag time for this effect was clearly reduced (Fig. 7D solid bar) and the ER $[Ca^{2+}]$ was lower at 1 min after the addition of TG (Fig. 7A dashed line, E solid bar). Interestingly, the rate of reduction of the $[Ca^{2+}]_{ER}$ after TG was practically the same (Fig. 7A, C), the only difference was the onset, after TG, for the reduction in the $[Ca^{2+}]_{ER}$. It appears then that Orai3 channels are not involved in the ER Ca^{2+} leak after TG.

3.6. Overloading the ER Ca^{2+} store with bafilomycin unveiled a role for Orai3 channels

We have hypothesized that Orai3 channels might be functioning as a safety mechanism to avoid or reduce the overloading of the ER Ca^{2+} store. To test this, we have overloaded the ER Ca^{2+} store by applying bafilomycin to eliminate the acidic intracellular Ca^{2+} stores, a maneuver that results in a clear elevation of the $[Ca^{2+}]_{ER}$ (Fig. 8A, lower panel) and compared with $[Ca^{2+}]_i$ response after TG in cells transfected with control siRNA (Fig. 8A, dotted line) and those transfected with an Orai3 siRNA (solid line). In this condition, the ER Ca^{2+} store is overloaded because the $[Ca^{2+}]_i$ responses after TG are larger than in the absence of bafilomycin (compare Fig. 1D, E vs Fig. 8A, B) and this was also reflected in an increase of the $[Ca^{2+}]_{ER}$ (Fig. 8C, left bars). In the presence of bafilomycin, the reduction in Orai3 channel expression, further increased the level of ER Ca^{2+} store because the TG-induced $[Ca^{2+}]_i$ response was clearly larger (Fig. 8A, B) although we could not detect that in the $[Ca^{2+}]_{ER}$ (Fig. 8C). Alternatively, this larger $[Ca^{2+}]_i$ response after TG might be due to the new ER Ca^{2+} leak that appears to be present (Fig. 8A, solid line in lower panel) when Orai3 expression had been reduced. Collectively, these data suggest that Orai3 channels are located in the ER and function as a safety mechanism to avoid overloading of the ER Ca^{2+} store in HeLa cells. This idea is different to the proposed one that Orai channels function as leak channels in the ER, the main difference is that leak channels are permanently open and balancing the activity of SERCA pumps, while it appears that Orai3 channel is not active all the time.

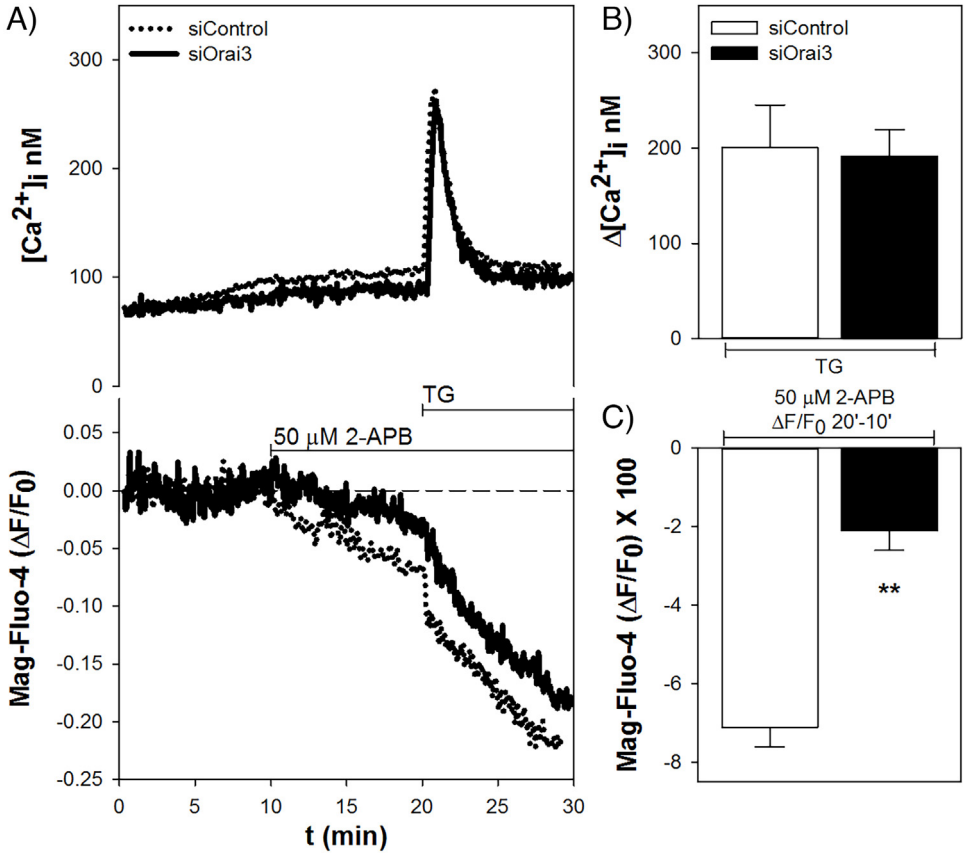

Fig. 6. Reducing the expression of Orai3 reduced the 2-APB-induced ER Ca^{2+} leak. A) Cells transfected with a siRNA control (dotted line) or with an Orai3 siRNA (solid line) were placed in 1.8 mM external [Ca^{2+}] and exposed to 2-APB followed by TG at the indicated times. B) The $[Ca^{2+}]_i$ response after TG was not altered by this condition. C) The 2-APB-induced ER Ca^{2+} leak was significantly inhibited by Orai3 siRNA (solid bar).

4. Discussion

Our data corroborate previous reports showing that Orai3 channel is expressed in the ER membrane of HeLa cells [10]. Additionally, we show that this channel appears to be activated by 2-APB and reduces the luminal $[Ca^{2+}]_{ER}$ but it does not seem to be involved in the ER Ca^{2+} leak observed after TG. Actually, it appears that the sensitivity of Orai3 channel to 2-APB depends on the luminal [Ca^{2+}] in the ER [19]. Additionally, we have observed that Orai3 regulates the ER Ca^{2+} level but only in conditions that the ER store was overloaded with Ca^{2+} by inhibiting with bafilomycin the Ca^{2+} buffering capacity of the acidic Ca^{2+} stores. These data suggest that Orai3 channel is not a bona fide ER Ca^{2+} leak that is open all the time, but it appears that this channel functions as a mechanism to avoid overloading of the ER Ca^{2+} store.

Application of TG, which potently and specifically inhibits SERCA pump activity, should result in a transient increase of the $[Ca^{2+}]_i$ due to Ca^{2+} release from the ER via leak channels that are permanently open. However, this outcome does not occur in all the cases because freshly isolated smooth muscle cells that were exposed to TG showed neither a transient increase of the $[Ca^{2+}]_i$ nor a clear reduction in the $[Ca^{2+}]_{ER}$ [28,32], suggesting that these cells do not have an SR Ca^{2+} leak at least under those recording conditions. The major limitation here is that the molecular nature of the ER Ca^{2+} leak has not been identified yet. It appears that the ER Ca^{2+} leak is carried out by at least two different types of Ca^{2+} permeable ion channels. If this is the case then it might be the explanation behind the generalized observation that application of TG produces a basically immediate and transient increase in the $[Ca^{2+}]_i$ while a delayed reduction in the $[Ca^{2+}]_{ER}$ [33–37]. This lack of correlation between the $[Ca^{2+}]_i$ response and the reduction in the $[Ca^{2+}]_{ER}$ might be explained by the existence of at least two different types of ER Ca^{2+} leak channels. It has been proposed that IP_3R participates in the ER Ca^{2+} leak [14,38–40] and it has been found that cells with knocked-out IP_3Rs type II and III basically eliminates the $[Ca^{2+}]_i$ response to TG but still SOCE is turned on by TG, suggesting that other channel was involved in decreasing the ER Ca^{2+} store [41]. In this sense we have observed that 2-APB, an inhibitor of IP_3Rs, is able to partially inhibit the ER Ca^{2+} leak, which is revealed by inhibition of SERCA pump with TG. Fig. 2 shows that 2-APB reduces by half the $[Ca^{2+}]_i$ response after TG although it fully inhibits the reduction in the $[Ca^{2+}]_{ER}$ in those cells expressing the dominant negative Orai1 E106A mutant. These data suggest that ER Ca^{2+} leak involves at least two different channels, one that is inhibited by 2-APB and is evident in the late phase of ER depletion by eliminating the activating effect of 2-APB on Orai channels, while the other channel would not be that sensitive to 2-APB and participates in the early phase of ER depletion and to a large extent in the $[Ca^{2+}]_i$ response.

This scenario of a permanently open ER Ca^{2+} leak channel should result in an immediate reduction in the $[Ca^{2+}]_{ER}$ with a mono-exponential time course together with a transient increase of the $[Ca^{2+}]_i$, the latter is in general observed while the former is seldom seen when the $[Ca^{2+}]_i$ and the $[Ca^{2+}]_{ER}$ are simultaneously recorded in response to TG. Here, we have observed that the application of this inhibitor produces an immediate increase of the $[Ca^{2+}]_i$ with no or minimal reduction of the $[Ca^{2+}]_{ER}$. Nevertheless, this situation has been observed in many different cell types [35–37,42–44]. To us this observation is completely unexpected and there is no explanation for it, except that IP_3Rs might be involved. In any event this

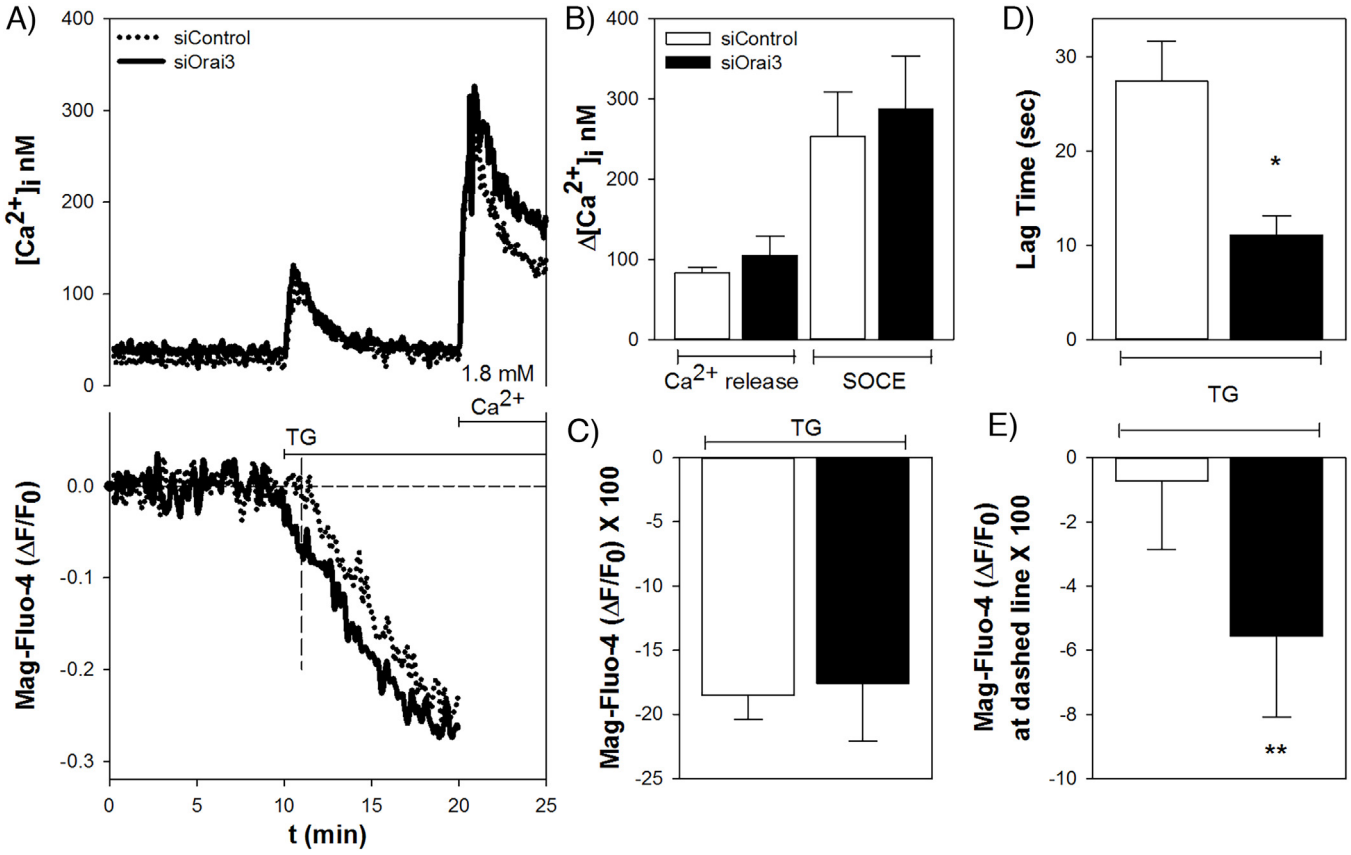

Fig. 7. Reducing the expression of Orai3 does not affect SOCE. A) $[Ca^{2+}]_i$ response and the $[Ca^{2+}]_{ER}$ from cells transfected with a siRNA control (dotted line) or with an Orai3 siRNA (solid line) were recorded in the absence of external $[Ca^{2+}]$ (0.1 mM EGTA) while being exposed to TG followed by the addition of 1.8 mM $[Ca^{2+}]$ at the indicated times. B) Neither TG-induced $[Ca^{2+}]_i$ response nor SOCE were modified by reducing the expression of Orai3. C) The extent of ER Ca^{2+} leak was not affected either. D) Reduction in the $[Ca^{2+}]_{ER}$ by TG has a characteristic long delay that was clearly decreased by decreasing the expression of Orai3 channel (solid bar). E) The reduction in the $[Ca^{2+}]_{ER}$ after TG was larger at 1 min after the addition of TG (dashed line in A).

means that we are still far from understanding the ER Ca^{2+} leak revealed by TG.

It is important to distinguish between a true ER Ca^{2+} leak that would be open irrespective of the luminal $[Ca^{2+}]_{ER}$ as opposed to a channel that would open once the luminal $[Ca^{2+}]_{ER}$ has reached certain threshold. The main difference is for SERCA pump because in the former case the pump will have an inefficient activity since the Ca^{2+} stored is immediately leaked out by the permanently open channel, while this would not happen in the latter case, because the channel would not be open until the ER store has been filled. This scenario is not entirely new. It has been shown in pancreatic β cells that activation of an atypical CICR involves a still undetermined type of ER Ca^{2+} channel that eliminates the excess of Ca^{2+} to the cytoplasm once the ER store has been loaded and this type of channel deactivates after the ER Ca^{2+} store has been depleted [45,46]. Since the nature of this channel has not been determined, it is difficult to elaborate any further on this interesting regulatory mechanism. However, the direct measure of changes in the $[Ca^{2+}]_{ER}$ clearly shows that this channel is closed when the ER store has been depleted of Ca^{2+}, and it activates with certain delay when the ER store has been replenished with Ca^{2+} due to the action of SERCA pumps [46]. Again, this implies that this type of channel is somehow regulated by the luminal $[Ca^{2+}]_{ER}$.

It has been reported that TMCO1, a protein widely expressed in the ER, forms a Ca^{2+} permeable channel in response to the overloading with Ca^{2+} of the ER store [47]. Although in this case, overloading the ER Ca^{2+} store was achieved with an extremely high concentration of ethanol (8%), so it is not clear how exactly this channel is operating in physiological conditions. Nevertheless, this work stresses the idea that the activity of ER Ca^{2+} leak channels might be regulated by the $[Ca^{2+}]_{ER}$, in other words they are not active when $[Ca^{2+}]_{ER}$ is low and would activate to avoid overloading of the ER Ca^{2+} store.

It has been proposed that Orai2 might be functioning as an ER Ca^{2+} leak channel [14] because lowering its expression level increases the $[Ca^{2+}]_{ER}$ while its overexpression decreases the $[Ca^{2+}]_{ER}$. We have also observed that overexpression of Orai2 channel decreased the TG-induced $[Ca^{2+}]_i$ response, arguing for a smaller ER Ca^{2+} store. However, it appears that this situation is not that straightforward because Orai2 does not seem to localize in the ER. Additionally, the expression of the dominant negative Orai1 E106A mutant inhibited the 2-APB-induced ER Ca^{2+} leak but did not have any effect on the ER Ca^{2+} leak, arguing against the idea that Orai channels have any role in the ER Ca^{2+} leak revealed after inhibition of SERCA pumps. On the other hand we know that Orai channels can have leak activity (see Introduction) in the presence of Ca^{2+} and in the absence of STIM. Moreover, the overexpression of the dominant negative mutant Orai1 E106A results in a slightly higher $[Ca^{2+}]_{ER}$ determined with D1ER [9]. These data could be interpreted as Orai1 might be also an ER Ca^{2+} leak channel. However, our data show that this mutant inhibited the effect of 2-APB on the $[Ca^{2+}]_{ER}$ that is mediated by Orai3 channel, in agreement with the idea that Orai1 E106A can inhibit the activity of all three types of Orai channels because they form heteromultimers [4,31]. Therefore, these data suggest that Orai channel, is not clear whether Orai2 or Orai3, is involved in regulating the $[Ca^{2+}]_{ER}$. In the companion paper, we have shown that 2-APB opens an ER Ca^{2+} leak channel and that this effect can be inhibited when the ER Ca^{2+} store has been

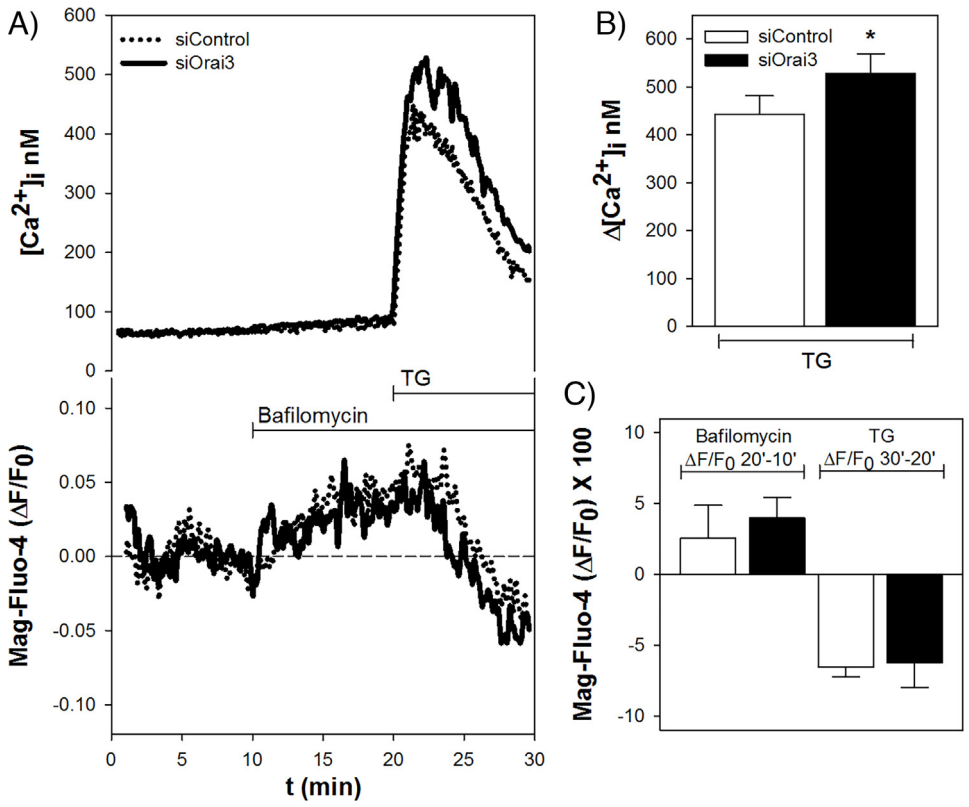

Fig. 8. Reduction of Orai3 expression increased the $[Ca^{2+}]_i$ response to TG when the acidic Ca^{2+} buffering system has been eliminated with bafilomycin. A) HeLa cells that were transfected with either siRNA control (dotted line) or Orai3 siRNA (solid line) and placed in 1.8 mM external $[Ca^{2+}]$ were exposed to 100 nM bafilomycin followed by TG at the time indicated. B) The $[Ca^{2+}]_i$ response after TG was increased by the application of bafilomycin (compare this with the response shown in Fig. 1E [open bar]) and was further increased by reducing the expression of Orai3 channels (solid bar). C) The application of bafilomycin resulted in an increase of the $[Ca^{2+}]_{ER}$ that was not affected by reducing the expression of Orai3 (solid bar).

partly depleted by incubating cells in the absence of external $[Ca^{2+}]$ or with TG [19]. We now know, based on the work shown here, that this channel responding to 2-APB is Orai3 in the ER of HeLa cells. It appears then that Orai2 and Orai3 channels can alter luminal $[Ca^{2+}]$ of intracellular compartments, but how do they do that is not that clear, at the very least we can discard that these channels are permanently open and functioning as a true leak channels. Our data show that Orai3 and IP_3R colocalize suggesting that the former channel locates in the ER. This is not new since it has been shown that Orai3 colocalizes with BAP31 protein, another marker of ER [10]. Additionally, the overexpression of Orai3 channel decreased the TG-induced $[Ca^{2+}]_i$ response, suggesting that this channel, similarly to Orai2, decreases the ER Ca^{2+} store when is overexpressed. Therefore, it can be concluded that Orai3 resides in the ER membrane of HeLa cells. It has been shown that Orai3 needs Orai1 to reach the plasma membrane because it lacks a tripeptide EFA [12]. In our case the overexpression of Orai1 produced an increase in the $[Ca^{2+}]_i$ in response to 2-APB, but only in the presence of external Ca^{2+} (data not shown). However, Orai3 did not show any increase in the $[Ca^{2+}]_i$ in response to 2-APB. These data argue for, in our recording conditions, Orai1 goes readily to the plasma membrane while Orai3 stays in intracellular compartments, most likely the ER.

It has been found that Orai3 is overexpressed in different types of cancer cells [48–51] and apparently this situation increases survival of these cells [52]. However, it is not clear how Orai3 is doing this. Furthermore, VEGF-induced Ca^{2+} release from intracellular stores requires the expression of Orai3 channels [53]. In conclusion it appears that Orai3 channel might be functioning as an ER Ca^{2+} release channel under very particular conditions because is not permanently open and is not involved in the ER Ca^{2+} leak revealed by TG.

Funding

Daniel Leon-Aparicio (233231), Jonathan Pacheco (372743) and Jesus Chavez-Reyes (348233) are Ph. D. students supported by fellowships (indicated in parenthesis) by Conacyt. This work was partly supported by Conacyt grant 128735 to AGH, grant 236104 to JV and grants from the Dirección General de Asuntos del Personal Académico (DGAPA) and CONACYT 127822 to LV.

Acknowledgment

We thank Norma Cirnes for the design of graphical abstract.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ceca.2017.01.012.

References

[1] R.M. Luik, M.M. Wu, J. Buchanan, R.S. Lewis, The elementary unit of store-operated Ca^{2+} entry: local activation of CRAC channels by STIM1 at ER-plasma membrane junctions, J. Cell Biol. 174 (2006) 815–825, http://dx.doi.org/10.1083/jcb.200604015.
[2] M.M. Wu, J. Buchanan, R.M. Luik, R.S. Lewis, Ca2+ store depletion causes STIM1 to accumulate in ER regions closely associated with the plasma membrane, J. Cell Biol. 174 (2006) 803–813, http://dx.doi.org/10.1083/jcb.200604014.
[3] J.C. Mercer, W.I. Dehaven, J.T. Smyth, B. Wedel, R.R. Boyles, G.S. Bird, et al., Large store-operated calcium selective currents due to co-expression of Orai1 or Orai2 with the intracellular calcium sensor, Stim1, J. Biol. Chem. 281 (2006) 24979–24990, http://dx.doi.org/10.1074/jbc.M604589200.
[4] A. Lis, C. Peinelt, A. Beck, S. Parvez, M. Monteilh-Zoller, A. Fleig, et al., CRACM1, CRACM2, and CRACM3 are store-operated Ca^{2+} channels with distinct

functional properties, Curr. Biol. 17 (2007) 794–800, http://dx.doi.org/10.1016/j.cub.2007.03.065.
[5] Y. Gwack, S. Srikanth, S. Feske, F. Cruz-Guilloty, M. Oh-hora, D.S. Neems, et al., Biochemical and functional characterization of orai proteins, J. Biol. Chem. 282 (2007) 16232–16243, http://dx.doi.org/10.1074/jbc.M609630200.
[6] I. Frischauf, M. Muik, I. Derler, J. Bergsmann, M. Fahmer, R. Schindl, et al., Molecular determinants of the coupling between STIM1 and Orai channels: differential activation of Orai1-3 channels by a STIM1 coiled-coil mutant, J. Biol. Chem. 284 (2009) 21696–21706, http://dx.doi.org/10.1074/jbc.M109.018408.
[7] M. Prakriya, S. Feske, Y. Gwack, S. Srikanth, A. Rao, P.G. Hogan, Orai1 is an essential pore subunit of the CRAC channel, Nature 443 (2006) 230–233, http://dx.doi.org/10.1038/nature05122.
[8] S. Feske, Y. Gwack, M. Prakriya, S. Srikanth, S.-H. Puppel, B. Tanasa, et al., A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function, Nature 441 (2006) 179–185, http://dx.doi.org/10.1038/nature04702.
[9] E.J. Dickson, J.G. Duman, M.W. Moody, L. Chen, B. Hille, Orai-STIM-mediated Ca^{2+} release from secretory granules revealed by a targeted Ca^{2+} and pH probe, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) E3539–E3548, http://dx.doi.org/10.1073/pnas.1218247109.
[10] S. Varadarajan, K. Tanaka, J.L. Smalley, E.T.W. Bampton, M. Pellecchia, D. Dinsdale, et al., Endoplasmic reticulum membrane reorganization is regulated by ionic homeostasis, PLoS One 8 (2013) e56603, http://dx.doi.org/10.1371/journal.pone.0056603.
[11] M. Ikeya, K. Yamanoue, Y. Mochizuki, H. Konishi, S. Tadokoro, M. Tanaka, et al., Orai-2 is localized on secretory granules and regulates antigen-evoked Ca^{2+} mobilization and exocytosis in mast cells, Biochem. Biophys. Res. Commun. 451 (2014) 62–67, http://dx.doi.org/10.1016/j.bbrc.2014.07.060.
[12] D. Alansary, I. Bogeski, B.A. Niemeyer, Facilitation of Orai3 targeting and store-operated function by Orai1, Biochim. Biophys. Acta Mol. Cell Res. 1853 (2015) 1541–1550, http://dx.doi.org/10.1016/j.bbamcr.2015.03.007.
[13] Y. Zhou, P. Meraner, H.T. Kwon, D. Machnes, M. Oh-hora, J. Zimmer, et al., STIM1 gates the store-operated calcium channel ORAI1 in vitro, Nat. Struct. Mol. Biol. 17 (2010) 112–126, http://dx.doi.org/10.1038/nsmb.1724.
[14] S. Bandara, S. Malmersjö, T. Meyer, Regulators of calcium homeostasis identified by inference of kinetic model parameters from live single cells perturbed by siRNA, Sci. Signal. 6 (2013) ra56, http://dx.doi.org/10.1126/scisignal.2003649.
[15] T. Maruyama, T. Kanaji, S. Nakade, T. Kanno, K. Mikoshiba, 2APB 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of ins(1,4,5)P3-induced Ca^{2+} release, J. Biochem. 122 (1997) 498–505.
[16] W.I. DeHaven, J.T. Smyth, R.R. Boyles, G.S. Bird, J.J.W. Putney, Complex actions of 2-aminoethyldiphenyl borate on store-operated calcium entry, J. Biol. Chem. 283 (2008) 19265–19273, http://dx.doi.org/10.1074/jbc.M801535200.
[17] X. Xu, S. Ali, Y. Li, H. Yu, M. Zhang, J. Lu, et al., 2-Aminoethoxydiphenyl borate potentiates CRAC current by directly dilating the pore of open Orai1, Sci. Rep. 6 (2016) 29304, http://dx.doi.org/10.1038/srep29304.
[18] R. Schindl, J. Bergsmann, I. Frischauf, I. Derler, M. Fahrner, M. Muik, et al., 2-Aminoethoxydiphenyl borate alters selectivity of Orai3 channels by increasing their pore size, J. Biol. Chem. 283 (2008) 20261–20267, http://dx.doi.org/10.1074/jbc.M803101200.
[19] D. León-Aparicio, A. Guerrero-Hernandez, Activation of endoplasmic reticulum calcium leak by 2-APB depends on the luminal calcium concentracion, Cell Calcium (2016), http://dx.doi.org/10.1016/j.ceca.2017.01.013.
[20] G. Grynkiewicz, M. Poenie, R.Y. Tsien, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, J. Biol. Chem. 260 (1985) 3440–3450.
[21] M. Poenie, Alteration of intracellular Fura-2 fluorescence by viscosity: a simple correction, Cell Calcium 11 (1990) 85–91, http://dx.doi.org/10.1016/0143-4160(90)90062-Y.
[22] A.V. Shmigol, D.A. Eisner, S. Wray, Simultaneous measurements of changes in sarcoplasmic reticulum and cytosolic $[Ca^{2+}]$ in rat uterine smooth muscle cells, J. Physiol. 531 (2001) 707–713, http://dx.doi.org/10.1111/j.1469-7793.2001.0707h.x.
[23] M.K. Park, K.K. Lee, D.-Y. Uhm, Slow depletion of endoplasmic reticulum Ca^{2+} stores and block of store-operated Ca^{2+} channels by 2-aminoethoxydiphenyl borate in mouse pancreatic acinar cells, Naunyn. Schmiedebergs. Arch. Pharmacol. 365 (2002) 399–405, http://dx.doi.org/10.1007/s00210-002-0535-0.
[24] A. Shmygol, S. Wray, Modulation of agonist-induced Ca2+ release by SR Ca2+ load: direct SR and cytosolic Ca2+ measurements in rat uterine myocytes, Cell Calcium 37 (2005) 215–223, http://dx.doi.org/10.1016/j.ceca.2004.10.002.
[25] A.J. Laude, S.C. Tovey, S.G. Dedos, B.V.L. Potter, S.C.R. Lummis, C.W. Taylor, Rapid functional assays of recombinant IP3 receptors, Cell Calcium 38 (2005) 45–51, http://dx.doi.org/10.1016/j.ceca.2005.04.001.
[26] M. Flourakis, F. Van Coppenolle, V. Lehen'kyi, B. Beck, R. Skryma, N. Prevarskaya, Passive calcium leak via translocon is a first step for iPLA 2 −pathway regulated store operated channels activation, FASEB J. 20 (2006) E409–E416, http://dx.doi.org/10.1096/fj.05-5254fje.
[27] Y.M. Choi, S.H. Kim, S. Chung, D.Y. Uhm, M.K. Park, Regional interaction of endoplasmic reticulum Ca^{2+} signals between soma and dendrites through rapid luminal Ca^{2+} diffusion, J. Neurosci. 26 (2006) 12127–12136, http://dx.doi.org/10.1523/JNEUROSCI.3158-06.2006.
[28] A. Dagnino-Acosta, A. Guerrero-Hernández, Variable luminal sarcoplasmic reticulum Ca^{2+} buffer capacity in smooth muscle cells, Cell Calcium 46 (2009) 188–196, http://dx.doi.org/10.1016/j.ceca.2009.07.005.
[29] A. Zweifach, R.S. Lewis, Rapid inactivation of depletion-activated calcium current (ICRAC) due to local calcium feedback, J. Gen. Physiol. 105 (1995) 209–226, http://dx.doi.org/10.1085/jgp.105.2.209.
[30] L. Fierro, A.B. Parekh, Fast calcium-dependent inactivation of calcium release-activated calcium current (CRAC) in RBL-1 cells, J. Membr. Biol. 168 (1999) 9–17, http://dx.doi.org/10.1007/s002329900493.
[31] O. Mignen, J.L. Thompson, T.J. Shuttleworth, Both Orai1 and Orai3 are essential components of the arachidonate-regulated Ca^{2+}-selective (ARC) channels, J. Physiol. 586 (2008) 185–195, http://dx.doi.org/10.1113/jphysiol.2007.146258.
[32] M. Potier, J.C. Gonzalez, R.K. Motiani, I.F. Abdullaev, J.M. Bisaillon, H.A. Singer, et al., Evidence for STIM1- and Orai1-dependent store-operated calcium influx through ICRAC in vascular smooth muscle cells: role in proliferation and migration, FASEB J. 23 (2009) 2425–2437, http://dx.doi.org/10.1096/fj.09-131128.
[33] A.E. Palmer, C. Jin, J.C. Reed, R.Y. Tsien, Bcl-2-mediated alterations in endoplasmic reticulum Ca^{2+} analyzed with an improved genetically encoded fluorescent sensor, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 17404–17409, http://dx.doi.org/10.1073/pnas.0408030101.
[34] K. Ishii, K. Hirose, M. Iino, Ca^{2+} shuttling between endoplasmic reticulum and mitochondria underlying Ca^{2+} oscillations, EMBO Rep. 7 (2006) 390–396, http://dx.doi.org/10.1038/sj.embor.7400620.
[35] D.S.T. Ong, T.-W. Mu, A.E. Palmer, J.W. Kelly, Endoplasmic reticulum Ca^{2+} increases enhance mutant glucocerebrosidase proteostasis, Nat. Chem. Biol. 6 (2010) 424–432, http://dx.doi.org/10.1038/nchembio0810-630a.
[36] P. Gilon, H.-Y. Chae, G.A. Rutter, M.A. Ravier, Calcium signaling in pancreatic β-cells in health and in Type 2 diabetes, Cell Calcium 56 (2014) 340–361, http://dx.doi.org/10.1016/j.ceca.2014.09.001.
[37] S. Fu, A. Yalcin, G.Y. Lee, P. Li, J. Fan, A.P. Arruda, et al., Phenotypic assays identify azoramide as a small-molecule modulator of the unfolded protein response with antidiabetic activity, Sci. Transl. Med. 7 (2015) 292ra98, http://dx.doi.org/10.1126/scitranslmed.aaa9134.
[38] S.A. Oakes, L. Scorrano, J.T. Opferman, M.C. Bassik, M. Nishino, T. Pozzan, et al., Proapoptotic BAX and BAK regulate the type 1 inositol trisphosphate receptor and calcium leak from the endoplasmic reticulum, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 105–110, http://dx.doi.org/10.1073/pnas.0408352102.
[39] K. Szlufcik, L. Missiaen, J.B. Parys, G. Callewaert, H. De Smedt, Uncoupled IP3 receptor can function as a Ca2+-leak channel: cell biological and pathological consequences, Biol. Cell 98 (2006) 1–14, http://dx.doi.org/10.1042/BC20050031.
[40] B. Boutin, N. Tajeddine, G. Monaco, J. Molgo, D. Vertommen, M. Rider, et al., Endoplasmic reticulum Ca^{2+} content decrease by PKA-dependent hyperphosphorylation of type 1 IP3 receptor contributes to prostate cancer cell resistance to androgen deprivation, Cell Calcium 57 (2015) 312–320, http://dx.doi.org/10.1016/j.ceca.2015.02.004.
[41] G. Lur, M.W. Sherwood, E. Ebisui, L. Haynes, S. Feske, R. Sutton, et al., $InsP_3$ receptors and Orai channels in pancreatic acinar cells: co-localization and its consequences, Biochem. J. 436 (2011) 231–239, http://dx.doi.org/10.1042/BJ20110083.
[42] N. Li, J.-Y. Sul, P.G. Haydon, A calcium-induced calcium influx factor, nitric oxide, modulates the refilling of calcium stores in astrocytes, J. Neurosci. 23 (2003) 10302–10310 (23/32/10302[pii]).
[43] Y.M. Usachev, A.J. Marsh, T.M. Johanns, M.M. Lemke, S.A. Thayer, Activation of protein kinase C in sensory neurons accelerates Ca2+ uptake into the endoplasmic reticulum, J. Neurosci. 26 (2006) 311–318, http://dx.doi.org/10.1523/jneurosci.2920-05.2006.
[44] C. Betz, D. Stracka, C. Prescianotto-baschong, M. Frieden, N. Demaurex, mTOR complex 2-Akt at mitochondria-associated endoplasmic reticulum membranes (MAM) regulates mitochondrial physiology, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 12526, http://dx.doi.org/10.1073/pnas.1302455110.
[45] M.C. Beauvois, A. Arredouani, J.-C. Jonas, J.-F. Rolland, F. Schuit, J.-C. Henquin, et al., Atypical Ca^{2+}-induced Ca^{2+} release from a sarco-endoplasmic reticulum Ca^{2+}-ATPase 3-dependent Ca^{2+} pool in mouse pancreatic β-cells, J. Physiol. 559 (2004) 141–156, http://dx.doi.org/10.1113/jphysiol.2004.067454.
[46] M.A. Ravier, D. Daro, L.P. Roma, J.-C. Jonas, R. Cheng-Xue, F.C. Schuit, et al., Mechanisms of control of the free Ca^{2+} concentration in the endoplasmic reticulum of mouse pancreatic β-cells: interplay with cell metabolism and $[Ca^{2+}]$c and role of SERCA2b and SERCA3, Diabetes 60 (2011) 2533–2545, http://dx.doi.org/10.2337/db10-1543.
[47] Q.-C. Wang, Q. Zheng, H. Tan, B. Zhang, X. Li, Y. Yang, et al., TMCO1 is an ER Ca2+ load-activated Ca2+ channel, Cell 165 (2016) 1454–1466, http://dx.doi.org/10.1016/j.cell.2016.04.051.
[48] R.K. Motiani, I.F. Abdullaev, M. Trebak, A novel native store-operated calcium channel encoded by Orai3: selective requirement of Orai3 versus Orai1 in estrogen receptor-positive versus estrogen receptor-negative breast cancer cells, J. Biol. Chem. 285 (2010) 19173–19183, http://dx.doi.org/10.1074/jbc.M110.102582.
[49] M. Faouzi, P. Kischel, F. Hague, A. Ahidouch, N. Benzerdjeb, H. Sevestre, et al., ORAI3 silencing alters cell proliferation and cell cycle progression via c-myc pathway in breast cancer cells, Biochim. Biophys. Acta 1833 (2013) 752–760, http://dx.doi.org/10.1016/j.bbamcr.2012.12.009.
[50] R.K. Motiani, X. Zhang, K.E. Harmon, R.S. Keller, K. Matrougui, J. a Bennett, et al., Orai3 is an estrogen receptor α-regulated Ca^{2+} channel that promotes

tumorigenesis, FASEB J. 27 (2013) 63–75, http://dx.doi.org/10.1096/fj.12-213801.
[51] C. Dubois, F. Vanden Abeele, V. Lehen'kyi, D. Gkika, B. Guarmit, G. Lepage, et al., Remodeling of channel-forming ORAI proteins determines an oncogenic switch in prostate cancer, Cancer Cell. 26 (2014) 19–32, http://dx.doi.org/10.1016/j.ccr.2014.04.025.
[52] M. Faouzi, F. Hague, M. Potier, A. Ahidouch, H. Sevestre, H. Ouadid-Ahidouch, Down-regulation of Orai3 arrests cell-cycle progression and induces apoptosis in breast cancer cells but not in normal breast epithelial cells, J. Cell. Physiol. 226 (2011) 542–551, http://dx.doi.org/10.1002/jcp.22363.
[53] J. Li, A.F. Bruns, B. Hou, B. Rode, P.J. Webster, M.A. Bailey, et al., Orai3 surface accumulation and calcium entry evoked by vascular endothelial growth factor, Arterioscler. Thromb. Vasc. Biol. 35 (2015) 1987–1994, http://dx.doi.org/10.1161/ATVBAHA.115.305969.