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# Overexpression of TRPC3 Reduces the Content of Intracellular Calcium Stores in HEK-293 Cells

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The mammalian canonical transient receptor channels (TRPCs) are considered to be candidates for store-operated calcium channels (SOCCs). Many studies have addressed how TRPC3 channels are affected by depletion of intracellular calcium stores. Conflicting results have been shown for TRPC3 regarding its function, and this has been linked to its level of expression in various systems. In the present study, we have investigated how overexpression of TRPC3 interferes with the regulation of intracellular calcium stores. We demonstrate that overexpression of TRPC3 reduces the mobilization of calcium in response to stimulation of the cells with thapsigargin (TG) or the G-protein coupled receptor agonist sphingosine-1-phosphate (S1P). Our results indicate that this is the result of the expression of TRPC3 channels in the endoplasmic reticulum (ER), thus depleting ER calcium stores. OAG evoked calcium entry in cells overexpressing TRPC3, indicating that functional TRPC3 channels were also expressed in the plasma membrane. Taken together, our results show that overexpression of the putative SOCC, TRPC3, actually reduces the calcium content of intracellular stores, but does not enhance agonist-evoked or store-dependent calcium entry. Our results may, in part, explain the conflicting results obtained in previous studies on the actions of TRPC3 channels.

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Cells are controlled by calcium signaling during their whole lifespan. From the fertilization of the egg cell to the signaling that initiates cell death, the fate of the cell relies on spatiotemporal calcium signaling (Berridge et al., 2000, 2003). The primary intracellular calcium stores are in the endoplasmic reticulum (ER). After the initial stimuli of a cell, calcium is released from these stores, by an inositol 1,4,5-trisphosphate ($IP_3$) sensitive mechanism. This initial release of calcium is followed by a secondary rise in cytosolic calcium derived from an extracellular origin, which is called store-operated calcium entry (SOCE) (Berridge, 1995). The exact nature of the channels involved in SOCE has remained elusive despite intensive investigations.

The canonical transient receptor potential (TRPC) family may contain candidates for store-operated calcium channels (SOCCs). The TRPCs share substantial homology with the TRP channel in *Drosophila* (Montell et al., 2002a,b). Some TRPCs, as well as the *Drosophila* TRP channel, are activated downstream of phospholipase C (PLC) (Montell et al., 2002a). PLC activation results in cleaving of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) into $IP_3$ and diacylglycerol (DAG). Of these two second messengers, $IP_3$ releases calcium from the ER by binding to the $IP_3$ receptor. The main function for DAG is to activate protein kinase C (PKC). When the ER is locally depleted of calcium, it signals by an unknown mechanism to SOCCs to open, and replenish calcium stores. Recent investigations have put forward strong evidence for the involvement of two membrane proteins, Orai1 and STIM1, in regulating SOCE and the calcium release-activated current ($I_{CRAC}$) (Huang et al., 2006; Mercer et al., 2006; Peinelt et al., 2006; Spassova et al., 2006). Of these proteins, Orai1 apparently functions as a plasma membrane calcium channel, or as a channel subunit. STIM1 resides in the ER, and has been considered an ER calcium sensor, regulating SOCE and $I_{CRAC}$ upon depletion of the ER calcium stores. Further studies have shown that in addition to activating native SOCCs and $I_{CRAC}$, STIM1 also activates native TRPC1 channels (Huang et al., 2006; Lopez et al., 2006; Ong et al., 2007). STIM1 also binds to TRPC2, 4, and 5, but not to TRPC3, 6, 7 (Huang et al., 2006).

One of the most studied TRPCs is TRPC3. Conflicting results have been obtained regarding their function in SOCE. These results have been explained by differences in the expression level of the channels. Overexpression of TRPC3 has resulted in three main responses: constitutive activity (Zitt et al., 1997; McKay et al., 2000), a PLC-dependent activation (McKay et al., 2000; Venkatachalam et al., 2001) or a channel that is activated after store depletion (Vazquez et al., 2001; Trebak et al., 2002). Interestingly, the SOCC properties of TRPC3 are not present when the channel is transiently transfected in HEK-293 cells (McKay et al., 2000). A possible explanation for this is that when TRPC3 is expressed at a high concentration, which is often the case when transfecting cells transiently, other channel subunits are not present in sufficient amounts. This leads to mostly homomeric channels that are not activated by depletion of intracellular calcium stores (Vazquez et al., 2003; Putney, 2004). TRPC3 is also activated by the DAG analog 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) via a mechanism independent of PKC (Hofmann et al., 1999; Ma et al., 2000). In fact, PKC negatively regulates TRPC3 by phosphorylation of the channel protein at serine 712 (Trebak et al., 2004). Protein kinase G (PKG) has been shown to inhibit TRPC3 activity by phosphorylating the channel at threonine 11 and serine 263 (Kwan et al., 2004). As PKG is apparently activated by PKC,

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PKC may indirectly inhibit TRPC3 via activation of PKG (Kwan et al., 2006). Furthermore, the tyrosine kinase Src has been shown to modulate the activation of TRPC3 via DAG and receptor activation (Vazquez et al., 2004).

Earlier investigations on the regulation of TRPC3 have rendered conflicting results: overexpression of TRPC3 has indicated that it may function in a receptor-operated mode or as a SOCC, depending on the level of expression (Vazquez et al., 2003; Putney, 2004). In the present study, we wanted to further investigate the effects of overpression of TRPC3 on cellular calcium homeostasis. Several reports have indicated a localization of TRP channels in intracellular compartments (Turner et al., 2003; Wisnoskey et al., 2003; Sours et al., 2006). Furthermore, overexpression of TRP-channels has resulted in the increased localization of channel proteins in the ER (Hofmann et al., 2000, 2002; Hassock et al., 2002). We thus wanted to investigate how overexpression of TRPC3 affects the content of intracellular calcium stores. We used two plasmid constructs of TRPC3, one which was a fusion protein with green fluorescent protein (GFP), and demonstrated that overexpression of the constructs reduced the thapsigargin- (TG) or sphingosine-1-phosphate- (S1P) evoked calcium response. The cellular localization of TRPC3 has been shown to be the plasma membrane (Singh et al., 2004), but we show here that when overexpressed, apparently functional TRPC3 channels can also be expressed in intracellular calcium stores, mainly the ER. The expression of functional TRPC3 channels in the ER reduced the amount of sequestered calcium, resulting in decreased agonist-evoked calcium responses.

## Materials and Methods

### Materials

Fura-2 AM and mag-fura-2 AM were from Molecular Probes (Eugene, OR). Sphingosine-1-phosphate and inositol-1,4,5-trisphosphate were from Biomol (Plymouth Meeting, PA). Poly-L-lysine, 1-oleyl-2acetyl-*sn*-glycerol (OAG) and digitonin were from Sigma (St. Louis, MO). Ionomycin was from Calbiochem (Darmstadt, Germany). MEM-medium (with Earle's salts), L-glutamine, fetal bovine serum, penicillin and streptomycin were from GIBCO (Grand Island, NY). Thapsigargin (TG) was from Alexis Corporation (San Diego, CA).

### Cell culture and transfections

HEK-293 cells were cultured in MEM, containing 2 mM L-glutamine, 6% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were grown in a humidified incubator with 5% $CO_2$ and 95% air at 37°C. Transient transfection was performed using the calcium phosphate precipitation method (Sambrook and Russell, 2001) with 2 μg of pCAGGSM2_GFP (TRPC3) or pcDNA3hTRP3topaz (TRPC3-GFP) per 35-mm Petri dish. The plasmids pCAGGSM2_GFP and pcDNA3hTRP3topaz were kindly provided by Dr. Bernd Nilius (KU Leuven, Leuven, Belgium) and Dr. James Putney, Jr. (NIH/NIEHS, Research Triangle Park, NC), respectively.

### Calcium measurements

Cells cultured on poly-L-lysine coated coverslips were washed twice with HEPES-buffered salt solution (HBSS) consisting of 118 mM NaCl, 4.6 mM KCl, 1 mM $CaCl_2$, 10 mM glucose and 20 mM HEPES (pH 7.4) and incubated with 2 μM Fura-2 AM for 30 min at room temperature. The cells were then washed with HBSS. The coverslip was placed in a perfusion chamber that was mounted on a Zeiss Axiovert 35 microscope. Experiments were initiated in $Ca^{2+}$-free HBSS ($CaCl_2$ omitted and 150 μM EGTA added), unless otherwise noted. The 340 and 380 nm excitation filters were used and emission was measured at 510 nm. Light was obtained from an XBO 75W/2 xenon lamp. The shutter was controlled by a Lambda 10-2 control device (Sutter Instruments, Novato, CA), and images were collected with a SensiCam CCD camera (PCO/CD Imaging, Kelheim, Germany). The images were processed using Axon Imaging Workbench 5.1 (2.1 for Figs. 4A,B and 6) software (Axon Instruments, Foster City, CA). The experiments were performed at room temperature. The $F_{340}/F_{380}$ ratio was used to determine intracellular calcium concentration. The cells had negligible autofluorescence and a correction was considered unnecessary. GFP fluorescence at 488 nm was used to identify transfected cells, which were compared against non-transfected control cells on the same coverslip.

For experiments on permeabilized cells, HEK-293 cells were plated on poly-L-lysine coated coverslips. Cells were loaded with 3 μM mag-Fura-2 AM for 45 min at 37°C and then washed with KCl rinse buffer (125 mM KCl, 25 mM NaCl, 10 mM HEPES and 0.2 mM $MgCl_2$, pH 7.25). Cells were then permeabilized with intracellular buffer (KCl rinse buffer with 200 μM $CaCl_2$ and 500 μM EGTA, pH 7.25) containing 10 μg/ml digitonin. The permeabilization process was monitored continuously. When approximately 80% of the cells were permeabilized, excess digitonin was rinsed away by perfusion with intracellular buffer.

### Confocal microscopy

For confocal microscopy, HEK-293 cells were transfected as above with TRPC3. The cells were washed with cold PBS, and then fixed using 4% paraformaldehyde in PBS for 20 min. Next the cells were washed three times with PBS and treated with 1% Triton-X in PBS for 3 min, followed by three washes with PBS. After incubation with blocking solution (PBS containing 2% milk) for 60 min, the cells were incubated overnight in 4°C with rabbit-anti-TRPC3 (Alomone Labs, Jerusalem, Israel) and mouse-anti-calnexin (Abcam, Cambridge, UK) primary antibodies in blocking solution (1:1,000). After three washes with PBS, the secondary Alexa 568 anti-rabbit and Alexa 488 anti-mouse antibodies were added (1:1,000) and the coverslips were incubated in a humidified chamber for 1 h. The coverslips were washed three times with PBS, and were then mounted on a slide with Mowiol. For each staining there was a negative control that was not incubated with primary antibody. The images were acquired using a Leica TCS SP confocal microscope (Leica, Heidelberg, Germany) equipped with an Argon-Krypton laser (Omnichrome, Melles Griot, Carlsbad, CA).

### Statistics

Data is expressed as the mean ± SEM. Statistical analysis was made using Student's *t*-test for independent observations. Area under the curve analysis was performed with Graphpad Prism 3.03 (Graphpad, San Diego, CA).

## Results

### Characterization of transiently expressed TRPC3 channels

Transiently overexpressed TRPC3 channels have been shown to have constitutive activity (Zitt et al., 1997; McKay et al., 2000). In accordance with this, we show that overexpression of TRPC3 has a significant effect on calcium fluxes in the cells (Fig. 1). First, in cells held in a calcium-containing buffer, the resting intracellular calcium concentration was significantly higher in TRPC3-overexpressing cells, compared to control cells ($F_{340}/F_{380}$ was 0.66 ± 0.06 for TRPC3 overexpressing cells vs. 0.35 ± 0.01 for control cells, $P < 0.01$). When TRPC3 overexpressing cells were perfused with a calcium-containing buffer, and then switched to a calcium-free buffer, the resulting calcium efflux was significantly higher than non-transfected control cells (Fig. 1A,B). When the cells were held in a calcium-free buffer, and then perfused with buffer containing 1 mM calcium, there was a significant increase in the influx of calcium in TRPC3 overexpressing cells as compared to control

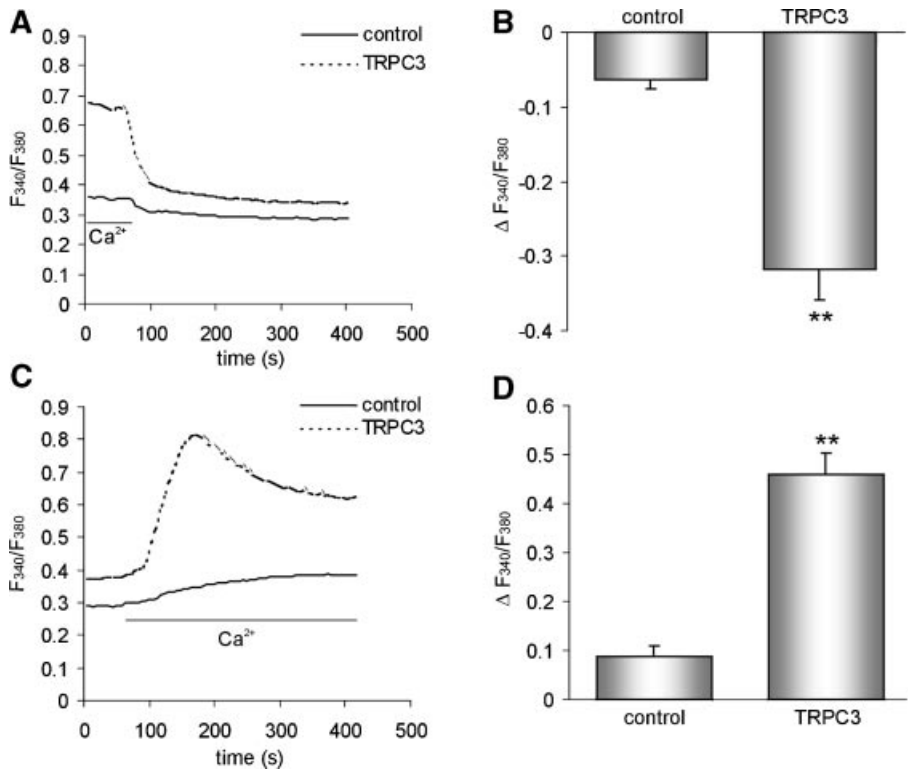


**Fig. 1. Basal activity of TRPC3 overexpressed in HEK cells. A: Control cells, or cells overexpressing TRPC3 were kept in HBSS containing 1 mM $Ca^{2+}$. When a stable baseline was obtained, the fluorescence was measured ($Ca^{2+}$), and then the medium was changed to $Ca^{2+}$-free HBSS, and the fluorescence was measured when a new stable baseline was obtained. The traces shown are mean traces of 20 measurements. B: The bars are a summarization of the traces (A) and give the mean ± SEM. $^{**}P < 0.01$ when compared to control. C: The cells were kept in $Ca^{2+}$-free HBSS and then perfused with HBSS containing 1 mM $Ca^{2+}$. The traces shown are mean traces of 22 measurements. D: The bars give the mean ± SEM of 22 cells ($^{**}P < 0.01$), which are a summarization of the traces in (C).**

cells (Fig. 1C,D). The difference in basal calcium levels and in calcium fluxes seen in TRPC3 cells, suggested that the cells expressed constitutively active channels.

To further assess if the overexpressed TRPC3 channels were functional, we stimulated the cells with 1-oleyl-2acetyl-*sn*-glycerol (OAG). The direct activation of TRPC3 with the DAG analog OAG has been shown in numerous systems (Hofmann et al., 1999; Ma et al., 2000). We noticed a significant $Ca^{2+}$-influx after stimulation of TRPC3 cells (Fig. 2) with 90 μM OAG in calcium-containing HBSS, compared with control cells. Thus, although the results above suggest a constitutive activity of the TRPC3 channels in our cells, transfection of the cells with TRPC3 also resulted in stimulation-activated channel proteins.

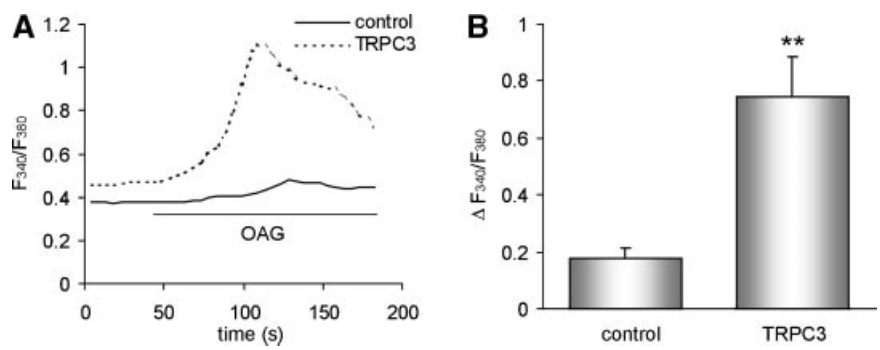


**Fig. 2. OAG activates calcium entry in HEK cells expressing TRPC3. A: Control cells, or cells expressing TRPC3 were kept in HBSS containing 1 mM $Ca^{2+}$. When a stable baseline was obtained the cells were stimulated with 90 μM OAG and the change in fluorescence was measured. The traces shown are mean traces of 44 measurements. B: The bars give the mean ± SEM of 44 cells. $^{**}P < 0.01$.**

### Overexpression of TRPC3 does not increase agonist-induced calcium entry or store-operated calcium influx

To investigate if TRPC3 overexpression promotes agonist-induced calcium entry in our cells, we stimulated the cells with 1 μM sphingosine-1-phosphate (S1P) in calcium-free HBSS, where after we added 1 mM calcium to the perfusion medium to stimulate calcium influx. We were not able to show any enhanced calcium entry in cells stimulated with S1P in either control cells or TRPC3 cells, compared to respective vehicle-treated cells (Fig. 3A,B). Thus, transfection with TRPC3 does not result in an increase in agonist-induced calcium entry in our cells. It must be noted, however, that the calcium entry per se after readdition of calcium was increased in transfected cells, compared with control cells, in a manner similar to what is shown in Figure 1. To further investigate the calcium influxes in TRPC3 overexpressing cells, we repeated the experiment, but instead of S1P, we stimulated the cells with 1 μM thapsigargin (TG). Again, we were unable to show any enhanced calcium entry in TRPC3 overexpressing cells, compared to vehicle-treated cells (Fig. 3C,D). To rule out that the lack of SOCE in TRPC3 overexpressing cells was not due to calcium-pumps masking the effects we also did barium-entry experiments (Fig. 3E,F). We could not find any differences in barium-entry between TG and vehicle-treated cells, either. Taken together, overexpression of TRPC3 did not increase agonist-induced calcium entry or SOCE in the cells.

A most interesting observation in the above experiments was that both the S1P- and the TG-evoked mobilization of sequestered calcium were decreased in cells overexpressing TRPC3 (Fig. 3). The calcium release (quantified as $\Delta F_{340}/F_{380}$) in response to S1P was significantly less in TRPC3 overexpressing cells than in control cells ($0.05 \pm 0.01$ vs. $0.49 \pm 0.1$, $P < 0.01$). The TG-evoked responses was also significantly reduced in cells overexpressing TRPC3 ($\Delta F_{340}/F_{380}$ was $0.16 \pm 0.02$ for TRPC3

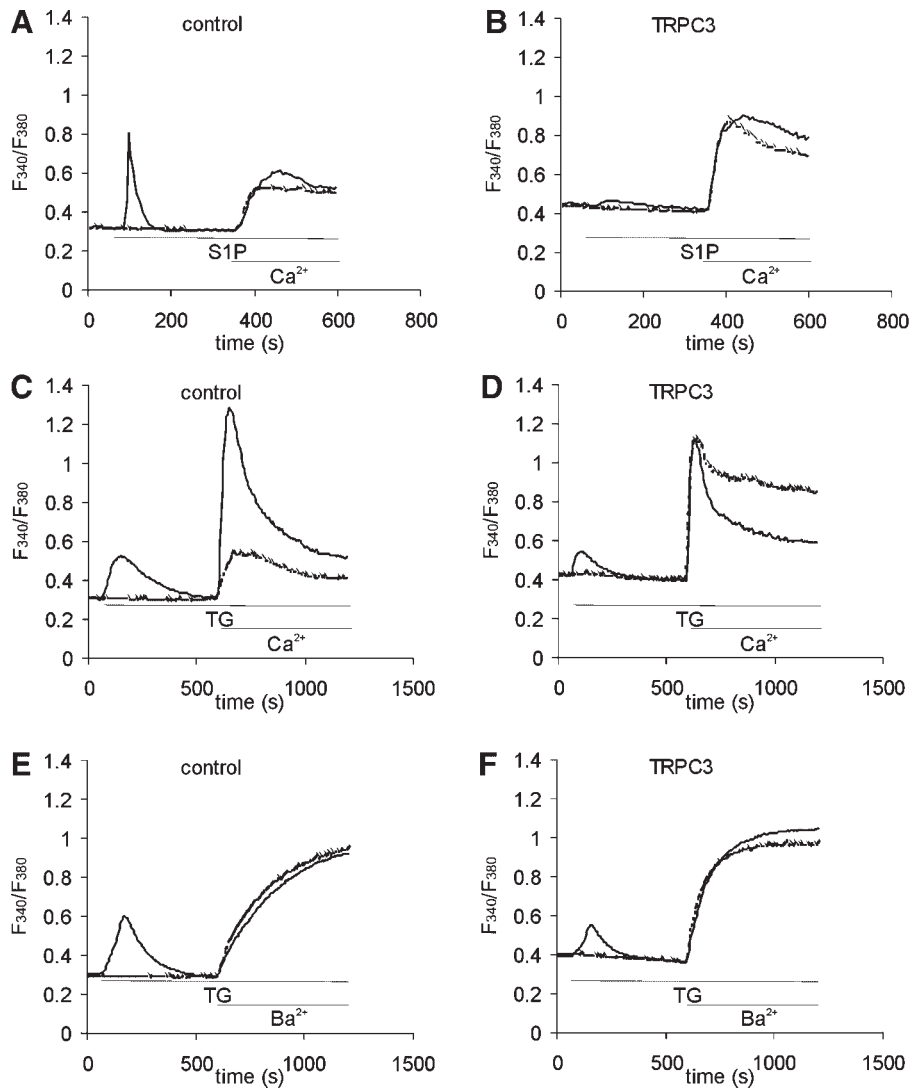


**Fig. 3. Overexpression of TRPC3 does not increase agonist induced calcium entry or SOCE while it hampers the release of sequestered calcium. A,B: Control cells and TRPC3 overexpressing cells were stimulated with 1 μM S1P or vehicle in $Ca^{2+}$-free HBSS where after 1 mM $Ca^{2+}$ was added, n ≥ 19. C,D: Control cells and cells overexpressing TRPC3 where stimulated with 1 μM TG or vehicle in $Ca^{2+}$-free HBSS where after 1 mM $Ca^{2+}$ was added, n ≥ 17. E,F: The same experiment as (C) and (D) but $Ba^{2+}$ entry was monitored instead of $Ca^{2+}$ entry, n ≥ 23. Please note the difference in the ordinate between (A), (B) and (C–F). All traces shown are means of respective treatments.**

cells and $0.23 \pm 0.01$ for control cells, $P < 0.01$). Area under the curve analysis for the 0–580 sec period was also done on the cells in Figure 3C,D, and this confirmed that less calcium is in fact released from TRPC3 overexpressing cells ($18.4 \pm 2.8$ for TRPC3-cells and $43.3 \pm 2.9$ for control cells, $P < 0.01$). Taken together, our results show that TRPC3 does not form SOCCs in our cells, and that overexpression of TRPC3 channel proteins strongly interferes with mobilization of sequestered calcium from the ER.

### Intracellular calcium stores are reduced when TRPC3 is overexpressed

The decreased mobilization of sequestered calcium in TRPC3-overexpressing cells could be due to reduced storage of calcium in the ER. To investigate this possibility, we first loaded the intracellular stores with mag-Fura-2 and then permeabilized the cells with digitonin to measure the concentration of sequestered calcium in intracellular calcium stores. As can be seen in Figure 4A,B, the concentration of calcium in the ER was significantly reduced in cells transfected with TRPC3, compared with control cells. After stimulation with 2 μM ionomycin, to completely deplete the agonist-sensitive calcium stores, the levels of residual calcium were equal in control cells and transfected cells. In an attempt to monitor the rate of calcium loss from intracellular calcium stores we repeated the above experiment but instead of ionomycin we treated the cells with 1 μM TG. In the time period tested TG had no effect on the intracellular stores, whereas 5 μM $IP_3$ had an immediate effect. Once again the releasable calcium pool was significantly reduced in cells overexpressing TRPC3. Presently we have no explanation for the lack of an effect of TG, but the result indicates that SERCA is not a major contributor to calcium leak in our system. Similar results have been observed in other cell types as well (see, e.g., Gomez-Viquez et al., 2003).

The above results led us to believe that TRPC3 may form a functional channel in the ER. To determine whether TRPC3 was localized in the ER, HEK-293 cells expressing TRPC3 were stained for TRPC3 and for calnexin, which is commonly used as a marker for the ER. Although the TRPC3 protein was found in the plasma membrane, a substantial amount of TRPC3 proteins was found in intracellular membranes and a partial colocalization with native calnexin was found (Fig. 5). Taken together, the result suggest that both proteins may reside in the ER, and that TRPC3 proteins may act as release channels in the ER, thus depleting calcium stores.

### Overexpression with a TRPC3-GFP fusion protein perturbs intracellular calcium stores

To further confirm our results, we tested another TRPC3 construct. Unlike the first construct, this was a fusion protein between TRPC3 and GFP. We found that overexpression of TRPC3-GFP affected the intracellular stores in similar ways as did overexpression of TRPC3. First, stimulation with 1 μM S1P or 1 μM TG in a calcium-free buffer resulted in significantly smaller release of sequestered calcium in TRPC3-GFP transfected cells, compared with control cells, indicating that the intracellular stores may be depleted in TRPC3-GFP cells (Fig. 6A–D). Second, after permeabilization with digitonin we

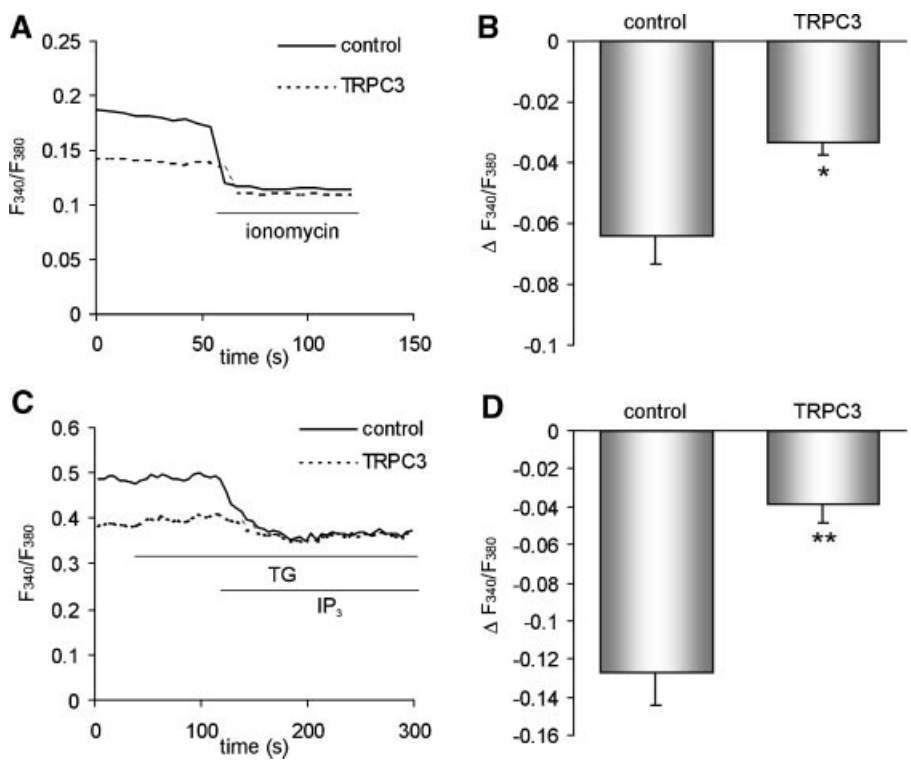


**Fig. 4. Overexpression of TRPC3 decreases the releasable calcium pool in intracellular calcium stores. A: The endoplasmic reticulum was loaded with Mag-Fura 2 as described in Materials and Methods Section. After permeabilization of control cells or cells overexpressing TRPC3 with digitonin, the cells were kept in an intracellular buffer. After obtaining a stable baseline, the fluorescence was measured, and then 2 μM ionomycin was added. When a new stable baseline was obtained, fluorescence was again measured. The traces shown are mean traces of all measurements. B: The bars give the mean ± SEM of at least 19 separate measurements. *$P < 0.05$ compared with control. C: Mean traces of cells treated as the above experiment but stimulated with 1 μM thapsigargin and 5 μM $IP_3$. D: Is a summarization of the traces in (C) and the bars give the mean ± SEM of 19 separate measurements. **$P < 0.01$ compared with control.**

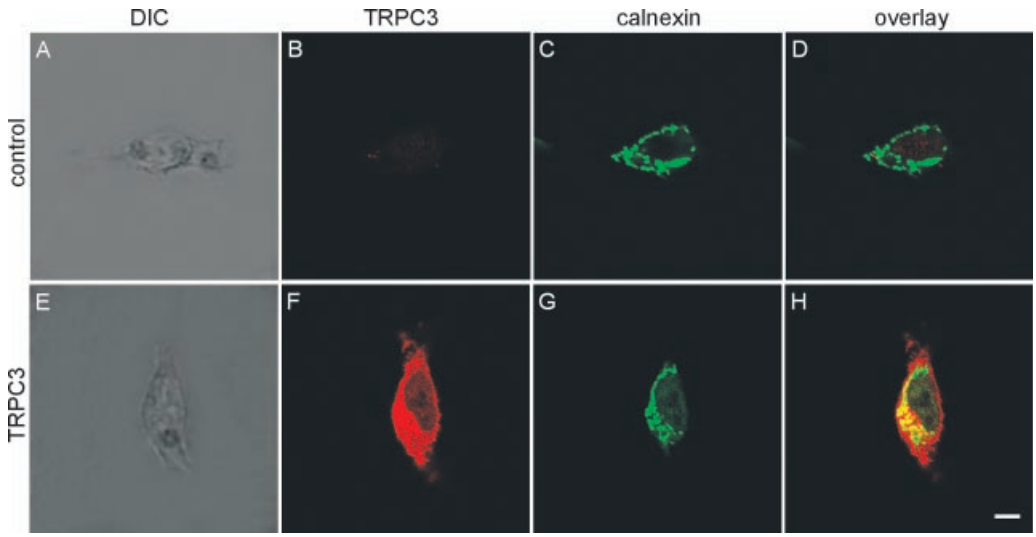


**Fig. 5. TRPC3 and calnexin staining in HEK-293 cells. A: A differential interference contrast (DIC) image of a control cell. B: A Control cell stained with the TRPC3 antibody. C: A control cell stained with the calnexin antibody. D: Overlay picture of (B) and (C). E: A DIC image of a TRPC3 overexpressing cell. F: Cellular distribution of overexpressed TRPC3. G: Cells overexpressing TRPC3 stained with the calnexin antibody. H: Overlay picture of TRPC3 (F) and calnexin (G), the colocalization is seen as yellow. A single optical section is shown for each channel and in the overlay picture. The scale bar represents 10 μm.**

measured the amount of releasable calcium in intracellular stores, and found that the intracellular calcium stores where severely reduced in TRPC3-GFP overexpressing cells, compared with control cells (Fig. 6E,F). In accordance with the TRPC3 results, we did not see a significant calcium influx after readdition of calcium to cells transfected with TRPC3-GFP and stimulated with either S1P or TG in a calcium-free buffer, compared to vehicle-treated cells (data not shown).

## Discussion

In the present report we have shown that overexpression of either TRPC3 or a TRPC3-GFP construct in HEK-293 cells results in a remarkable perturbation of cellular calcium homeostasis. First, overexpression of TRPC3 results in constitutively active channels in the plasma membrane. Second, although the channels are constitutively active, they can be further activated by OAG. Thirdly, and most remarkably, a substantial amount of TRPC3 channels are sequestered in the ER, as evidenced by colocalization with calnexin, resulting in a substantial depletion of the releasable calcium pool in the ER. As a result of this depletion, stimulating the cells with agonists known to mobilize sequestered calcium resulted in markedly attenuated calcium responses. There were some differences between the two constructs of TRPC3. The TRPC3-GFP fusion protein seemed to have an accentuated pathophysiological effect on the intracellular stores when compared with the other construct where TRPC3 and GFP are expressed separately. The reason for this is unknown but we speculate that the fusion protein is more easily retained in the intracellular stores due to its larger size. We concentrated mainly on the construct where TRPC3 and GFP are expressed separately to get more accurate results of the effects of TRPC3 on the intracellular calcium stores.

To our knowledge our report is the first to show that TRPC3 can be sequestered in the ER, and there apparently form calcium release channels. This property of overexpressed TRPC3 is, however, not unique. Recent studies have shown that other members of the TRP superfamily can reside in the ER. The TRPV1 channel has been shown to function as an intracellular release channel, but unlike our results, do not mediate a global depletion of the ER calcium stores (Turner et al., 2003; Wisnoskey et al., 2003). Furthermore, the TRPC2 channel, the TRPC1A channel, and the TRPC1 channel, were retained in the ER (Hofmann et al., 2000, 2002; Hassock et al., 2002). However, TRPC1A could be transported to the plasma membrane, but only if cotransfected with TRPC4β (Hassock et al., 2002). In all these studies, none of the channels were shown to be involved in SOCE or $I_{CRAC}$ when present in the intracellular membranes.

The possible involvement of TRPC3 channels in SOCE is controversial. Several groups have shown that overexpression of, for example, TRPC3 has resulted in different modes of action of the channel: some studies indicate a store-operated mode (Vazquez et al., 2001; Trebak et al., 2002), whereas other suggests a receptor-operated mode (McKay et al., 2000; Venkatachalam et al., 2001). A constitutive activity of the channel is also observed in some investigations (Zitt et al., 1997; McKay et al., 2000). Our results clearly suggest that TRPC3 in the plasma membrane is constitutively active, and further activation is obtained upon stimulation with OAG. Whether this is due to OAG-evoked activation of plasma membrane TRPC3 only, activation of TRPC3 in both the plasma membrane and the ER, or different modes of gating of the channel protein in the plasma membrane, was not investigated. However, the evidence for depleted calcium stores in cells expressing TRPC3, apparently due to TRPC3 channels in the ER, may explain the constitutive activity of the channels. Whether this depletion is limited to a subset of ER compartments is not clear at present, but the results obtained with thapigargin in permeabilized cells suggest that the channels exist in a subpopulation of ER stores. Evidence by Turner et al. (2003) regarding TRPV1 channels suggests the localization of channel proteins in different subcompartments. We cannot either exclude the possibility that the depletion of the ER resulted in activation of endogenous plasma membrane SOCC. However, a TRPC3 channel functioning as a leak channel in the ER could explain the fact that calcium responses are reduced. Furthermore, due to the reduced calcium content in the ER, the resting intracellular

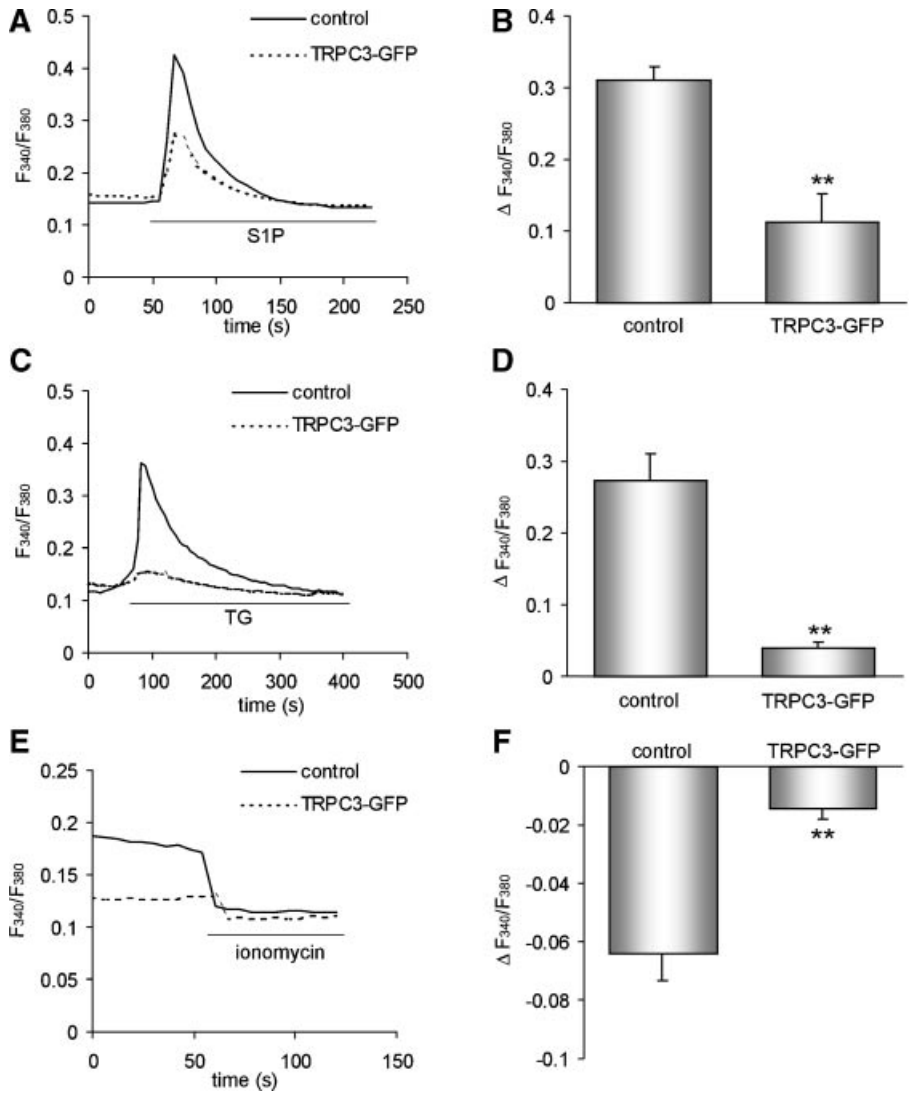


**Fig. 6. Overexpression with the TRPC3-GFP fusion protein depletes intracellular calcium stores. A: Mean traces of control cells and TRPC3-GFP expressing cells stimulated with 1 μM S1P in $Ca^{2+}$-free HBSS. B: Summarization of the traces from (A), the bars denote the mean ± SEM of at least 19 separate determinations. *$P < 0.01$ compared with control. C–D: Same as (A) and (B) but cells were stimulated with 1 μM TG in $Ca^{2+}$-free HBSS, n ≥ 10. Please note the difference in the ordinate in (A) and (C). E: Permeabilization of control cells or cells overexpressing TRPC3-GFP loaded with Mag-Fura 2. After obtaining a stable baseline, the fluorescence was measured, and then 2 μM ionomycin was added. When a new stable baseline was obtained, fluorescence was again measured. The traces shown are mean traces of at least 14 cells. Summarized in (F) is the traces from (E), the bars give the mean ± SEM. **$P < 0.01$.**

calcium concentration in the cells overexpressing TRPC3 are higher than in control cells due to SOCE. The observations regarding the localization of members of the TRP superfamily in the ER, suggest that these channels indeed may participate as leak channels. Recent data show that overexpression of the channel protein pannexin 1, which is of importance in regulating intercellular calcium movements, also results in the formation of a calcium channel in the ER (Vanden Abeele et al., 2006). Another viable explanation could be that the elevated resting intracellular calcium concentration in the TRPC3 overexpressing cells could modify ER calcium stores via regulating endogenous pump- and leak proteins via transcriptional events.

Presently the physiological nature of calcium leak channels is controversial. Possible candidates are the $IP_3$-receptors, the ryanodine receptors, the anti-apoptotic protein Bcl-2, and calcium ATPases in the ER working in the reverse mode (see Camello et al., 2002; Szlufcik et al., 2006; for recent reviews). Furthermore, the $IP_3$- and ryanodine receptors have been suggested to participate in pathological calcium leak from the ER (Szlufcik et al., 2006). In addition, results obtained by Wissing et al. (2002) suggested that a specialized calcium channel mediates the basal leak. As several members of the TRP-superfamily of channels can be found in the ER, it cannot be excluded that this specialized calcium channel could be, for example, the TRPC3 channel. If these observations are of physiological significance is still not known, but they could be of importance in regulating spatially localized calcium signals, or in regulating calcium-induced calcium release.

An examination of earlier reports on TRPC3 channels and calcium fluxes in TRPC3-overexpressing cells also give at hand that, for example, the agonist-evoked release of sequestered calcium is hampered, compared with that seen in control cells (Zhu et al., 1998; Trebak et al., 2002). Our results thus also shed

some light upon the possible pathophysiological effects of overexpression studies, urging to caution when overexpression systems are used. Furthermore, the recent findings that Stim 1, the novel regulator of SOC and $I_{CRAC}$, interacts with TRPC1, 2 and 4, but not with TRPC3, 6 or 7, strongly suggest that TRPC3 is not part of a channel complex regulating SOCE (Huang et al., 2006; Lopez et al., 2006; Ong et al., 2007).

In conclusion, we show that the overexpression of TRPC3 may form calcium leak channels in the ER, and thus reduces the content of intracellular calcium stores. This results in an increase in the intracellular cytosolic free calcium concentration due to constitutive calcium entry, and attenuated agonist-evoked calcium signals.

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