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TRUSS, TNF-R1, and TRPC Ion Channels Synergistically Reverse Endoplasmic Reticulum Ca^{2+} Storage Reduction in Response to m1 Muscarinic Acetylcholine Receptor Signaling

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Abstract

Although most signaling responses initiated by tumor necrosis factor-α (TNF-α) occur in a Ca^{2+}-independent fashion, TNF-α receptor signaling augments Ca^{2+} entry induced by $G\alpha_{q/11}$ G-protein coupled receptors (GPCRs) in endothelial cells and increases transendothelial permeability. The signaling events involved in GPCR-induced Ca^{2+} influx have been characterized and involve store-operated Ca^{2+} entry facilitated by the Ca^{2+} permeable ion channel, transient receptor potential canonical 4 (TRPC4). Little is known about the mechanisms by which TNF-α receptor signaling augments GPCR-induced Ca^{2+} entry. *T* NF-α *R* eceptor *U* biquitous *S* ignaling and *S* caffolding protein (TRUSS) is a tumor necrosis factor receptor-1 (TNF-R1)-associated protein whose gene name is TRPC4-associated protein (TRPC4AP). The goal of our study was to test the hypothesis that TRUSS serves to link TNF-R1 and GPCR-signaling pathways at the level of TRPC4 by: (i) determining if TRUSS and TNF-R1 interact with TRPC4, and (ii) investigating the role of TRUSS, TNF-R1, and TRPC4 in GPCR-induced Ca^{2+} signaling. Here, we show that TRUSS and TNF-R1 interact with a sub-family of TRPC channels (TRPC1, 4, and 5). In addition, we show that TRUSS and TNF-R1 function together with TRPC4 to elevate endoplasmic reticulum Ca^{2+} filling in the context of reduced endoplasmic reticulum Ca^{2+} storage initiated by G-protein coupled m1 muscarinic acetylcholine receptor (m1AchR) signaling. Together, these findings suggest that TNF-R1, TRUSS, and TRPC4 augment Ca^{2+} loading of endoplasmic reticulum Ca^{2+} stores in the context of m1AchR stimulation and provide new insights into the mechanisms that connect TNF-R1 to GPCR-induced Ca^{2+} signaling.

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Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine involved in innate and adaptive immunity, inflammation, apoptosis, and cell survival. Although a limited repertoire of TNF-α-induced responses are initiated by TNF-receptor-2 (Peschon et al., 1998; Wang et al., 2009a,b), most are mediated by tumor necrosis factor receptor-1 (TNF-R1), which signals the activation of nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPK), Akt, and apoptosis (Locksley et al., 2001; Chen and Goeddel, 2002). TNF-R1 signaling events are activated in a Ca^{2+}-independent fashion. However, TNF-α signaling does affect Ca^{2+} entry and signaling by $G\alpha_{q/11}$-linked G-protein coupled receptors (GPCRs). Studies initially reported by Amrani et al. (1995, 1996) showed that incubation of tracheal smooth muscle cells with TNF-α augmented bradykinin and histamine-induced Ca^{2+} influx leading to increased smooth muscle cell proliferation and tracheal smooth muscle contraction. Similarly, Tiruppathi et al. (2001) revealed a fundamentally similar signaling response in human umbilical vein endothelial cells that were incubated with TNF-α for 2 h prior to stimulation with the PAR-1 agonist, thrombin. Thus, while TNF-α signaling occurs independently of changes in intracellular Ca^{2+}, it promotes Ca^{2+} entry and signaling by some $G\alpha_{q/11}$-linked GPCRs.

GPCR-stimulated Ca^{2+} entry in diverse non-excitable cells utilizes $G\alpha_{q/11}$-dependent activation of phospholipase C-β, which leads to the generation of diacylglycerol and inositol 1,4,5-trisphosphate (IP3) (Tiruppathi et al., 2002b; Bird et al., 2004). Binding of IP3 by IP3 receptors located on the endoplasmic reticulum stimulates Ca^{2+} release into the cytosol, which in turn promotes store-operated Ca^{2+} entry via STIM1, a sensor of endoplasmic reticulum Ca^{2+} depletion, and Orai1, a STIM1-linked Ca^{2+} channel located on the plasma membrane (Liou et al., 2005; Roos et al., 2005; Feske et al., 2006; Vig et al., 2006; Putney, 2007). In addition, several members of the mammalian transient receptor potential canonical (TRPC) family have been implicated in store-operated Ca^{2+} entry in non-excitable cells, including TRPC1, 4, and 5 (Liu et al., 2007; Worley et al., 2007; Yuan et al., 2007). Of these, targeted disruption of the *trpc4* gene in mice has been shown to inhibit store-operated Ca^{2+} entry in endothelial cells (Freichel et al., 2001; Tiruppathi et al., 2002a). Despite the fundamental importance of these events in endothelial barrier disruption in acute lung injury and smooth muscle contraction in airway hyper-responsiveness, little is known about the molecular mechanisms that link TNF-R1 signaling and GPCR-induced Ca^{2+} signaling.

TNF-R1 ubiquitous scaffolding signaling protein (TRUSS) was originally identified in a two-hybrid screen as a TNF-R1-associated protein and has since been shown to be a component of TNF-R1 signaling complexes involved in NF-κB and JNK activation (Soond et al., 2003, 2006; Rual et al., 2005). The gene name for TRUSS is TRPC4-associated protein (TRPC4AP). In view of the role of TRPC4 in promoting store-operated Ca^{2+} entry in endothelial cells, together with the effect of TNF-α signaling in augmenting this response, we investigated the potential role of TRUSS in coupling TNF-R1 to the augmentation in GPCR-induced Ca^{2+} responses. The goals of our study were: (i) to determine if TRUSS and TNF-R1 interact with TRPC channels, and (ii) to evaluate if TRUSS and TNF-R1, along with TRPC channels, influence GPCR-induced Ca^{2+} signaling. These goals were addressed using an HEK293 cell model since Ca^{2+} signaling has been extensively studied in this cell line and because of their suitability for studies aimed at manipulating the expression of

genes of interest. In addition, we addressed these goals in the context of Ca^{2+} signaling induced by the G-protein coupledm1muscarinic acetylcholine receptor (m1AcR). As we will show, TRUSS and TNF-R1 interact with a subset of TRPC channels and serve to promote the filling of endoplasmic reticulum Ca^{2+} stores. Together, these findings suggest that TRUSS and TNF-R1 work together to affect $G\alpha_{q/11}$-linked GPCR-induced Ca^{2+} signaling.

Methods

Cell culture and transfection

HEK293 cells, purchased from ATCC (Manassas, VA), were cultured at 37°C in 5% CO_2, in Dulbecco's modified Eagle's medium plus 10% heat-inactivated fetal bovine serum supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100μg/ ml streptomycin. Twenty-four to 48 h before transfection, $3–6 \times 10^5$ cells/well were plated onto poly-D-lysine-coated, six-well culture dishes. Transfections were accomplished with 5 μl of Lipofectamine 2000 (Invitrogen Life Technology, Carlsbad, CA) per well according to the manufacturer's instructions. Generally, cells were transfected with the following amounts of pcDNA3.1 plasmids: 1 μg mTRUSS, 0.5 μg h-Flag-TNF-R1, 0.25 μg HATRPC1, -C4 or -C5, and 1μg HA-TRPC3, -C6, -C7. One microgram of m1AchR and m3AchR in pCMV were transfected per well. Transfections were controlled for DNA amounts by adding empty pcDNA3.1. Lipid with DNA complexes were incubated with cells in 2 ml/well Optimem (Gibco Invitrogen, Carlsbad, CA) for 4–6 h before addition of an equal amount of DMEM containing 20% FBS. Co-immunoprecipitation and intracellular Ca^{2+} measurements were carried out 24–48 h post-transfection.

Co-immunoprecipitation and Western blotting

HEK293 cells were plated at a density of $0.5–1.0 \times 10^6$ cells per well, 24 h before transfection as described above. The cells were lysed with lysis buffer (20mM Tris/HCl buffer, pH 8.0, containing 150mMNaCl, 0.5% (w/v) deoxycholate, 1% (v/v) Triton-X 100 and a final concentration of the protease inhibitors: 5μg/ml leupeptin, 5 μg/ml aprotinin, 1mM NaF, and 1mM Na_3VO_4), then passed 10 times through a 20-gauge needle and 5 times through a 26-gauge needle. Insoluble nuclear material was then removed cleared centrifugation at $2 \times 10^4 g$ for 10 min to obtain a whole cell lysate (WCL). Immunoprecipitations were conducted by adding 2 μg of anti-HA (Roche Diagnostics Corp., Indianapolis, IN), anti-TRUSSC terminal (Soond et al., 2003), 1.5 μg goat anti-TNF-R1 (R&D Systems, Minneapolis, MN) antibodies or an equivalent amount of murine, rabbit, or goat non-immune IgG (Santa Cruz Biotechnology, Santa Cruz, CA) together with Protein G PLUS agarose beads (20–50 μl) (Santa Cruz) to 400–500 μg of WCL protein. The tubes were rotated overnight at 4°C and the agarose-complexes washed three times with 400 μl of lysis buffer containing 150–500mM NaCl. Finally, the agarose beads were resuspended in 2 × Laemmli sample buffer, boiled for 5 min, resolved by SDS–PAGE and the bound proteins were detected by Western blotting with anti-TRUSS C-terminal, anti-FLAG-M2 (Sigma-Aldrich, St. Louis, MO), or anti-HA (Covance, Emoryville, CA) antibodies. To control for the level of protein expression, 15 μg of WCL protein, obtained prior to immunoprecipitation, was included on each gel.

Intracellular Ca^{2+} measurements

Transfected HEK293 cells were removed from poly-D-lysine-coated plates with 0.25% (w/v) trypsin–EDTA and pelleted by centrifugation at 1,000*g* for 10 min. Supernatants were removed and the cells were resuspended in 3ml of Ca^{2+} buffer (10mM HEPES, pH 7.5, containing 135mM NaCl, 5mM KCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 5.6mM glucose, and 0.1% (w/v) BSA). The cells were loaded in Ca^{2+} buffer with 0.5–0.75 μM Fura-2 AM for 20 min at room temperature, de-esterified in 5ml of fresh Ca^{2+} buffer for 30 min at room temperature, and collected centrifugation at $2 \times 10^3 g$ for 8–10 min using a modification of a previously described procedure (Lussier et al., 2005). The buffer was removed and the cells were resuspended in 2ml of fresh Ca^{2+} buffer before analysis in a QM-6/2003 fluorometer (Photon Technology International Inc., Birmingham, NJ). Generally, baseline measurements were collected and, when indicated, EGTA was added to a final concentration of 625 μM before stimulation with a final concentration of 50μM carbachol (Cch) or 1.25 μM ionomycin. For store-operated Ca^{2+} entry experiments, $CaCl_2$ was added to a final concentration of 2.5 mM.

To quantify the Ca^{2+} content of the endoplasmic reticulum, appropriately transfected HEK293 cells were loaded with 1.5 μM Mag-Fura-2 AM in Ca^{2+} buffer for 45 min at 37°C as described (Hofer and Machen, 1993; Hofer et al., 1995; Hofer and Schulz, 1996). Cells were washed with Ca^{2+} buffer, pelleted and buffer was aspirated from the cells. Immediately prior to analysis, the cells were permeabilized with digitonin (10μg/ml for 5–10 min) in intracellular KCl buffer (10mMHEPES, pH 7.25 containing 125mM KCl, 25mM NaCl, 0.15mM $MgCl_2$, and 170 nM free Ca^{2+}). Fresh KCl buffer was added and cells were immediately analyzed. As a control, cells were loaded with Fura-2AMand examined in parallel. For these experiments, baseline data were collected for 1.5 min prior to the addition of ionomycin (1.25 μM). Data were collected for an additional 1.5 min after the addition of ionomycin.

The area under the curve was determined by summing the fluorescence-ratio (F340/F380) value for each point collected within the indicated phase of the response. This value was then subtracted by the average baseline value multiplied by the number of time points analyzed in the response phase. All samples within an experiment were normalized to the pcD control. The mean of each condition was calculated and a one-way ANOVA with Newman–Keuls multiple comparison post-test was performed on at least three independent experiments.

Results

TRUSS interacts with TRPC1, -C4, and -C5 but not TRPC3, -C6, or -C7

To determine if TRUSS interacts with TRPC channels, we co-transfected HEK293 cells with constructs encoding untagged TRUSS and HA-tagged TRPC channels. The cells were then lysed, immunoprecipitated with anti-TRUSS antibody, separated by SDS–PAGE and co-immunoprecipitating TRPC channels were detected by immunoblotting with anti-HA antibody. Figure 1A (upper parts) shows that TRPC1, 4, and 5 were specifically co-immunoprecipitated with TRUSS, whereas TRPC3, 6, and 7 were not. To confirm these

findings we performed the experiments in the reverse direction. Figure 1B (upper parts) shows that TRUSS co-immunoprecipitated with TRPC1, 4, and 5, but not TRPC3, 6, or 7 (Fig. 1B, lower parts). To verify specificity, all immunoprecipitations were performed in parallel with species-matched non-immune IgG (Fig. 1A,B). These data confirm that TRUSS associates specifically with the TRPC1, 4, and 5 subfamily of ion channels, while interactions with TRPC3, 6, and 7 were not detected above that seen with the non-immune IgG control.

TNF-R1 associates with TRPC ion channels

We initially cloned TRUSS through its association with TNF-R1 (Soond et al., 2003, 2006). Since TRUSS associates with a sub-family of TRPC channels, and because TNF-R1 ligation augments GPCR-induced Ca^{2+} entry (Amrani et al., 1995; Tiruppathi et al., 2001), we used a co-immunoprecipitation approach to determine if HA-tagged TRPC channels also interact with FLAG-tagged TNF-R1. Figure 2 shows that TRPC1, 4, and 5 co-immunoprecipitated with TNF-R1, whereas TRPC6 did not. While TNF-R1 expression was below the level of detection in the WCL, it was enriched to equivalent levels upon immunoprecipitation. Control immunoprecipitations with non-immune goat IgG were performed in parallel and confirmed the specificity of the immunoprecipitations (Fig. 2). Thus, the TRPC1, 4, and 5 sub-family of TRPC channels are also capable of forming heteromeric protein complexes with TNF-R1.

m1AchR-induced reduction in endoplasmic reticulum store Ca^{2+} content is reversed by co-expression of TNF-R1, TRUSS, and TRPC1 or 4

Next, we investigated the roles of TRUSS, TNF-R1, and TRPC channels in GPCR-induced increases in Ca^{2+} entry. Cch, a well-characterized agonist for muscarinic acetylcholine receptors (mAchR), signals via $G\alpha_{q/11}$ to generate IP3 from phosphatidylinositol-4,5-bisphosphate. IP3 initiates a rapid, transient increase in cytoplasmic Ca^{2+} following Ca^{2+} release from the endoplasmic reticulum Ca^{2+} stores. In turn, the depletion of endoplasmic reticulum Ca^{2+} stores initiates store-operated Ca^{2+} entry by Ca^{2+} channels located at the plasma membrane (Parekh and Putney, 2005). To investigate the function of TRUSS, TNF-R1, and TRPC channels in m1AchR-induced Ca^{2+} entry, we initially measured changes in $[Ca^{2+}]_i$ in response to stimulation of HEK293 cells with the m1AchR agonist, Cch. Since the HEK293 cells responded poorly to Cch stimulation, we transfected HEK293 cells with an m1AchR expression construct. Under these conditions, Cch-induced an increase in $[Ca^{2+}]_i$ that was observed in m1AchR-transfected cells but not in cells transfected with the control empty pcDNA3.1 (pcD) (Fig. 3A) indicating that the transfected receptor was functional. As the endoplasmic reticulum is a pivotal organelle for Ca^{2+} signaling, we next applied the Ca^{2+} ionophore, ionomycin, to m1AchR and control pcD-transfected cells to pharmacologically induce maximal release of Ca^{2+} from the endoplasmic reticulum and thereby assess how m1AchR expression affected endoplasmic reticulum Ca^{2+} content. Figure 3B,C shows that expression of m1AchR significantly decreased ionomycin-elicited Ca^{2+} release ($40.3 \pm 4.3\%$, $P < 0.0001$) compared to pcD control, suggesting that the transfected m1AchR was capable of signaling a reduction in endoplasmic reticulum Ca^{2+} store content. To confirm this finding, we loaded cells with Mag-Fura 2AM to directly measure endoplasmic reticulum Ca^{2+} content in m1AchR versus control pcD cells (Hofer

and Machen, 1993; Hofer et al., 1995; Hofer and Schulz, 1996). Figure 3D shows that when Mag-Fura 2 was released by permeabilization with digitonin, control cells transfected with vector only (pcD) had a higher basal level of $[Ca^{2+}]_i$ compared to cells transfected with m1AchR, indicating that the expression of m1AchR reduced the Ca^{2+} content of the endoplasmic reticulum. Figure 3D,E also shows that the change in fluorescence after the addition of ionomycin was significantly decreased by 32.6 ± 4.8% ($P < 0.001$) in cells expressing m1AchR compared to control cells transfected with empty vector (pcD), consistent with the measurements made using Fura-2 (Fig. 3B,C). The mechanism of signaling by the transfected m1AchR is not known and could involve ligand-independent signaling as has been reported for overexpressed TNF-R1 (Wajant et al., 2003). Alternatively, it is possible that the depletion of endoplasmic reticulum Ca^{2+} stores in m1AchR-expressing cells is initiated by m1AchR partial agonists, such as choline, that are present in tissue culture medium (Carriere and El-Fakahany, 2000). Consistent with either possibility, we also observed that m1AchR-transfected HEK293 cells exhibited increased extracellular regulated kinase (ERK)-phosphorylation (Fig. 3F). Collectively, these experiments identify a novel m1AchR-induced effect on endoplasmic reticulum Ca^{2+} storage and provide a system with which we evaluated the functions of TRUSS, TNF-R1, and TRPC channels in GPRC-induced Ca^{2+} signaling.

Enforced expression of TNF-R1 has previously been shown to activate signaling in the absence of TNF-α ligand (Wajant et al., 2003). Therefore, we determined the role of TRUSS, TNF-R1, and TRPC on the m1AchR signaling-induced reduction in endoplasmic reticulum Ca^{2+} content by enforced expression of m1AchR in combinations with TNF-R1, TRUSS, and TRPC4. Figure 4A,B shows that co-expression of m1AchR singly with TRPC1 (C1), TRPC4 (C4), TRUSS (TR), or TNF-R1 (R1) did not alter the m1AchR (m1)-induced reduction in endoplasmic reticulum Ca^{2+} content. Similarly, co-expression of m1AchR with TRPC4 or the related TRPC1 (C1) plus TRUSS did not alter ionomycin-elicited endoplasmic reticulum Ca^{2+} release (Fig. 4C,D). However, when TNF-R1 was co-expressed with TRPC1, TRPC4, or TRUSS, the m1AchR-induced reduction in endoplasmic reticulum Ca^{2+} content was significantly reversed ($P < 0.05$, Fig. 4C,D). Maximal reversal of m1AchR-dependent endoplasmic reticulum Ca^{2+} storage depletion was achieved when m1AcR was co-expressed together with either TNF-R1 and TRUSS, or with the combination of TNF-R1, TRPC4 (or TRPC1), and TRUSS, ($P < 0.0001$) (Fig. 4E,F). As a negative control, we co-expressed combinations of m1AchR, TNF-R1, TRUSS, and TRPC4 with the unrelated vesicular stomatitis virus glycoprotein (VSV-G) and found that the expression of this protein with m1AchR, TRUSS, and TRPC4 did not elevate the intracellular Ca^{2+} content above that of m1AchR alone (Fig. 4G,H). In addition, VSV-G did not act synergistically with TNF-R1 to elevate endoplasmic reticulum Ca^{2+} storage in the presence of m1AchR (Fig. 4G,H) suggesting that m1AchR specifically signals a reduction in endoplasmic reticulum Ca^{2+} content and that TRPC1, TRPC4, and TRUSS act together with TNF-R1 to reverse this response and promote endoplasmic reticulum Ca^{2+} filling.

To verify: (i) the specificity of the reduced Ca^{2+} content induced by m1AchR expression, and (ii) the specificity of TNF-R1, TRUSS, and TRPC channels in elevating endoplasmic reticulum Ca^{2+} store content, we examined ionomycin responses to the related $G\alpha_{q/11}$

coupled muscarinic acetylcholine receptor, m3AchR. Ionomycin evoked maximal responses in all cells expressing pcD and combinations of TRPC, TRUSS, and TNF-R1 (Fig. 5A,D). m1AchR expression resulted in the expected reduction in endoplasmic reticulum Ca^{2+} store content (Fig. 5B,D) and TNF-R1 together with TRUSS and TRPC4 co-expression significantly relieved the m1AchR-dependent reduction in endoplasmic reticulum Ca^{2+} store content ($P < 0.01$) (Fig. 5B,D). In contrast, endoplasmic reticulum Ca^{2+} store content was not significantly altered in m3AchR-expressing cells compared to pcD control conditions. In addition, co-expression of TNF-R1, with TRUSS and TRPCs did not significantly change these values ($P > 0.05$) (Fig. 5C,D). Dose response experiments were performed to ensure that the alteration in endoplasmic reticulum Ca^{2+} content observed between m1AchR and m3AchR expression were not due to differences in receptor expression levels. Figure 5E shows that even at the lowest concentration of m1AchR plasmid (0.25 μg), m1AchR elicited an ~40% reduction in endoplasmic reticulum Ca^{2+} content. In contrast, expression of m3AchR, between 0.25 and 2 μg, did not alter endoplasmic reticulum Ca^{2+} content compared to pcD control cells. Attempts to quantify the level of expression of the transfected receptor by Western blotting were unsuccessful. These data suggest that expression of m1AchR, but not the m3AchR, activates a distinct signal that reduces endoplasmic reticulum Ca^{2+} store content. In the absence of m1AchR, that is, when endoplasmic reticulum Ca^{2+} stores are at their normal filling capacity, TNF-R1, TRUSS, and TRPCs do not further enhance endoplasmic reticulum Ca^{2+} storage. Taken together, these findings suggest that TRUSS, TNF-R1, and TRPC4 (or TRPC1) interact to form a signaling complex that stimulates increased endoplasmic reticulum Ca^{2+} content in response to m1AchR signaling.

To further explore this notion, we investigated the ability of TNF-R1, TRUSS, and TRPC4 to form heteromeric signaling complexes. In addition, since these molecules contribute to the reversal of the m1AchR-induced reduction in endoplasmic reticulum Ca^{2+} content, we questioned if expression of the m1AchR affected complex formation. To address these questions, we first transfected HEK293 cells with constructs encoding FLAG-tagged TNF-R1, and HA-tagged TRPC4 in the presence and absence of untagged TRUSS. Complex formation was detected by immunoprecipitation with anti-TNF-R1 antibody followed by Western blotting with anti-HA, anti-TRUSS, and anti-FLAG antibodies. Figure 6A shows that a heteromeric protein complex composed of TNF-R1, TRPC4, and TRUSS was formed in cells transfected with constructs encoding all three proteins. However, in the absence of transfected TRUSS, complex formation between TNF-R1 and TRPC4 was still detected suggesting that the bimolecular interaction between each partner contributes to the formation of the trimolecular TNF-R1–TRPC4–TRUSS complex. Figure 6B shows that expression of TNF-R1, TRPC4, and TRUSS in the presence of m1AcR did not affect these interactions.

M1AchR impairs store-operated Ca^{2+} entry while expression of TNF-R1, TRUSS, and TRPCs enhances store-operated Ca^{2+} entry

Brandman et al. (2007) have shown that basal store-operated Ca^{2+} entry feeds Ca^{2+} into the endoplasmic reticulum to maintain Ca^{2+} homeostasis. Recently, Sternfeld et al. (2007) reported that acetylcholine application acts through mAchRs to reduce store-operated Ca^{2+} entry in HEK293 cells. To determine if store-operated Ca^{2+} entry is altered among cells

expressing: (i) control pcD (ii) m1AchR alone, or the combination of m1AchR with either (iii) TRUSS, TNF-R1, and TRPC4 or (iv) TRUSS and TNF-R1, we first depleted endoplasmic reticulum Ca^{2+} stores with ionomycin (1.25 μM) in Ca^{2+} free buffer-containing EGTA and then added Ca^{2+} back to the extracellular medium to reveal and quantify store-operated Ca^{2+} entry, as described (Kwan et al., 1990). Figure 7A,B shows that m1AchR-expressing cells exhibited a significant decrease ($P < 0.01$) in store-operated Ca^{2+} entry compared to pcD control cells. These data confirm the findings of Sternfeld et al. (2007) and support a model wherein the m1AchR-dependent decrease in basal store-operated Ca^{2+} entry contributes to the observed reduction in endoplasmic reticulum Ca^{2+} content. Next, we investigated the role of store-operated Ca^{2+} entry in reversing the reduction in endoplasmic reticulum Ca^{2+} content. We observed that transfection of m1AchR (m1), TRUSS (TR), TNF-R1 (R1), and TRPC4 (C4) or m1AchR, TRUSS, and TNF-R1 resulted in a significant increase ($P < 0.0001$ and $P < 0.01$, respectively) in store-operated Ca^{2+} entry compared to cells expressing m1AchR alone (Fig. 7A,B). These data suggest that in the context of m1AchR signaling, TNF-R1, TRUSS, and TRPCs elevate store-operated Ca^{2+} entry to promote endoplasmic reticulum Ca^{2+} filling.

We performed control experiments to determine if the cells exhibited general alterations in Ca^{2+} permeability that were not attributable to store-operated Ca^{2+} entry. In Figure 7C extracellular Ca^{2+} was acutely removed by the addition of EGTA, then extracellular Ca^{2+} was added back to evaluate if Ca^{2+} permeability was altered among the conditions examined. Figure 6C shows that none of the conditions (pcD, m1AcR alone or m1AchR (m1), TRUSS (TR), and TNF-R1 (R1)-transfected cells) resulted in changes in Ca^{2+} permeability following this protocol of extracellular Ca^{2+} depletion and readdition. The subsequent ionomycin application demonstrates that the cells were properly transfected and Fura-2 AM loaded. These data suggest that the transfection conditions do not alter basal Ca^{2+} permeability, and further support the interpretation that alterations in store-operated Ca^{2+} entry contribute to the changes in endoplasmic reticulum Ca^{2+} storage observed with m1AchR, TNF-R1, TRUSS, and TRPC4.

Discussion

Synergistic interactions between the pro-inflammatory cytokine TNF-α and the GPCR-agonists bradykinin and thrombin have been shown to play an important role in breaches in lung microvascular endothelial cell barrier function and airway smooth muscle cell hyperplasia and contraction through augmentation of GPCR-induced store-operated Ca^{2+} entry (Amrani et al., 1995; Tiruppathi et al., 2001). Little is known about how TNF-α signaling, acting through the receptor TNF-R1, intersects with GPCR-induced Ca^{2+} entry. In this study, we investigated: (i) the ability of TNF-R1 and its signaling adaptor TRUSS to interact with members of the Ca^{2+}-permeable TRPC family; and (ii) the consequences of TRUSS, TNF-R1, and TRPC4 expression on GPCR-induced filling of endoplasmic reticulum Ca^{2+} stores and store-operated Ca^{2+} entry using m1 muscarinic acetylcholine receptor (m1AchR)-induced endoplasmic reticulum Ca^{2+} depeletion as a model. For the first time, we show that both TRUSS and TNF-R1 specifically associate with the TRPC1, 4, and 5 sub-family of TRPC channels. In addition, we show TNF-R1, TRUSS, and TRPC channels influence Ca^{2+} homeostasis by altering store-operated Ca^{2+} entry that contributes

to setting the basal Ca^{2+} concentration within the endoplasmic reticulum (Brandman et al., 2007).

Using a transient expression and co-immunoprecipitation approach, TRUSS was found to form heteromeric protein complexes with TRPC1, 4, and 5, but not with TRPC3, 6, or 7. In addition, TRPC1, 4, and 5 were found to specifically associate with TNF-R1. This pattern of specificity among protein interaction partners is noteworthy as the endoplasmic reticulum Ca^{2+} sensor and master regulator of store-operated Ca^{2+} entry, STIM1, also interacts with TRPC1, 4, and 5, but not with TRPC3, 6, or 7 (Huang et al., 2006). Interestingly, TNF-R1, TRUSS, and TRPC4 were found to associate and form a trimolecular heteromeric complex. However, the formation of this complex was not affected by the presence of the m1AchR. Collectively, these findings suggest that distinct protein binding partners between these two sub-families of TRPCs may contribute to functional regulation of the channels. Our data also suggest that TRUSS, TNF-R1, and a sub-family of TRPC channels may function together to control biological Ca^{2+} responses.

We investigated the role of TRUSS, TNF-R1, and TRPC channels in GPCR-induced Ca^{2+} signaling using the m1AchR as a representative $G\alpha_{q/11}$-coupled GPCR. In the presence of m1AchR-induced endoplasmic reticulum Ca^{2+} depletion, TNF-R1 appeared to be an essential factor to confer an increase in endoplasmic reticulum Ca^{2+} storage since increased endoplasmic reticulum Ca^{2+} storage was only observed with TNF-R1 co-expression with either TRUSS, TRPC1, TRPC4, or both, but not in the absence of TNF-R1. While this system is potentially confounded by the expression of endogenous TRUSS and TRPC channels (Riccio et al., 2002 and K.E. Mace, J.L. Terry Powers, and D.W.H. Riches, unpublished observations), we propose a model where protein complexes composed of TNF-R1, TRUSS, and TRPC1 or 4 mediate an increase in store-operated Ca^{2+} entry in the presence of an m1AchR-dependent reduction in basal endoplasmic reticulum Ca^{2+} content. Our data are consistent with such a model where the transfection of exogenous components shifts the balance of protein complex formation to promote the observed alterations in Ca^{2+} signaling. Intriguingly, we also found that neither endoplasmic reticulum Ca^{2+} storage capacity nor store-operated Ca^{2+} entry was enhanced by TNF-R1, TRUSS, and TRPC expression in the absence of m1AchR (data not shown), suggesting that the ability of TNF-R1, TRUSS, and TRPCs to alter Ca^{2+} signaling may be context dependent. The dependency on m1AchR for revealing this phenomenon might contribute to the differential susceptibility of various cell types to increases in Ca^{2+} signaling after exposure to TNF-α. However, our data do not exclude the possibility that m1AchR activates a common signaling pathway to alter store-operated Ca^{2+} entry. Thus, exclusive reliance on m1AchR activation for the TNF-R1, TRUSS, and TRPC-dependent enhancement of store-operated Ca^{2+} entry and subsequent endoplasmic reticulum Ca^{2+} homeostasis might not be necessary.

We have also demonstrated that reductions in store-operated Ca^{2+} entry by m1AchR expression correlated with decreased endoplasmic reticulum Ca^{2+} storage capacity. These data are interesting considering recent findings that link basal store-operated Ca^{2+} entry with homeostatic maintenance of endoplasmic reticulum Ca^{2+} content, a phenomenon dependent on STIM2, which serves as a sensitive sensor to activate Ca^{2+} influx upon small decreases in endoplasmic reticulum Ca^{2+} (Brandman et al., 2007). Our data substantiate recent findings

showing that acetylcholine impairs store-operated Ca^{2+} entry in HEK293 through a mechanism involving the activation of m1AchR (Sternfeld et al., 2007). Our work expands on this to suggest that endoplasmic reticulum homeostasis is directly influenced by m1AchR-mediated changes in store-operated Ca^{2+} entry. It is unclear how m1AchR expression impairs store-operated Ca^{2+} entry and Ca^{2+} accumulation in the endoplasmic reticulum. One possibility is that mere over-expression of m1AchR is sufficient to promote signaling in a ligand-independent fashion, as has been seen with TNF-R1 (Wajant et al., 2003). Alternatively, transfected m1AcR could bind a ligand present in the tissue medium, which in turn, promotes receptor signaling. For example, choline, a component of tissue culture medium used in our study, has been shown to activate signaling by m1AchR (Carriere and El-Fakahany, 2000). Regardless of the mechanism, our data, together with those of Sternfeld et al. (2007) suggest that ligand-induced activation of endogenous m1AchR might have the capacity to alter both store-operated Ca^{2+} entry and endoplasmic reticulum Ca^{2+} content.

In a broader setting, our findings may also have potentially important implications for Alzheimer's disease. Impaired Ca^{2+} signaling in neurons is a hallmark of Alzheimer's disease and proteins linked to this disease either promote over-filling or depletion of endoplasmic reticulum Ca^{2+} stores (Ito et al., 1994; Bezprozvanny and Mattson, 2008). In addition, cholinergic neurodegeneration occurs early in Alzheimer's disease and attempts to improve cholinergic neuronal function has spawned a significant interest in generating therapeutic m1AchR agonistic drugs (Langmead et al., 2008). Our finding that TNF-R1, TRUSS, and TRPC proteins increase endoplasmic reticulum Ca^{2+} storage raise the intriguing possibility that disruption of TRUSS, TNF-R1, and TRPC signaling may contribute to the impaired Ca^{2+} signaling seen in patients with Alzheimer's disease. In support of this possibility, a recent genome-wide association study revealed an association between several single nucleotide polymorphisms in the coding sequence of TRUSS and increased susceptibility to the development of late-onset Alzheimer's disease (Poduslo et al., 2009a,b). Furthermore, TNF-R1 deficient mice have been shown to be resistant to the development of neurodegeneration in an amyloid-dependent model of Alzheimer's disease (He et al., 2007). Together with the results of our study, these findings suggest that links between TNF-R1 and Ca^{2+} signaling may have broad significance in health and disease.

In summary, the findings presented herein suggest that TRUSS and TRPC1, 4, and 5 are essential components of a signaling complex that serves to link TNF-R1 signaling to increased loading of endoplasmic reticulum Ca^{2+} stores due to increased store-operated Ca^{2+} entry in the context of m1AchR-receptor signaling. Future studies in mice bearing a targeted disruption of the TRUSS gene are expected to provide novel insights into the physiologic role of TRUSS in the TNF-R1-induced augmentation of GPCR-stimulated Ca^{2+} entry.

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Infectious Diseases of the National Institutes of Health. Kimberly E. Mace was supported in part by T32 training grant AI07405 from the National Institute of Allergy and Infectious Diseases.

Abbreviations

TNF-α	tumor necrosis factor-α
TNF-R1	tumor necrosis factor receptor-1
TRUSS	TNF-R1 ubiquitous scaffolding signaling protein
TRPC	transient receptor potential canonical
TRPC4AP	TRPC4-associated protein
mAchR	muscarinic acetylcholine receptor
Cch	carbachol
ERK	extracellular regulated kinase
IP3	inositol 1, 4, 5-trisphosphate
IP3R	IP3 receptor
pcD	pcDNA3.1 transfection control
VSV-G	vesicular stomatitis virus glycoprotein
NF-κB	nuclear factor-κB
JNK	c-Jun-NH_2-terminal kinase
$[Ca^{2+}]_i$	concentration of intracellular Ca^{2+}.

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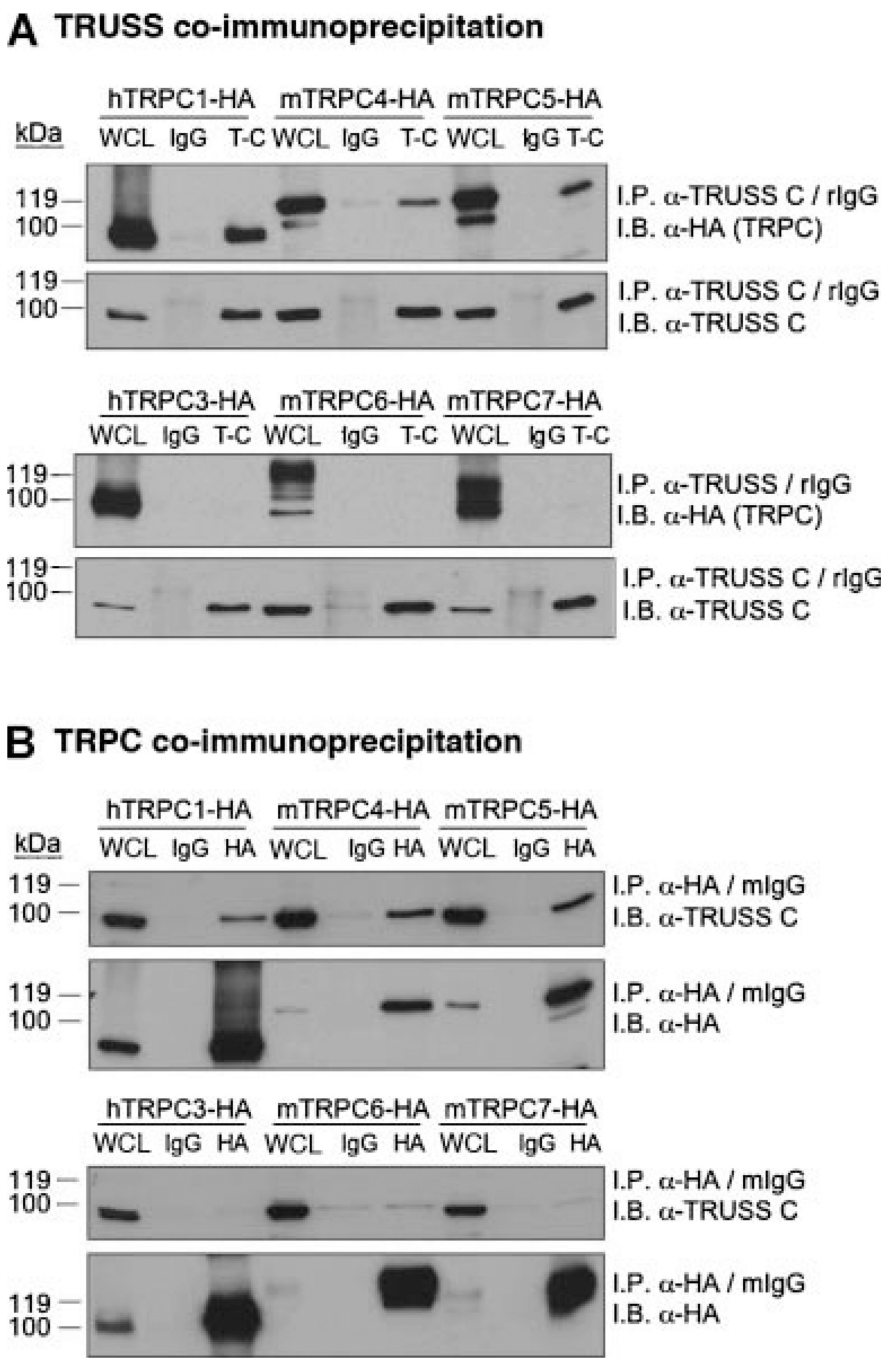

Figure 1.

TRUSS (TRPC4AP) interacts with TRPC1, -C4, and -C5, but not TRPC3, -C6, or -C7 in intact cells. A: TRUSS was expressed with HA-tagged TRPC1, -C4, -C5 (top two panels), -C3, -C6, or -C7 (bottom two panels) in HEK293 cells, immunoprecipitated with anti-TRUSS antibody (TR), or rabbit non-immune IgG (rIgG) and immunoblotted with anti-HA (upper panels) or anti-TRUSS antibodies (lower panels). B: TRUSS was expressed with HA-tagged TRPC1, -C4, -C5 (top two panels), -C3, -C6, or -C7 (bottom two panels) in HEK293 cells, immunoprecipitated with anti-HA antibody (HA) or non-immune IgG

(mIgG) and immunoblotted with anti-TRUSS (upper panels) or anti-HA antibodies (lower panels).

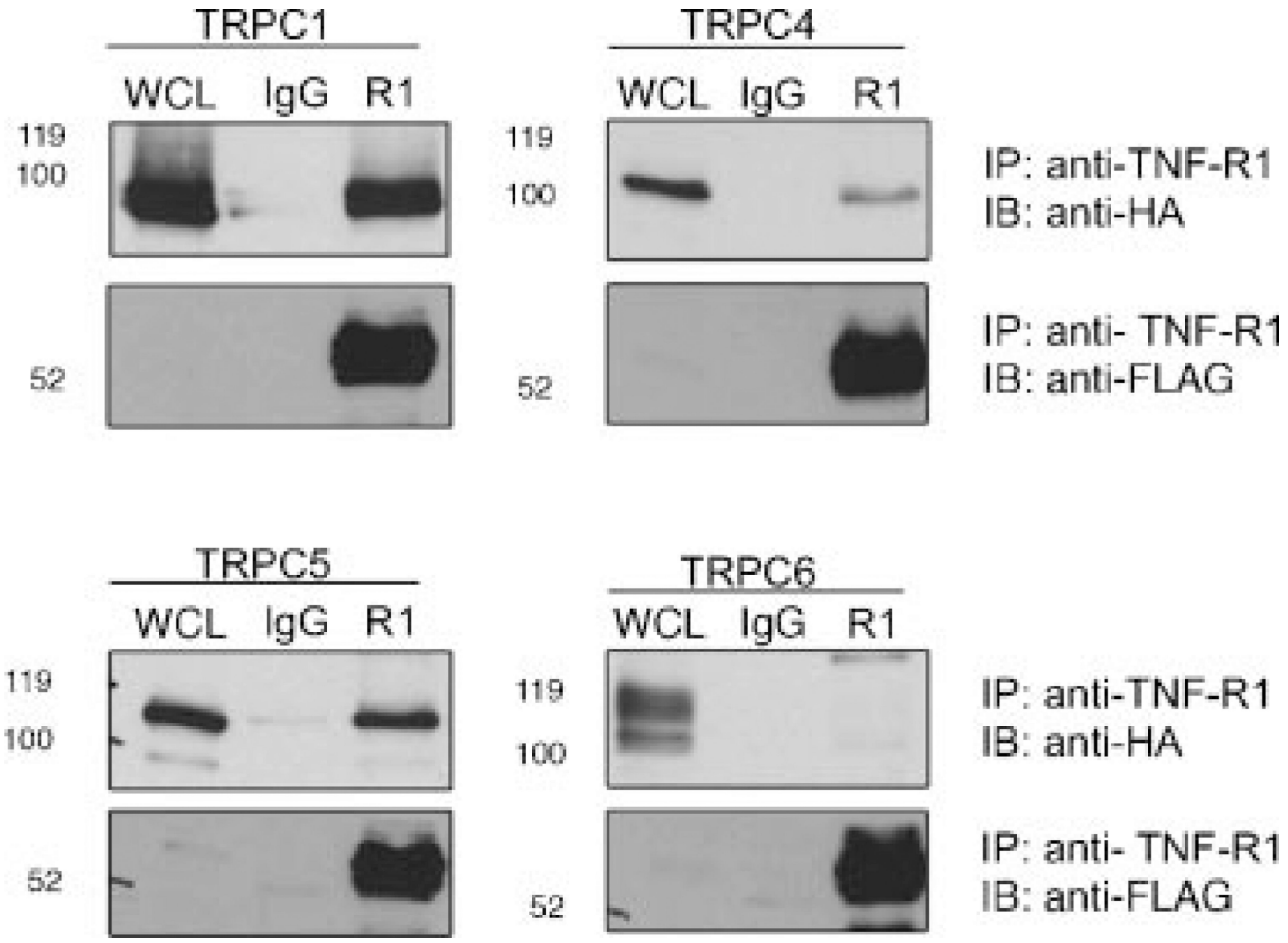

Figure 2.

TNF-R1 interacts with TRPC1, -C4, and -C5, but not TRPC6. TNF-R1 was co-expressed with HA-tagged TRPC1, -C4, -C5, or C6 in HEK293 cells and TNF-R1 was immunoprecipitated as discussed in Methods Section with a goat-anti-TNF-R1 antibody or non-immune IgG (IgG). The immunoprecipitates and post-nuclear supernatants (PNS) were separated by SDS–PAGE and subjected to Western blotting with anti-HA (upper panels) or anti-FLAG antibodies (lower panels).

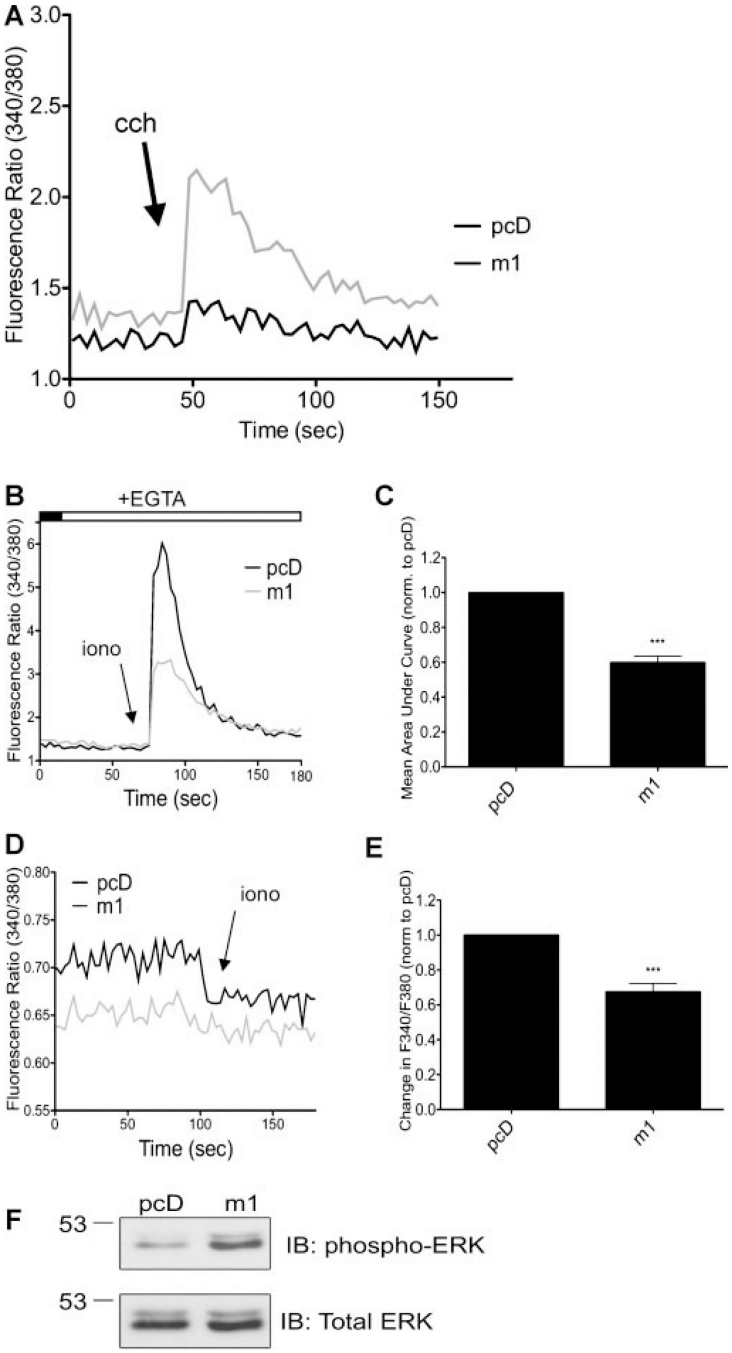

Figure 3.

A: Transfection of m1AchR into HEK293 cells increases intracellular Ca^{2+} ($[Ca^{2+}]_i$) following stimulation with carbachol (50 μM). B: Expressionofm1AchRdecreasesionomycin-sensitiveendoplasmic reticulum Ca^{2+} stores. C: Quantification of (**B**) where bars represent the mean areas under the curves, normalized to pcD ± SEM; ***$P < 0.0001$. D: Confirmation that expression of m1AchR decreases the Ca^{2+} content of endoplasmic reticulum Ca^{2+} stores as measured with Mag-Fura 2. E: Quantification of (D) showing the change in fluorescence after stimulation with ionomycin,

normalized to pcD. Data shown are the mean ± SEM; ***$P < 0.0001$. F: Transfection of m1AchR into HEK293 cells promotes ERK phosphorylation. HEK293 were transfected with empty vector (pcD), or m1AchR (m1) for 24 h. Total ERK and phospho-ERK were quantified by Western blotting of whole cell lysates.

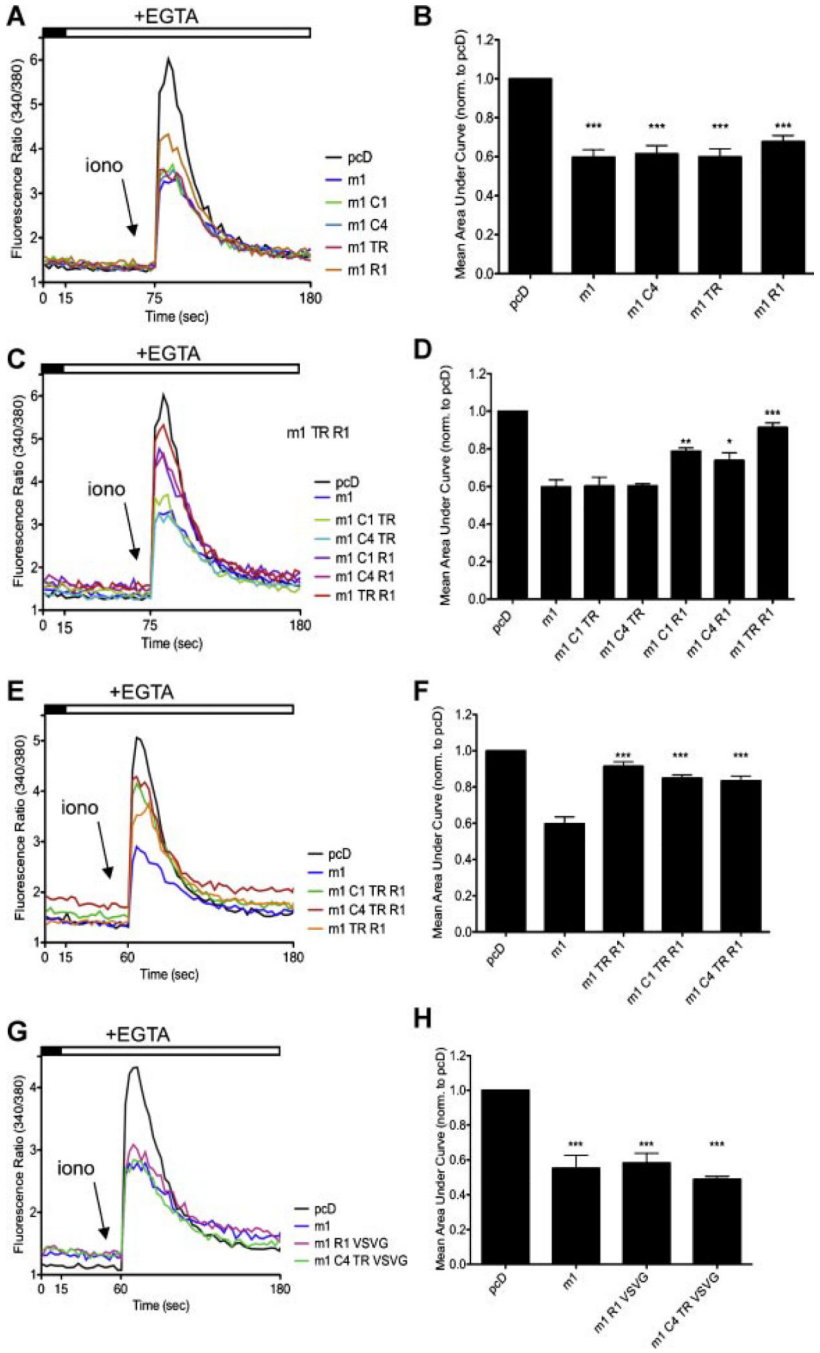

Figure 4.

m1AchR decreases basal endoplasmic reticulum Ca^{2+} and co-expression of TNF-R1, TRUSS, and TRPC1 or TRPC4 reverses this response. The fluorescence ratios (340/380) of Fura-2 AM-loaded HEK293 cells expressing the indicated combinations of pcD, m1AchR, TRUSS, TRPC4, TNF-R1, or VSV-G was determined. Parts (A,C,E,G) show representative fluorescence 340/380 ratio measurements of the indicated transfection conditions. Parts (B,D,F,H) show quantification of the ionomycin responses in Panels (A,C,E,G) where bars represent the mean area under the curves, normalized to pcD ± SEM. B: Comparison to pcD,

***$P < 0.0001$; (D) comparison to m1AchR, ***$P < 0.0001$; **$P < 0.01$, and *$P < 0.05$; (F) comparison to m1AchR, ***$P < 0.0001$; (H) comparison to pcD, ***$P < 0.0001$.

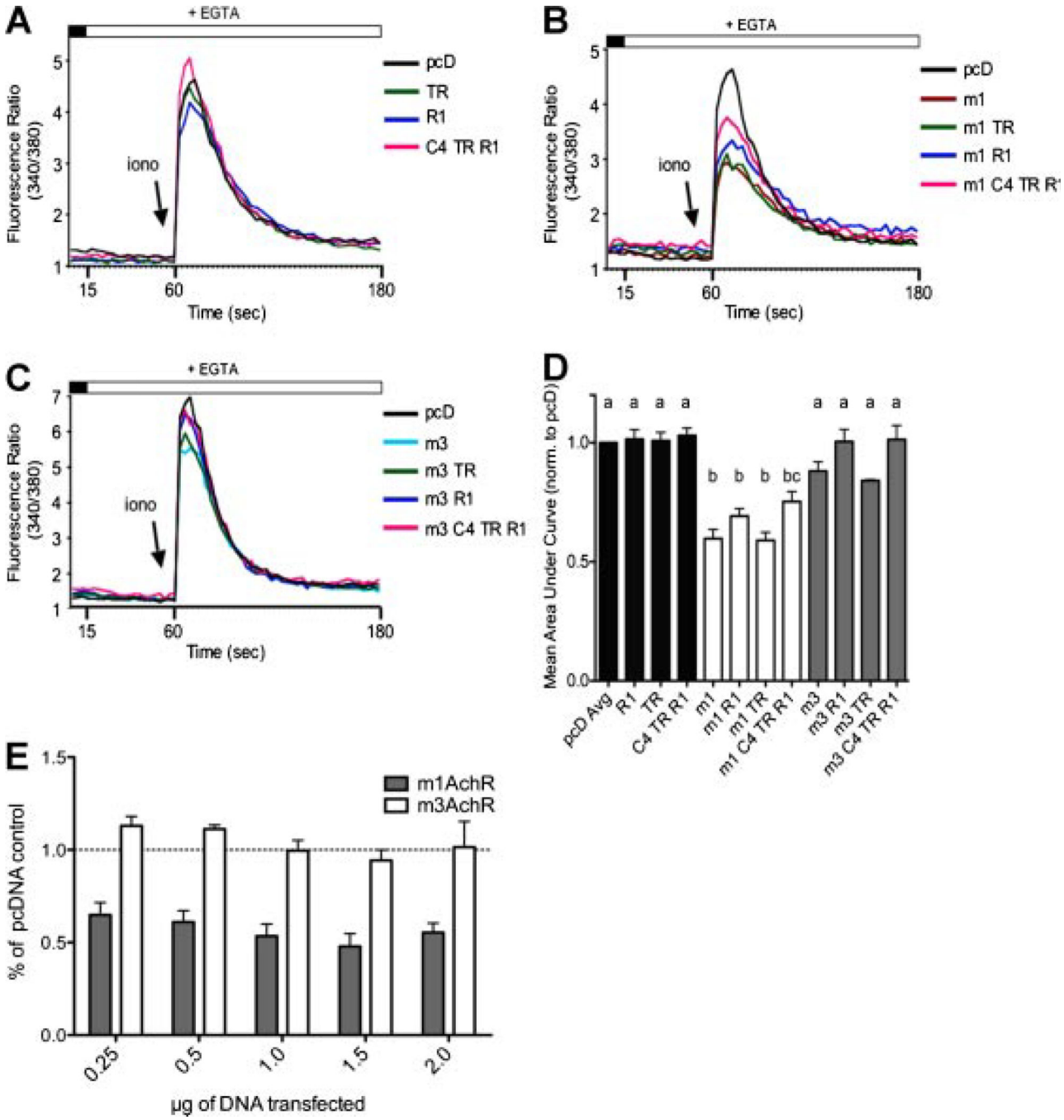

Figure 5.

m1AchR, but not m3AchR, reduces basal endoplasmic reticulum Ca^{2+} levels and reveals TNF-R1, TRUSS, TRPC-dependent enhanced endoplasmic reticulum Ca^{2+} storage. Representative fluorescence ratios (340/380) of Fura-2-loaded HEK293 cells expressing combinations of TRUSS, TRPC4, or TNF-R1 was determined with: (A) pcD, (B) m1AchR, or (C) m3AchR. D: Quantification of the ionomycin-induced responses from panels (A–C). Bars represent the mean area under the curve, normalized to pcD control ± SEM, $^{a}P > 0.05$ compared to pcD; $^{b}P < 0.0001$ compared to pcD; $^{c}P < 0.01$ compared to m1AchR. E:

Quantification of the ionomycin-induced endoplasmic reticulum Ca^{2+} release from Fura-2 AM loaded cells transfected with 0.25–2.0 μg of m1AchR or m3AchR plasmid. Bars represent the mean area under the curve, normalized to pcD (dotted line) ± SEM.

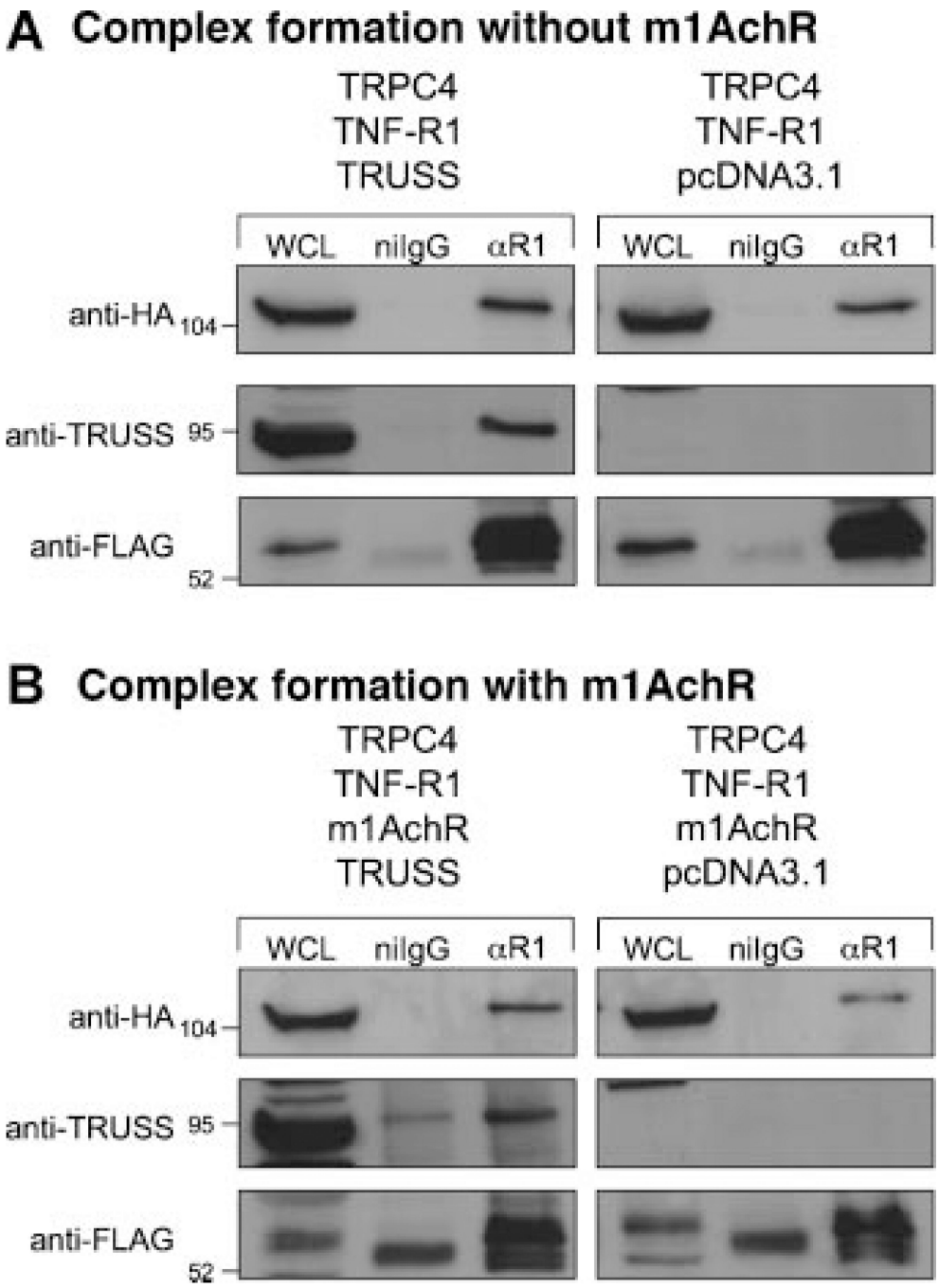

Figure 6.

TRUSS, TNF-R1, and TRPC4 associate to form a trimolecular complex in the presence and absence of m1AchR. A: HEK 293 cells were co-transfected with FLAG-tagged TNF-R1 and HA-tagged TRPC4 in the presence and absence of TRUSS. Cell lysates were immunoprecipitated with anti-TNF-R1 antibody and TNF-R1-associated TRPC4 and TRUSS detected by immunoblotting with anti-HA and anti-TRUSS C-terminal antibodies, respectively. B: Complex formation between TNF-R1, TRPC4, and TRUSS was determined in the presence of transfected m1AchR using the same approach to that shown in panel (A).

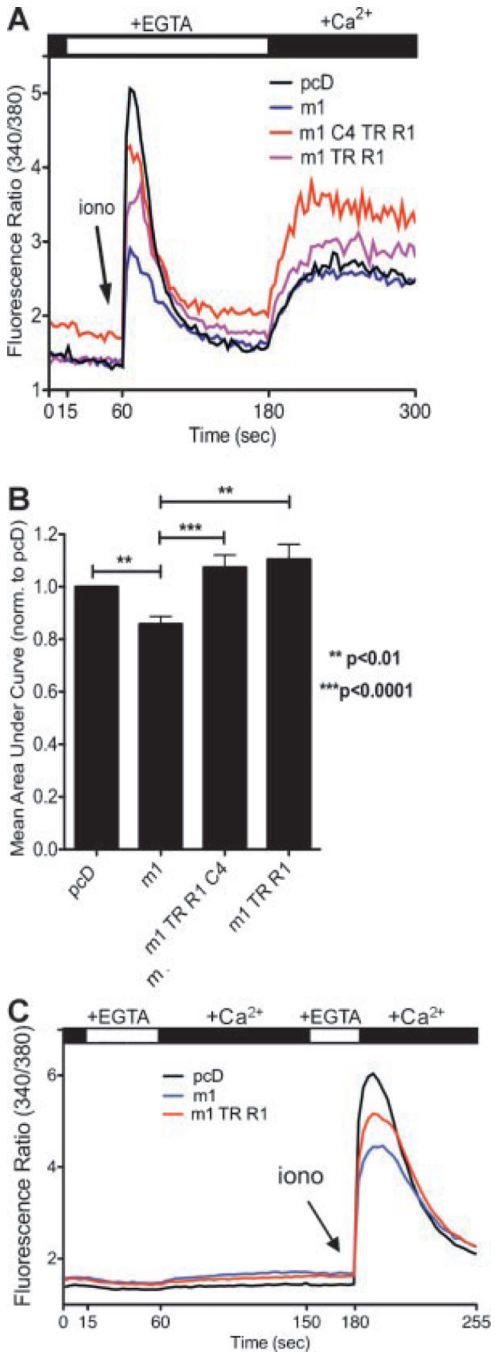

Figure 7.

m1AchR impairs store-operated Ca^{2+} entry in HEK293 cells, while co-expression of TNF-R1, TRUSS, and TRPC4 enhances store-operated Ca^{2+} entry. A: Representative fluorescence ratios (340/380) of Fura-2 AM-loaded HEK293 expressing the indicated proteins. B: Quantification of responses from panel (A). Bars represent the mean area under the curve normalized to pcD ± SEM; $^{**}P < 0.01$ and $^{***}P < 0.0001$. C: The fluorescence

ratios (340/380) of Fura-2 AM loaded HEK293 cells expressing pcD, m1AchR (m1) or the combination of m1AchR (m1) TRUSS (TR), and TNF-R1 (R1).