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

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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.

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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-