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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Chow JY, Estrema C, Orneles T, Dong X, Barrett KE, Dong H. Calcium-sensing receptor modulates extracellular Ca\(^{2+}\) entry via TRPC-encoded receptor-operated channels in human aortic smooth muscle cells. Am J Physiol Cell Physiol 301: C461–C468, 2011. First published May 11, 2011; doi:10.1152/ajpcell.00389.2010.—Ca-sensing receptor (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 G66976. Depletion of membrane cholesterol by pretreatment with methyl- \(\beta\)-cyclodextrin markedly decreased CaSR-induced increase in [Ca\(^{2+}\)]\(_{cyt}\). Blockade of TRPC channels with 2-aminoethoxydiphenyl 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\(^{2+}\)]\(_{cyt}\), in HASMC.

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

* J. Y. C. Chow, C. Estrema, and T. Orneles contributed equally to this work.

Address for reprint requests and other correspondence: H. Dong, Dept. of Medicine, UCSD, 9500 Gilman Drive, La Jolla, CA 92093 (e-mail: hdong@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^{2+}]_{cyt} measurement and membrane protein extraction for Western blots were performed 48 h after transfection.

[Ca^{2+}]_{cyt} measurement. [Ca^{2+}]_{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 acetoxymethyl 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^{2+}-free or Ca^{2+}-containing solutions (0.1% DMSO in normal physiological salt solution (PSS), described above) at 22°C with the use of a M fura-2 Fluor Imaging System (Universal Imaging, Downingtown, PA) and recorded for later analysis. PSS used in digital Ca^{2+} measurement and membrane protein extraction for Western blots was achieved within 24 h. They were then loaded with 5 μM fura-2 acetoxymethyl 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^{2+}-free or Ca^{2+}-containing solutions (0.1% DMSO in normal physiological salt solution (PSS), described above) at 22°C with the use of a M fura-2 Fluor Imaging System (Universal Imaging, Downingtown, PA) and recorded for later analysis. PSS used in digital Ca^{2+} measurement contained the following (in mM): 140 Na^+, 5.0 K^+, 2 Ca^{2+}, 147 Cl^-, 10 HEPES, and 10 glucose, pH 7.4. For the Ca^{2+}-free PSS solution, Ca^{2+} was omitted, and 0.5 mM EGTA was added to prevent possible Ca^{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_003305) forward strand, 5′-TGCTTACCAAACTGCTGTG-3′ and reverse strand, 5′-AACTGTITTTCGCTTTTGGACC-3′; TRPC3 (accession no. NM_003505) forward strand, 5′-CAATCCGAGAGAAGC-3′ and reverse strand, 5′-CAAACTGCTGGTG-3′; TRPC4 (accession no. NM_016179) forward strand, 5′-GCTGAGGAGAAGACCATGCG-3′ and reverse strand, 5′-GACCTGTCGATGTGCTGAGA-3′; and TRPC6 (accession no. NM_004621) forward strand, 5′-GCAATGAGACCATGCTGAAATG-3′ and reverse strand, 5′-AACCCTTTCGCTCCCTGACAAA-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′-ACCACAGTCCATGACATCC-3′ and reverse strand, 5′-TCCACCACCTGTGCTGCTA-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 then photographed under UV light.

Western blot analysis. HASMC cells were washed three times with ice-cold PBS. Cells were then lysed with total lysis buffer [150 mM NaCl, 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 0.5% Triton X-100, and 1 mM sodium orthovanadate] containing protease inhibitors (1 μg/ml leupeptin and 100 μg/ml PMSF) and incubated at 4°C for 30 min with constant shaking. The cells were then scraped into microcentrifuge tubes and centrifuged at 12,000 g for 15 min to remove insoluble material. The protein content in each sample was determined 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 mem-

brane (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 (1:1,000, Abcam, Cambridge, MA), TRPC1 -4, and -6 (1:500, Alomone Lab, Israel), phospho-PKCα 1:1,000 (Upstate Biotechnology, Lake Placid, NY), PKCε 1:1,000 (BD Biosciences, San Jose, CA), or GAPDH 1:5,000 (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 (Fivephoton 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, nifedipine, chelerythrine, and G60976 were purchased from Sigma. 2-Amino-6-phenyl diphenyl 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^{2+} signaling in HASMC. Although activation of CaSR resulted in ERK1/2 phosphorylation in HASMC (6, 21), little is known about Ca^{2+} signaling downstream of CaSR activation in this cell type. Therefore, [Ca^{2+}]_{cyt} levels in HASMC were measured, examining the dose-response of [Ca^{2+}]_{cyt} changes in HASMC stimulated with various levels of [Ca^{2+}]_{o}. Following a short exposure to Ca-free solutions (2–3 min), cells were superfused with different concentrations of [Ca^{2+}]_{o} (0.2–2.0 mM) (Fig. 1). While [Ca^{2+}]_{o} at 0.2 mM did not affect the basal [Ca^{2+}]_{cyt}, obvious increases were seen at higher levels of [Ca^{2+}]_{o} (Fig. 1A). Elevated [Ca^{2+}]_{cyt} was dependent on [Ca^{2+}]_{o} because it was promptly reversed when [Ca^{2+}]_{o} was removed (Fig. 1A). CaSR activation appeared to be cooperative, with a steep dose-response relationship (Fig. 1B). The half-maximal response (EC_{50}) was seen when [Ca^{2+}]_{o} was 0.52 mM and the Hill coefficient for [Ca^{2+}]_{o} stimulation was 5.5 (Fig. 1B).

Although [Ca^{2+}]_{o} stimulates CaSR, it may enter healthy cells through store-operated Ca^{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^{2+}]_{cyt} in normal PSS, obvious increases were seen at 3 mM (Fig. 1C). In addition, spermine (3 mM) did not affect the basal [Ca^{2+}]_{cyt} in Ca^{2+}-free PSS, but significantly increased [Ca^{2+}]_{cyt} in normal Ca^{2+} PSS (Fig. 1D). These results provide further evidence for a functional role of CaSR in the regulation of [Ca^{2+}]_{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. 2D 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

![Fig. 1. Extracellular Ca\(^{2+}\) (\([\text{Ca}^{2+}]_{\text{o}}\)) and spermine stimulate an increase in free cytosolic Ca\(^{2+}\) (\([\text{Ca}^{2+}]_{\text{cyt}}\)) in human aortic smooth muscle cells (HASMC). After HASMC were loaded with Fura-2 AM, \([\text{Ca}^{2+}]_{\text{cyt}}\) in the cells was measured by a digital Ca\(^{2+}\) imaging system. A: time course of \([\text{Ca}^{2+}]_{\text{cyt}}\) changes in HASMC induced by different concentrations of \([\text{Ca}^{2+}]_{\text{o}}\). B: summary data showing a dose-dependent relationship for the effect of \([\text{Ca}^{2+}]_{\text{o}}\) stimulation or peak \([\text{Ca}^{2+}]_{\text{cyt}}\) responses. C: time course of \([\text{Ca}^{2+}]_{\text{cyt}}\) changes in HASMC induced by different concentrations of spermine in normal Ca\(^{2+}\)-physiological salt solution (PSS) D: time course of \([\text{Ca}^{2+}]_{\text{cyt}}\) changes in HASMC induced by same concentration of spermine (3 mM) in Ca\(^{2+}\)-free or normal Ca\(^{2+}\) PSS. Data are shown as means ± SE of 30–40 cells.

![Fig. 2. Protein expression and function of Ca-sensing receptor (CaSR) wild type and mutants in HASMC. A: HT29 (a human colonic cancer cell line used as a positive control) and HASMC were lysed and then Western blot analysis was performed to detect protein expression of CaSR wild type. B–E: after transfection of Flag-tagged empty (MT) vector, R185Q, or R795W construct in HASMC for 48 h, Western blot analysis was performed to detect protein expression of CaSR mutants (B), and digital Ca\(^{2+}\) imaging was performed to determine \([\text{Ca}^{2+}]_{\text{o}}\)-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) signaling in HASMC transfected with MT (C), R185Q (D), or R795W construct (E). Data are shown as means ± SE of 30–40 cells.]

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were superfused with \([\text{Ca}^{2+}]_o\) with or without pertussis toxin (PTX, 100 ng/ml) (Fig. 3A), a Gi/0 protein inhibitor (37). PTX significantly reduced the \([\text{Ca}^{2+}]_o\)-induced \([\text{Ca}^{2+}]_o\) signal (Fig. 3B). We next investigated the role of PLC using a selective PLC inhibitor (20), U-73122 (30 μM) (Fig. 3C). As shown in Fig. 3, C and D, the \([\text{Ca}^{2+}]_o\)-induced \([\text{Ca}^{2+}]_o\) signal was also significantly reduced by U-73122, at least at the lower level of \([\text{Ca}^{2+}]_o\). Taken together, both G proteins and PLC are involved in CaSR-mediated \([\text{Ca}^{2+}]_o\) signaling in HASMC, which is consistent with other reports (5, 11).

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

PKCe activation plays a role in CaSR-mediated \([\text{Ca}^{2+}]_o\) 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 PKCα, -β, and -γ in the conventional family, PKCe, -μ, and -δ in the novel family, and PKCc in the atypical family (Fig. 5).

Since PKCe was previously implicated in the regulation of \([\text{Ca}^{2+}]_o\) 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 μM)-induced serine phosphorylation of PKCe in HASMC was reversed by chelerythrine (10 μM). Similarly, \([\text{Ca}^{2+}]_o\)-induced (0.5 mM) PKCe serine phosphorylation was also prevented by chelerythrine (10 μM) (Fig. 6), suggesting that CaSR activation results in PKCe phosphorylation in HASMC (35).

**Source of \([\text{Ca}^{2+}]_o\) mobilized by CaSR activation.** Since GPCR activation may mobilize different \([\text{Ca}^{2+}]_o\) sources in different cell types, we investigated whether CaSR activation induces intra-

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**Fig. 3.** Inhibition of G proteins or phospholipase C (PLC) attenuates \([\text{Ca}^{2+}]_o\)-induced increase in \([\text{Ca}^{2+}]_o\) in HASMC. **A**: time course of \([\text{Ca}^{2+}]_o\) changes in HASMC induced by different concentrations of \([\text{Ca}^{2+}]_o\), in control cells. **B** and **C**: time course of \([\text{Ca}^{2+}]_o\) changes in HASMC induced by different concentrations of \([\text{Ca}^{2+}]_o\), in the presence of pertussis toxin (PTX, 100 ng/ml) or U-73122 (30 μM). **D**: summary data showing inhibition of \([\text{Ca}^{2+}]_o\)-induced increase in \([\text{Ca}^{2+}]_o\) by PTX or U-73122. Data are shown as means ± SE of 40–50 cells for each group. **P < 0.01, ***P < 0.001 vs. control in the absence of inhibitors.

**Fig. 4.** \([\text{Ca}^{2+}]_o\)-induced increase in \([\text{Ca}^{2+}]_o\) in HASMC is inhibited by chelerythrine (CHE) but not by Gö6976. **A**: time course of \([\text{Ca}^{2+}]_o\) changes in HASMC induced by different concentrations of \([\text{Ca}^{2+}]_o\), in control cells. **B** and **C**: time course of \([\text{Ca}^{2+}]_o\) changes in HASMC induced by different concentrations of \([\text{Ca}^{2+}]_o\), in the presence of chelerythrine (10 μM) or Gö6976 (10 μM). **D**: summary data showing effect of chelerythrine or Gö6976 on \([\text{Ca}^{2+}]_o\)-induced increase in \([\text{Ca}^{2+}]_o\). Data are shown as means ± SE of 40–50 cells for each group. ***P < 0.001 vs. control in the absence of inhibitors.

**Fig. 5.** Expression profiles of PKC isoforms in HASMC. HASMC and BxPc3 (a pancreatic ductal cell line used as a positive control) were lysed with lysis buffer and then Western blot analysis was performed. These data are representative of 3 experiments with similar results.

**Table:** PKC isoform expression in HASMC and BxPc3.
cellular Ca\(^{2+}\) release, [Ca\(^{2+}\)]\(_{o}\) entry, or both in HASMC. To test whether intracellular Ca\(^{2+}\) release from the sarcoplasmic/ endoplasmic reticulum (S/ER) plays a role, we used cyclopiazonic acid (CPA, 30 μM) to deplete intracellular Ca\(^{2+}\) stores (41), or ionomycin (10 μM) and caffeine (10 mM) to release total intracellular Ca\(^{2+}\) stores (18) in Ca\(^{2+}\)-free solutions. None of these reagents induced any change in the basal [Ca\(^{2+}\)]\(_{cyt}\) (Fig. 7, B and C), but 1 mM [Ca\(^{2+}\)]\(_{o}\) induced significant [Ca\(^{2+}\)]\(_{cyt}\) signaling in HASMC (Fig. 7, A, C, and D). Interestingly, re-addition of [Ca\(^{2+}\)]\(_{o}\) after application of CPA plus caffeine did not induce a significant [Ca\(^{2+}\)]\(_{cyt}\) increase in HASMC (Fig. 7, B vs. A). Together, these results suggest that neither intracellular Ca\(^{2+}\) release mechanisms nor store-operated Ca\(^{2+}\) entry plays a significant role in regulating [Ca\(^{2+}\)]\(_{cyt}\) in HASMC.

Although voltage-gated Ca\(^{2+}\) channels (VGCC) in plasma membrane play an important role in regulating Ca\(^{2+}\) entry into VSMC, nifedipine (10 μM), which blocks VGCC, did not significantly affect [Ca\(^{2+}\)]\(_{o}\)-induced [Ca\(^{2+}\)]\(_{cyt}\) signaling in HASMC (data not shown), suggesting a minor role for VGCC in CaSR-mediated [Ca\(^{2+}\)]\(_{cyt}\) signaling.

**TRPC-encoded nonselective cation channels are involved in CaSR-mediated Ca\(^{2+}\) entry.** Since neither intracellular Ca\(^{2+}\) release from the S/ER nor VGCC appeared to be involved in CaSR-mediated [Ca\(^{2+}\)]\(_{cyt}\) signaling in HASMC, we further investigated whether other Ca\(^{2+}\) pathways, such as plasma membrane nonselective cation channels (NSCC), are involved. All of the TRPC-encoded NSCC blockers, including La\(^{3+}\) (10 μM), SKF-96365 (50 μM), or 2-APB (100 μM) (16), significantly inhibited CaSR-mediated [Ca\(^{2+}\)]\(_{cyt}\) signaling in HASMC (Fig. 8), while flufenamic acid (100 μM), an activator of TRPC6 channels (16), did not alter basal [Ca\(^{2+}\)]\(_{cyt}\) in Ca\(^{2+}\)-free solutions, it significantly elevated [Ca\(^{2+}\)]\(_{cyt}\) in a [Ca\(^{2+}\)]\(_{o}\)-dependent manner, suggesting the involvement of TRPC6-encoded NSCC in CaSR-mediated Ca\(^{2+}\) entry into HASMC (Fig. 9).

We then examined the expression of TRPC channels 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 CaS in caveolae plays a role in [Ca\(^{2+}\)]\(_{o}\)-mediated [Ca\(^{2+}\)]\(_{cyt}\) signaling in HASMC.

**DISCUSSION**

Here we found that 1) [Ca\(^{2+}\)]\(_{o}\) 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 pathway is involved in the CaSR-mediated [Ca\(^{2+}\)]\(_{cyt}\) rise in HASMC. Our study demonstrates a role for CaSR in regulating [Ca\(^{2+}\)]\(_{o}\) entry via ROC, and advances our understanding of the molecular mechanisms underlying CaSR-mediated [Ca\(^{2+}\)]\(_{cyt}\) 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 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\(^{2+}\)\(_{\text{cyt}}\) entry; 3) [Ca\(^{2+}\)\(_{\text{cyt}}\) entry; 4) ROC blockers reduced, but a TRPC activator potentiated, CaSR-mediated [Ca\(^{2+}\)\(_{\text{cyt}}\) entry; and 5) mRNA and protein for TRPC were identified in HASMC.

Our data show a Hill coefficient for CaSR activation by [Ca\(^{2+}\)\(_{\text{cyt}}\)], 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\(^{2+}\)\(_{\text{cyt}}\)], in HASMC (1, 31). However, the EC\(_{50}\) for [Ca\(^{2+}\)\(_{\text{cyt}}\) entry; 3) [Ca\(^{2+}\)\(_{\text{cyt}}\) entry; and 5) mRNA and protein for TRPC were identified in HASMC.

CaSR-mediated [Ca\(^{2+}\)\(_{\text{cyt}}\) entry; has previously been studied mostly in vascular endothelial cells (3, 42), but not in vascular smooth muscle cells. Moreover, little was known about CaSR activation 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 (31), the precise mechanisms underlying CaSR-mediated [Ca\(^{2+}\)\(_{\text{cyt}}\) entry; and 5) mRNA and protein for TRPC were identified in HASMC.

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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 [Ca2+]o-induced [Ca2+]cyt increase we observed in the present study because C) the [Ca2+]cyt signal was not only induced by [Ca2+]o, but also induced by spermine in Ca2+-containing solutions; 2) [Ca2+]o-induced [Ca2+]cyt increase was abolished in HASMC transfected with two different CaSR dominant negative mutants; and 3) [Ca2+]o re-addition did not induce a significant [Ca2+]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 [Ca2+]o-induced [Ca2+]cyt increase in HASMC although Orai1 is another molecular candidate of SOC (2).

We therefore tested whether ROC participate in [Ca2+]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 [Ca2+]o 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 [Ca2+]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 [Ca2+]o entry into HASMC.

In summary, we demonstrate that [Ca2+]o activates CaSR in HASMC, and mediates [Ca2+]o entry into the cell interior likely through TRPC6-encoded ROC. We have also provided evidence that CaSR-mediated [Ca2+]o entry is regulated by the PLC/PKCε signaling cascade. Further studies are needed to assess the physiological role of CaSR-mediated [Ca2+]o 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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