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Store-operated CRAC channels regulate PAR2-activated Ca^{2+} signaling and cytokine production in airway epithelial cells

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Abstract

The G-protein coupled protease-activated receptor 2 (PAR2) plays an important role in the pathogenesis of various inflammatory and auto-immune disorders. In airway epithelial cells (AECs), stimulation of PAR2 by allergens and proteases triggers the release of a host of inflammatory mediators to regulate bronchomotor tone and immune cell recruitment. Activation of PAR2 turns on several cell signaling pathways of which the mobilization of cytosolic Ca^{2+} is likely a critical but poorly understood event. Here, we show that Ca^{2+} release-activated Ca^{2+} (CRAC) channels encoded by STIM1 and Orai1 are a major route of Ca^{2+} entry in primary human AECs and drive the Ca^{2+} elevations seen in response to PAR2 activation. Activation of CRAC channels induces the production of several key inflammatory mediators from AECs including TSLP, IL-6 and PGE_2, in part through stimulation of gene expression via NFAT (nuclear factor of activated T-cells). Furthermore, PAR2 stimulation induces the production of many key inflammatory mediators including PGE_2, IL-6, IL-8 and GM-CSF in a CRAC-channel dependent manner. These findings indicate that CRAC channels are the primary mechanism for Ca^{2+} influx in AECs and a vital checkpoint for the induction of PAR2-induced proinflammatory cytokines.

INTRODUCTION

The epithelial cells of the lung are directly exposed to inhaled air and form the first line of defense against environmental hazards (1–3). In addition to serving as a physical barrier to protect the lung, airway epithelial cells (AECs) play an active role in orchestrating inflammatory effector responses to inhaled substances through the production of a wide array of secreted cytokines and through their interactions with many immune cells (3, 4). Effector responses in AECs are coordinated through a multitude of interactions between extrinsic signaling molecules and intrinsic signal transduction programs activated within the AECs (1, 3). In this signaling repertoire, protease-activated receptor 2 (PAR2) is of particular importance in regulating allergic inflammatory responses that are characteristic of diseases like asthma. PAR2 receptors belong to a family of seven-transmembrane G-protein

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coupled receptors (GPCRs) that are widely expressed in a variety of cell types and are activated by cleavage of the extracellular N-terminus through the serine protease activity of PAR2 proteolytic agonists. In the airway epithelium, PAR2 receptors are activated by several types of allergens derived from dust mites, cockroach and fungi, all well-known triggers of asthma, and also by endogenous protease molecules such as trypsin and mast-cell tryptase (5–7). PAR2 activation in AECs stimulates the production of several proinflammatory cytokines (IL-6, GM-CSF and TSLP) and chemokines (IL-8 and eotaxin) (8–10). Moreover, asthmatic patients show increased expression of PAR2 receptors in their airway epithelium and PAR2 lacking mice show reduced eosinophilic infiltration and airway hyper-responsiveness (11, 12). These findings underscore the importance of PAR2 proteins in mediating allergic inflammatory responses in the airway.

Despite the well-defined importance of PAR2 receptors in driving inflammatory responses, the signal transduction mechanisms involved in PAR2-mediated effector responses are not well-understood. PAR2 activation stimulates a multi-component signal transduction cascade within which the mobilization of Ca^{2+} by phospholipase-C (PLCβ) activation and subsequent IP_3-mediated release of Ca^{2+} from endoplasmic reticulum (ER) Ca^{2+} stores is a key signaling process (13, 14). As a multifunctional second messenger, Ca^{2+} activates distinct genetic programs and enzymatic cascades to regulate many processes in the immune system including lymphocyte activation, mast-cell degranulation and neutrophil mediated bacterial killing (15–18). There is growing interest in the role of cellular Ca^{2+} as a key second messenger regulating effector responses in the airway (19–21). Yet the functional architecture of the Ca^{2+} signaling network: the molecular entities and their organization, and how this machinery regulates Ca^{2+} signaling and PAR2 evoked effector responses remains poorly understood in airway epithelial cells.

In many non-excitable cells, mobilization of cellular Ca^{2+} signaling occurs through the opening of store-operated Ca^{2+} release-activated Ca^{2+} (CRAC) channels (17, 18). These highly Ca^{2+} selective ion channels are encoded by the Orai genes (Orai1–3) and activated through direct physical interactions with the ER Ca^{2+} sensors, STIM1 and STIM2 (22). Mechanistically, it is now known that STIM1 and STIM2 sense the $[Ca^{2+}]_{ER}$, and, in response to ER Ca^{2+} store-depletion, translocate to the junctional ER to interact with Orai channels (22, 23). In immune cells, previous studies have established that CRAC channels encoded by STIM1/Orai1 proteins play a central role in driving Ca^{2+} signaling that controls the function of T-cell, mast cells, B-cells and neutrophils (15–18). However, the role of CRAC channels in regulating immune functions of the airway epithelium, and in particular, their contributions to the production and release of inflammatory mediators, are unknown.

In this study, we show that primary human AECs show robust store-operated Ca^{2+} entry (SOCE) mediated by the CRAC channel proteins STIM1 and Orai1. Activation of CRAC channels stimulates several critical effector functions in AECs including gene transcription and production of a range of inflammatory modulators. We further find that stimulation of PAR2 receptors produces intracellular Ca^{2+} elevations that are critically dependent on CRAC channel activity. In turn, CRAC channel-mediated Ca^{2+} signals induce the production of several pro-inflammatory mediators IL-8, IL-6, GM-CSF and PGE_2 in part through stimulation of gene transcription via the NFAT-calcineurin signaling axis. These

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results demonstrate that CRAC channels are a major route of Ca^{2+} entry in AECs and serve as a key check-point for PAR2 mediated generation of inflammatory mediators.

MATERIALS AND METHODS

Cells and media

NHBE (Normal human bronchial epithelial) cells were purchased from Lonza, Walkersville, MD and grown in bronchial epithelial cell growth medium (BEGM) containing various growth factors. Cells from passages 2–3 were used for experiments. BEAS-2B, a bronchial epithelial cell-line was a kind gift from Curtis Harris, National Cancer Institute and were cultured in DMEM/F12 medium containing 5% FBS (cell passages 44–49 were used for experiments). A549, an alveolar epithelial cell line was a kind gift of Dr. Jacob Sznajder, Northwestern University (cell passages 12–18 were used for experiments). 1Haeo- cells, a bronchial epithelial cell-line was a kind gift from Dr. Alice Prince, Columbia University. Both A549 and 1Haeo- cells were cultured in DMEM containing 10% FBS. All the cells were maintained at 37°C and in 5% CO_2.

Plasmids and transfections

The plasmids and RNAi constructs used in this study were transfected into primary cells and cell-lines using Lipofectamine2000 (Invitrogen) according to manufacturer instructions. E106A Orai1-YFP plasmid has been described previously (24). Human NFATc3-GFP construct was a kind gift of Dr. Anjana Rao (UC San Diego). NFAT-luciferase and renilla luciferase plasmids were obtained from Dr. Richard Lewis (Stanford University). RNAi constructs used to down regulate Stim1 and Orai1 protein expression as well as scrambled siRNA control was purchased from Ambion, Life Technologies (SilencerSelect predesigned siRNA). Transfected cells were used for experiments 24–36 hours after transfection for cDNA constructs and 48–72 hours for RNAi treated cells.

Reagents and chemicals

The standard extracellular Ringers solution had the following composition (in mM): 150 NaCl, 4.5 KCl, 10 D-glucose, 1 $MgCl_2$, 2 $CaCl_2$ and 5 Na-HEPES. pH was adjusted to 7.4 using NaOH. For the Ca^{2+} free Ringers solution, $CaCl_2$ was excluded from the above composition and $MgCl_2$ was increased to 3mM. The 20 mM Ca^{2+} solution used for electrophysiological recordings contained 20 mM $CaCl_2$ and 130 mM NaCl, other components of this solution were identical to the standard Ringer's solution. The divalent-free (DVF) Ringer's solution contained (in mM): 150 NaCl, 10 HEDTA, 1 EDTA and 10 Hepes (pH 7.4). Stock solutions of 2-APB (2-Aminoethoxydiphenylborane), BTP2 (3,5-bis (trifluroromethyl) pyrazole) , thapsigargin (TG), RO2959, Cyclosporin A (CsA) and FK-506 were dissolved in DMSO. PAR2-agonists including type IX trypsin (Sigma) as well as the agonistic peptides SLIGRL and SLIGKV, control peptide LRGILS and the PAR1 peptide TFLLR (all from Tocris Bioscience) were constituted in water. 2-APB, CsA and U73122 were from Sigma Aldrich. RO2959 (Difluoro-N-(5-(4-methyl-1-(5-methyl-thiazol-2-yl)-1,2,5,6-tetrahydro-pyridin-3-yl)-pyrazin-2-yl)-benzamide) was from Synta Pharmaceuticals.

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Intracellular Ca^{2+} measurements

NHBE cells and various cell lines were grown on poly-L lysine coated glass bottom dishes (MatTek corp). Cells were loaded with 2.5 μM Fura-2 AM (Invitrogen) in dark for 40 minutes at room temperature in the appropriate culture medium containing 5–10% FBS. After washing away excess dye, cells were incubated in media for an additional 10 minutes before initiating Ca^{2+} imaging. Single cell $[Ca^{2+}]_{cyt}$ measurements were done according to the protocol described previously (25). Image acquisition and analysis was performed using IPLab (Scanalytics, Rockville, MD, USA) and Slidebook (Denver, CO, USA). For data analysis, regions of interest (ROIs) were drawn around single cells, background subtracted, and the F_{340}/F_{380} intensity ratios were determined for each time point. The F_{340}/F_{380} intensity ratios were converted to $[Ca^{2+}]_{cyt}$ using the formula:

$$[Ca^{2+}]_{cyt} = \beta * K_d (R - R_{min}) / (R_{max} - R)$$

where R is the F_{340}/F_{380} fluorescence intensity ratio and R_{max} (=9.645) and R_{min} (=0.268) were determined by *in-vitro* calibration of fura-2. β (=20.236) was determined from the F_{min}/F_{max} ratio at 380 nm and K_d is the apparent dissociation constant of fura-2 binding to Ca^{2+} (135 nM). Where applicable, rate of SOCE, "$\Delta[Ca^{2+}]_{cyt}/\Delta t$" was calculated as the slope of a line fitted to the 1st three points following addition of 2 mM Ca^{2+} in the Ca^{2+} imaging trace. Slope was averaged over several cells in the imaging field to generate the relevant bar graphs.

Western blots

NHBE and BEAS-2B cells were cultured in 6-well plates. At ~70–80% confluency, cells were washed with cold PBS following which cell lysis and Western blotting was done using protocols described previously (25). Orai1 and STIM1 proteins were detected using affinity purified polyclonal antibodies and peroxidase labelled secondary antibodies (26, 27).

Patch clamp measurements

Patch clamp recordings and analysis of CRAC currents were performed using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) using standard methods as previously described (28). The holding potential was +30 mV. The standard voltage stimulus consisted of a 100-ms step to −100 mV followed by a 100 ms ramp from −100 to +100 mV applied at 1-s intervals. Cells were pretreated with 1 μM thapsigargin (TG) prior to establishment of the seal to deplete stores and activate CRAC channels.

NFAT-luciferase assay

Endogenous NFAT activity in cells was measured using the PGL3-NFAT luciferase reporter construct (29). NFAT activity was normalized to the activity of Renilla luciferase (pRL-Tk-luc). Plasmids were transfected into AECs in a ratio of 20:1 (NFAT: Renilla). 24 hours after transfection, cells were pre-treated with the CRAC channel inhibitors BTP-2 and RO2959 for 1 hour followed by treatment with 0.5 μM TG and 50 nM PDBu for 8–10 hours. Cells were lysed and the luciferase activity was determined using the Dual-luciferase Reporter Assay Kit (Promega) and a single tube luminometer (Berthold Instruments, Germany).

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NFAT translocation assay

BEAS-2B cells were transfected with NFATc3-GFP construct and imaged for GFP 24- 36 hours later. Images were taken in resting 2 mM Ca^{2+} solution and after depleting ER Ca^{2+} stores with TG (1 μM, 20 min). The percentage of cells showing NFAT localization to the nucleus was calculated and averaged between several fields and reported as a bar graph.

Analysis of cytokine secretion

AECs (NHBE, BEAS, A549 and 1Haeo- cells) were cultured in 24 well plates. Twelve hours before treatment, media was changed to a 2 mM Ca^{2+} media without growth factors. Cells were treated with specific CRAC channel activating stimuli (either TG and PDBu or PAR2 agonists: SLIGRL and SLIGKV) for 2- 24 hours and the levels of various inflammatory mediators was measured using custom made ELISA kits (RayBiotech for TNFα, IL-6, IL1α and GM-CSF, Cayman chemicals for PGE_2, R&D for TSLP, LifeTechnologies for IL-8 and IL1β).

Data analysis

All bar-graphs summarizing the data are reported as mean ± SEM. For data sets involving more than two groups, initial statistical analysis was performed using ANOVA with a confidence interval of 5%. This was followed by two-tailed paired student t-test for comparing different treatment conditions within the set. For data sets with only two groups, two-tailed paired student t-test was used to compare between control and test conditions.

RESULTS

A screen for GPCR agonists identifies PAR2 receptors as activators of store-operated Ca^{2+} entry in the airway epithelium

In what context would CRAC channels be relevant for Ca^{2+} signaling in AECs? CRAC channels are voltage independent and require depletion of ER Ca^{2+} stores for activation (17). There are numerous extracellular stimuli (e.g., growth factors, pathogens, GPCR ligands, etc.) that act in this manner and could be regarded as potential candidates that stimulate CRAC channels in AECs. To examine this, we screened several ligands that are coupled to the generation of IP_3 in NHBE cells. Although several receptors coupled to PLC-IP_3 signaling are thought to be expressed in AECs (30), this screen revealed that only a subset of ligands, including agonists for PAR2 and purinergic P2Y receptors (and to a smaller extent, thrombin and bradykinin) evoked Ca^{2+} signals consistent with store-operated Ca^{2+} entry (Table I). PAR2 receptors are important for the ability of AECs to sense allergens and endogenous proteases and for mediating the subsequent inflammatory responses in the lung (31). Moreover, aberrant PAR2 signaling has been implicated in several allergic respiratory disorders including asthma (31). We therefore sought to define the mechanisms and functional consequences of CRAC channel signaling in AECs following PAR2 activation.

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PAR2 activation stimulates Ca^{2+} signaling in AECs by activating CRAC channels

PAR2 activation initiates many cell signaling cascades including IP_3 mediated mobilization of $[Ca^{2+}]_{cyt}$ (13). However the pathways involved in generating PAR2 mediated $[Ca^{2+}]_{cyt}$ elevations and the functional significance of these Ca^{2+} elevations in AECs are unclear. To address the potential role of store-operated CRAC channels in generating PAR2 mediated Ca^{2+} signals in AECs, we examined the ability of PAR2 ligands to elicit SOCE by imaging cytosolic Ca^{2+} transients using the Ca^{2+} indicator, fura2. Activation of PAR2 by the PAR2 activating peptides, SLIGRL and SLIGKV, caused a biphasic $[Ca^{2+}]_{cyt}$ elevation that included a rapid initial rise in $[Ca^{2+}]_{cyt}$ followed by sustained Ca^{2+} oscillations in NHBE cells (Fig. 1A, E and data not shown). The rise in $[Ca^{2+}]_{cyt}$ following add-back of extracellular Ca^{2+} indicates that Ca^{2+} entry across the plasma membrane is necessary for sustaining PAR2-mediated Ca^{2+} elevations (Fig. 1A). The Ca^{2+} response was specific to PAR2 activation, since administration of a control peptide LRGILS or a PAR1-specific peptide TFLLR, failed to elicit Ca^{2+} signaling (Fig. 1E). Response to SLIGKV was also abolished when cells were pre-treated with the phospholipase C (PLC) inhibitor U73122 consistent with PAR2 activating a G_q mediated PLC signaling pathway (Fig. 1E). Importantly, the CRAC channel inhibitors BTP2 and RO2959 (32, 33) attenuated both the number of oscillations and the amplitude of the Ca^{2+} response to PAR2 peptides (Fig. 1B–E). These results indicate that PAR2 stimulation evokes a biphasic Ca^{2+} signal whose sustained component arises from CRAC channel activation.

Previous evidence indicates that the CRAC channel proteins Orai1 and STIM1 are expressed in the lung (21, 34), raising the possibility that these proteins may contribute to Ca^{2+} signaling in AECs. We therefore sought to investigate the contribution of STIM1 and Orai1 to PAR2 evoked Ca^{2+} signals. siRNA mediated knock-down of STIM1 and Orai1 proteins significantly reduced their expression and inhibited the sustained Ca^{2+} signals in response to the PAR2 activating peptide SLIGKV in the bronchial cell-line BEAS-2B (Figs. 1F and G, Supplemental Fig. 1A). Similarly over-expression of a dominant negative pore mutant of CRAC channels, E106A Orai1, (35), significantly reduced SLIGKV activated Ca^{2+} response (data not shown). These results indicate that CRAC channels encoded by STIM1 and Orai1 are essential for mobilizing PAR2 evoked Ca^{2+} signals.

PAR2 activation by the serine protease trypsin stimulates CRAC channel mediated Ca^{2+} signals in AECs

In the airway epithelium, PAR2 is activated by endogenous proteases including trypsin, mast cell tryptase and human airway trypsin-like proteases that are released during inflammation and injury (31). We therefore tested the effects of trypsin on Ca^{2+} signaling in AECs. Administration of type IX trypsin produced a sustained Ca^{2+} signal only when Ca^{2+} was present in the external bath solution. The sustained component of the $[Ca^{2+}]_{cyt}$ elevation was abrogated by the CRAC channel inhibitor BTP2 (Figs. 2A and B) without affecting store-release. Likewise, over-expression of E106A Orai1, which functions as a dominant negative inhibitor of CRAC channels (35) as well as the knockdown of CRAC channel proteins STIM1 and Orai1 attenuated trypsin induced Ca^{2+} signal (Figs. 2C and D). Taken together, these results show that PAR2 activation in response to both synthetic peptides and

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to enzymatic cleavage by trypsin mobilizes Ca^{2+} signaling in primary human AECs through activation of CRAC channels.

STIM1 and Orai1 contribute to SOCE in human AECs

The ability of CRAC channel antagonists to suppress PAR2-evoked Ca^{2+} signals implies that human AECs express the machinery for CRAC channels. To directly address this issue, we next examined the pharmacology, biophysical characteristics, and molecular basis of CRAC channels in AECs. Following the depletion of ER Ca^{2+} stores with the SERCA pump inhibitor thapsigargin (TG) in a nominally Ca^{2+} free solution, robust Ca^{2+} influx was seen when Ca^{2+} was added to the external bath in primary bronchial (NHBE) cells (Fig. 3A). SOCE in NHBE cells exhibited pharmacological properties consistent with CRAC channels including acute inhibition by 2 μM La^{3+}, potentiation by 5 μM 2-APB and inhibition by a higher dose of 2-APB (50 μM) (Fig. 3A to 3C). Moreover, SOCE was significantly inhibited by the CRAC channel antagonists BTP2 and RO2959 (Fig. 3D and 3F). Similar results were obtained in the bronchial cell lines BEAS-2B and 1Haeo- as well as the alveolar epithelial cell line A549, suggesting that SOCE is widespread in lower airway epithelial cells (Fig. 3I, Supplemental Fig. 1B and C). The rates of Ca^{2+} influx following Ca^{2+} add-back reflect store-operated channel activity and are summarized in Figure 3E. These findings are consistent with recent reports indicating presence of CRAC channels in the bronchial cell lines 16HBE and $CFBE410^{-}$ (21, 34).

We next sought to resolve the molecular machinery of CRAC channels in AECs. CRAC channels are activated by STIM proteins (STIM1 and 2) and encoded by the Orai proteins (Orai1, 2 and 3) (36). Western blots showed the expression of both STIM1 and Orai1 proteins in NHBE and BEAS-2B cells (Fig. 3H and Supplemental Fig. 1A). Over-expression of dominant negative E106A Orai1 significantly abrogated SOCE in various AECs (Figs. 3F and 3I). Moreover, knockdown of STIM1 and Orai1 using siRNA significantly reduced the protein expression and inhibited both the rate and the amplitude of SOCE in NHBE cells and various cell-lines (Figs. 3E, 3G and 3I, Supplemental Fig. 1B, C and data not shown), indicating that these isoforms do not appreciably contribute to SOCE in human AECs. siRNA mediated knockdown of Orai2, Orai3 and STIM2 did not significantly affect SOCE (Fig. 3G). We were unable to directly study the expression of Orai2 and Orai3 isoforms due to non-specificity of the commercially available antibodies we tested (data not shown). Nevertheless, together with the functional evidence presented in the previous section, these results indicate that the CRAC channel proteins STIM1 and Orai1 are essential for conferring SOCE in human AECs. We therefore focused our studies on the functional roles of STIM1 and Orai1 in the airway epithelium.

CRAC channels have a distinct electrophysiological profile characterized by an inwardly rectifying current-voltage relationship, low permeability to large monovalent cations such as Cs^{+} and depotentiation in divalent free solutions (37). Patch-clamp recordings of store-operated currents in BEAS-2B cells following treatment with TG to deplete ER Ca^{2+} stores showed the presence of a cation current consistent with these well-known properties of CRAC channels (17, 25) (Fig. 3J). In 20 mM Ca^{2+}_{o}, the current-voltage (I-V) relationship showed strong inward rectification and an extremely positive reversal potential. In divalent-

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free solution, the I-V relationship revealed a reversal potential of ~+50 mV, indicating low permeability to internal Cs^+ (Fig. 3J). Similar results were seen in the airway epithelial cell line A549 (data not shown). These results indicate that SOCE in airway cells exhibits the canonical biophysical properties of CRAC channels encoded by STIM1 and Orai1.

CRAC channel activation stimulates cytokine and chemokine production in AECs

AECs play a vital role in host defense by producing inflammatory mediators such as TSLP, IL-6, PGE_2, IL-33, IL-8 and RANTES in response to a variety of stimuli including infectious agents, allergens and other bioactive molecules (38–41). Although Ca^{2+} signals have been implicated in the generation of inflammatory mediators from AECs, the Ca^{2+} signaling pathways that mediate this process remain poorly understood (7, 20, 42).

To address a potential role for CRAC channels in the production of inflammatory mediators from AECs, we initially performed a multiplex cytokine screen in NHBE cells following activation of CRAC channels by the SERCA inhibitor TG (Supplemental Fig. 2). Previous studies have shown that Ca^{2+} signaling pathways in immune cells including T-cells often work in synergy with protein kinase-C (PKC) dependent signaling to induce cytokines (43). Therefore, in these experiments, we also co-stimulated PKC by administering the phorbol ester, PDBu. Key hits from this screen were further examined by individual ELISA kits. These tests revealed that several key inflammatory mediators are regulated by CRAC channel activation in NHBE cells, including TSLP, IL-6, TNFα, IL1β and GM-CSF (Fig. 4A, 4C to 4F). CRAC channel activation also induced the production of the arachidonic acid metabolite PGE_2 (Prostaglandin E_2) from AECs (Fig. 4B). We also confirmed IL-6, TNFα and PGE_2 production in various airway epithelial cell lines (Fig. 4D, Supplemental Fig. 3A–C). The production of these inflammatory mediators was prevented in Ca^{2+} free medium and by the CRAC channel inhibitor BTP2 (Fig. 4A to 4F). Furthermore, knock down of STIM1 and Orai1 inhibited CRAC channel induced production of TNFα and PGE_2 (Fig. 4B and C). Thus, these results implicate a causal role for Ca^{2+} influx through CRAC channels in the production of inflammatory cytokines from AECs. Interestingly, while maximal induction of TNFα and IL-6 required co-stimulation of NHBE cells with both TG and PDBu, TSLP and PGE_2 were induced maximally by TG alone, suggesting that Ca^{2+} signals differentially regulate cytokine production depending on the presence of specific co-stimulatory signals.

In addition to the cytokines, CRAC channel activation also caused significant increase in the production of chemokines IL-8 and RANTES which was blocked by the CRAC channel inhibitors BTP2 and RO2959 (Figs. 4G and H). The multiplex cytokine screen further revealed that while AECs also produce IFNγ and CXCL1, these factors are not induced by CRAC channels, indicating that only a subset of inflammatory mediators produced by AECs are regulated by CRAC channels. Collectively, these results establish a key role for CRAC channels as an important route of Ca^{2+} entry for the production of cytokines and chemokines in the airway epithelium.

PAR2 agonists activate CRAC channels to induce cytokine production in AECs

Previous studies have established that PAR2 activation in AECs induces the production of a wide range of cytokines including IL-6, GM-CSF, TSLP and chemokines IL-8 and eotaxin,

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the growth factor PDGF, arachidonic metabolites PGE_2 and PGD_2 as well as the enzyme MMP-9 (8–10, 40, 44). The dual findings that PAR2 activation mobilizes Ca^{2+} signals through CRAC channels (Fig. 1 and 2) and that CRAC channel activation with TG stimulates cytokine production (Fig. 4 and 5) therefore led us to consider a role for CRAC channels in the production of inflammatory mediators by PAR2 agonists. These tests revealed that administration of the PAR2 agonist peptide SLIGRL resulted in a significant increase in the production of PGE_2, IL-6, GM-CSF and IL-8 (Fig. 5A, C, E and F). PGE_2 was induced in 2 hours while induction of IL-6, GM-CSF and IL-8 occurred after 8- 24 hours of PAR2 activation. The PAR2 mediated production of these factors was significantly inhibited by the CRAC channel antagonist, BTP2 (Figs. 5A, C, E and F). Interestingly, PAR2 activation by SLIGRL did not evoke TNFα induction (not shown). This is in contrast to cell stimulation with TG/PDBu, which was very effective in evoking TNFα production (Figure 4D). The reasons for this difference need to be further investigated, but could be because the amplitude and duration of Ca^{2+} elevations in response to PAR2 activation are insufficient to induce TNFα. PGE_2 and IL-6 induction in response to SLIGRL were significantly inhibited by combined knockdown of STIM1 and Orai1 (Figs. 5B and D). Knockdown of STIM1 or Orai1 alone, however, did not significantly diminish the production of these cytokines (data not shown). We suspect that this is due to the low knockdown efficiency of protein expression in NHBE cells (~50% for both proteins as seen by Western Blot, Fig. 3H) which would be expected to only partially inhibit SOCE. Similarly, trypsin produced a significant increase in PGE_2 production that was attenuated by the CRAC channel blocker BTP2 (Fig. 5A). Thus, these findings strongly suggest that CRAC channels contribute to the production and release of inflammatory modulators in response to PAR2 activation.

PAR2 receptor stimulation results in IL-6 and IL-8 production through the activation of NFAT

By what mechanism does Ca^{2+} influx through CRAC channels regulate PAR2 mediated production of cytokines? In many immune cells, cytokine induction is regulated transcriptionally by the transcription factor NFAT (nuclear factor of activated T-cells) (45, 46). Ca^{2+} elevations activate NFAT through the protein phosphatase, calcineurin, which then causes NFAT to move into the nucleus and bind to target DNA sequences (43). While AECs express NFAT proteins (47), the specific targets of NFAT and the physiological relevance of this signaling remains unknown. To explore whether induction of cytokines by PAR2 activation occurs through the calcineurin-NFAT pathway, we measured PAR2 induced cytokine levels in the presence of the calcineurin inhibitor Cyclosporin A (CsA), a widely used small molecule inhibitor of calcineurin/NFAT signaling (48). Induction of both IL-6 and IL-8 was significantly inhibited by CsA (Fig. 6A). By contrast, the NF-kB inhibitor, caffeic acid, had no effect on induction of IL-6 or IL-8 indicating that under these experimental conditions, the induction of the cytokines primarily occurs through NFAT-dependent gene transcription (Supplemental Fig. 3D). Moreover, induction of PGE_2 and GM-CSF was not affected by CsA, suggesting that the induction of these cytokines in response to PAR2 activation is not significantly regulated by calcineurin/NFAT signaling. These findings highlight a novel role for NFAT dependent gene expression in the induction of specific cytokines and chemokines in response to PAR2 signaling.

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NFAT regulates cytokine and chemokine production in response to CRAC channel activation

To explore the role of NFAT in CRAC-channel mediated cytokine production more directly, we stimulated NHBE cells with TG and PDBu for 12 hours in the presence or absence of CsA and evaluated inflammatory mediator production by ELISA. CsA caused an almost complete inhibition of TNFα and IL-6 production, implicating a key role for NFAT in induction of these cytokines (Fig. 6B). CsA also partially inhibited production of PGE_2 and IL-8, suggesting that NFAT contributes to the production of these mediators (Fig. 6B). The partial inhibition of PGE_2 induction in this case stands in contrast to the lack of CsA effect seen following two hours of PAR2 stimulation (Fig. 6A), suggesting that at longer time points, PGE_2 production is influenced by NFAT-mediated gene expression, possibly through transcriptional regulation of the enzymes mediating PGE_2 generation (49). Interestingly, although the promoter elements of GM-CSF is believed to exhibit NFAT binding, the induction of this cytokine in response to both PAR2 and TG/ PDBu was unaffected by CsA, suggesting that not all cytokines induced by CRAC channels are regulated through the calcineurin- NFAT pathway (Fig. 6B).

NFAT is normally phosphorylated at rest and resides in the cytoplasm in the inactive state. Ca^{2+} elevations mobilize NFAT activation through dephosphorylation of conserved serine/ threonine residues by the Ca^{2+}-activated protein phosphatase, calcineurin, which causes NFAT to move into the nucleus and bind to target DNA sequences (43). Consistent with this property of NFAT, examination of a fluorescently tagged NFAT isoform (GFP-NFATc3) revealed that activation of CRAC channels by TG resulted in robust NFAT import into the nucleus (Figs. 7A and B). The import was blocked in a nominally Ca^{2+} free solution and by BTP2 indicating that Ca^{2+} entry through CRAC channels was required to trigger nuclear translocation of NFAT (Figs. 7A and B). Moreover, examination of NFAT-dependent gene transcription using a luciferase reporter assay indicated that CRAC channel activation stimulated NFAT-dependent luciferase expression in NHBE cells (Fig. 7C). Luciferase activity was abrogated in the absence of external Ca^{2+}, and by the CRAC channel antagonists BTP2 and RO2959 (Fig. 7C) without affecting cell viability (Supplemental Fig. 4). Importantly, siRNA mediated suppression of STIM1 and Orai1 expression significantly inhibited NFAT-dependent gene transcription (Fig. 7D). The calcineurin inhibitors CsA and FK506 also strongly inhibited induction of NFAT-luciferase activity consistent with the well-known role of calcineurin in controlling NFAT activation (Fig. 7C). Thus, these results highlight a broader role for NFAT in the generation of inflammatory mediators from the airway epithelium.

DISCUSSION

Ca^{2+} signals regulate many key physiological processes in airway epithelial cells (AECs) including activation of Ca^{2+} activated Cl^- conductances, ciliary beat frequency, and mucous and surfactant production (19, 50, 51). There is now growing evidence that Ca^{2+} signals also play an important role in the induction of inflammatory mediators from the airway epithelium in response to pathogens and allergens and may be involved in orchestrating inflammatory responses in several airway diseases including cystic fibrosis, asthma, and

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acute lung injury (7, 20, 52). Despite these observations, our understanding of how Ca^{2+} signals are generated in human AECs and how they are linked to effector functions remains incomplete. Knowledge of molecules that comprise the functional architecture of the Ca^{2+} signaling network and the mechanisms by which these proteins shape Ca^{2+} signals is important to gain a full understanding of how Ca^{2+} signals modulate downstream inflammatory responses in human AECs. In this study we show that CRAC channels encoded by Orai1 and STIM1 mediate a critical role in generating Ca^{2+} signals in response to PAR2 activation and regulate the production of several key inflammatory mediators in the airway epithelium, in part by activating NFAT dependent gene expression. These results highlight CRAC channels as a key checkpoint for transducing responses from proteases and for the generation of inflammatory mediators from the airway epithelium and identify CRAC channels as a potential target for therapeutic treatment of allergic and inflammatory airway diseases.

CRAC channels are a major route of Ca^{2+} entry in AECs

We show that primary human AECs exhibit SOCE and store-operated currents that share the pharmacological and biophysical hallmarks of CRAC channels (37). These features include block by low concentrations of (1–2 μM) La^{3+}, modulation by 2-APB, inhibition by the CRAC channel inhibitors BTP2 and RO2959, an inwardly rectifying I-V, fast inactivation, and depotentiation of the Na^{+} CRAC current (Fig. 3). Analysis of the underlying molecular machinery further reveals that the CRAC channel proteins, STIM1 and Orai1 make essential contributions to SOCE in primary human AECs (Figs. 3E to G). Both bronchial and alveolar epithelial cells exhibited STIM1-Orai1 dependent SOCE, suggesting that this Ca^{2+} influx pathway is broadly conserved throughout the lower airway epithelium (Fig. 3 and Supplemental Fig. 1). These findings confirm and broaden observations from past studies that have described STIM1 and Orai1 mediated SOCE in various airway epithelial cell-lines and corroborate the emerging viewpoint that CRAC channels are a well conserved mechanism for Ca^{2+} influx in the lower airways (21, 34, 53). Our data however do not rule out a role for other CRAC channel isoforms. Indeed, SOCE was not fully abolished by STIM1 and Orai1 knockdown. Moreover, STIM2 and Orai3 knockdown resulted in a modest inhibition of SOCE (Fig. 3G), raising the possibility that these proteins may be expressed and make additional contributions to SOCE in AECs. More studies using isoform specific antibodies and knockout mice would be needed to test this issue. These issues notwithstanding, our findings unequivocally show that STIM1 and Orai1 are essential for conferring SOCE in human AECs.

CRAC channels mediate the downstream effects of PAR2 signaling

A screen for ligands that activate SOCE revealed that only a very specific set of receptors stimulate CRAC channel activation in AECs (Table 1). A prominent hit in this screen was PAR2, an important sensor of allergens and endogenous proteases in the airway epithelium (5, 6, 9). While many elements of the PAR2 signaling pathway have been studied in AECs, it is unclear whether PAR2 activation evokes CRAC channel-mediated Ca^{2+} signals. Here, we find that Ca^{2+} influx through CRAC channels encoded by STIM1 and Orai1 is needed to maintain the sustained Ca^{2+} signal that arises from PAR2 activation (Fig. 1 and 2). Furthermore, we find that PAR2 activation induces production of PGE_2, IL-6, IL-8 and GM-

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CSF in AECs in a CRAC channel-dependent manner. These findings highlight a novel role for CRAC channels as key control element in the PAR2 signaling response. It is worth noting that while PGE_2 production is seen following brief activation of PAR2 receptors (2 hours), IL-6, IL-8 and GM-CSF production required prolonged stimulation of PAR2 (8–24 hours). Because PGE_2 evokes bronchodilation through effects on the airway smooth muscle, these results are consistent with the emerging idea that acute responses to PAR2 tend to be protective largely, while more sustained responses to PAR2 activation result in induction of cytokines that are pro-inflammatory thereby contributing to the pathogenesis of diseases like asthma (12, 54).

CRAC channels induce the production of inflammatory mediators from AECs

In the repertoire of inflammatory mediators secreted by AECs, several including PGE_2, TSLP, IL-6, IL-8 and GM-CSF are thought to be induced by cytosolic Ca^{2+} elevations (7, 55, 56). However, the source of Ca^{2+} signals driving the induction of these proinflammatory mediators remains unclear. We find that CRAC channel activation induces the production of a host of inflammatory mediators from primary bronchial cells including the cytokines IL-6, TSLP, TNFα and IL1β, the arachidonic acid metabolite PGE_2, the chemokines IL-8 and RANTES, and the growth factor GM-CSF (Fig. 6). Further examination shows that TNFα, IL-6 and GM-CSF were specifically secreted only by co-stimulation of CRAC channels with protein-kinase C (PKC) activation while TSLP, PGE_2 and IL1β are maximally induced by CRAC channel stimulation alone (Figs 6 A-F). These results indicate that cross-talk between signaling pathways can change the specific repertoire of inflammatory mediators released from AECs.

Among the inflammatory mediators that regulate airway inflammation, TSLP and PGE_2 are produced primarily by the airway epithelium. TSLP plays a vital role in effecting a Th2 type airway inflammatory response that is characteristic of allergic diseases like asthma and is known to be induced by various bacterial, viral and fungal products (1). On the other hand, the arachidonic acid metabolite PGE_2 has immunoprotective effects in the airway that includes attenuation of broncho-constriction in exercise and allergen induced asthma, and inhibition of lymphocytic proliferation (57, 58). Here, we find that CRAC channel activation induces both TSLP and PGE_2 in AECs but at very different time scales: PGE_2 is induced in less than 2 hours while TSLP induction requires at least 48 hours of AEC stimulation. These temporal differences indicate that CRAC channels regulate multiple stages of the inflammatory response in AECs and the net consequences for airway function will depend on balance between the ensuing pro- and anti-inflammatory effector responses. Curiously, while CRAC channel activation produced PGE_2, we saw no induction of the leukotrienes (LTB_4, LTC_4-E_4) which are pro-inflammatory products of the arachidonic acid pathway (Fig. 5 and data not shown). This is in contrast to findings in mast cells, where CRAC channel activation is a potent and specific trigger for the induction of LTC_4 production (46). CRAC channels may thus activate different arachidonic acid metabolites and produce distinct biological responses depending on the cell type.

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CRAC channels activate NFAT dependent gene-expression in AECs

Induction of IL-6, TNFα, PGE_2 and IL-8 in response to CRAC channel activation was suppressed by CsA (Fig. 6), indicating that these cytokines are transcriptionally regulated by the calcineurin-NFAT pathway. This is consistent with observations that the promoter regions of a number of cytokine genes including IL-6, IL-8 and TNFα harbor NFAT binding sites (43) and reveals an important role for NFAT as a CRAC channel dependent regulator of cytokine production in AECs. In line with this interpretation, PAR2 mediated induction of IL-6 and IL-8 was also significantly inhibited by blocking the NFAT signaling pathway. Interestingly, while CsA inhibited IL-8 production in response to both PAR2 agonists and TG/PDBu to the same extent, the induction of IL-6 by PAR2 activation was only partially inhibited by CsA. We speculate that this difference may reflect the possibility that additional NFAT independent mechanisms activated by PAR2 signaling come into play to drive IL-6 induction. Moreover, not all inflammatory mediators activated by PAR2 signaling require NFAT gene expression (e.g., PGE_2 and GM-CSF; Fig. 6A) suggesting that the induction of these mediators occurs through alternate Ca^{2+}-regulated pathways. One possibility is that Ca^{2+}-mediated stimulation of $cPLA_2$ may drive the induction of PGE_2 (59), something that remains to be tested. Taken together, our results highlight a hitherto unappreciated role for NFAT as an important mediator of downstream PAR2 signaling in the airway epithelium.

Based on these results, we propose a model for PAR2 receptor signaling in the airway epithelium wherein activation of these receptors, either by peptides or endogenous proteases, causes G_q-PLCβ-IP_3 mediated store-release, leading to the generation of long-lasting Ca^{2+} signals through the activation of store-operated CRAC channels. The ensuing Ca^{2+} influx triggers the activation of transcription factor NFAT which then translocates to the nucleus and drives expression of IL-6 and IL-8 genes in conjunction with other signaling mechanism (Fig. 8). In conclusion, our study reports a novel Ca^{2+} signaling pathway in the airway epithelium mediated by CRAC channels which likely constitute an important regulatory element in the induction of inflammatory mediators from the airway epithelium.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

AEC	airway epithelial cells
BTP2	3,5-bis(trifluoromethyl)pyrazole
CRAC	Calcium release-activated Ca^{2+}
ER	endoplasmic reticulum

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GPCR	G-protein coupled receptor
IP_3	Inositol triphosphate
NFAT	nuclear factor of activated T-cells
PAR	Protease-activated receptor
PKC	protein kinase C
PLC	phospholipase C
SOCE	store-operated Ca^{2+} entry
STIM	Stromal interaction molecule
TSLP	thymic stromal lymphopoietin

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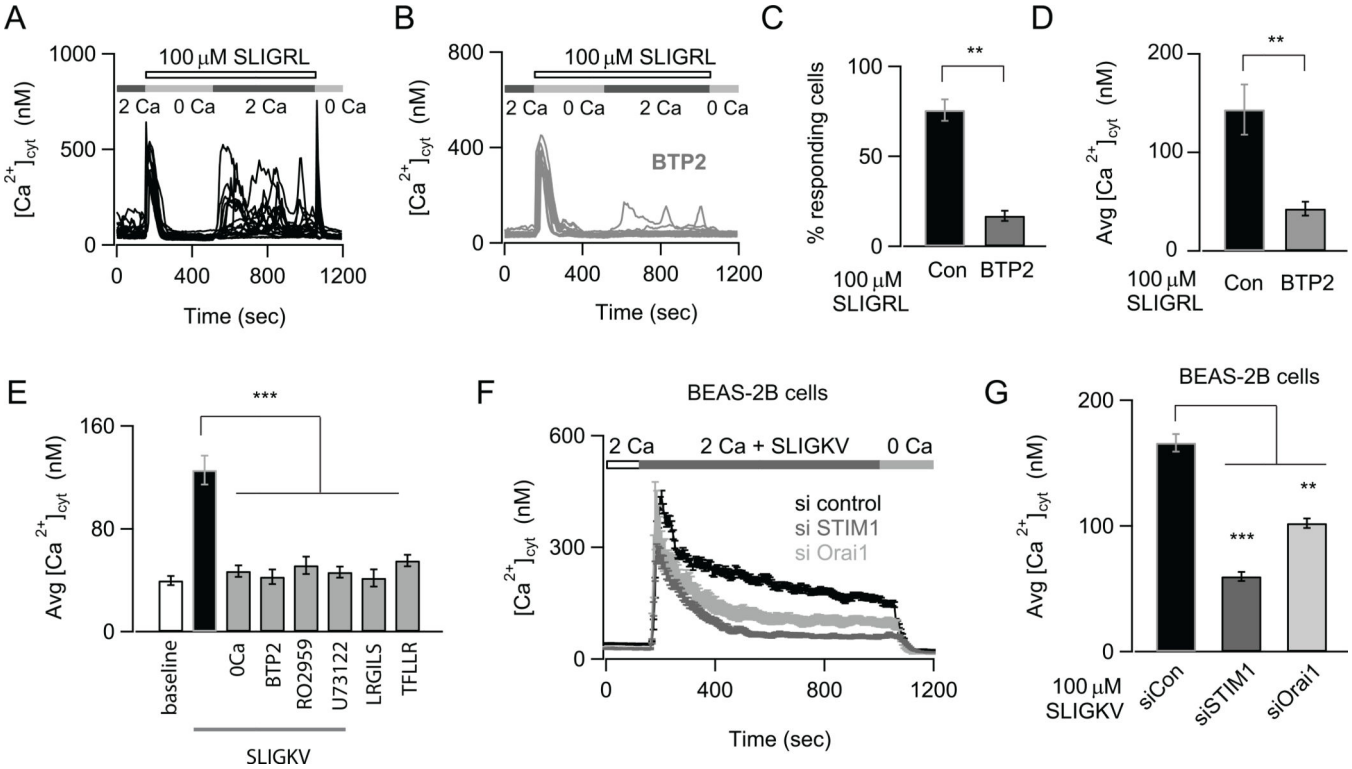

FIGURE 1.

Store-operated CRAC channels regulate Ca^{2+} signals induced by PAR2 activation. (**A**) The PAR2-agonist SLIGRL induces oscillatory Ca^{2+} signals due to SOCE. NHBE cells were treated with 100 μM SLIGRL in a nominally Ca^{2+} free solution to deplete internal stores. 2 mM Ca^{2+} was re-added to the external bath solution to activate SOCE. The Ca^{2+} traces show responses of individual cells in the imaging field. (**B**) Pre-treatment with the CRAC channel inhibitor BTP2 does not affect store-release but abrogates SOCE. Cells were treated with 500 nM BTP2 for 1.5 hours (A, B; data are mean ± SEM of N=18–21 cells; representative of 3 independent experiments). (**C**) Bar-graph summarizing the percentage of cells showing Ca^{2+} responses after re-addition of external Ca^{2+}. (**D**) Summary of the amplitude of Ca^{2+} response at 840 sec (data are mean ± SEM of N=38–45 cells; 3 independent experiments). (**E**) Summary of the average elevation in $[Ca^{2+}]_{cyt}$ in response to the PAR2 activating peptide SLIGKV in Ca^{2+} and nominally Ca^{2+} free solutions, control peptide LRGILS and PAR1 activating peptide TFLLR. The effects of CRAC channel inhibitor RO2959 and PLC inhibitor U73122 on SLIGKV mediated Ca^{2+} elevation is also indicated. The $[Ca^{2+}]_{cyt}$ was measured 156–192 sec after addition of various peptides to NHBE cells with 2mM Ca^{2+} in the external bath (data are mean ± SEM of N=12–23 cells; 3 independent experiments). (**F**) Effects of siRNA knockdown of STIM1 and Orai1 on SLIGKV-induced Ca^{2+} signals in BEAS-2B cells (data are mean ± SEM of N=24–31 cells; representative of 3 independent experiments). The traces reflect the mean Ca^{2+} concentration of all cells in the imaging field. (**G**) Summary of mean $[Ca^{2+}]_{cyt}$ levels 748 sec after addition of ligand. Statistical significance was determined by unpaired two-sample student t-test). The following standardized notation for statistical significance (P-values) is used in all the figures, * $P< 0.05$, ** $P< 0.01$, *** $P< 0.001$, n.s. (not significant).

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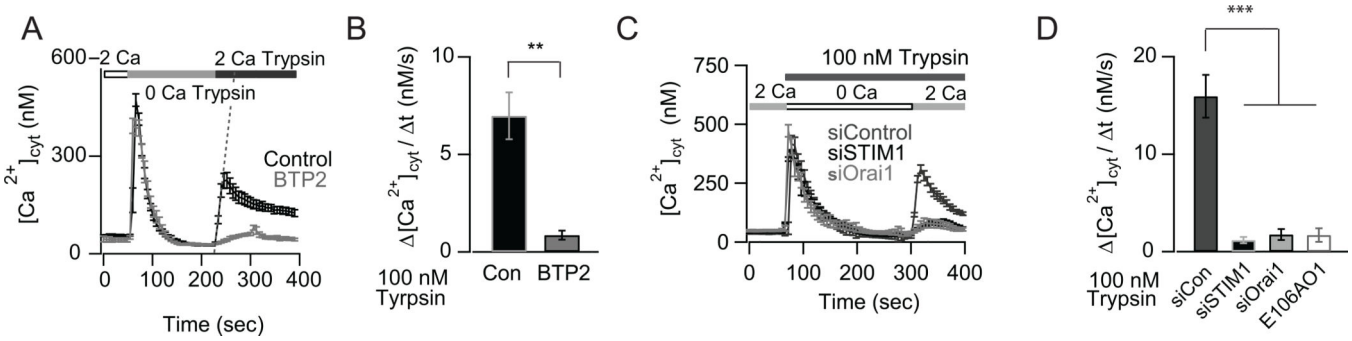

FIGURE 2.

The PAR2 agonist, type IX trypsin, evokes Ca^{2+} elevations by activating CRAC channels. (**A**) Type IX trypsin (100 nM) activates SOCE in NHBE cells. SOCE was activated using the same protocol as in Figure 1A. The CRAC channel inhibitor, BTP2, abrogates trypsin induced Ca^{2+} elevations in NHBE cells (mean ± SEM of N= 17–25 cells, 3 independent experiments). Slope of the line (used to calculate rate of SOCE) following addition of 2 mM Ca^{2+} is indicated for the control condition. (**B**) Summary of the rate of Ca^{2+} influx for SOCE shown in Fig. 2A. (**C**) siRNA knockdown of STIM1 or Orai1 inhibits trypsin mediated SOCE in BEAS-2B cells. (**D**) Summary of the rates of trypsin mediated Ca^{2+} influx shown in Fig. 2C and in cells transfected with dominant negative E106A Orai1 (mean ± SEM of N=16–26 cells; 3 independent experiments).

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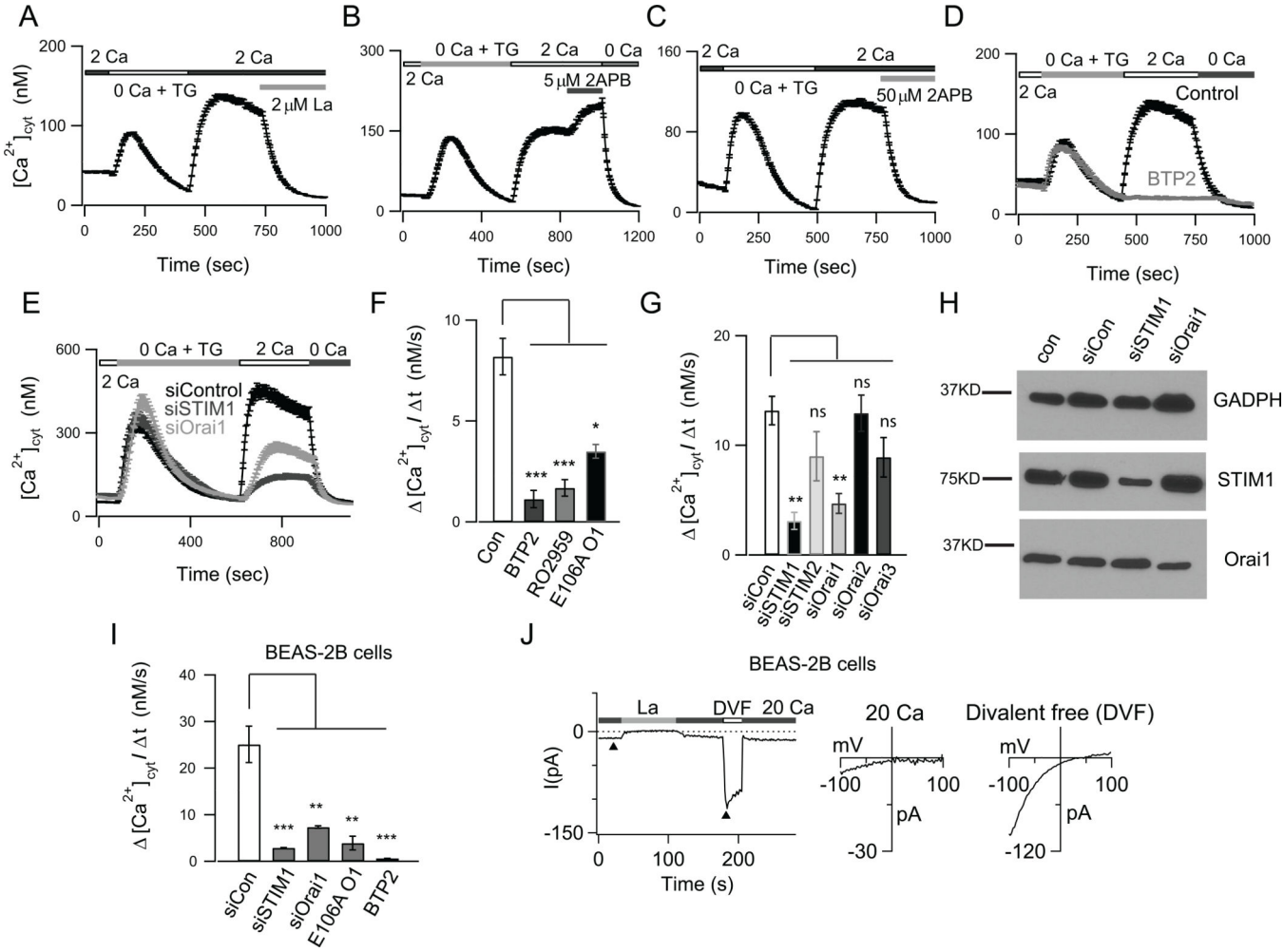

FIGURE 3.

Characterization of store-operated CRAC channels in human airway epithelial cells. (**A–C**) ER Ca^{2+} stores were depleted with 1 μM TG in a nominally Ca^{2+} free solution. Re-addition of 2 mM Ca^{2+} to the external bath solution evoked SOCE. (**A**) SOCE is acutely blocked by 2 μM La^{3+}. (**B**) A low dose of (5 μM) 2-APB facilitated SOCE. (**C**) A high dose of 2-APB (50 μM) inhibited SOCE. (**D**) Pre-treatment with the CRAC channel inhibitor BTP2 (500 nM) abolished SOCE in NHBE cells (A-D; mean ± SEM of N=18–29; representative of 3 independent experiments). (**E**) Knockdown of STIM1 and Orai1 expression in NHBE cells significantly inhibited SOCE (N=21–26 cells; 3 independent experiments). (**F–G**) Summary of the rates of SOCE in NHBE cells following treatment with the CRAC channel inhibitors BTP2 (500 nM), RO2959 (500 nM) and expression of dominant negative E106A Orai1 (N=21–42 cells; 3 experiments) (**F**), and following siRNA mediated knockdown of STIM1–2, Orai 1–3 (mean ± SEM of N=22- 34; 3 experiments) (**G**). (**H**) Western blots showing expression of Orai1 and STIM1 in NHBE cells. (**I**) Summary of rates of SOCE in BEAS-2B cells (N=31–48 cells; 3 independent experiments). (**J**) Whole-cell patch-clamp recordings of I_{CRAC} in BEAS-2B cells. Cells were pretreated with TG to deplete ER Ca^{2+} stores. The external solution was periodically switched between 20 mM Ca^{2+} and a divalent-free (DVF solution). In 20 mM $Ca^{2+}{}_{o}$, the current exhibits a current-voltage (I-V) relationship with

strong inward rectification and positive reversal potential (>+60 mV), consistent with the known biophysical hallmarks of CRAC channels.

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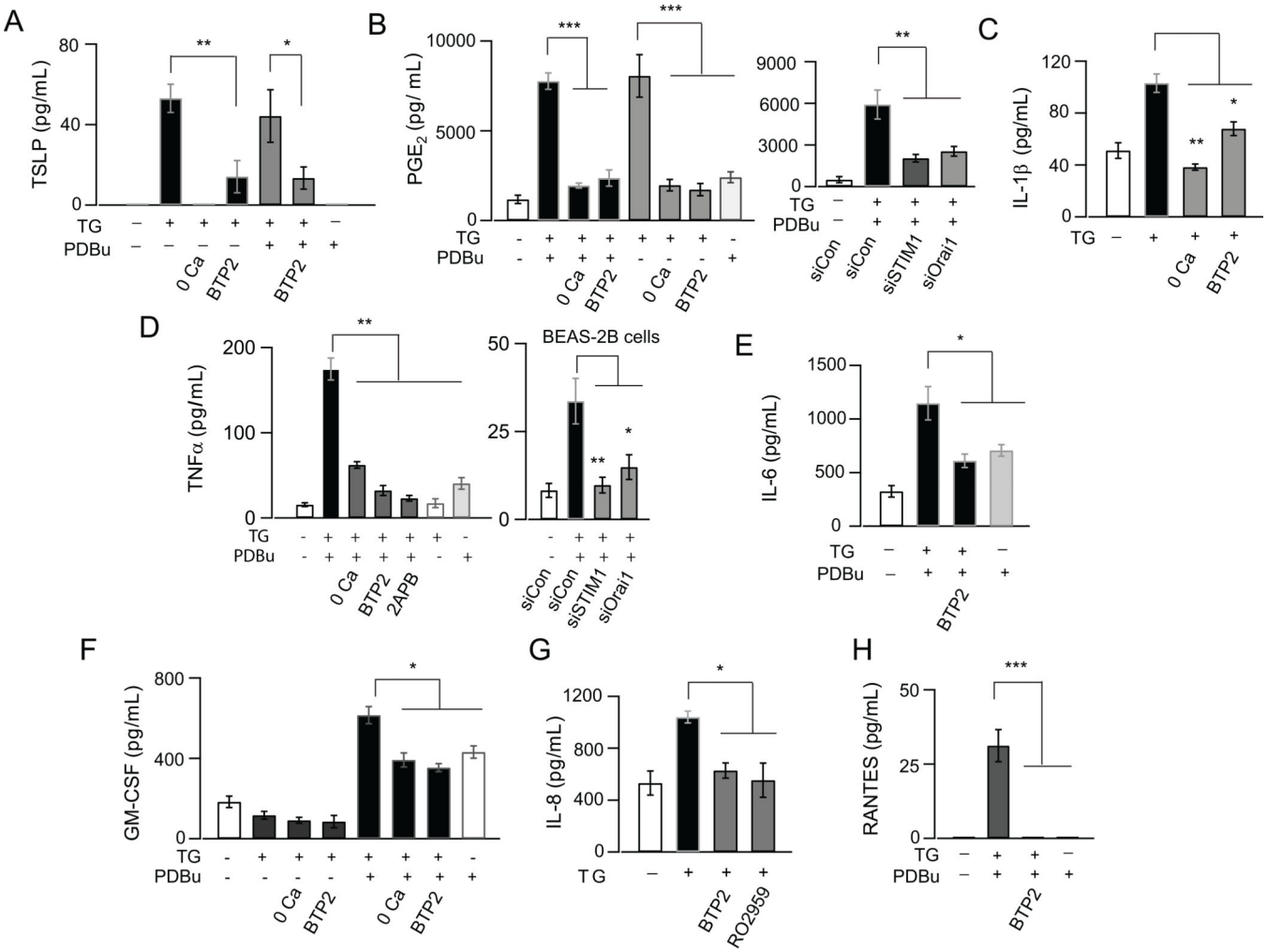

FIGURE 4.

CRAC channel activation induces production of inflammatory mediators in human airway epithelial cells. (**A-H**) Production of cytokines and chemokines as detected by ELISA. AECs were stimulated with either TG alone (0.25 μM) or TG (0.25 μM) and PDBu (50 nM), in the absence or presence of the CRAC channel inhibitor BTP2 (250 nM) and in nominally Ca^{2+} free media. Cells treated with solvent alone (DMSO) were used as control. Cell-culture supernatants were collected 8–12 hours after treatment for all cytokines and after 48 hours for TSLP. N=3–4 replicates; representative of 3 independent experiments. Unless otherwise noted, all experiments were in NHBE cells.

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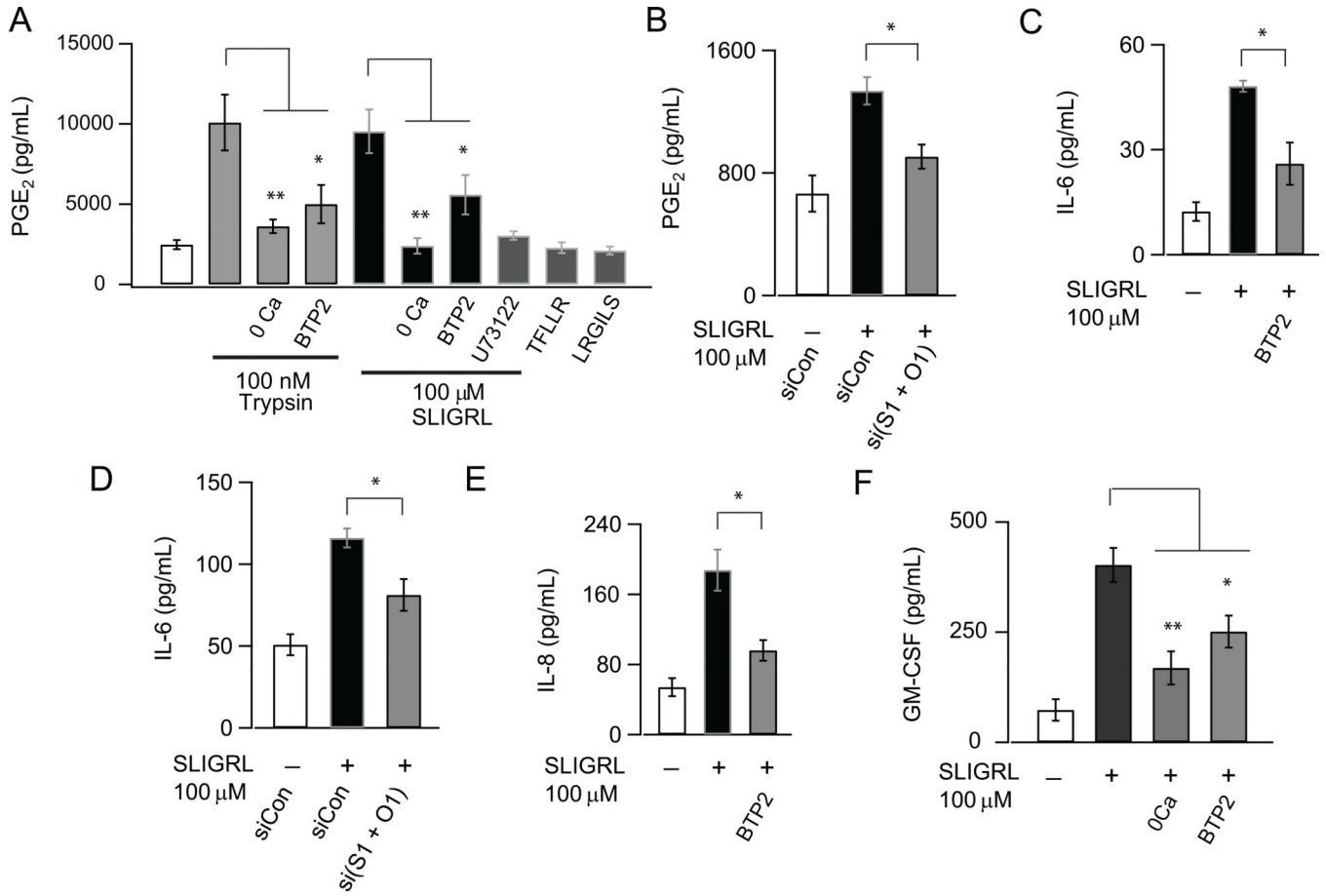

FIGURE 5.

CRAC channels regulate generation of inflammatory mediators in response to PAR2 activation in human airway epithelial cells. NHBE cells were stimulated with the PAR2-activating peptide SLIGRL (100 μM) in the absence or presence of BTP2 or following knockdown of both STIM1 and Orai1. Cell-culture supernatants were collected after 2 hours (for PGE_2) or 24 hours (for IL-6, IL-8 and GM-CSF measurements) and levels of PGE_2, IL-6, IL-8 and GM-CSF were determined by cytokine-specific ELISA kits. (**A**) PGE_2 induction by SLIGRL and Type IX trypsin (100 nM) is inhibited by BTP2 and U73122. A scrambled peptide (LRGILS) and a PAR1-specific peptide (TFLLR) had no effect on PGE_2 induction. (**B**) Inhibition of PGE_2 secretion by knockdown of STIM1 and Orai1. (**C**) Inhibition of IL-6 secretion by BTP2 and (**D**) siRNA mediated knockdown of STIM1 and Orai1. (**E-F**) Effect of BTP2 on IL-8 and GM-CSF induction (N=3 replicates, 3 independent experiments).

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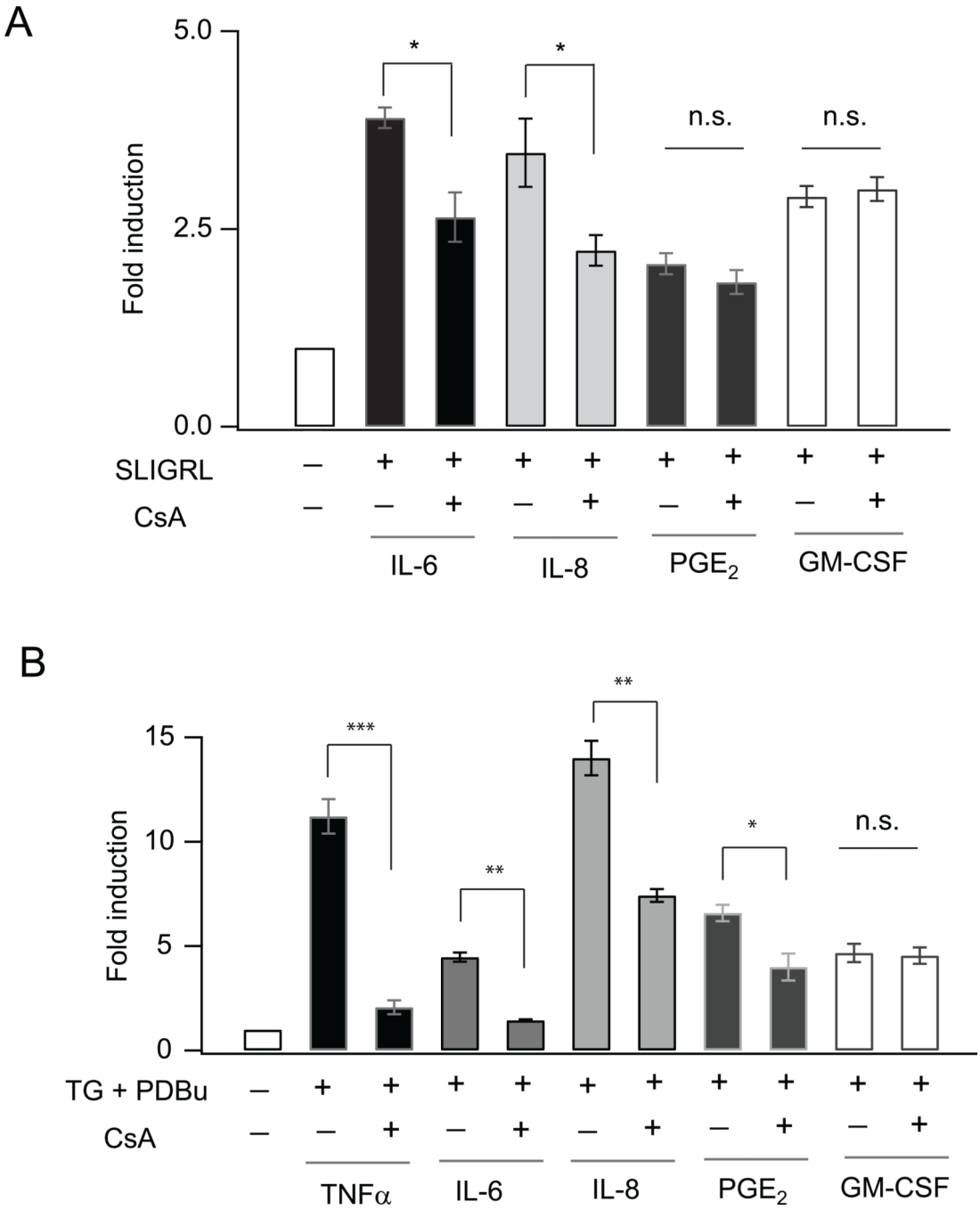

FIGURE 6.

Calcineurin-NFAT signaling regulates PAR2 and CRAC channel mediated induction of inflammatory mediators from AECs. (**A**) Summary of the effect of calcineurin inhibitor CsA (500 nM) on IL-6, IL-8, PGE_2 and GM-CSF induction in response to the PAR2 agonistic peptide SLIGRL (100 μM) in NHBE cells. Cells were stimulated for 2 hours for PGE_2 induction, 8 hours for IL-6 and IL-8 induction and 24 hours for GM-CSF induction. Data is shown as fold change in cytokine production (actual concentrations: IL-6: 18–70 pg/mL; IL-8: 55–200 pg/mL; PGE_2: 2300–4500 pg/mL; GM-CSF: 75–220 pg/mL). (**B**) Summary of

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the change in cytokine levels in NHBE cells (12 hour stimulation) evoked by CRAC channel activation. NHBE cells were treated with TG (250 nM) and PDBu (50 nM) in the absence and presence of CsA (250 nM). N=3 replicates, 3 independent experiments.

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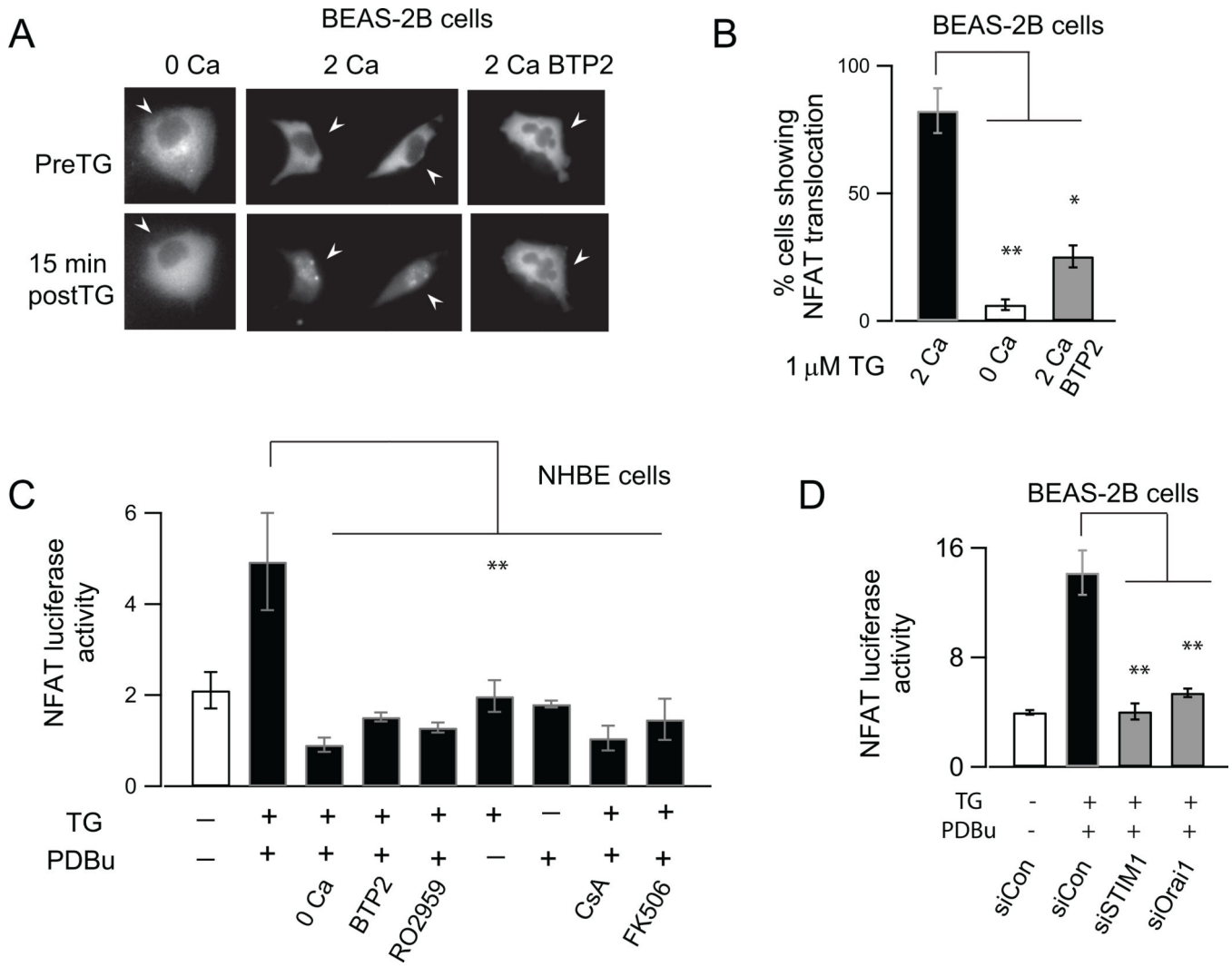

FIGURE 7.

CRAC channels activate NFAT dependent gene expression in AECs. (**A**) Wide-field images showing the localization of NFATc3-GFP protein under resting conditions and after CRAC channel activation with TG (1 μM, 20 min.) in the presence or absence of Ca^{2+} in the external bath and in the presence of BTP2 (500 nM). In each case, the pre- and post-TG images show the same cells. (**B**) Summary of the percentage of cells showing NFAT translocation in BEAS-2B cells following CRAC channel activation. (mean ± SEM of N=12–15 cells from 3 separate coverslips for each condition). (**C**) Summary of NFAT-luciferase activity in the presence and absence of CRAC channel (BTP2 and RO2959: 500 nM) and calcineurin inhibitors (CsA 500 nM, FK506 1 μM. Data is mean ± SEM of 4 replicates; 3 independent experiments in NHBE cells). NFAT-luciferase activity was measured using a pGL3 NFAT luciferase reporter construct (29). Cells were stimulated with TG and PdBu to activate CRAC channels and PKC, respectively. (**D**) siRNA knockdown of STIM1 and Orai1 significantly inhibits NFAT luciferase activity in BEAS-2B cells (mean ± SEM of 3 replicates; 3 independent experiments). NFAT luciferase activity normalized to renilla luciferase in order to correct for variations in cell density as described in the methods.

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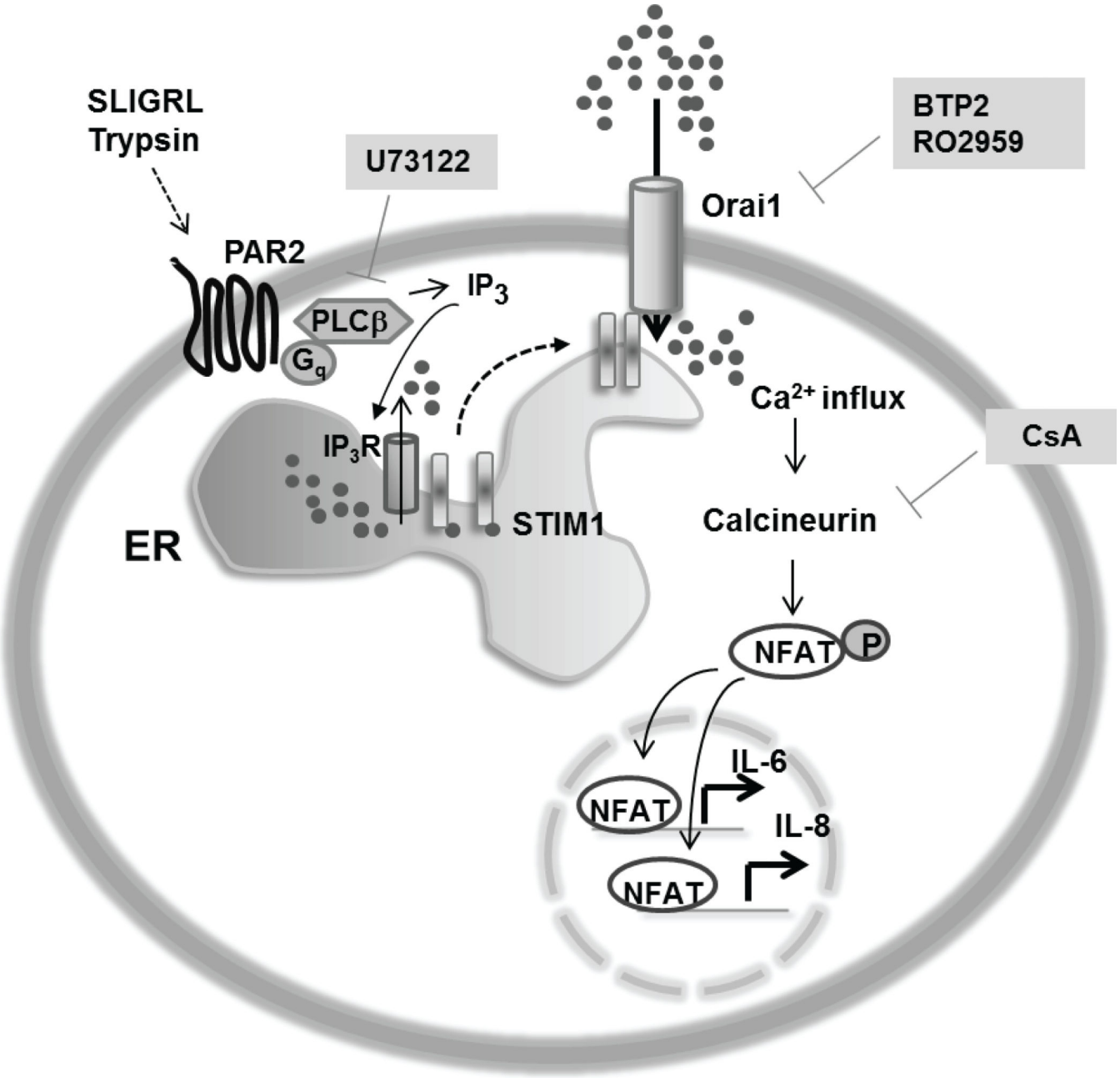

FIGURE 8.

Model for the Ca^{2+} dependent signaling pathway activated by PAR2. Activation of PAR2 receptors by serine proteases (trypsin) and peptide agonists (SLIGRL and SLIGKV) evokes phospholipase C (PLC) stimulation leading to IP_3 production and ER store-release through the opening of IP_3 receptors. Release of Ca^{2+} from intracellular stores leads to activation of STIM1 which opens CRAC channels comprised of Orai1 subunits. Ca^{2+} influx through CRAC channels activates calcineurin-NFAT signaling to transcriptionally induce IL-6 and IL-8 production, likely in conjunction with other pathways. CsA, Cyclosporin A; U73122, PLCβ inhibitor; BTP2 and RO2959, CRAC channel inhibitors.

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Table I

Effect of GPCR and Toll-Like Receptor (TLR) agonists on SOCE in AECs.

LIGAND	KNOWN RECEPTOR	RECEPTOR TYPE	SOCE ACTIVATION?
PROTEASE ACTIVATED RECEPTORS			
Type IX Trypsin	PAR2	GPCR	++
SLIGKV, SLIGRL	PAR2	GPCR	++
TFLLR	PAR1	GPCR	−
Thrombin	PAR1, 3, 4	GPCR	+
PATHOGENS			
PamC3, LTA	TLR2	TLR	−
pI:C	TLR3	TLR	−
LPS	TLR4	TLR	−
PURINERGIC AGONISTS			
ATP	P_2X, P_2Y	GPCR	++
UTP	P_2Y_2, $P2Y_4$	GPCR	++
UDP-glucose	P_2Y_{14}	GPCR	−
OTHER LIGANDS			
Bradykinin	BK1, 2	GPCR	+
PGE_2	EP1	GPCR	−
PAF (platelet activating factor)	PAFR	GPCR	−
EGF (epidermal growth factor)	EGFR	GPCR	−
Salbutamol	β2 adrenergic	GPCR	−
Denatonium	Bitter taste receptors	GPCR	−
tBHP (oxidative stress)	−	STIM1, IP_3	−

Table I: NHBE cells were treated with the indicated ligands in a Ca^{2+}-free Ringer's solution. Extracellular Ca^{2+} (2 mM) was then restored and the amplitude of the $[Ca^{2+}]_{cyt}$ rise was examined. Cells were considered responders if the $[Ca^{2+}]_{cyt}$ elevation was > 2 x SEM above the resting $[Ca^{2+}]_{cyt}$.