Calcium-sensing receptor modulates extracellular Ca\(^{2+}\) entry via TRPC-encoded receptor-operated channels in human aortic smooth muscle cells

**Jimmy Y. C. Chow,* Christine Estrema,* Tiffany Orneles,* Xiao Dong, Kim E. Barrett, and Hui Dong**

Department of Medicine, University of California San Diego, La Jolla, California

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CaSR, a member of the G protein-coupled receptor family, regulates the synthesis of parathyroid hormone in response to changes in serum Ca\(^{2+}\) concentrations. The functions of CaSR in human vascular smooth muscle cells are largely unknown. Here we sought to study CaSR activation and the underlying molecular mechanisms in human aortic smooth muscle cells (HASMC). Extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{cyt}\)) dose-dependently increased free cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{cyt}\)) in HASMC, with a half-maximal response (EC\(_{50}\)) of 0.52 mM and a Hill coefficient of 5.50. CaSR was expressed in HASMC, and the [Ca\(^{2+}\)]\(_{cyt}\)-induced [Ca\(^{2+}\)]\(_{cyt}\) rise was abolished by dominant negative mutants of CaSR. The CaSR-mediated increase in [Ca\(^{2+}\)]\(_{cyt}\) was also significantly inhibited by pertussis toxin, the phospholipase C inhibitor U-73122, or the general protein kinase C (PKC) inhibitor Gö6976. Depletion of membrane cholesterol by pretreatment with methyl-b-cyclodextrin markedly decreased CaSR-induced increase in [Ca\(^{2+}\)]\(_{cyt}\). Blockade of TRPC channels with 2-aminooxydiphenyl borate, SKF-96365, or La\(^{3+}\) significantly inhibited [Ca\(^{2+}\)]\(_{cyt}\) entry, whereas activation of TRPC6 channels with flufenamic acid potentiated [Ca\(^{2+}\)]\(_{cyt}\) entry. Neither cyclopiazonic acid nor caffeine or ionomycin had any effect on [Ca\(^{2+}\)]\(_{cyt}\) in [Ca\(^{2+}\)]\(_{cyt}\)-free solutions. TRPC6 and PKCe mRNA and proteins were detected in HASMC, and [Ca\(^{2+}\)]\(_{cyt}\)-induced PKCe phosphorylation, which could be prevented by chelerythrine. Our data suggest that CaSR activation mediates [Ca\(^{2+}\)]\(_{cyt}\) entry, likely through TRPC6-encoded receptor-operated channels that are regulated by a PLC/PKC cascade. Our study therefore provides evidence not only for functional expression of CaSR, but also for a novel pathway whereby it regulates [Ca\(^{2+}\)]\(_{cyt}\) entry in HASMC.

Ca\(^{2+}\) signaling; TRPC6 channels; PKCe phosphorylation; vascular smooth muscle cells

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Address for reprint requests and other correspondence: H. Dong, Dept. of Medicine, UCSD, 9500 Gilman Drive, La Jolla, CA 92093 (e-mail: h2dong@ucsd.edu).

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its respective G protein(s) but fail to activate them. HASMC were transfected with the plasmid DNA constructs with Fugene 6. Six-well plates containing 80–90% confluent cells in each well were transfected with a total of 2 μg/well plasmid DNA. [Ca\textsuperscript{2+}]\textsubscript{cyt} measurement and membrane protein extraction for Western blots were performed 48 h after transfection.

[Ca\textsuperscript{2+}]\textsubscript{cyt} measurement. [Ca\textsuperscript{2+}]\textsubscript{cyt} levels in HASMC were measured by fura-2 fluorescence ratio digital imaging as described previously (36). Briefly, HASMC were trypsinized and replated onto 10-mm round glass coverslips that had been precoated with 1 mg/ml poly-D-lysine (Sigma) at a density such that 70–90% confluence was achieved within 24 h. They were then loaded with 5 μM fura-2 acetoxyethyl ester (AM) [dissolved in 0.01% Pluronic F-127 plus 0.1% DMSO] in normal physiological salt solution (PSS), described below, at room temperature for 50 min, then washed in PSS for at least 20 min. Thereafter, the coverslips with HASMC were mounted in a perfusion chamber on a Nikon microscope stage. Cells were initially superfused with PSS for 5 min and then switched to Ca\textsuperscript{2+}-free or Ca\textsuperscript{2+}\textsuperscript{-}containing solutions during drugs of interest. Fura-2 fluorescence ratio (510-nm light emission excited by 340- or 380-nm illumination), as well as background fluorescence, was collected at room temperature (22°C) with the use of a X40 Nikon UV-Flour objective and an intensified CCD camera (ICCD200). The fluorescence signals emitted from the cells were monitored continuously using a MetaFluor Imaging System (Universal Imaging, Downingtown, PA) and recorded for later analysis. PSS used in digital [Ca\textsuperscript{2+}] measurement contained the following (in mM): 140 Na\textsuperscript{+}, 5.0 K\textsuperscript{+}, 2 Ca\textsuperscript{2+}, 147 Cl\textsuperscript{-}, 10 HEPES, and 10 glucose, pH 7.4. For the Ca\textsuperscript{2+}-free PSS solution, Ca\textsuperscript{2+} was omitted, and 0.5 mM EGTA was added to prevent possible Ca\textsuperscript{2+} contamination. The osmolalities for all solutions were ~300 mosM/L.

**RT-PCR analysis of TRPC.** Briefly, total RNA (5 μg) from HASMC was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions and our previous publication (2). RNA were converted into cDNA with reverse transcriptase and PCR was performed using the following primers: TRPC1 (accession no. NM_003304) forward strand, 5'-CTGCTACCAACTGCTGGTGG-3' and reverse strand, 5'-AAGTCTTGTGCTGTGACCC-3'; TRPC3 (accession no. NM_003305) forward strand, 5'-CAATCCGAGAGAAGCAAGC-3' and reverse strand, 5'-GTACCAGCTGCCAAGAGC-3'; TRPC4 (accession no. NM_016179) forward strand, 5'-GCTGGAGGAGGAGAAGACTCGG-3' and reverse strand, 5'-GACCCTTGCGATGCTGAGA-3'; and TRPC6 (accession no. NM_004621) forward strand, 5'-GCAACATGACATCTGGAAT-3' and reverse strand, 5'-AACCTCCTGCTGGTTGAAAAG-3'. The conditions for PCR reactions for these genes were similar to those previously described (9). Primers for GAPDH were used as a control (forward strand, 5'-ACCACGTCTCAGATCCATCTG-3' and reverse strand, 5'-TCCACACCTTGGTGGTGA-3'). The samples were amplified in an automated thermal cycler (GeneAmp 2400; Applied Biosystems). DNA amplification conditions included an initial 3-min denaturation step at 94°C, 35 cycles of 30 s at 94°C, 30 s at 57°C, 40 s at 72°C, and a final elongation step of 10 min at 72°C. The products were separated on a 1.5% agarose gel, stained with ethidium bromide, and normalized. Cell lysates were then resuspended in 2× gel loading buffer, boiled for 5 min, and then separated by SDS-polyacrylamide gel electrophoresis (9% polyacrylamide). Resolved proteins were transferred overnight at 4°C onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). Membranes were then blocked with a 5% solution of skim milk for 30 min at room temperature, followed by further incubation with monoclonal antibodies specific for CaSR (1:1000, Abcam, Cambridge, MA), TRPC1, -4, and -6 (1:500, Alomone Lab, Israel), phospho-PKC\textalpha 1:1000 (Upstate Biotechnology, Lake Placid, NY), PKC\epsilon 1:1000 (BD Biosciences, San Jose, CA), or GAPDH 1:5000 (Ambion, Austin, TX). After being washed with PBS with 1% Tween (PBST), the rabbit anti-mouse secondary antibody was applied to the membrane. After washing with PBST, the membrane was treated with a chemiluminescent solution (Five photon Biochemicals, San Diego, CA) according to manufacturer’s instructions and exposed to X-ray film. Densitometric analysis of the blots was performed with the use of an Alphalager digital imaging system (Alpha Innotech, San Leandro, CA).

**Chemicals and solutions.** SKF-96365, U-73122, nifedipine, chelerythrine, and Go6976 were purchased from Sigma, 2-Aminoethoxydiphenyl borate (2-APB) was purchased from Tocris Bioscience (Ellisville, MO), Chiral enantiomers of BEL (S- and R-BEL) were from Cayman Chemical (Ann Arbor, MI). Fura 2-AM was from Molecular Probes (Eugene, OR). The other chemicals were obtained from Fisher Scientific (Santa Clara, CA).

**Statistical analysis.** Results are expressed as means ± SE. Differences between means were considered to be statistically significant at P < 0.05 using Student’s t-test or one-way ANOVA followed by Newman-Keuls post hoc test, as appropriate.

**RESULTS**

**CaSR activation induces Ca\textsuperscript{2+} signaling in HASMC.** Although activation of CaSR resulted in ERK1/2 phosphorylation in HASMC (6, 21), little is known about Ca\textsuperscript{2+} signaling downstream of CaSR activation in this cell type. Therefore, [Ca\textsuperscript{2+}]\textsubscript{cyt} levels in HASMC were measured, examining the dose-response of [Ca\textsuperscript{2+}]\textsubscript{cyt} changes in HASMC stimulated with various levels of [Ca\textsuperscript{2+}]\textsubscript{o}. Following a short exposure to Ca-free solutions (2–3 min), cells were superfused with different concentrations of [Ca\textsuperscript{2+}]\textsubscript{o} (0.2–2.0 mM) (Fig. 1). While [Ca\textsuperscript{2+}]\textsubscript{o} at 0.2 mM did not affect the basal [Ca\textsuperscript{2+}]\textsubscript{cyt}, obvious increases were seen at higher levels of [Ca\textsuperscript{2+}]\textsubscript{o} (Fig. 1A). Elevated [Ca\textsuperscript{2+}]\textsubscript{cyt} was dependent on [Ca\textsuperscript{2+}]\textsubscript{o} because it was promptly reversed when [Ca\textsuperscript{2+}]\textsubscript{o} was removed (Fig. 1A). CaSR activation appeared to be cooperative, with a steep dose-response relationship (Fig. 1B). The half-maximal response (EC\textsubscript{50}) was seen when [Ca\textsuperscript{2+}]\textsubscript{cyt} was 0.52 mM and the Hill coefficient for [Ca\textsuperscript{2+}]\textsubscript{o} stimulation was 5.5 (Fig. 1B).

Although [Ca\textsuperscript{2+}]\textsubscript{o} stimulates CaSR, it may enter healthy cells through store-operated Ca\textsuperscript{2+} entry pathway (26), or may even directly leak into unhealthy cells through nonspecific pathways. We therefore tested these possibilities using spermine, another direct activator of CaSR (6, 21). While spermine at 1 mM did not affect the basal [Ca\textsuperscript{2+}]\textsubscript{cyt} in normal PSS, obvious increases were seen at 3 mM (Fig. 1C). In addition, spermine (3 mM) did not affect the basal [Ca\textsuperscript{2+}]\textsubscript{cyt} in Ca\textsuperscript{2+}-free PSS, but significantly increased [Ca\textsuperscript{2+}]\textsubscript{cyt} in normal Ca\textsuperscript{2+} PSS (Fig. 1D). These results provide further evidence for a functional role of CaSR in the regulation of [Ca\textsuperscript{2+}]\textsubscript{cyt} in HASMC.

To demonstrate the presence of CaSR in HASMC, we performed Western blot analysis of lysates from HASMC and HT29, a human colonic cancer cell line used as a positive control. HASMC lysate produced a band of 160 kDa (Fig. 2A), consistent with the mature, full-size CaSR (21). To confirm the functional role of CaSR in the regula-
tion of \([\text{Ca}^{2+}]_{\text{cyt}}\) in HASMC, we used the CaSR mutants R185Q and R795W. The former showed a prominent dominant negative effect on the coexpressed wild-type receptor, while the latter could potentially still bind to its respective G protein(s) but fail to activate them (1). After transfection of dominant negative-CaSR constructs in HASMC for 48 h, expression of Flag-R185Q and Flag-R795W, but not empty (MT) vector, was seen (Fig. 2B). \([\text{Ca}^{2+}]_{\text{o}}\) (0.3–1.0 mM) increased \([\text{Ca}^{2+}]_{\text{cyt}}\) in MT-transfected HASMC (Fig. 2C) but not in R185Q- or R795W-transfected HASMC (Fig. 2, D and E). Together, these results confirm the functional role of CaSR in the regulation of \([\text{Ca}^{2+}]_{\text{cyt}}\) in HASMC.

CaSR functions as a GPCR. CaSR activation elicits intracellular signaling events, including those mediated by G proteins, PLC and PKC, that in turn modulate \([\text{Ca}^{2+}]_{\text{cyt}}\) (6, 15). Therefore, we sought to test the involvement of these pathways following CaSR activation in HASMC, using \([\text{Ca}^{2+}]_{\text{o}}\) at both the threshold concentration (0.5 mM) and maximal concentration (1.0 mM). We first examined whether CaSR activation stimulates G proteins. HASMC
were superfused with [Ca\(^{2+}\)]\(_o\) with or without pertussis toxin (PTX, 100 ng/ml) (Fig. 3A), a G\(_{i0}\) protein inhibitor (37). PTX significantly reduced the [Ca\(^{2+}\)]\(_o\)-induced Ca\(^{2+}\) signal (Fig. 3B). We next investigated the role of PLC using a selective PLC inhibitor (20), U-73122 (30 \(\mu\)M) (Fig. 3C). As shown in Fig. 3, A and B, the [Ca\(^{2+}\)]\(_o\)-induced Ca\(^{2+}\) signal was also significantly reduced by U-73122, at least at the lower level of [Ca\(^{2+}\)]\(_o\). Taken together, both G proteins and PLC are involved in CaSR-mediated Ca\(^{2+}\) signaling in HASMC, which is consistent with other reports (5, 11).

To test whether CaSR activation stimulates PKC, HASMC were superfused with [Ca\(^{2+}\)]\(_o\) in the absence (Fig. 4A) or the presence of chelerythrine (10 \(\mu\)M) (Fig. 4B), an inhibitor of the majority of PKC isoforms (39), or Gö6976 (10 \(\mu\)M) (Fig. 4C), a relatively selective inhibitor for conventional isoforms of PKC (39). As shown in Fig. 4D, the [Ca\(^{2+}\)]\(_o\)-induced Ca\(^{2+}\) signal in HASMC was prevented by chelerythrine, but not by Gö6976, suggesting that PKC isoforms other than conventional isoforms are involved in CaSR-mediated Ca\(^{2+}\) signaling in HASMC (35).

PKCe activation plays a role in CaSR-mediated Ca\(^{2+}\) signaling. More than 11 isoforms of PKC have been identified (23). To further our understanding of the expression of different isoforms of PKC in HASMC, we screened for expression of PKC isoforms using Western blot analysis of whole cell lysates. Like BxPc3 cells, pancreatic ductal cells that were used as positive controls (9), HASMC contained proteins immunoreactive with antibodies to PKC\(\alpha\), -\(\beta\), and -\(\gamma\) in the conventional family, PKCe, -\(\mu\), and -\(\delta\) in the novel family, and PKC\(\xi\) in the atypical family (Fig. 5).

Since PKCe was previously implicated in the regulation of Ca\(^{2+}\) signaling in rabbit aortic smooth muscle cells (23), we focused on PKCe phosphorylation in HASMC in response to CaSR activation. As shown in Fig. 6, PMA (1 \(\mu\)M)-induced serine phosphorylation of PKCe in HASMC was reversed by chelerythrine (10 \(\mu\)M). Similarly, [Ca\(^{2+}\)]\(_o\)-induced (0.5 mM) PKCe serine phosphorylation was also prevented by chelerythrine (10 \(\mu\)M) (Fig. 6), suggesting that CaSR activation results in PKCe phosphorylation in HASMC (35).

Source of Ca\(^{2+}\) mobilized by CaSR activation. Since GPCR activation may mobilize different Ca\(^{2+}\) sources in different cell types, we investigated whether CaSR activation induces intra-
Data further support that TRPC-encoded NSCC may contribute to CaSR-mediated Ca\(^{2+}\) entry in HASMC. Our RT-PCR data revealed transcripts for TRPC1, -3, -4, and -6 in HASMC (Fig. 9E), and Western blotting identified protein expression of TRPC1 and -6 (Fig. 9F). These data further support that TRPC-encoded NSCC may contribute to CaSR-mediated Ca\(^{2+}\) entry in HASMC.

Caveolae are involved in CaSR-mediated Ca\(^{2+}\) signaling. Spatially organized complexes of signaling molecules, such as caveolae, were found recently in microdomains of the plasma membrane in VSMC (13). These are important microstructures within the plasma membrane that contain signaling molecules such as caveolins, GPCR, and TRPC, all of which are involved in multiple cellular processes including Ca\(^{2+}\) homeostasis and signal transduction (27). Since caveolae are enriched in cholesterol, we tested whether caveolae were involved in CaSR-mediated Ca\(^{2+}\) entry by pretreating HASMC with MβCD (5 mM) for 1 h to deplete membrane cholesterol (27). Indeed, MβCD decreased CaSR-mediated Ca\(^{2+}\) entry in HASMC (Fig. 10), suggesting that CaSR in caveolae plays a role in [Ca\(^{2+}\)]\(_{\text{c}}\) mediated [Ca\(^{2+}\)]\(_{\text{cyt}}\) signaling in HASMC.

**DISCUSSION**

Here we found that 1) [Ca\(^{2+}\)]\(_{\text{c}}\) induces Ca\(^{2+}\) entry into HASMC likely via activation of CaSR; 2) CaSR activation mediates Ca\(^{2+}\) entry through receptor-operated channels (ROC); and 3) the PLC/PKC\(\varepsilon\) pathway is involved in the CaSR-mediated [Ca\(^{2+}\)]\(_{\text{cyt}}\) rise in HASMC. Our study demonstrates a role for CaSR in regulating [Ca\(^{2+}\)]\(_{\text{cyt}}\) entry via ROC, and advances our understanding of the molecular mechanisms underlying CaSR-mediated [Ca\(^{2+}\)]\(_{\text{cyt}}\) rise in human VSMC.

Although CaSR was cloned from and characterized in bovine parathyroid cells in 1993 (5), it was recently shown also...
to be expressed in human VSMC and to function via the ERK signaling pathway (21). However, the proximal mechanism(s) by which CaSR transduces calcium-dependent signaling in VSMC are largely unknown. CaSR has physiological roles in the cardiovascular system by modulating myogenic tone in small arteries (24), arterial blood pressure (12, 34), and VSMC proliferation (32), through Ca\(^{2+}\) and/or ERK1/2 pathways (21, 32). In the present study, we found that both [Ca\(^{2+}\)]\(_{o}\) and spermine, two direct CaSR agonists with different molecular structures (33, 34), raised [Ca\(^{2+}\)]\(_{cyt}\) through CaSR activation in HASMC. We have also demonstrated that 1) CaSR proteins are expressed in HASMC, 2) [Ca\(^{2+}\)]\(_{o}\)-induced increase in [Ca\(^{2+}\)]\(_{cyt}\) was abolished in HASMC transfected with two CaSR mutant constructs that are mutated at different locations of the wild-type CaSR, and 3) [Ca\(^{2+}\)]\(_{o}\)-induced increase in [Ca\(^{2+}\)]\(_{cyt}\) was attenuated by inhibition of G\(_{i/o}\) protein, PLC or PKC. Together, our results confirm the functional role of CaSR in the regulation of [Ca\(^{2+}\)]\(_{cyt}\) in HASMC. Similar to other GPCR, CaSR activation stimulates PLC, which cleaves phosphatidylinositol 4,5-bisphosphate into 1,2-diacylglycerol (DAG) and IP\(_3\) (6, 8, 33, 34). While DAG activates PKC\(_{\varepsilon}\), and Ca\(^{2+}\) entry via receptor-operated calcium channels (ROC) (19, 29, 35, 38), IP\(_3\) causes intracellular Ca\(^{2+}\) release from S/ER stores and depletion of intracellular Ca\(^{2+}\) stores leads to Ca\(^{2+}\) entry via store-operated calcium channels (SOC) in different cell types (26, 30).

Both [Ca\(^{2+}\)]\(_{o}\) and [Ca\(^{2+}\)]\(_{cyt}\) play essential roles in numerous physiological process (10). [Ca\(^{2+}\)]\(_{o}\) is controlled by a complex mechanism with GER, exchangers, and Ca\(^{2+}\) sequestration into the ER (6, 10). Our data suggest that [Ca\(^{2+}\)]\(_{o}\) specifically activates CaSR to induce Ca\(^{2+}\) entry via a specific PLC/PKC\(_{\varepsilon}\) pathway, rather than nonspecifically causing [Ca\(^{2+}\)]\(_{o}\) to leak into HASMC because 1) either [Ca\(^{2+}\)]\(_{o}\), or spermine dose-dependently raised [Ca\(^{2+}\)]\(_{cyt}\) in HASMC, which was inhibited by a G protein inhibitor or a PLC inhibitor; 2) a PKC inhibitor reduced CaSR-mediated [Ca\(^{2+}\)]\(_{cyt}\); 3) [Ca\(^{2+}\)]\(_{o}\) induced PKC phosphorylation in HASMC, which was prevented by chelerythrine; 4) ROC blockers reduced, but a TRPC activator potentiated, CaSR-mediated [Ca\(^{2+}\)]\(_{cyt}\); and 5) mRNA and protein for TRPC were identified in HASMC.

Our data show a Hill coefficient for CaSR activation by [Ca\(^{2+}\)]\(_{o}\) of 5.50 in HASMC, which is close to that of 4.73 in HEK-293 cells transfected with the human CaSR as reported by Quinn et al. (31), suggesting that multiple CaSR binding sites for [Ca\(^{2+}\)]\(_{o}\) in HASMC (1, 31). However, the EC\(_{50}\) for [Ca\(^{2+}\)]\(_{o}\) to raise [Ca\(^{2+}\)]\(_{cyt}\) in HASMC (0.52 mM) is about fourfold lower than seen in CaSR-transfected human embryonic kidney (HEK)-293 cells (~2.0 mM) (31), suggesting that CaSR endogenously expressed in native HASMC might be more sensitive to [Ca\(^{2+}\)]\(_{o}\) than that heterologously overexpressed in HEK-293 cells. Our findings in HASMC also support an important role for the PLC/PKC\(_{\varepsilon}\) cascade during CaSR activation in different types of VSMC (34, 35, 38).

CaSR-mediated [Ca\(^{2+}\)]\(_{cyt}\) entry has previously been studied mostly in vascular endothelial cells (3, 42), but not in vascular smooth muscle cells. Moreover, little was known about [Ca\(^{2+}\)]\(_{o}\) entry mechanisms upon activation of CaSR. Although CaSR activation was found to enhance the activity of permeable NSCC in HEK-293 cells stably transfected with CaSR (40), the precise mechanisms underlying CaSR-mediated [Ca\(^{2+}\)]\(_{cyt}\) homeostasis in VSMC were largely unknown. After excluding the involvement of VGCC, we focused on SOC and ROC, both of which exist in VSMC. The major differences between SOC and ROC are 1) SOC is known to be functionally activated by depletion of intracellular Ca\(^{2+}\) stores, but ROC is...
activated via a PLC/PKCε cascade (19, 29, 35, 38); and 2) TRPC1 is a molecular candidate for SOC in VSMC, compared with TRPC6 for ROC (16, 19, 29, 38). SOC is unlikely involved in the [Ca^{2+}]_{o} induced [Ca^{2+}]_{cyt} increase we observed in the present study because 1) the [Ca^{2+}]_{cyt} signal was not only induced by [Ca^{2+}]_{o} in Ca^{2+}-free solutions, but also induced by spermine in Ca^{2+}-containing solutions; 2) [Ca^{2+}]_{o} induced [Ca^{2+}]_{cyt} increase was abolished in HASMC transfected with two different CaSR dominant negative mutants; and 3) [Ca^{2+}]_{o} re-addition did not induce a significant [Ca^{2+}]_{cyt} signal in HASMC after application of CPA plus caffeine. Together, our data suggest that TRPC1- or Orai1-mediated SOC does not play a major role in [Ca^{2+}]_{o} induced [Ca^{2+}]_{cyt} increase in HASMC although Orai1 is another molecular candidate of SOC (2).

We therefore tested whether ROC participate in [Ca^{2+}]_{o} entry because they are NSCC that have been characterized in VSMC. Here, we showed that ROC blockers with different chemical structures significantly inhibited CaSR-mediated Ca^{2+} entry in HASMC, suggesting an involvement of ROC in this process. Moreover, we also found that TRPC6 is expressed in HASMC and that CaSR-mediated [Ca^{2+}]_{o} entry into HASMC was significantly enhanced by flufenamic acid, an activator of TRPC6 channels (16). Since TRPC6 satisfies many of the functional criteria of ROC and thus has been considered a ROC candidate in VSMC (16, 19, 29, 38), our findings support that TRPC6-encoded ROC may play a key role in CaSR-mediated [Ca^{2+}]_{o} entry into HASMC.

In summary, we demonstrate that [Ca^{2+}]_{o} activates CaSR in HASMC, and mediates [Ca^{2+}]_{cyt} entry into the cell interior likely through TRPC6-encoded ROC. We have also provided evidence that CaSR-mediated Ca^{2+} entry is regulated by the PLC/PKCε signaling cascade. Further studies are needed to assess the physiological role of CaSR-mediated [Ca^{2+}]_{cyt} in modulating cardiovascular function, and eventually to discover a novel therapeutic target for the treatment of cardiovascular diseases.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).
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