

M6:09435-Revision 2

**Relocalization of STIM1 for activation of store-operated  $\text{Ca}^{2+}$  entry is determined by the depletion of subplasma membrane endoplasmic reticulum  $\text{Ca}^{2+}$  store.**

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**Running Title: Regulation of SOCE**

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**Abstract**

Stromal interacting molecule 1 (STIM1), an endoplasmic reticulum (ER) protein that controls store-operated  $\text{Ca}^{2+}$  entry (SOCE), redistributes into punctae at the cell periphery after store depletion. This redistribution is suggested to have a causal role in activation of SOCE. However, whether peripheral STIM1 punctae that are involved in regulation of SOCE are determined by depletion of peripheral or more internal ER has not yet been demonstrated. Here we show that  $\text{Ca}^{2+}$  depletion in subplasma membrane ER is sufficient for peripheral redistribution of STIM1 and activation of SOCE. 1  $\mu\text{M}$  Tg induced substantial depletion of intracellular  $\text{Ca}^{2+}$  stores and rapidly activated SOCE. In comparison, 1 nM Tg induced slower, about 60-70% less  $\text{Ca}^{2+}$  depletion, but similar SOCE. SOCE was confirmed by measuring  $I_{\text{SOCE}}$  in addition to  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Ba}^{2+}$

entry. Importantly, 1nM Tg caused redistribution of STIM1 only in the ER-plasma membrane junction while 1  $\mu\text{M}$  Tg caused a relatively global relocalization of STIM1 in the cell. During the time taken for STIM1 relocalization and SOCE activation, 1nM BD-Tg primarily labeled the subplasma membrane region while 1  $\mu\text{M}$  Tg labeled the entire cell. The localization of Tg in the subplasma membrane region was associated with depletion of ER in this region and activation of SOCE. Together, these data suggest that peripheral STIM1 relocalization that is causal in regulation of SOCE is determined by the status of  $[\text{Ca}^{2+}]$  in the ER in close proximity to the plasma membrane. Thus the mechanism involved in regulation of SOCE is contained within the ER-plasma membrane junctional region.

## Introduction

Store-operated calcium entry (SOCE) is activated in response to depletion of  $\text{Ca}^{2+}$  in intracellular  $\text{Ca}^{2+}$  store(s). SOCE not only ensures refilling of the intracellular  $\text{Ca}^{2+}$  stores but also provides sustained elevation of cytosolic  $[\text{Ca}^{2+}]$  ( $[\text{Ca}^{2+}]_i$ ). Physiologically, SOCE is activated by agonist-stimulated generation of inositol-1, 4, 5-trisphosphate ( $\text{IP}_3$ ), and  $\text{IP}_3$ -mediated release of  $\text{Ca}^{2+}$  from ER- $\text{Ca}^{2+}$  stores via  $\text{IP}_3\text{R}$ . It has been clearly demonstrated that during refilling of internal  $\text{Ca}^{2+}$  stores,  $\text{Ca}^{2+}$  entering the cell is rapidly taken up into the ER by SERCA activity with minimal diffusion in the subplasma membrane region. These studies indicate close apposition of the ER and plasma membrane at the site of SOCE (1-6). Thus, it has been suggested that ER localized in the subplasma membrane region is likely to be coupled to SOCE and that depletion of these local  $\text{Ca}^{2+}$  stores triggers activation of SOCE. The presently proposed mechanisms for activation of SOCE suggest either direct physical coupling between components in the ER and plasma membrane, generation of diffusible components, or recruitment of the SOC channel. The exact mechanism involved in regulation of SOCE as well as the molecular components of the channel(s) mediating  $\text{Ca}^{2+}$  entry in different types of cells has not been clearly identified.

Recent studies have suggested stromal interacting molecule 1 (STIM1) as a novel regulator for SOCE. STIM1, which is a  $\text{Ca}^{2+}$  binding protein localized diffusely in the ER, has been shown to relocate into a peripheral region of the cell in response to internal  $\text{Ca}^{2+}$  store

depletion (7,8). STIM1 displays punctate subplasma membrane localization in response to depletion of intracellular  $\text{Ca}^{2+}$  stores which has been reported to be causal in activation of SOCE and define the elementary unit that is involved in SOCE. While several studies have clearly established involvement of ER-plasma membrane junctional domains in the regulation of SOCE, the exact site of store depletion has not yet been demonstrated. Based on all the data available presently, it is logical to propose that since the EF hand domain of STIM1 is localized in the lumen of the ER and STIM1 punctae involved in SOCE activation are localized in subplasma membrane  $\text{Ca}^{2+}$  stores very close to the surface membrane (likely interacting with SOCE channels), this ER  $\text{Ca}^{2+}$  store would have to be depleted for activation of SOCE. Further, these stores would have to remain in a depleted state in order to retain the punctate localization of STIM1 which is required to maintain SOCE in an activated state. However conditions typically used for activating SOCE and assessing STIM1 function (8-13) have used high concentrations of thapsigargin (Tg) or agonists which induce substantial and global depletion of internal  $\text{Ca}^{2+}$  stores. Thus, it is unclear whether changes in the local  $\text{Ca}^{2+}$  store, i.e. in the vicinity where regulation of SOCE seems to be taking place, regulate relocation of STIM1 and activation of SOCE or whether signals from more interior regions of the cell are required.

In this study we have assessed whether STIM1 regulation of SOCE is dependent on the  $[\text{Ca}^{2+}]$  in subplasma membrane ER or in more internal ER. We have used low concentrations of Tg to slow down the process of ER depletion and

activation of SOCE. It has been previously shown that low concentrations of Tg which induce only partial depletion of internal  $\text{Ca}^{2+}$  stores, result in a slow but almost complete activation of  $I_{\text{CRAC}}$  (14,15), although these previous studies do not clarify the location of the SOCE-coupled ER  $\text{Ca}^{2+}$  store. We demonstrate that as Tg diffuses into the periphery of the cell,  $\text{Ca}^{2+}$  stores in this region are depleted and this is associated with activation of SOCE. SOCE is not further enhanced by depletion of more internal ER. Further we show that mobilization of the SOCE regulatory protein STIM1 in the subplasma membrane region of the cells is associated with depletion of ER in this region. Together our data suggest that STIM1 senses  $[\text{Ca}^{2+}]_{\text{i}}$  within ER localized in the ER-plasma membrane junctional regions and that the status of this peripheral ER- $\text{Ca}^{2+}$  store determines relocation of peripheral STIM1 that is required for activation of SOCE.

### Experimental Procedures

**Materials:** Bodipy-fluorescein thapsigargin (BD-Tg), Fura-2AM, Mag-Fluo4AM, Fluo-4AM and the ER-Tracker Red dye and the Vybrant staining assay were obtained from Invitrogen (Carlsbad, CA, USA). Thapsigargin (Tg) was obtained from Calbiochem (San Diego, CA, USA). Carbachol (CCh) and 2-aminoethyldiphenylborinate (2-APB) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Glass-bottomed 35mm petri dishes were obtained from MatTek Corporation (Ashland, MA, USA). Glass coverslips were obtained from Fisher Scientific (Pittsburgh, PA, USA). The rabbit antibody against phosphorylated PERK (phospho-PERK)

was obtained from Cell Signaling Technology (Danvers, MA, USA). The mouse anti-STIM1 antibody was obtained from BD Biosciences (San Jose, CA, USA), whereas the FITC-conjugated donkey anti-mouse antibody was from Jackson ImmunoResearch (West Grove, PA, USA). The cDNA for untagged STIM1 was obtained from OriGene (Rockville, MD, USA).

**Cell culture and transient transfection:** HSG cells were cultured on glass coverslips and were transiently transfected with untagged STIM1 (1  $\mu\text{g}$ ) or YFP-STIM1 (1  $\mu\text{g}$ ) for 48 h using the method described previously (16). For  $[\text{Ca}^{2+}]_{\text{i}}$  measurements and live cell imaging experiments, cells were plated on glass-bottomed 35 mm petri dishes. For patch clamp measurement and TIRF, cells were plated on glass coverslips.

**$[\text{Ca}^{2+}]_{\text{i}}$  measurements:** Measurements with 1  $\mu\text{M}$  Fura-2AM and analyses of data were conducted as described previously (17,18).

**Electrophysiological measurements:** Whole cell-attached patch clamp measurements were performed as described previously (1,17-19). For  $\text{Ca}^{2+}$  current measurements, the external solution was composed as follows: 145 mM NaCl, 5 mM CsCl, 1 mM  $\text{MgCl}_2$ , 10 mM  $\text{CaCl}_2$ , 10 mM HEPES, 10 mM glucose, pH 7.4 (NaOH). For DVF- $\text{Na}^+$  current measurements, the external solution was composed as follows: 165 mM NaCl, 5 mM CsCl, 10 mM EDTA, 10 mM HEPES, 10 mM glucose, pH 7.4 (NaOH). The patch pipette contained the standard intracellular solution, which was composed as follows: 145 mM cesium methane-sulfonate, 8 mM NaCl, 5 mM  $\text{MgCl}_2$ , 10 mM HEPES, 10 mM EGTA; pH 7.2 (CsOH).

**Immunodetection of phosphorylated PERK:** Lysates from HSG cells were

prepared and immunoblotting using phospho-PERK antibody was carried out as described previously (20,21).

**Immunofluorescence and confocal microscopy:** Immunofluorescence was conducted in a similar manner as described previously (22). Cells were stained with the mouse anti-STIM1 antibody (1:100 dilution) and probed using a donkey anti-mouse antibody conjugated to FITC (1:100 dilution). Fluorescence images were acquired using a confocal laser scanning microscope Leica TCS-SP2 attached to an upright Leica DM-RE7 microscope as described earlier (22).

**Live cell imaging using Bodipy-fluorescein thapsigargin (BD-Tg):** HSG cells were scanned using Leica TCS SP2 attached to an inverted Leica DM-IRE2 microscope. BD-Tg was added to the cells at 1  $\mu\text{M}$  and 1 nM concentrations. Fluorescence intensity in selected regions of the cell was measured. Images were acquired every 3 s for 3 min. Background fluorescence was subtracted from each data point.

**$[\text{Ca}^{2+}]_{\text{ER}}$  Measurements:** HSG cells were loaded with 3  $\mu\text{M}$  Mag-Fluo4AM in a  $\text{Ca}^{2+}$ -containing standard extracellular solution ( $\text{Ca}^{2+}$ -SES) for 1h at 37°C in 5%  $\text{CO}_2$ . The  $\text{Ca}^{2+}$ -SES was composed as follows: 145 mM NaCl, 5 mM CsCl, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , 10 mM HEPES, 10 mM glucose, pH 7.4 (NaOH) (23). After incubation, cells were then rinsed with  $\text{Ca}^{2+}$ -free SES and used in live cell imaging experiments on Leica DM-IRE2 microscope as described above or in TIRF imaging as described below.

**Vybrant staining assay:** Vybrant staining assay was used to evaluate apoptosis, using the method described previously (24).

**TIRF imaging:** HSG cells were seeded and cultured overnight on glass coverslips, which formed the base of a perfusion chamber that was mounted on the stage of an Olympus IX81 motorized inverted microscope (OPELCO, Dulles, VA, USA). Prior to the start of imaging, cells were loaded with 4  $\mu\text{M}$  Fluo-4AM in a  $\text{Ca}^{2+}$ -SES for 1h at 37°C in 5%  $\text{CO}_2$ . To determine ER localization in the subplasma membrane regions, cells were loaded with 1  $\mu\text{M}$  ER-Tracker Red dye for 1 h at 37°C. TIRF imaging was then conducted as described previously (25). Changes in Fluo-4 fluorescence were calculated using KaleidaGraph software (Red Rock Software, Salt Lake City, UT, USA) and were expressed as  $\Delta F/F_0$ , where F is the fluorescence captured at a particular time point and  $F_0$  is the mean fluorescence intensity from the initial 20 captured images.

## Results

***Submaximal depletion of ER- $\text{Ca}^{2+}$  store activates SOCE.*** Stimulation of HSG cells by the endoplasmic  $\text{Ca}^{2+}$  pump inhibitor thapsigargin (Tg; 1  $\mu\text{M}$ ) induces an initial rapid increase in  $[\text{Ca}^{2+}]_i$  (represented by 340/380 fluorescence ratio, Fig 1A) due to internal  $\text{Ca}^{2+}$  release which is detected as a transient  $[\text{Ca}^{2+}]_i$  increase in the absence of external  $\text{Ca}^{2+}$ . Addition of  $\text{Ca}^{2+}$  to the external medium after internal store depletion induces a second increase in  $[\text{Ca}^{2+}]_i$  due to  $\text{Ca}^{2+}$  influx. At this concentration Tg induces almost complete depletion of the internal  $\text{IP}_3$ -sensitive  $\text{Ca}^{2+}$  store (addition of Tg after carbachol induced some additional  $\text{Ca}^{2+}$  release but not *vice versa*, data not shown). Further, caffeine does not affect Tg- or carbachol-induced  $[\text{Ca}^{2+}]_i$  increase in HSG cells (data not shown).

Contribution of mitochondrial  $\text{Ca}^{2+}$  transport to the Tg-stimulated  $[\text{Ca}^{2+}]_i$  changes was not assessed.

To determine the extent of  $[\text{Ca}^{2+}]_{\text{ER}}$  depletion required for activation of SOCE, we examined internal  $\text{Ca}^{2+}$  release and  $\text{Ca}^{2+}$  entry induced by lower [Tg]. 0.5  $\mu\text{M}$ , 10 nM, and 1 nM Tg induced slower and less internal  $\text{Ca}^{2+}$  release than 1  $\mu\text{M}$  Tg (Fig 1B). Importantly, substantial  $\text{Ca}^{2+}$  entry was seen at the lower [Tg] and neither the rates nor amplitudes of  $[\text{Ca}^{2+}]_i$  increase at the various [Tg] were significantly different from each other (see Fig 1D, and also traces in E and G). The extent of store depletion was assessed by addition of 1  $\mu\text{M}$  Tg after treatment of cells with lower [Tg] (Fig 1C and D). 1  $\mu\text{M}$  Tg-induced  $[\text{Ca}^{2+}]_i$  increase was reduced by <50% in cells pretreated with 1 nM Tg for 500 s, suggesting that at least 50% of internal  $\text{Ca}^{2+}$  store was not associated with activation of  $\text{Ca}^{2+}$  entry. Similarly, 10 nM and 0.5  $\mu\text{M}$  also induced less store-depletion compared to 1  $\mu\text{M}$  Tg (see Fig 1D). These data demonstrate that  $\text{Ca}^{2+}$  entry is activated even when internal  $\text{Ca}^{2+}$  stores are incompletely depleted. To confirm that SOCE was activated by 1 nM Tg, we examined the effects of  $\text{Gd}^{3+}$  and 2-APB, both of which are used to block SOCE (26,27).  $\text{Ca}^{2+}$  entry stimulated by 1  $\mu\text{M}$  and 1 nM Tg was inhibited by 1  $\mu\text{M}$   $\text{Gd}^{3+}$  and 10  $\mu\text{M}$  2-APB (Figs 1E to H, respectively). The inhibitory effect of 1  $\mu\text{M}$   $\text{Gd}^{3+}$  was seen whether it was added before or after 1 mM  $\text{CaCl}_2$  (inset in Fig 1E, data with 1 nM Tg not shown). These findings strongly suggest that SOCE is activated under conditions where there is relatively less depletion of ER- $\text{Ca}^{2+}$  store.

1 nM Tg, like 1  $\mu\text{M}$  Tg, also activated the previously described inwardly-rectifying store-operated  $\text{Ca}^{2+}$  current,  $I_{\text{SOC}}$  in HSG cells (1) (Figs 2 A-C). However, the magnitude of the current with 1 nM Tg was about 60% less than that with 1  $\mu\text{M}$  Tg and there was also a longer lag before an increase in current was detected ( $209 \pm 55$  s compared to  $20 \pm 4$  s with 1  $\mu\text{M}$  Tg). Notably, the current developed rapidly after this lag period and appeared to be quite stable, unlike the current with 1  $\mu\text{M}$  Tg which inactivated more rapidly. These data are inconsistent with the Fura-2 data which demonstrate that 1  $\mu\text{M}$  and 1 nM Tg activate similar magnitude of SOCE although activation is slower at the lower [Tg]. We hypothesized that this discrepancy could be due to a contribution of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release to the Fura-2 fluorescence measurements (28). Alternatively, it could be a result of recycling of  $\text{Ca}^{2+}$  into the ER store by residual SERCA activity in cells treated with the lower [Tg]. It should be noted that since  $I_{\text{SOC}}$  measurements were done in the presence of 10 mM external  $\text{Ca}^{2+}$  it is possible that, despite the buffering of  $\text{Ca}^{2+}$  in the pipette solution,  $\text{Ca}^{2+}$  entering the cell is taken back into the ER to partially refill the store (3). However, removal of external  $\text{Ca}^{2+}$  (as was the case in the Fura-2 assay) would allow this store to be substantially depleted. Uptake of  $\text{Ca}^{2+}$  into the ER by the residual SERCA activity in cells stimulated with low [Tg] will also result in lower ambient  $[\text{Ca}^{2+}]_i$  which would attenuate  $\text{Ca}^{2+}$ -dependent inactivation of SOCE and account for the relatively stable  $I_{\text{SOC}}$  in these cells.

To determine the possible contribution of  $\text{Ca}^{2+}$  entry and ER  $\text{Ca}^{2+}$  recycling  $I_{\text{SOC}}$  was measured in a divalent cation free

external medium (Figs 2 D-F). Under these conditions (i) the magnitudes of the steady-state currents induced by 1 nM and 1  $\mu$ M Tg were similar, and (ii) the lag time for current activation with 1 nM Tg was reduced (140 compared to 209 s). The possible contribution of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release was confirmed by measuring  $\text{Ba}^{2+}$  entry stimulated by 1 nM or 1  $\mu$ M Tg. As seen in the traces shown in Figures 2 G and H, similar levels of  $\text{Ba}^{2+}$  entry were induced under the two conditions (basal  $\text{Ba}^{2+}$  entry into these cells was not detectable). In aggregate, these data suggest that  $\text{Ca}^{2+}$  entry and reuptake into ER account for the lower magnitude and in part for the longer lag time for activation of  $I_{\text{SOC}}$  (with  $\text{Ca}^{2+}$  as the charge carrier) in cells treated with 1 nM Tg. The longer lag for activation of  $I_{\text{SOC}}$  at the lower [Tg] is also due to the longer diffusion time and likely reflects the time required to inhibit enough SERCA to achieve sufficient depletion of ER- $\text{Ca}^{2+}$ . Once this is achieved,  $I_{\text{SOC}}$  is rapidly activated.

The relatively slow internal  $\text{Ca}^{2+}$  release induced by 1 nM Tg allowed us to examine the temporal characteristics of SOCE activation. Re-addition of external  $\text{Ca}^{2+}$  at 20, 50, 150, and 250 s after stimulation with 1 nM Tg in  $\text{Ca}^{2+}$ -free medium resulted in substantial elevations of  $[\text{Ca}^{2+}]_i$  (Figs 3 A-D respectively) which was blocked by 2-APB (Fig 3C, red trace). Figure 3E demonstrates that SOCE is half-maximally activated within 20 s and maximally activated by 150 s. The magnitude of SOCE activated by 1 nM Tg at 150 s was similar to that activated by 1  $\mu$ M Tg for 300 s (red \* in Fig 3E). Note that longer incubation with either the low or high Tg did not induce any

further increase in SOCE. Addition of 1  $\mu$ M Tg after stimulation with 1 nM Tg for 150 s demonstrated that there was about 30% depletion of the internal  $\text{Ca}^{2+}$  store (data not shown, but compare with Fig 1C). Together these data demonstrate that SOCE is maximally activated by 150 s after 1 nM Tg treatment. To confirm this, SOCE was measured using  $\text{Mn}^{2+}$  as a  $\text{Ca}^{2+}$  surrogate (29). 50 s after addition of 1 nM Tg the rate of Fura-2 quenching was substantially less than that induced by 1  $\mu$ M Tg (Fig 3F). However, similar  $\text{Mn}^{2+}$  entry was seen in cells stimulated for 150 and 250 s with either 1 nM or 1  $\mu$ M Tg (Figs 3 G and H). In aggregate, these data suggest that depletion of SOCE-coupled stores with 1 nM Tg is slow at first due to recycling of  $\text{Ca}^{2+}$  into the ER by residual SERCA activity. This would also account for the transient increase in  $[\text{Ca}^{2+}]_i$  at the 20 s time point (Fig 3A). With time enough SERCA are inactivated to prevent this recycling, thus resulting in complete activation of SOCE (similar magnitude seen with 1 nM Tg and 1  $\mu$ M Tg).

#### ***Translocation of STIM1 by low and high concentrations of thapsigargin.***

STIM1 has been proposed to be an essential regulatory protein for SOCE (10-13). It is translocated to the plasma membrane region of cells upon depletion of internal  $\text{Ca}^{2+}$  stores and this translocation has been associated with activation of SOCE. We have used TIRFM to examine localization of heterologously expressed YFP-STIM1 in HSG cells. Both low and high concentrations of Tg stimulated movement of YFP-STIM1 in the subplasma membrane in cells maintained in a  $\text{Ca}^{2+}$ -containing medium in contrast to control cells expressing mYFP alone

which does not change (Fig 4A). However, while there was a fast and almost uniform increase in STIM1 punctae with 1  $\mu\text{M}$  Tg (Fig 4B, punctae were detected at 2 min and maximum by 4 min), translocation of STIM1 with 1 nM Tg (Fig 4C) was relatively slower (4 and 6 min time points are shown). Addition of 1  $\mu\text{M}$  Tg after 1 nM Tg to these cells induced further increase in STIM1 in the subplasma membrane region (data not shown). Importantly, when cells were treated with 1 nM Tg in the absence of external  $\text{Ca}^{2+}$  the extent of STIM1 relocalization was similar to that induced by 1  $\mu\text{M}$  Tg (Fig 4D). The data in Figs 4 B-D are fully consistent with the functional data shown in Figs 1-3. In the presence of 1 nM Tg, rapid recycling of  $\text{Ca}^{2+}$  that enters cells incubated in a  $\text{Ca}^{2+}$ -containing medium prevents complete depletion of the ER  $\text{Ca}^{2+}$  store, thus accounting for the lower SOCE and less STIM1 redistribution into punctae. When  $\text{Ca}^{2+}$  is removed from the external solution, the store depletes more substantially and thus when  $\text{Ca}^{2+}$  is re-added to the external medium comparable influx is seen in the two conditions. These data clearly demonstrate that incomplete depletion of ER  $\text{Ca}^{2+}$  stores is sufficient for relocalization of STIM1 and activation of SOCE.

***Depletion of peripheral  $\text{Ca}^{2+}$  stores is associated with STIM1 relocation and SOCE activation.*** We assessed the localization of the ER stores involved in STIM1-regulation of SOCE by using several different experimental protocols. First, Bodipy-fluorescein thapsigargin (BD-Tg) and live cell confocal imaging were used to determine the extent of diffusion (and thus localization) of Tg in HSG cells during activation of SOCE. 1

$\mu\text{M}$  BD-Tg rapidly induced strong labeling of all the ER (Fig 5B, upper panel shows  $x$ - $y$  image). The  $x$ - $z$  image (Fig 5B, lower panel) demonstrates that this labeling is predominantly perinuclear. In comparison, the pattern of labeling seen with 1 nM BD-Tg was quite distinct. Strong punctate labeling was seen only in the subplasma membrane region (Fig 5A, upper panel shows  $x$ - $y$  image) with very little intracellular fluorescence. This was confirmed by the  $x$ - $z$  image of the cells (Fig 5A, lower panel) which clearly shows the peripheral localization of this signal. Overlay of Bodipy-fluorescein and DIC images of selected cells are shown in Fig 5C. Further, specificity of labeling was assessed by pre-incubation of cells with either 2 nM Tg for 3 min before treatment with 1 nM BD-Tg. This pre-incubation dampened the labeling of the cells with BD-Tg (Fig 5D, at higher [BD-Tg] initial fluorescence was similarly dampened by preincubation of cells with an equivalent concentration of non-fluorescent Tg). Fluorescence was measured in selected regions of the cell following stimulation with 1 nM Tg (Fig 5E, images were collected up to 3 min, data from the first 150 s are shown. Note that SOCE was maximally activated by 1 nM Tg within 150 s). BD-Tg fluorescence in the subplasma membrane region increased within the 5 s after Tg addition and continued to rise, leveling out after 150 s. Signal in the nucleus did not significantly increase until about 100 s. Fluorescence in the internal region was less than in the subplasma membrane ER and displayed a slow, somewhat linear increase after a longer initial lag. Thus, with 1 nM BD-Tg initial labeling was mostly limited to the subplasma membrane region of the cell, suggesting

that SOCE activation by 1 nM Tg is coincident with the presence of Tg in the subplasma membrane region of the cell. With time there was further diffusion of Tg into the more internal region of the cell, and more global inactivation of SERCA in the cells, but without corresponding increase in SOCE. The peripheral localization of 1 nM BD-Tg during activation of  $\text{Ca}^{2+}$  entry suggests that the ER in this region is depleted rather than the more internal ER. This was examined using a mathematical model based on the data in Fig. 5 and in a previous report (15). We wanted to test whether a model with two ER compartments that are functionally distinct but which slowly equilibrate with each through diffusion of  $\text{Ca}^{2+}$  between them (see supplemental section for details) could account for the data in Fig. 3 A – C. Supplemental Fig 1 A-C confirms that this simple model reproduces those observation. The simulations further predict (Supplemental Fig 1 D – F) that when cells are treated with 1 nM Tg there is greater depletion of the local subplasma membrane  $\text{Ca}^{2+}$  store than the internal ER within the time frame for activation of  $\text{Ca}^{2+}$  entry. Thus, the simulations agree well with our hypothesis that activation of  $\text{Ca}^{2+}$  entry is associated with the depletion of peripheral ER- $\text{Ca}^{2+}$  stores.

This was directly confirmed by measuring the depletion of  $\text{Ca}^{2+}$  stores in the subplasma membrane region of the cells ( $[\text{Ca}^{2+}]_{\text{ER}}$ ) by using Mag-Fluo4 and confocal microscopy. Images of the whole cell were taken and changes in the fluorescence of selected regions of interest in the cell were determined. 1 nM or 1  $\mu\text{M}$  Tg induced a faster decrease of the fluorescence in the

subplasma membrane region ER ( $\text{ER}_{\text{sub}}$ ) than in the internal ER (Figs 6 A-B; C shows fluorescence in unstimulated cells). Furthermore, the decrease with 1  $\mu\text{M}$  Tg in both ER regions was faster than with 1 nM Tg. It should be noted that the decrease in Mag-Fluo4 fluorescence (representing ER  $[\text{Ca}^{2+}]$ ) is relatively slower than the increase in Fura-2 fluorescence (representing  $[\text{Ca}^{2+}]_{\text{i}}$ ) under the same conditions. This is difference is most likely due to the higher  $[\text{Ca}^{2+}]$  in ER compared to the cytosol. Thus, the relative changes in fluorescence are larger in the cytosol than in the ER. Similar slow decrease in ER- $[\text{Ca}^{2+}]$  but faster increase in  $[\text{Ca}^{2+}]_{\text{i}}$  have been reported previously in cells treated with Tg or other SERCA inhibitors (30-32). In aggregate, SOCE appears to be fully activated at <50% depletion of the  $\text{ER}_{\text{sub}}$  (at this time point there is <15% decrease in the internal ER). These data agree well with the predictions made by the model.

ER stress response is a hallmark of global depletion of ER  $\text{Ca}^{2+}$  and has been reported to occur in cells treated with relatively high concentrations of Tg and agonists (within the range used to activate SOCE, (33,34). One of the earliest events in the unfolded protein response to ER stress is activation of the kinase PERK, which leads to inhibition of protein synthesis. Figure 6D shows that PERK is activated within 3 min after stimulation with either 100  $\mu\text{M}$  carbachol (CCh) or 1  $\mu\text{M}$  Tg, detected as phosphorylated PERK. More significantly, treatment of cells with 1  $\mu\text{M}$  Tg significantly increased cell death within 5 min (Fig 6E; the effect of CCh on cell viability was not examined). However, low concentrations of Tg (1 and 10 nM) did not induce did not

induce activation of PERK (Fig 6D) or cell death for up to 15 min (Fig 6E). Consistent with these findings, 1  $\mu$ M Tg induced global redistribution of STIM1 into punctae following stimulation when compared to control cells (Fig 6F), while 1 nM induced minimal global redistribution of STIM1. It should be noted that 1 nM Tg does induce relocalization of STIM1 in the periphery of the cells (see Fig 4). Similar results were also obtained using the muscarinic agonist CCh which induces SOCE in HSG cells (1,2,16). While global redistribution of STIM1 was observed following stimulation with 100  $\mu$ M CCh, minimal redistribution was observed with 1  $\mu$ M CCh (Supplemental Fig 2A). However, both high and low [CCh] induced peripheral STIM1 relocalization as detected by TIRFM (Supplemental Fig 2B). Previous reports show that low [agonist] and [IP<sub>3</sub>] induce incomplete depletion of internal Ca<sup>2+</sup> stores (14). However, TIRFM demonstrated that similar Ca<sup>2+</sup> entry was induced by 300 nM and 10  $\mu$ M CCh in exocrine gland acinar cells (35). Together, these data suggest that subplasma membrane ER depletion is involved in SOCE activation by CCh as well as Tg.

**Localization of ER and Tg-stimulation of [Ca<sup>2+</sup>]<sub>i</sub> changes in the subplasma membrane region.** The data presented above strongly suggest that depletion of subplasma membrane ER is involved in relocalization of STIM1 and activation of SOCE. We measured this more directly using TIRFM. ER-tracker dye labeling in this region as well as of the entire cytosol by epifluorescence (shown in Fig 7A) conclusively demonstrated that ER is present in close proximity to the plasma membrane. These data agree

well with recent studies reported by Lewis and co-workers (7,8). Importantly, both 1 nM and 1  $\mu$ M Tg induced sustained [Ca<sup>2+</sup>]<sub>i</sub> increases in this region (Figs 7 B and C, relative changes in Fluo-4 fluorescence measured using TIRFM are shown) in cells maintained in a normal Ca<sup>2+</sup>-containing external medium. Fluorescence increase was greater and faster with the higher [Tg]. When 1  $\mu$ M Gd<sup>3+</sup> was included in the external solution to block SOCE, the increase was smaller and more transient in both cases (Figs 7 D and E), suggesting that both Ca<sup>2+</sup> release and Ca<sup>2+</sup> entry contributed to the [Ca<sup>2+</sup>]<sub>i</sub> increases seen in Figs 7 B and C. However, internal Ca<sup>2+</sup> release in the subplasma membrane region appears to be less when cells are treated with low Tg since the change in Fluo-4 fluorescence induced by 1  $\mu$ M Tg was faster and larger (Fig 7D) than that seen with 1 nM Tg (Fig 7E). Ca<sup>2+</sup>-addback protocol was used to measure SOCE (Figs 7 F and G). The relative fluorescence changes induced by 1 nM and 1  $\mu$ M Tg induced in a Ca<sup>2+</sup>-free medium were similar to those seen in cells stimulated in the presence of 1  $\mu$ M Gd<sup>3+</sup> (note difference in scale for traces compared to those used for B-E). Importantly, re-addition of Ca<sup>2+</sup> induced comparable increases in Fluo-4 fluorescence in cells treated with 1  $\mu$ M and 1 nM Tg (Figs 7 F and G), suggesting that similar SOCE was induced under these two conditions.

Mag-Fluo4 and TIRFM were used to confirm the depletion of peripheral Ca<sup>2+</sup> stores by low and high [Tg]. As shown in Fig 7H, both 1 nM and 1  $\mu$ M Tg induced similar decrease (about 50-60%) in Mag-Fluo4 fluorescence in the subplasma membrane regions in cells.

Thus, peripheral ER depletion under these two conditions is similar. The apparently higher  $[Ca^{2+}]_i$  increase in this region in 1  $\mu$ M Tg-treated cells (Figs 7B-E) can be attributed to rapid diffusion of  $Ca^{2+}$  released more internally in the cells to the periphery (35).

### Discussion

The data presented above demonstrate that the peripheral relocalization of STIM1 and activation of SOCE are determined by the status of  $Ca^{2+}$  in ER localized in the subplasma membrane, rather than in more internal, regions of the cell. A key finding of this study is that low [Tg] (1 nM) activates SOCE to the same extent as high [Tg] (1  $\mu$ M) but with much less internal  $Ca^{2+}$  store depletion. Importantly, the SOCE regulatory protein STIM1 is relocalized primarily in the peripheral region of cells stimulated with 1 nM Tg in contrast to the more global relocation seen at the higher [Tg]. By using 1 nM BD-Tg to examine the extent of diffusion of Tg during activation of SOCE, we show that 1 nM Tg diffuses into the subplasma membrane region of the cells during the time required to maximally activate SOCE and relocalize STIM1 in this region. The presence of Tg in this region was temporally correlated with greater depletion of  $Ca^{2+}$  in ER localized in the periphery of the cells rather than in more internal regions. Finally, we show that under conditions where there is recycling of  $Ca^{2+}$  and incomplete depletion of peripheral ER there is less mobilization of STIM1 in the subplasma membrane region.  $Ca^{2+}$ -induced  $Ca^{2+}$  release has been suggested to contribute to  $[Ca^{2+}]_i$  increases and specifically  $Ca^{2+}$ -entry has been proposed to initiate  $Ca^{2+}$ -induced  $Ca^{2+}$  release in some cell types

(28). However, we can rule out the involvement of  $Ca^{2+}$ -induced  $Ca^{2+}$  release in our findings based on the following: (i)  $Ba^{2+}$  entry was similar with 1 nM and 1  $\mu$ M Tg, (ii)  $Mn^{2+}$  entry was also similar in both cases although the onset of SOCE was slower at the lower [Tg], (iii) blockers (ryanodine and caffeine, data not shown) of  $Ca^{2+}$ -induced  $Ca^{2+}$  release had no effect of 1 nM or 1  $\mu$ M Tg-stimulated internal  $Ca^{2+}$  release or  $Ca^{2+}$  entry components, and (iv)  $Ca^{2+}$  entry measured by TIRF, which would exclude contributions of internal ER, was similar with 1 nM and 1  $\mu$ M Tg. In aggregate, our data suggest that the formation of peripheral STIM1 punctae and SOCE activation is determined by the status of the local, subplasma membrane  $Ca^{2+}$  store and does not require depletion of more internal ER- $Ca^{2+}$  store. Decrease in the  $[Ca^{2+}]_i$  in the subplasma membrane ER is sensed by STIM1 localized in within the same domain which responds by the redistributing into punctae. These STIM1 punctae have been suggested as the elemental functional unit of SOCE (7,8) and likely represent homomeric complexes between STIM1 monomers as well as heteromeric interaction of STIM1 with proposed SOCE channels, TRPC1 and Orail (7,9,36-40). How exactly gating of the plasma membrane channel occurs is not yet clear, although STIM1 C-terminus has been suggested to be sufficient for SOCE activation (9).

The juxtaposition of ER with the plasma membrane  $Ca^{2+}$  entry channel facilitates  $Ca^{2+}$  entering the cell to be rapidly taken up into these peripheral stores without a substantial change in the global  $[Ca^{2+}]_i$ . This allows the cell to control the  $[Ca^{2+}]_i$  at the site of  $Ca^{2+}$  entry and thus minimize feedback inhibition of the

channel by local increases in  $[Ca^{2+}]_i$  (3-5). Thus while uptake into the ER is dependent on the activity of SERCA pumps, retention of the  $Ca^{2+}$  within the ER is determined by its "leakiness". Our data demonstrate that the extent of store depletion, and therefore the time required for activation of SOCE as well as its magnitude, is determined by recycling of  $Ca^{2+}$  into the ER, either from the cytosol or from the external medium via  $Ca^{2+}$  entry. When  $Ca^{2+}$  was used as the charge carrier, the lag for onset of the current was relatively longer with 1 nM Tg than with 1  $\mu$ M Tg and the magnitude of the current was also lower. However, when  $Na^+$  was used as the charge carrier, (i) the magnitude of the current was similar in the two conditions and (ii) although the lag with the lower [Tg] was reduced it was still larger than that seen with 1  $\mu$ M Tg. Similarly,  $Mn^{2+}$  entry was initially (<150 s) lower with 1 nM Tg. Further, we observed that  $I_{SOC}$  activated by low [Tg] is also relatively more stable than when stimulated by high [Tg]. These findings can be explained by the  $Ca^{2+}$  recycling that occurs at the lower [Tg] due to residual SERCA activity. Not only does this result in incomplete or delayed ER  $Ca^{2+}$  depletion but also lowers ambient  $[Ca^{2+}]_i$  in the vicinity of the  $Ca^{2+}$  entry channel, thus attenuating  $Ca^{2+}$ -dependent feedback inhibition of the channel (1,3,19). An important finding that demonstrates the relevance of the peripheral ER store status is that at the lower [Tg], STIM1 relocation is more prominent in cells stimulated in medium without external  $Ca^{2+}$ . We suggest that under these conditions,  $Ca^{2+}$  in the peripheral stores is sufficiently depleted (i.e. concentration is below that required for optimal binding to STIM1) to cause

STIM1 relocation and SOCE activation.

Based on our findings, we propose that activation of SOCE is determined by the  $[Ca^{2+}]$  in subplasma membrane ER. SOCE is activated by depletion of ER in this region and is not further increased by additional depletion of more internal ER. Our data also provide a possible explanation for activation of SOCE by agonist-dependent store depletion. Typically, low [agonist] induces  $[Ca^{2+}]_i$  oscillations, which in some cases do not appear to be associated with SOCE (41), while higher [agonist] or  $[IP_3]$  induces substantial ER depletion and maximally activates SOCE. We have shown here that low and high [CCh] induce similar relocation of STIM1 in the periphery of the cells but only high [CCh] induces global relocation of STIM1. This indicates that low [CCh] likely induces depletion of peripheral but not internal ER. As suggested by our observations with Tg, small depletions of  $Ca^{2+}$  in the peripheral, or internal, ER might not lead to detectable SOCE due to rapid recycling of  $Ca^{2+}$ . However, higher  $[IP_3]$  can induce substantial global  $Ca^{2+}$  depletion resulting in SOCE which is easily detected. Consistent with this suggestion, Won and Yule (35) have demonstrated that  $Ca^{2+}$  entry induced by low concentrations of carbachol are not detectable using wide-field microscopy but can be readily detected by using TIRFM. In aggregate, our findings agree well with the mechanism proposed by Berridge (5) to describe how the  $Ca^{2+}$  status of peripheral and internal ER regulates SOCE, according to which depletion of peripheral ER or sufficient release of  $Ca^{2+}$  in the internal ER (enough to drain  $Ca^{2+}$  from the peripheral ER) can result in activation of

SOCE. Further, the characteristic slow inactivation of SOCE that occurs as internal stores are refilled (6,29,42) can also be explained.  $\text{Ca}^{2+}$  entering the cell via SOCE is taken up into subplasma membrane ER from where it diffuses into the internal ER, if the latter is depleted. As the internal ER is refilled,  $[\text{Ca}^{2+}]$  in the subplasma membrane ER will increase, resulting in inactivation of SOCE by an as yet unidentified mechanism. Whether this is correlated with STIM1 disaggregation is not yet known. As long as the internal ER stores are depleted, SOCE will remain activated. Thus SOCE ensures refilling of internal  $\text{Ca}^{2+}$  stores, irrespective of the route of depletion, and protects against generation of ER stress and cell death.

In conclusion, STIM1, has been suggested to be an essential regulatory component of SOCE (7,10-13). Studies reported recently have shown that stimulation of cells induces STIM1 to aggregate into punctae in the subplasma membrane region of the cells (7,8,10,13) which appear to be causal in activation of SOCE. It is further suggested that these punctae indicate the localization of

the functional unit of SOCE. The present data demonstrate that mobilization of STIM1 and activation of SOCE are associated with the decrease of  $\text{Ca}^{2+}$  in ER which is localized within the subplasma region of the cell. Thus activation of SOCE by STIM1 is determined by the  $[\text{Ca}^{2+}]$  in the peripheral  $\text{Ca}^{2+}$  store where STIM1 is also localized. As long as  $[\text{Ca}^{2+}]$  in this store is below the threshold for binding to the STIM1-EF hand domain, STIM1 will be localized in punctae and SOCE will remain activated. Further studies are required to determine the exact molecular interactions involved in the generation of STIM1-containing punctae and how exactly these punctae regulate gating of the SOCE channel.

#### **Acknowledgments**

We are grateful to Dr. Tobias Meyer (Department of Molecular Pharmacology, Stanford University) for kindly providing us with the YFP-STIM1 DNA. We are also grateful to Dr. Vincent Schram (NICHD, NIH) for his assistance in TIRF, which was performed at the Microscopy and Imaging Core (NICHD, NIH).

## Figure Legends

**Figure 1. Activation of SOCE by incomplete depletion of ER Ca<sup>2+</sup> stores.** (A, B) Tg - induced intracellular Ca<sup>2+</sup> release and Ca<sup>2+</sup> entry in HSG cells. The various [Tg] used for activation of SOCE are indicated in the figure. (C) Ca<sup>2+</sup> release induced by 1 μM Tg in control cells (blue trace) and in cells previously treated with 1 nM Tg (black trace), indicating the extent of depletion of ER by 1 nM Tg. (D) Dose-response curve showing effect of [Tg] on Ca<sup>2+</sup> release (units; squares) and the rate of Ca<sup>2+</sup> influx (units/s; circles). Data were plotted as mean ± s.e.m. from 3-4 separate experiments and expressed in 340/380 fluorescence ratio units, error bars were within the size of the symbols. Ca<sup>2+</sup> influx induced by 1 μM and 1 nM Tg were blocked by 1 μM GdCl<sub>3</sub> (E and G, respectively) and 10 μM 2-APB (F and H, respectively). A trace showing the inhibition of Ca<sup>2+</sup> influx induced by 1 μM Tg by 1 μM GdCl<sub>3</sub> added before 1 mM CaCl<sub>2</sub> is shown as an inset (similar results were seen with 1 nM Tg, data not shown) (E). [Ca<sup>2+</sup>]<sub>i</sub> was measured in Fura-2 loaded cells and is expressed as 340/380 ratio. Each analog plot showing Ca<sup>2+</sup> release and Ca<sup>2+</sup> entry is representative of at least 4 experiments, each trace showing the average from at least 50 cells.

**Figure 2. Activation of I<sub>SOC</sub> and Ba<sup>2+</sup> influx by thapsigargin.** Activation of I<sub>SOC</sub> by high (A) and low [Tg] (B) in Ca<sup>2+</sup> and Mg<sup>2+</sup>-containing external medium (currents recorded at -80 mV are plotted). I-V relationships of the maximum currents shown in A and B are presented in (C). Activation of I<sub>SOC</sub> by high (D) and low (E) [Tg] in DVF medium (current recorded at -80 mV are plotted). I-V relationships of the maximum currents in D and E are shown in (F). These data represent results obtained with a minimum of 7-10 cells under each condition, with each trace showing the plot obtained with a single representative cell. Other details are provided in the text and Experimental Procedures. 1 μM (G) and 1 nM Tg (H)-induced Ba<sup>2+</sup> influx. Cells were stimulated with Tg in Ca<sup>2+</sup>-free medium and 1 mM BaCl<sub>2</sub> was added where indicated. Basal Ba<sup>2+</sup> entry, in unstimulated cells incubated in Ca<sup>2+</sup> free medium was minimal (data not shown).

**Figure 3. Temporal characteristics of SOCE activation.** Cells were stimulated with 1 nM Tg in Ca<sup>2+</sup>-free medium. 1 mM CaCl<sub>2</sub> added at 20 (A), 50 (B), 150 (C) and 250 s (D) after Tg addition. (E) Time-course of SOCE activation upon Ca<sup>2+</sup> addition after 1 nM Tg (rate of Ca<sup>2+</sup> influx (units/s) vs. time (s) after 1 nM Tg addition). The red \* shows the rate of maximal Ca<sup>2+</sup> influx (units/s) induced by 1 μM Tg with 1 mM CaCl<sub>2</sub> added 300 s after Tg addition. Data are plotted as mean ± s.e.m. (n=3). Mn<sup>2+</sup> influx induced by 1 μM (red trace) and 1 nM (black trace) Tg. Cells were stimulated with Tg in Ca<sup>2+</sup>-free medium, 100 μM MnCl<sub>2</sub> was added 50 (F), 150 (G) and 250 (H) s after Tg addition. Relative fluorescence at 365 nm is shown (these traces represent average fluorescence changes from more than 70 cells. Similar results were obtained in 4 separate experiments).

**Figure 4. Redistribution of YFP-STIM1 to the subplasma membrane region following Tg stimulation.** (A) Distribution of mYFP, measured using TIRFM in control and TG-treated cells. (B, C) Redistribution of YFP-STIM1 following stimulation with 1 μM and 1 nM Tg, respectively, in Ca<sup>2+</sup>-containing medium. (D) Redistribution of YFP-STIM1 following stimulation with 1 nM Tg in Ca<sup>2+</sup>-free medium. The time point at which each image was taken is noted in the figure.

**Figure 5. Localization of Tg during activation of SOCE. (A, B).** Labeling of HSG cells with 1 nM and 1  $\mu$ M BD-Tg detected by confocal microscopy. The images shown were taken 3 min after the addition of BD-Tg and displayed along the x-y (top panel) and x-z (bottom panel) axes. The arrow shows the bottom of the dish (C) Overlays of BD-Tg labeled cells and the DIC images of the same field. (D) Dampening of the BD-Tg signal when 1 nM BD-Tg was added following pre-incubation of cells with 2 nM unlabelled Tg. (E) Time-course of labeling the subplasma membrane, internal, and nuclear regions with 1 nM BD-Tg. Quantification was done as described in Experimental Procedures using specific regions of interest in the areas of the cell indicated. Similar data were obtained from 5 separate experiments.

**Figure 6. Tg-stimulated depletion of ER- $\text{Ca}^{2+}$ .** [ $\text{Ca}^{2+}$ ] in internal (squares) and subplasma membrane ER ( $\text{ER}_{\text{sub}}$ ; circles) calcium stores in cells treated with 1 nM (A) or 1  $\mu$ M thapsigargin (Tg, B) and control cells (C) measured using imaging of Mag-Fluo-4 fluorescence. Confocal microscopy (details are given in the Experimental Procedures section) was used to determine fluorescence changes. Fluorescence was determined in demarcated areas in the plasma membrane and internal regions (areas not shown). Traces shown are averages from at least 6-8 cells and represent similar data obtained from 3 separate experiments. (D) Detection of phosphorylated PERK (pPERK) in lysates of HSG cells treated with 100  $\mu$ M CCh, 1  $\mu$ M Tg, and 1 nM Tg by Western blotting using phospho-PERK antibody (1:1000 dilution). (E) The % of apoptotic cells detected using the Vybrant staining assay in control HSG cells and cells treated with 1 nM, 10 nM, and 1  $\mu$ M thapsigargin for 0, 5 and 15 min. Data are plotted as mean  $\pm$  s.e.m., where \* indicates  $P < 0.05$ . (F) Distribution of STIM1 in control HSG cells and following stimulation with 1  $\mu$ M and 1 nM Tg. STIM1 was detected using the mouse anti-STIM1 antibody (1:100 dilution) and the FITC-conjugated anti-mouse secondary antibody (1:100 dilution).

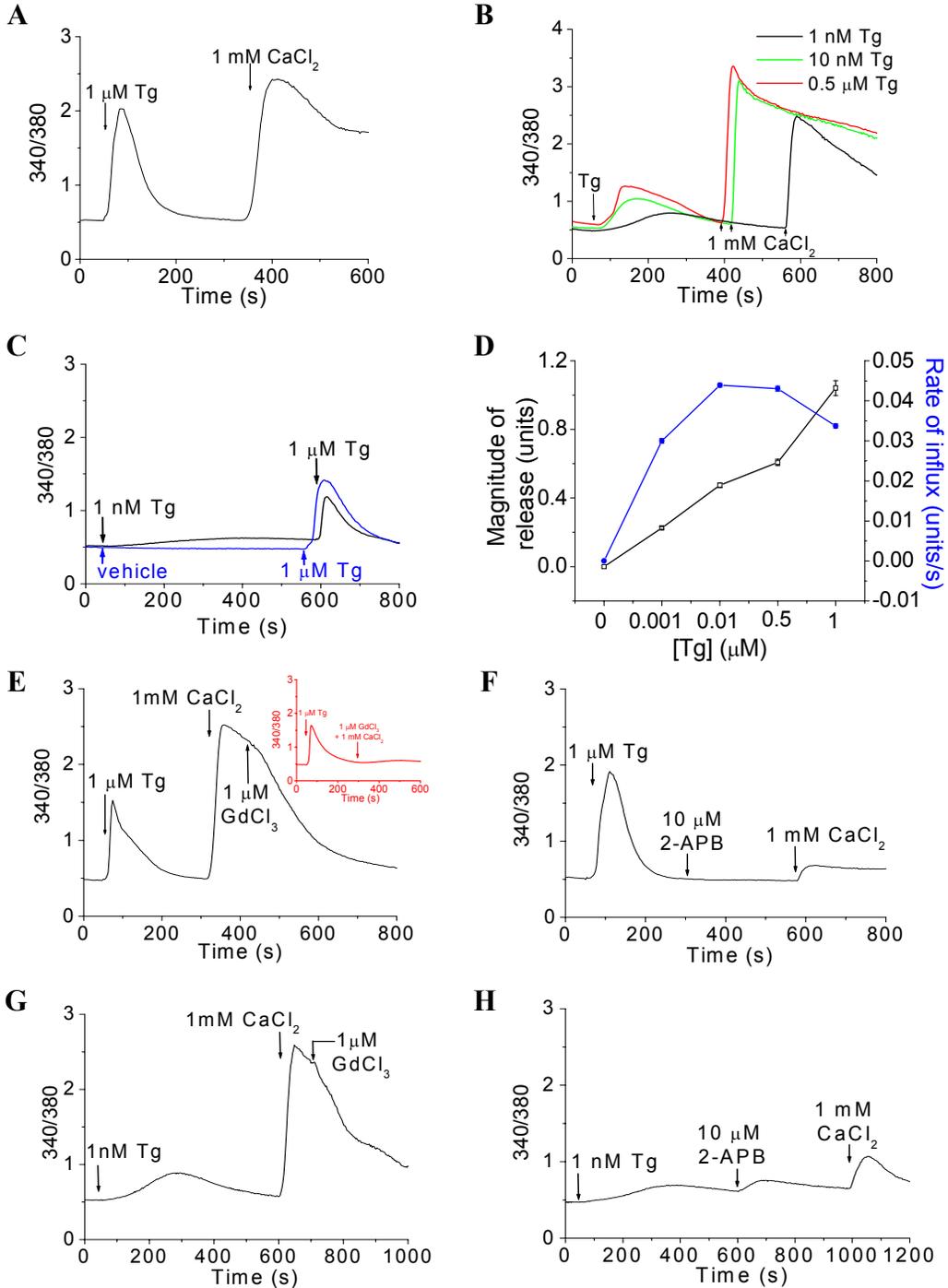
**Figure 7. Tg-stimulated [ $\text{Ca}^{2+}$ ] changes in the subplasma membrane region. (A)** Localization of the ER network within the subplasma membrane region using the ER-Tracker Red dye, left panel shown an epifluorescence image while the right panel shows a TIRF image. TIRFM was used to monitor [ $\text{Ca}^{2+}$ ]<sub>i</sub> with Fluo-4 (B-G) and [ $\text{Ca}^{2+}$ ] in the ER with Mag-Fluo4 (H) fluorescence in the subplasma membrane region of HSG cells (details in the Experimental Procedures section). Each analog plot showing  $\text{Ca}^{2+}$  release and  $\text{Ca}^{2+}$  entry is representative of at least 2 experiments, with each trace showing the average for at least 20 cells. (B-E) Cells were stimulated with thapsigargin (Tg) in  $\text{Ca}^{2+}$ -containing media, with or without 1  $\mu$ M  $\text{GdCl}_3$  ( $\text{Gd}^{3+}$ ). Basal  $\text{Ca}^{2+}$  influx is as shown by in (B). (F, G) Cells were initially stimulated with thapsigargin (Tg) in  $\text{Ca}^{2+}$ -free media, followed by re-addition of 1mM  $\text{CaCl}_2$  (fluorescence increase in each case was similar in the presence of  $\text{Gd}^{3+}$  or in  $\text{Ca}^{2+}$ -free medium, note that the scales in F and G are different from those in B-E). Basal  $\text{Ca}^{2+}$  influx is shown in F. (H) Cells were stimulated with Tg in  $\text{Ca}^{2+}$ -free media. Images at 0 and 4 min after Tg addition are as shown. For control cells, buffer was added instead of Tg.

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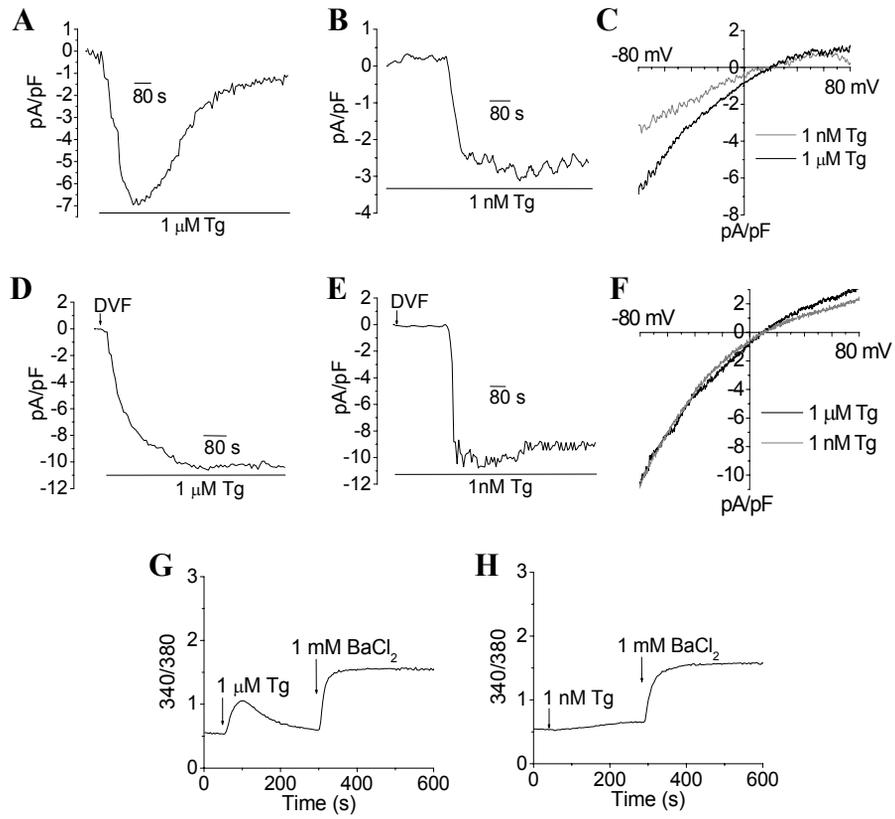
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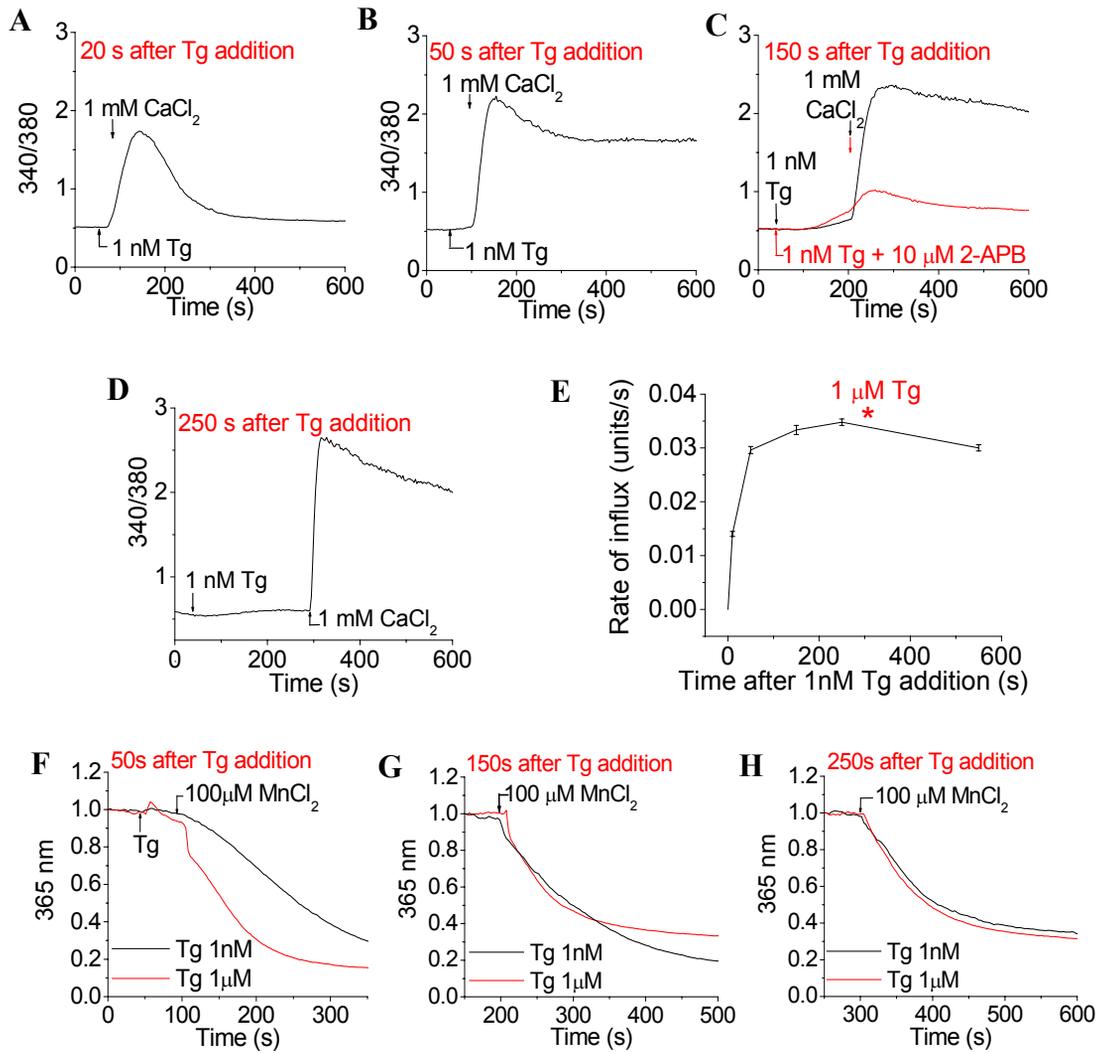
**FIGURE 1**



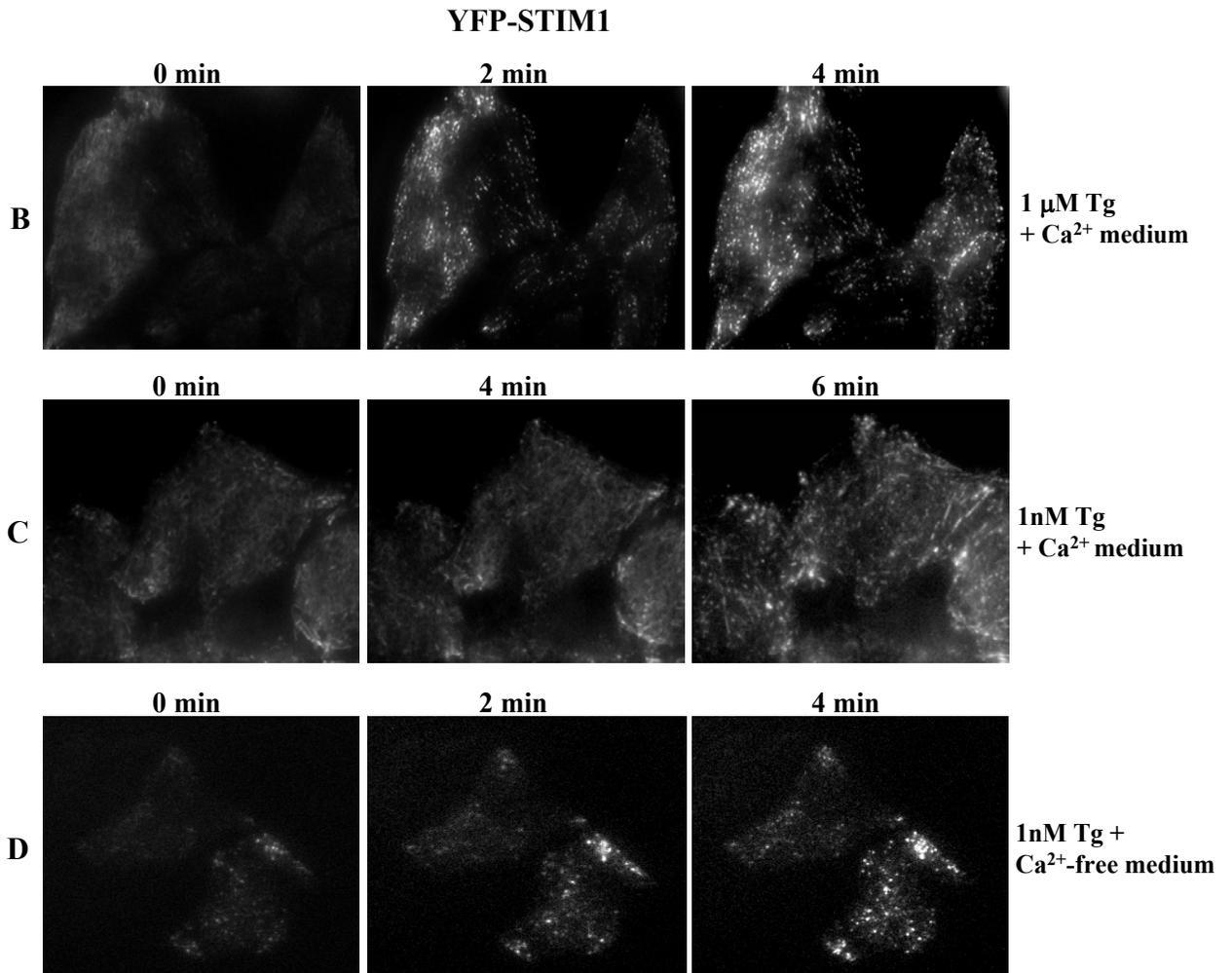
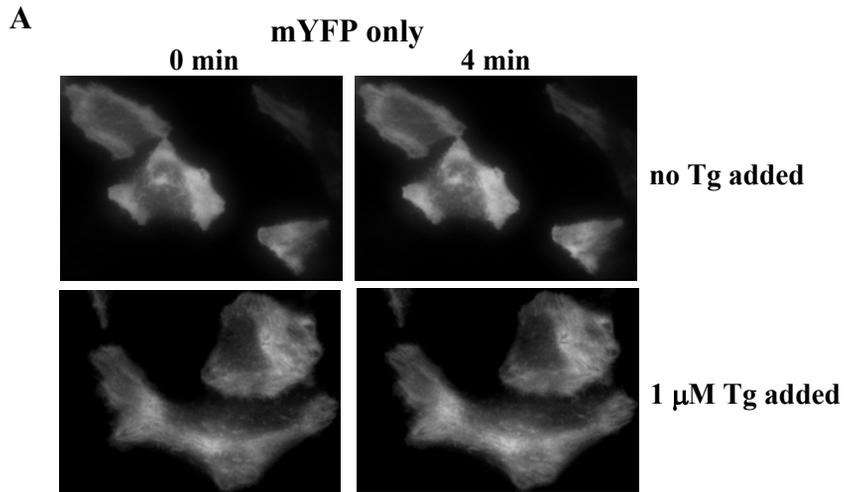
**FIGURE 2**



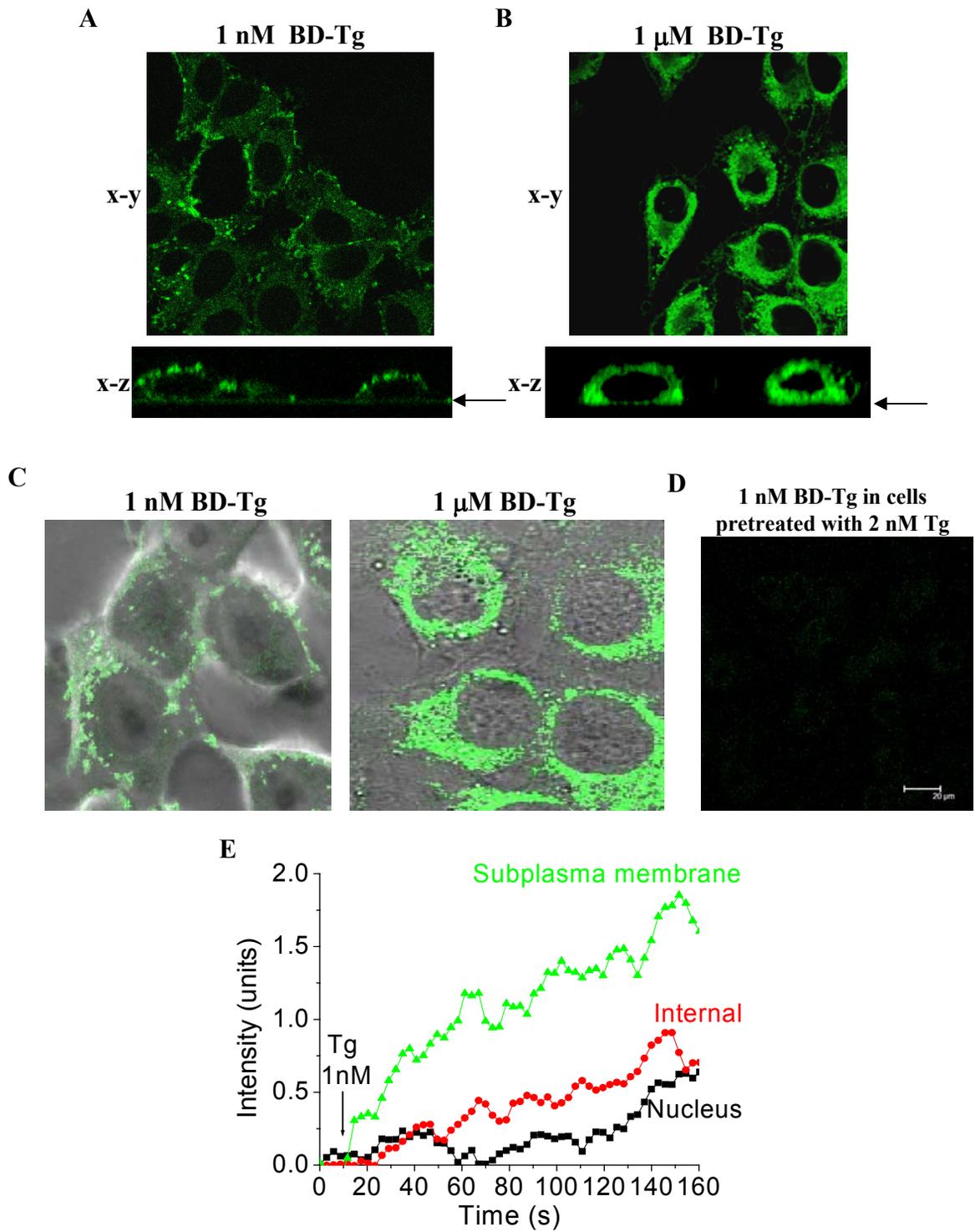
**FIGURE 3**



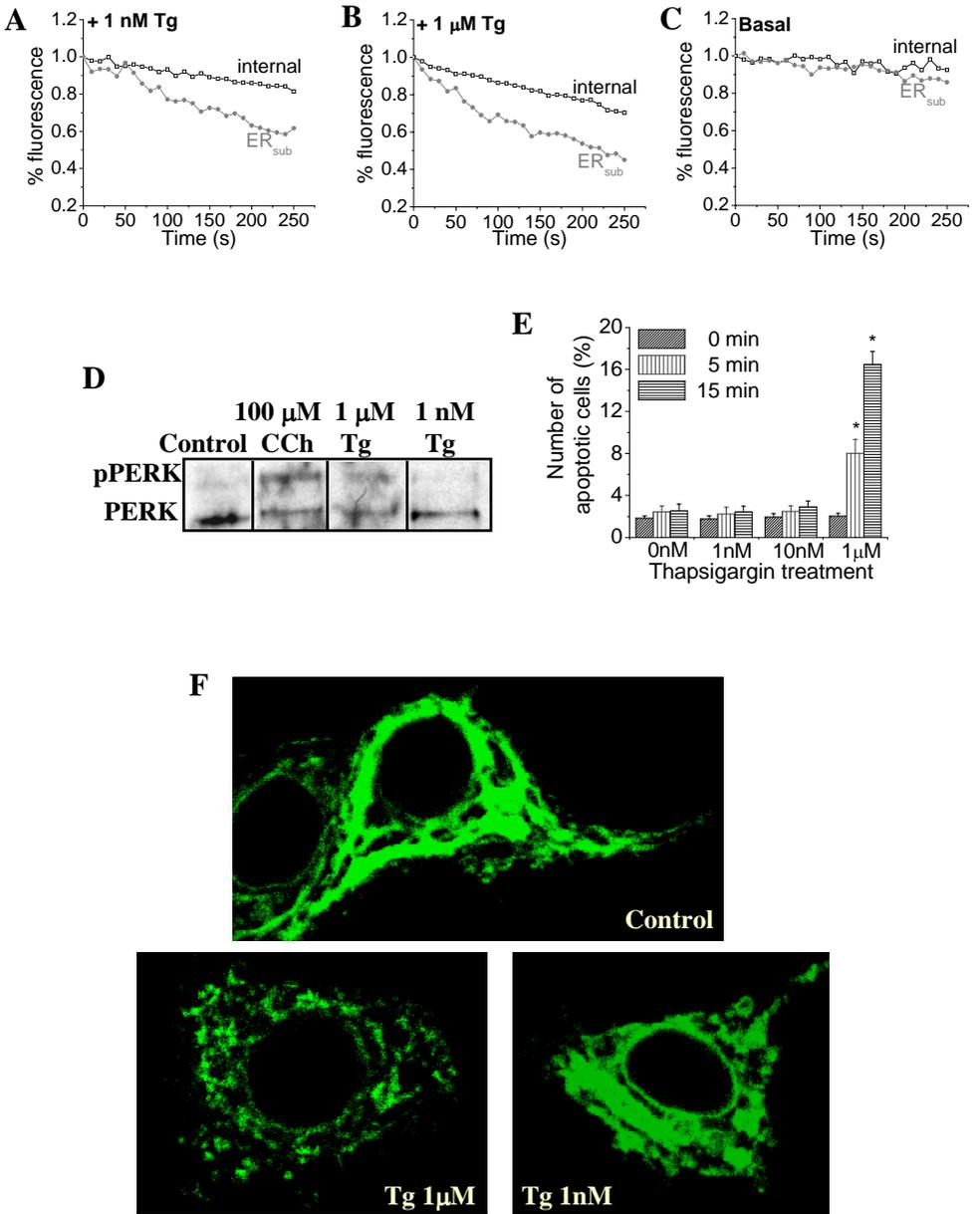
**FIGURE 4**



**FIGURE 5**



**FIGURE 6**



**FIGURE 7**