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Cardiac Microvascular Endothelial Cells Express a Functional Ca^{2+}-Sensing Receptor

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Abstract
The mechanism whereby extracellular Ca^{2+} exerts the endothelium-dependent control of vascular tone is still unclear. In this study, we assessed whether cardiac microvascular endothelial cells (CMEC) express a functional extracellular Ca^{2+}-sensing receptor (CaSR) using a variety of techniques. CaSR mRNA was detected using RT-PCR, and CaSR protein was identified by immunocytochemical analysis. In order to assess the functionality of the receptor, CMEC were loaded with the Ca^{2+}-sensitive fluorochrome, Fura-2/AM. A number of CaSR agonists, such as spermine, Gd^{3+}, La^{3+} and neomycin, elicited a heterogeneous intracellular Ca^{2+} signal, which was abolished by disruption of inositol 1,4,5-trisphosphate ($InsP_3$) signaling and by depletion of intracellular stores with cyclopiazonic acid. The inhibition of the Na^+/Ca^{2+} exchanger upon substitution of extracellular Na^+ unmasked the Ca^{2+} signal triggered by an increase in extracellular Ca^{2+} levels. Finally, aromatic amino acids, which function as allosteric activators of CaSR, potentiated the Ca^{2+} response to the CaSR agonist La^{3+}. These data provide evidence that CMEC express CaSR, which is able to respond to physiological agonists by mobilizing Ca^{2+} from intracellular $InsP_3$-sensitive stores.

Introduction

Extracellular Ca^{2+} has long been known to regulate vascular function. Small increases in extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$), for instance, cause the synthesis of nitric oxide (NO) from vascular endothelium, thus resulting in blood vessel dilatation [1]. The mechanism whereby $[Ca^{2+}]_o$ modulates endothelial activity is still unclear. Extracellular Ca^{2+} affects several cell types through a G-protein-coupled Ca^{2+}-sensing receptor (CaSR), which has originally been cloned in parathyroid cells and is ac-

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tivated by $[Ca^{2+}]_o$ in the millimolar range [2]. The human CaSR is a 1,078-amino-acid cell surface protein, consisting of a seven-membrane-spanning region and an extremely large extracellular venus flytrap domain, which contains the CaSR ligand-binding site [3]. CaSR exhibits a promiscuous pharmacology and, in addition to Ca^{2+}, is also sensitive to: (1) a variety of di- and trivalent cations including, in rank order of potency, $La^{3+} = Gd^{3+} > Ca^{2+} > Mg^{2+}$, and (2) organic polycations, e.g. polyamines (spermine, spermidine and putrescine) and aminoglycoside antibiotics (neomycin, streptomycin and gentamicin) [2]. Furthermore, L-amino acids endowed with aromatic side chains (L-tryptophan and L-phenylalanine) function as allosteric activators of CaSR, potentiating the CaSR response to sub-threshold doses of CaSR agonists [4]. Exposure to CaSR agonists elevates intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) due to recruitment of the heterotrimeric $G_{q/11}$ protein, which results in stimulation of phospholipase C (PLC) activity and subsequent generation of inositol 1,4,5-trisphosphate ($InsP_3$) [3]. The pattern of the intracellular Ca^{2+} signal occurring upon CaSR activation is extremely variable, ranging from a transient Ca^{2+} spike to repetitive Ca^{2+} oscillations [5, 6].

The best documented role of CaSR is to coordinate hormonally regulated systemic Ca^{2+} homeostasis, being mainly expressed on the cell surface of parathyroid, thyroid and kidney tubule cells [3]. Lower levels of the CaSR have been found in cells not known to play a role in Ca^{2+} homeostasis, e.g. pancreatic cells, cardiomyocytes, keratinocytes and fibroblasts [3]. In these cells, CaSR-mediated $[Ca^{2+}]_o$ sensing may contribute to the regulation of local ionic microenvironment or provide information necessary for non-homeostatic purposes [7]. A recent study has shown that CaSR mRNA and protein are also expressed in rat mesenteric and porcine coronary artery endothelial cells [8]. Stimulation of the receptor with the specific positive allosteric modulator calindol and with high $[Ca^{2+}]_o$ induced endothelium-dependent hyperpolarization of vascular smooth muscle cells [8]. Moreover, the CaSR has been reported in human aortic endothelial cells (HAEC) [9], although it does not respond to an increase in $[Ca^{2+}]_o$ in this preparation. Cardiac microvascular endothelial cells (CMEC) line the luminal side of coronary microvasculature and are in intimate contact with subjacent cardiomyocytes, which are not farther than 2–3 μm from a CMEC [10]. An elevation in $[Ca^{2+}]_i$ results in endothelial cell activation [11], causing the synthesis of NO and prostacyclin, which exert both vasoactive and cardioactive effects [10, 11]. In this study, we provide data confirming the presence of CaSR mRNA and protein in rat CMEC. Subsequently, we employed Fura-2 acetoxymethyl ester (Fura-2/AM)-loaded CMEC to assess the functionality of the receptor and to examine its sensitivity to a number of known CaSR agonists. Finally, we suggest that the interplay between CaSR and Na^+/Ca^{2+} exchanger (NCX) may mask the intracellular Ca^{2+} elevation induced by high $[Ca^{2+}]_o$ in endothelial cells.

Methods

Isolation, Culture and Identification of CMEC

Briefly, CMEC were enzymatically dissociated (0.02% collagenase type II for 20 min at 37°C and 0.027% trypsin for 20 min at 37°C in a shaking bath) from the left-ventricular muscle of 2- to 3-month-old Wistar rats *(Rattus norvegicus)* anesthetized with ether, according to a procedure described previously [12, 13] and very similar to the method originally developed by Nishida et al. [14]. The cells reached confluence after 7–9 days and always displayed a uniform 'cobblestone' morphology. Primary cultures (3–5 days) of CMEC were positively identified by three endothelial cell markers: (i) factor VIII-related antigen, (ii) lectin I *Griffonia (Bandeiraea) simplicifolia* and (iii) uptake of acetylated low-density lipoprotein. More than 90% of the cells were positive to the tests, as estimated by cell counting in microphotographs of randomly chosen visual fields.

RNA Isolation and Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR)

Total RNA was extracted from sub-confluent CMEC (2 flasks of 25 cm^2) and from rat heart homogenate using QIAzol Lysis reagent according to the manufacturer's instructions (Qiagen, Milan, Italy). cDNA was synthesized from 1 μg RNA using random hexamers and M-MLV RT (Invitrogen, Carlsbad, Calif., USA). PCR (30 s at 96°C, 30 s at 56°C and 30 s at 72°C for 38 cycles) was performed as previously described by Laforenza et al. [15] on 2.5 μl of cDNA using sense and antisense gene-specific, intron-spanning primers for rat CaSR (sense, 5′-CTATCATCAACTGGCACCTC-3′, and antisense, 5′-CCACGCCAGAAACTCAATCT-3′; GenBank/EMBL accession No. U20289). The expected RT-PCR product size was 356 bp. Reverse transcription was always performed in the presence or absence of the RT enzyme. RT-PCR was also performed for the housekeeping gene β-actin, as a quality control for the mRNA. PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide and acquired with the Image Master VDS (Amersham Biosciences Europe, Milan, Italy). The molecular weights of the PCR products were compared to the DNA molecular weight marker VIII (Roche Molecular Biochemicals, Milan, Italy).

Immunocytochemistry

Confluent CMEC grown on coverslips were fixed with 70% ethanol at –20°C for 30 min. Subsequently, cells were washed with PBS and treated with 3% hydrogen peroxide for 10 min at room temperature to block the endogenous peroxidases. After washing for 5 min with PBS, cells were blocked with 3% BSA in PBS for 30 min at room temperature. CMEC were incubated for 2 h at room temperature with a previously characterized [16–20] anti-CaSR

mouse monoclonal antibody (5C10, ADD clone, ABR Bioreagents, Golden, Colo., USA) diluted 1:500 in PBS. After three 5-min washes with PBS, cells were first incubated for 15 min at room temperature with biotinylated secondary antibody and then, after three washes with PBS, incubated for 15 min at room temperature with HRP-conjugated streptavidin (Universal DAKO LSAB®+ kit, peroxidase, K0679; DakoCytomation, Milan, Italy). The reaction was visualized by incubation with a DakoCytomation 3,3′-diaminobenzidine chromogen solution. The sections were counterstained with hematoxylin and mounted in DPX (Merck Eurolab, Milan, Italy). Control experiments were performed simultaneously omitting the primary antibody. The immunostained slides were examined by light microscopy using an Olympus BX41 and the digital images captured with an Olympus Camedia C-5050 zoom digital camera (Olympus Italia, Milan, Italy). Quantitative analysis of labeling intensity was performed by ScionImage software. For each microscopic field, single-cell mean intensity of the labeling was first measured. Then, the mean intensity of the background was evaluated and subtracted from the intensity of the labeling of the cells. The nuclear region was not considered in our analysis.

Microfluorimetry

Briefly, CMEC were loaded with 2 μM Fura-2/AM in physiological salt solution (PSS) for 25 min at room temperature (20–23°C). After washing in PSS, the coverslip was fixed to the bottom of a Petri dish and observed by an upright epifluorescence microscope (Zeiss, Axiolab, Oberkochen, Germany) equipped with a Zeiss ×40 water immersion objective. The exciting light was alternately passed through a 340- or 380-nm bandpass excitation filter (Chroma Technology, Brattleboro, Vt., USA) mounted on a filter wheel equipped with a shutter (Lambda 10, Sutter Instrument, Novato, Calif., USA). Emitted light was passed through a 510-nm filter and collected by a high-sensitivity camera (Extended ISIS Camera, Photonic Science, Millham, UK). Home-developed software was used to drive the camera, the filter wheel and the shutter, and to plot the fluorescence online from a number of rectangular regions of interest, each delimiting a single CMEC. $[Ca^{2+}]_i$ was monitored by evaluating the ratio of fluorescence signals emitted at 510 nm when exciting at 340 and 380 nm, respectively. Ratio measurements were performed every 3 or 5 s. Experiments were performed at the subconfluent stage and at room temperature.

Solutions

The composition of the extracellular PSS was (in mM): NaCl (150), KCl (6), $CaCl_2$ (1.5), $MgCl_2$ (1), glucose (10) and Hepes (10), titrated to pH 7.4 with NaOH. The osmolality of PSS, measured with an osmometer (Wescor 5500, Logan, Utah, USA), was 300–310 mmol/kg. In Ca^{2+}-free solution, Ca^{2+} was substituted with 2 mM NaCl, and 0.5 mM ethylene glycol-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid was added. The activity of NCX was prevented by replacing extracellular Na^+ with equimolar N-methyl-D-glucamine and HCl, as previously shown [21], to prevent any change in the ionic strength of the extracellular solution [3].

Chemicals

Acetylated low-density lipoprotein labeled with 1,1′-dioctadecyl-1-3,3,3′,3′-tetramethyl-indo-carbocyanine perchlorate was from Biomedical Technologies (Stoughton, Mass., USA). Fura-2/AM was obtained from Molecular Probes (Eugene, Oreg., USA) or Calbiochem (La Jolla, Calif., USA). DMEM and HBSS were purchased from Gibco. All other chemicals were from Sigma.

Presentation of Results

The traces shown in the figures represent single-cell signals. n is the number of tested cells, collected from at least three different hearts. For each experiment, a single-cell analysis has been performed and the percentage of cells showing a response similar to the reported single-cell tracing(s) has also been included.

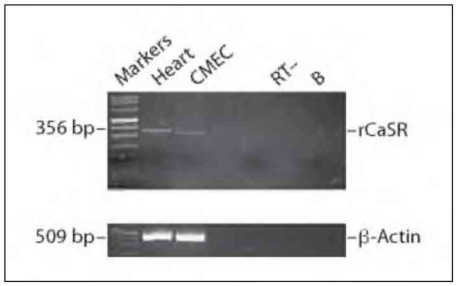


Fig. 1. CaSR mRNA expression in rat heart and CMEC. Detection of CaSR mRNA by semiquantitative RT-PCR of total RNA (1 μg) extracted from rat heart and CMEC in the presence and absence (RT–) of RT. RT-PCR was performed using specific primers for rat CaSR and for the housekeeping gene β-actin (a quality control for mRNA). The 356- and 509-bp bands correspond to the CaSR and β-actin-specific RT-PCR products, respectively. Similar results were obtained in 4 different experiments performed on 4 different hearts. B = Blank.

Results

Detection of CaSR mRNA and Immunocytochemical Localization of the Receptor Protein

The expression of CaSR mRNA both in whole rat cardiac tissue and, more specifically, in CMEC was explored by RT-PCR. The results of agarose gel electrophoresis of representative PCR reaction products are shown in figure 1. Single bands of the expected size of cDNA fragments were amplified (356 and 509 bp for CaSR and β-actin, respectively) both in whole rat cardiac tissue, as previously shown by Wang et al. [6], and in isolated CMEC. In the absence of RT, no PCR-amplified products were detected, indicating that the tested RNA samples were free of genomic contamination. These data are compatible with the presence of CaSR mRNA in rat CMEC. To determine whether CaSR protein is expressed in CMEC, immunostaining was performed using an anti-CaSR mouse monoclonal antibody. Figure 2a and 2c clearly show that CaSR protein is expressed in CMEC, both in the plasma membrane and in the cytoplasm, a finding

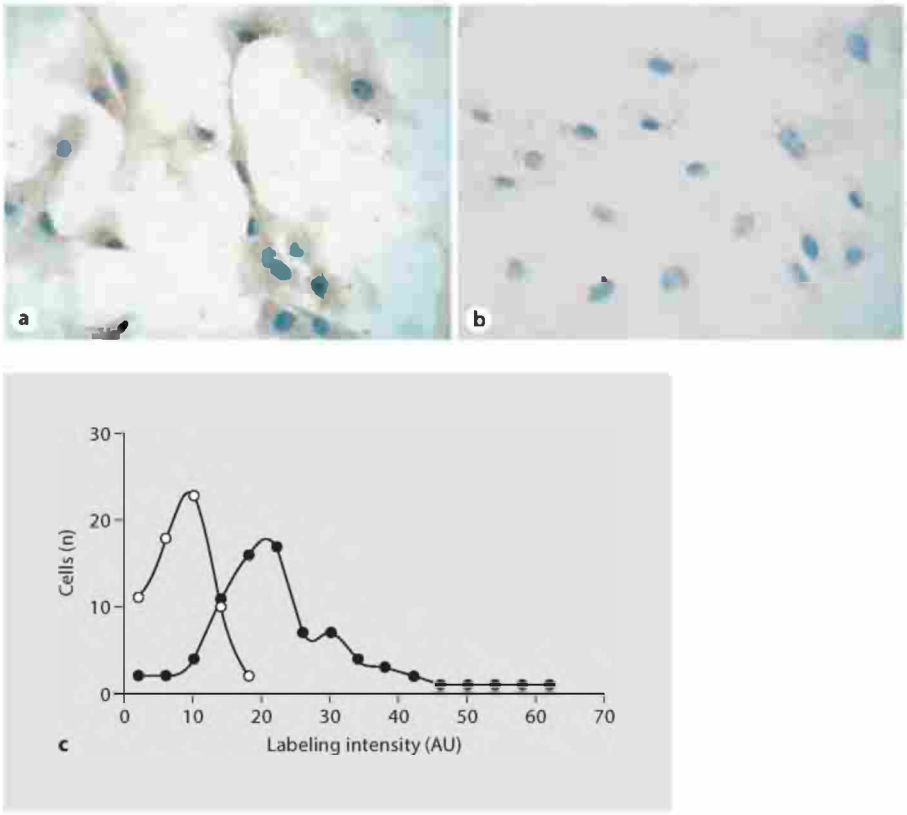


Fig. 2. Immunocytochemical localization of the CaSR protein in CMEC. **a** Immunocytochemical detection of CaSR in rat CMEC with an anti-CaSR antibody. The cells were incubated with anti-CaSR mouse primary monoclonal antibody and thereafter with a secondary antibody conjugated with HRP. **b** Controls cells, in which the primary antibody was omitted, showed only dim labeling. **a**, **b** ×630. **c** Distribution of HRP labeling intensity in isolated CMEC incubated with monoclonal antibody against mouse CaSR (filled dots) or not (empty dots).

which concurs with previous reports [3, 22]. Notably, control cells, i.e. cells not exposed to the primary antibody, always exhibited a dim labeling (fig. 2b). In quantitative analysis, mean staining was 23.40 ± 1.29 arbitrary units (AU; n = 80; 30 microscopic fields) and 8.33 ± 0.52 AU (n = 64; 17 microscopic fields) in the presence and absence of the anti-CaSR primary antibody ($p < 0.001$; fig. 2c). Taken together, these results clearly indicate that CaSR is produced in rat CMEC.

Agonists of CaSR Increase $[Ca^{2+}]_i$

To assess whether the expression of the CaSR protein is associated with the presence of functional receptors, Fura-2/AM-loaded CMEC were exposed to a number of CaSR agonists: spermine, Gd^{3+}, La^{3+} and neomycin [3]. As shown in figure 3a, 0.2 mM spermine increased $[Ca^{2+}]_i$ in 30 of 34 cells (88%). CMEC were also activated by 0.5 mM La^{3+} (fig. 3b; 61.1%, n = 18), 0.2 mM Gd^{3+} (fig. 3c; 61.9%, n = 21) and 2 mM neomycin (fig. 3d; 60%, n = 102). For each agonist, we used concentrations that stimulated CaSR in other cell types [23, 24]. The intracellular Ca^{2+} signal consisted of either a single Ca^{2+} spike (about 60% of responding cells; e.g. upper trace in fig. 3a) or a repetitive Ca^{2+} burst (about 40% of responding cells; e.g. lower trace in fig. 3a), independently of the agonist applied. Therefore, the CaSR protein expressed in CMEC is functional and capable of inducing an intracellular Ca^{2+} response.

Contribution of Intracellular Stores and Role of the PLC Pathway in the Ca^{2+} Response to CaSR Stimulation

The Ca^{2+} responses to 0.3 mM spermine (fig. 4a), 0.5 mM La^{3+} (fig. 4b), 1 mM Gd^{3+} (not shown; 54%, n = 13) and 2 mM neomycin (not shown; 77%, n = 52) were also present in the absence of extracellular Ca^{2+}, although the number of Ca^{2+} transients was reduced in oscillating cells. Therefore, CaSR agonists caused Ca^{2+} release from an intracellular source, as shown in other cell types [2]. Accordingly, no Ca^{2+} signal was ever observed after store depletion by 10 μM cyclopiazonic acid (CPA; fig. 4c, d), a reversible inhibitor of the sarcoendoplasmic reticulum Ca^{2+} pump [21]. As mentioned above, $InsP_3$ receptors ($InsP_3Rs$) mainly mediate intracellular Ca^{2+} discharge upon CaSR activation [2]. In agreement with this notion, U73122 (50 μM), a commonly employed blocker

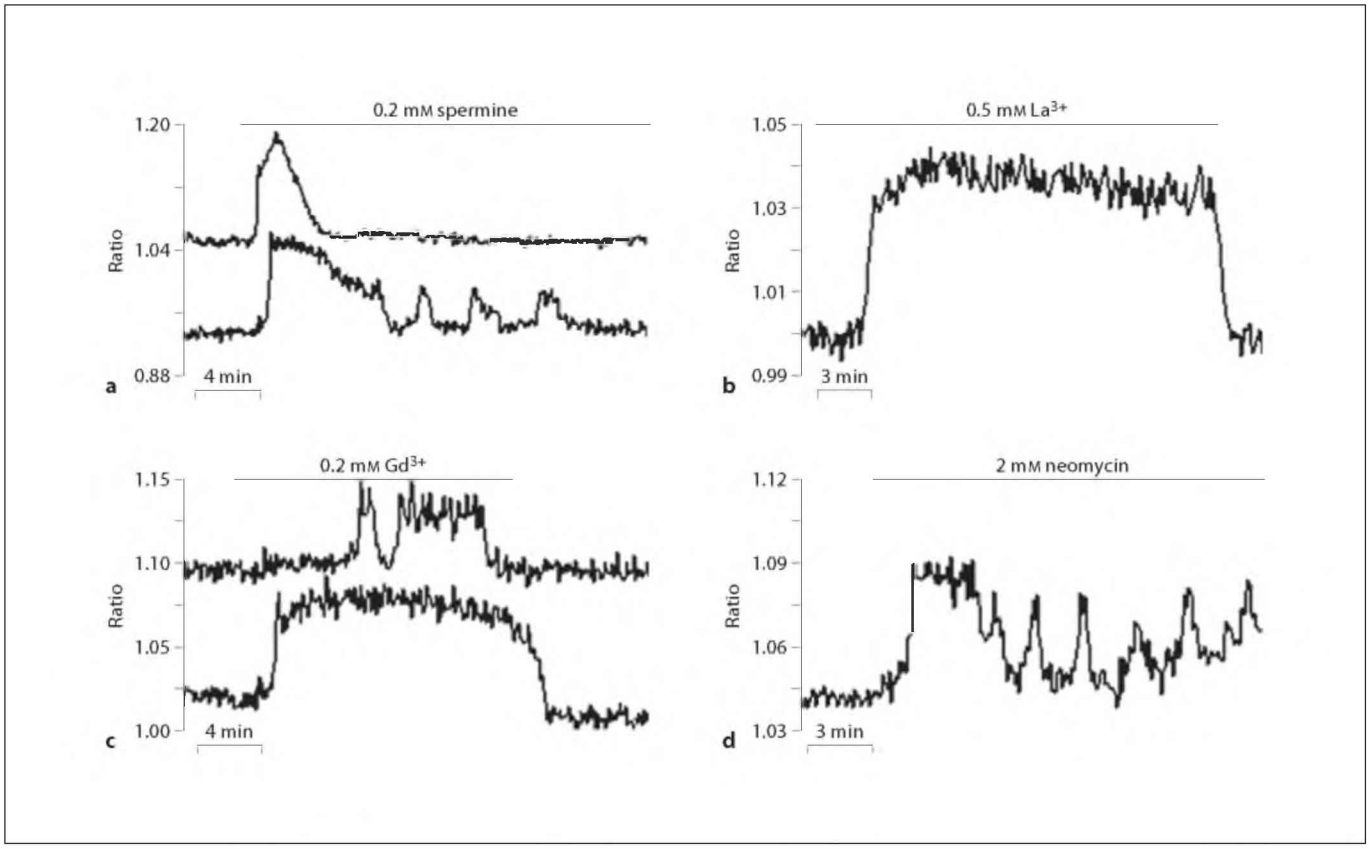


Fig. 3. CaSR agonists increased $[Ca^{2+}]_i$ in rat CMEC. **a** Cytosolic Ca^{2+} response of Fura-2/AM-loaded rat CMEC to known agonists of CaSR: 0.2 mM spermine (**a**), 0.5 mM La^{3+} (**b**), 0.2 mM Gd^{3+} (**c**) and 2 mM neomycin (**d**). **a**, **c** The traces display the different patterns of the Ca^{2+} signal elicited by the agonist in two cells from the same coverslip. In this and the following figures, perfusion of all substances is indicated by horizontal bars.

of PLC activity [6], caused a fast inhibition of spermine-induced $[Ca^{2+}]_i$ elevation in 30 of 30 cells (fig. 5a). The rather high concentration of U73122 was required in order to instantaneously block spermine-induced PLC stimulation, as discussed elsewhere [21]. Accordingly, a 20-min pre-incubation with a lower U73122 dose (5 μM) prevented the onset of the response to spermine in all cells tested (fig. 5b; n = 46). Similar results were obtained upon application of 5 mM La^{3+} (fig. 5c, d). Notably, addition of DMSO at the same dilution used for U73122 did not affect Ca^{2+} signaling in CMEC [21]. To further elucidate the transduction pathway leading to the increase in $[Ca^{2+}]_i$, we applied caffeine, a widely used antagonist of $InsP_3$-gated Ca^{2+} channels in many cell types [25–27], including mouse pancreatic acinar cells [28] and CMEC [13]. Figure 5e depicts that 2.5 mM of caffeine reversibly abolished the Ca^{2+} burst promoted by 5 mM La^{3+} in 28 of 28 cells, thus confirming that $InsP_3Rs$ mediated the Ca^{2+} signal upon CaSR stimulation. It is worth noting that low millimolar doses of caffeine might activate ryanodine receptors, however, a previous study from this laboratory has shown that caffeine-sensitive functional ryanodine receptors are not present in rat CMEC [13].

Removal of Extracellular Na^+ Unmasks the Intracellular Ca^{2+} Response to High $[Ca^{2+}]_o$

Increasing $[Ca^{2+}]_o$ from 0.5 to 2.5 mM is commonly used as a method for stimulating CaSR [6]. This procedure, however, did not evoke any apparent $[Ca^{2+}]_i$ elevation in CMEC (n = 50; data not shown), as also reported in HAEC [9]. We have previously shown that CMEC express a functional NCX, which plays a key role in extruding Ca^{2+} released from $InsP_3Rs$ upon agonist stimulation [12]. The activity of NCX in endothelial cells may be prevented by substituting extracellular Na^+ with N-methyl-

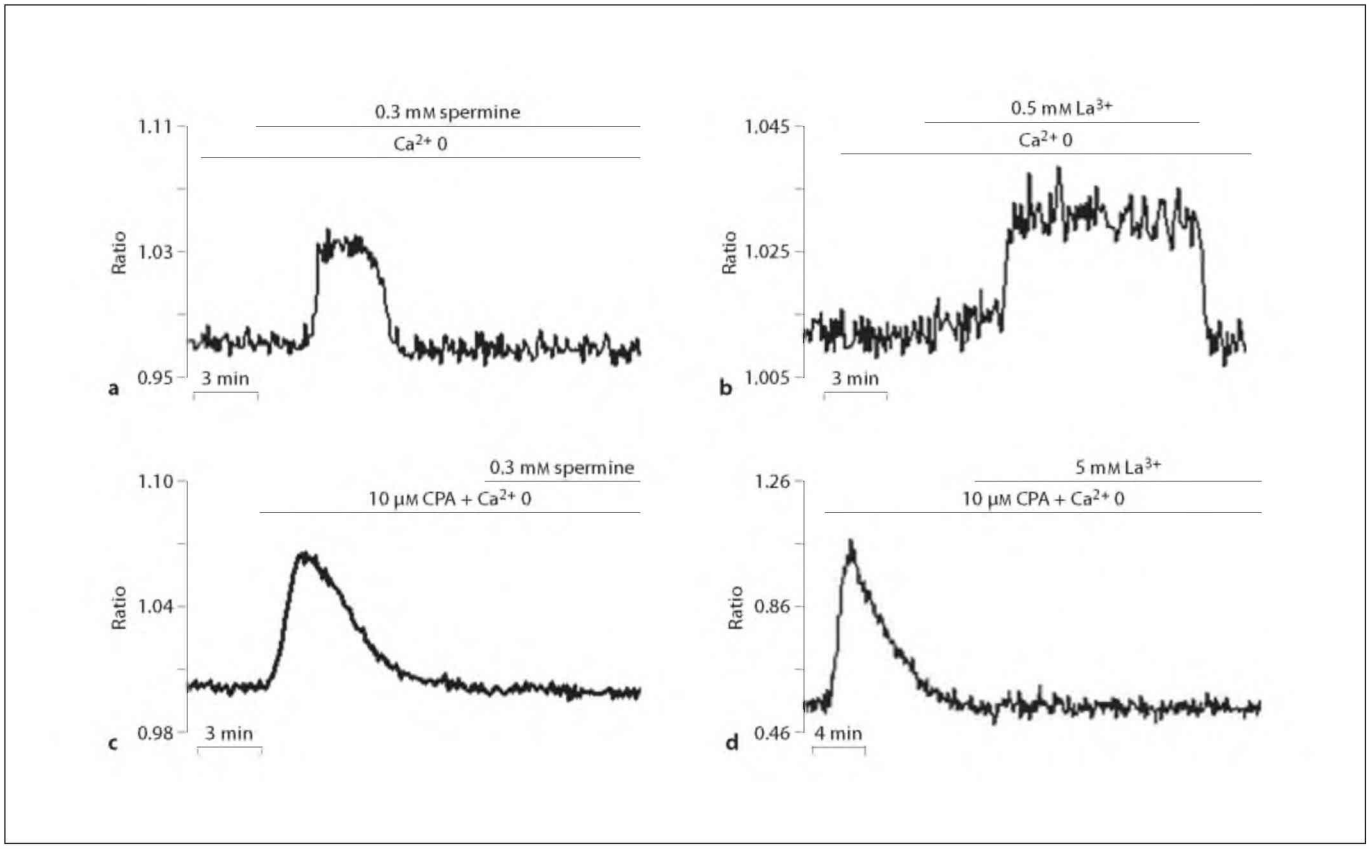
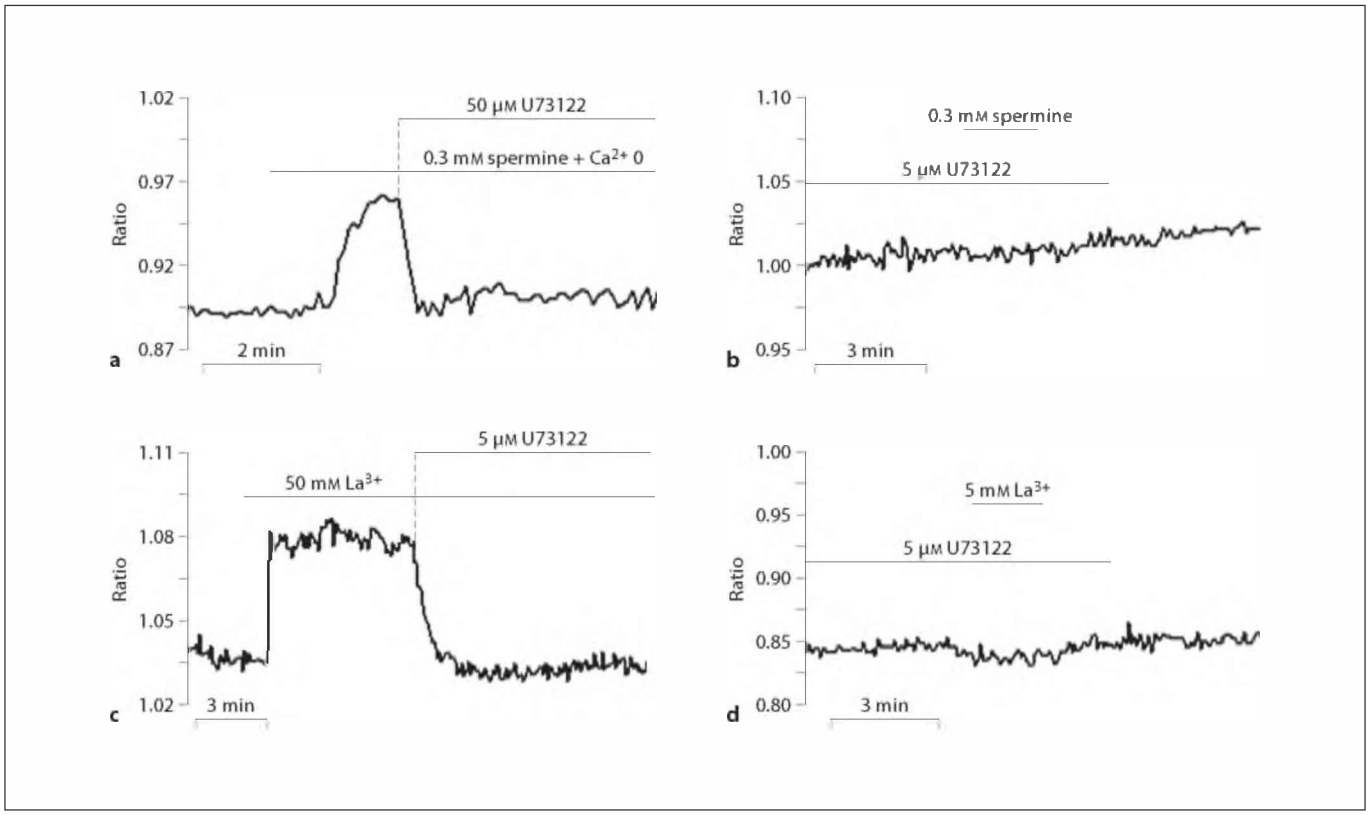


Fig. 4. The Ca^{2+} response to CaSR stimulation depended on intracellular Ca^{2+} release. **a** Removal of extracellular Ca^{2+} did not abolish the initiation of the response to 0.3 mM of spermine (43%, n = 39). **b** the Ca^{2+} burst induced by 0.5 mM La^{3+} was not prevented in Ca^{2+}-free solution (62%, n = 136). **c** Depletion of intracellular stores with CPA (10 μM) abrogated the Ca^{2+} signal promoted by 0.3 mM spermine in 18 of 18 cells. **d** Emptying of intracellular Ca^{2+} pools with CPA (10 μM) abolished the Ca^{2+} response to 5 mM La^{3+} in 56 of 56 CMEC. Under control conditions, 0.3 mM spermine and 5 mM La^{3+} mobilized intracellular Ca^{2+} in 103 of 107 cells (96%) and 89 of 122 cells (73%), respectively.

D-glucamine [21, 29]. Strikingly, such a manoeuvre unmasked the intracellular Ca^{2+} signal induced by high $[Ca^{2+}]_o$ in 37 of 41 cells (fig. 6a). Consistent with the aforementioned findings, the Ca^{2+} increase was abolished by pre-treatment with 10 μM CPA (n = 18; fig. 6b) and acute addition of 50 μM U73122 (fig. 6c; n = 17). Pre-incubation with 5 μM U73122 decreased the mean amplitude of the peak response to high $[Ca^{2+}]_o$ by 78% (not shown; n = 36). These data are compatible with the hypothesis that an elevation in $[Ca^{2+}]_o$ was able to activate Ca^{2+} release from an $InsP_3$-sensitive intracellular Ca^{2+} pool. Notably, after extracellular Na^+ removal, 0.1 mM La^{3+} evoked a Ca^{2+} increase in all cells tested (fig. 6d; n = 24), whereas 5 mM La^{3+} activated only 73% of cells under physiological conditions (fig. 4, legend). Collectively, all these data demonstrated that CMEC were able to respond to an increase in $[Ca^{2+}]_o$, but a bulk cytosolic response was prevented by the activity of NCX.

Aromatic Amino Acids Enhance the Sensitivity to the CaSR Agonist La^{3+}

Finally, we assessed the effect of the aromatic amino acids, L-phenylalanine and L-tryptophan, which may enhance the sensitivity of CaSR to its agonists [4]. These allosteric activators of CaSR were used at a concentration known to exert a maximal effect on the receptor activity [30, 31]. Aromatic amino acid application did not increase the $[Ca^{2+}]_i$ (n = 43; not shown), but potentiated the response to the CaSR agonist La^{3+}. Indeed, L-phenylalanine (10 mM) and L-tryptophan (10 mM) either increased the



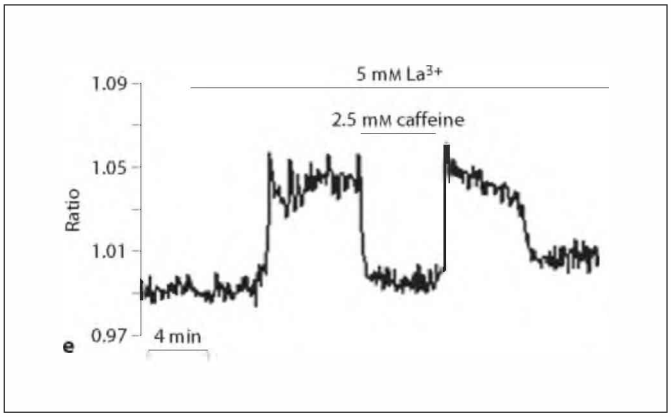


Fig. 5. The PLC/$InsP_3$ pathway mediated CaSR-dependent intracellular Ca^{2+} mobilization. **a** Acute addition of 50 μM U73122, an inhibitor of PLC, on top of the spermine-evoked Ca^{2+} response caused a fast decrease in $[Ca^{2+}]_i$. **b** A 20-min pre-treatment with 5 μM U73122 prevented the activation of CMEC by spermine (0.3 mM). **c** Acute application of 50 μM U73122 on top of the Ca^{2+} discharge triggered by La^{3+} (5 mM) caused a fast decrease in $[Ca^{2+}]_i$ in 29 of 30 cells (96.7%). **d** A 20-min incubation with 5 μM U73122 abolished the Ca^{2+} signal induced by 5 mM La^{3+} in all cells tested (n = 44). **e** 2.5 mM caffeine, a blocker of $InsP_3Rs$, reversibly abrogated the Ca^{2+} response to La^{3+} (5 mM).

response to La^{3+} (5 mM) in 21 of 24 responding cells or caused an immediate increase in $[Ca^{2+}]_i$ in 29 of 56 non-responding cells (fig. 7).

Discussion

It has long been known that changes in the plasma levels of Ca^{2+} may control the vascular tone through the production of NO [1, 32]. Recent studies have suggested a role for CaSR, the best-understood sensor of extracellular Ca^{2+}, in the regulation of vascular function by extracellular Ca^{2+} [8, 20]. Accordingly, CaSR is expressed both in macrovascular endothelial cells [8, 9], where it mediates NO synthesis through Ca^{2+} mobilization from intracellular stores [9], and in vascular smooth muscle cells [20]. The present communication extends these findings, providing evidence compatible with the presence of CaSR in microvascular endothelial cells. This was demonstrated by RT-PCR of RNA obtained from isolated cells, which

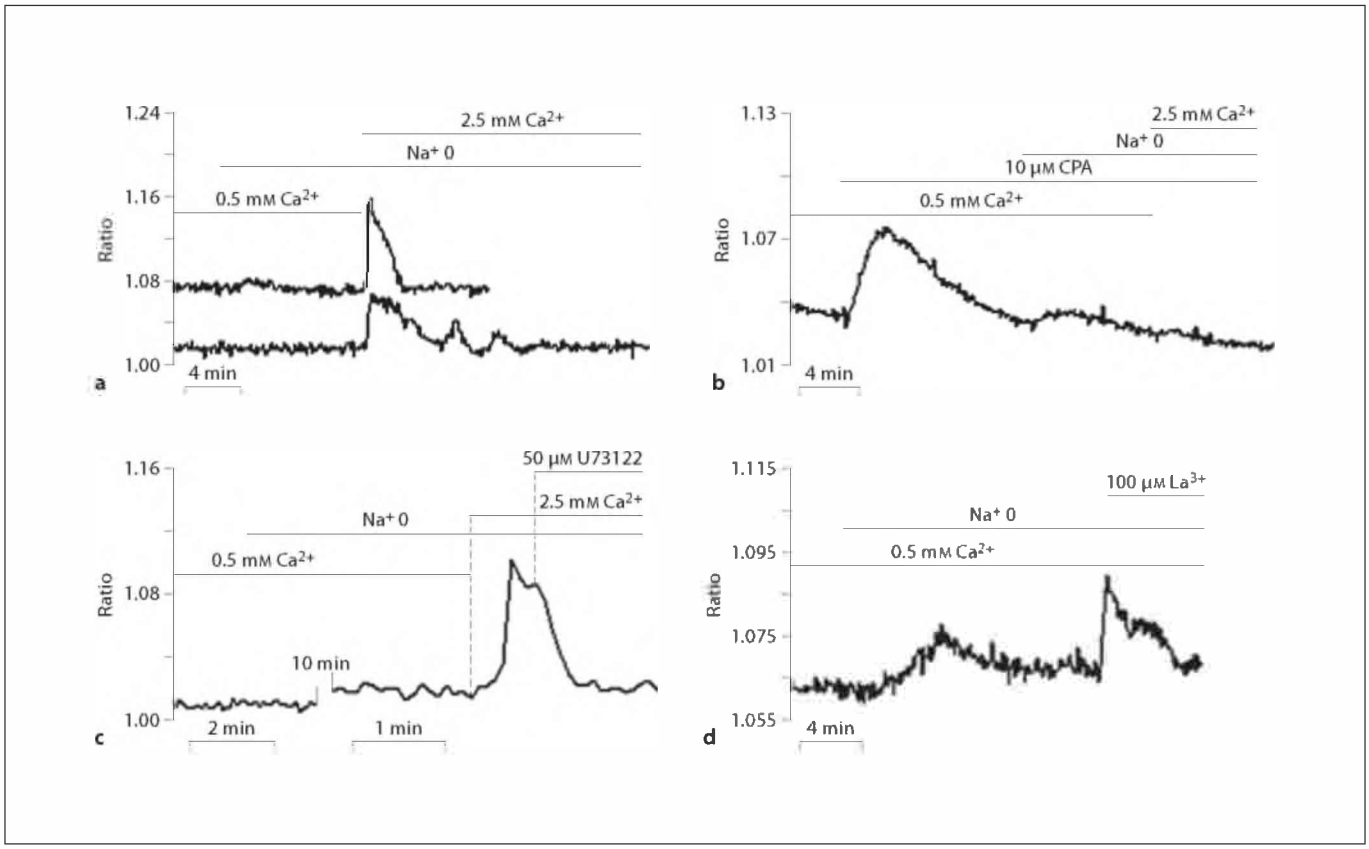


Fig. 6. Removal of extracellular Na^+ unmasked the Ca^{2+} response to a rise in $[Ca^{2+}]_o$. **a** Raising $[Ca^{2+}]_o$ from 0.5 to 2.5 mM in the absence of extracellular Na^+ induced an evident elevation in $[Ca^{2+}]_i$. The Ca^{2+} signal displayed a transient (upper trace) or an oscillatory pattern (lower trace). The cytosolic Ca^{2+} response was inhibited by 10 μM CPA (**b**) and acute addition of U73122 (50 μM; **c**). **d** Substitution of extracellular Na^+ with N-methyl-D-glucamine unmasked the Ca^{2+} mobilization elicited by low doses (100 μM) of La^{3+}. **b**, **d** Removal of extracellular Na^+ resulted in a transient increase in $[Ca^{2+}]_i$ due to reversal of NCX activity [12].

yielded the expected 356-bp product corresponding to rat CaSR. The immunocytochemical analysis confirmed that the CaSR protein is expressed in CMEC, both in the plasma membrane and in the cytoplasm. The intracellular localization of the receptor is not surprising, as it has been described in many cell types, including HAEC [9] and human keratinocytes [22], where CaSR was found both in endoplasmic reticulum and the Golgi apparatus [16]. The following observations support a role for the plasma membrane CaSR in intracellular Ca^{2+} signaling in rat CMEC: (1) a variety of CaSR agonists (spermine, Gd^{3+}, La^{3+}, neomycin and Ca^{2+}) increase $[Ca^{2+}]_i$ in these cells; (2) the pattern of the intracellular Ca^{2+} response displays a large heterogeneity from cell to cell, which is a hallmark of CaSR activation [33]; (3) the onset of the Ca^{2+} signal seems to require Ca^{2+} release from $InsP_3$-sensitive stores [3], and (4) aromatic amino acids (L-phenylalanine and L-tryptophan) may sensitize the response of CaSR, although they cannot directly elevate $[Ca^{2+}]_i$ [4]. These features are clearly different from those displayed by CaSR on HAEC, which is insensitive to Gd^{3+}, neomycin and Ca^{2+} and is activated by high doses of spermine (10 mM) [9]. These features of the CaSR in HAEC have been ascribed to the expression of a splice variant of the receptor in these cells [9].

The most striking result provided by the present report is that CMEC are able to detect an increase in $[Ca^{2+}]_o$, although the resulting increase in $[Ca^{2+}]_i$ is masked by NCX (fig. 6a), possibly suggesting an alternative explanation for the lack of the $[Ca^{2+}]_o$-induced $[Ca^{2+}]_i$ elevation in HAEC [9]. We speculate that the interplay between CaSR and NCX under physiological conditions might es-

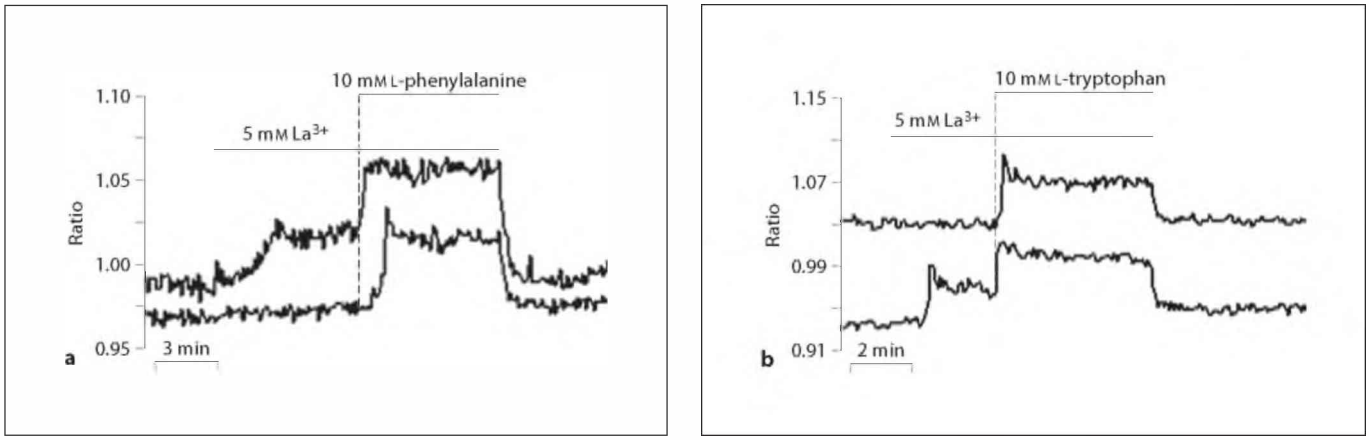


Fig. 7. L-Phenylalanine and L-tryptophan favor the stimulating activity of La^{3+}. **a** 10 mM L-phenylalanine either potentiated the Ca^{2+} increase induced by La^{3+} (5 mM) in responsive cells (upper trace) or promoted Ca^{2+} mobilization in unresponsive cells (lower trace). **b** A similar modulation of the Ca^{2+} response to La^{3+} (5 mM) was exerted by 10 mM L-tryptophan.

tablish a Ca^{2+} gradient between the sub-plasmalemmal space and the bulk cytoplasm. In agreement with this hypothesis, increasing $[Ca^{2+}]_o$ from 0.1 to 1.2 mM produced a subcortical Ca^{2+} wave in endothelial cells expressing a fluorescent Ca^{2+} sensor targeted at the plasma membrane [34]. Importantly, no $[Ca^{2+}]_i$ elevation was detected when the Ca^{2+} fluorochrome was expressed in the bulk cytosol [34]. The $[Ca^{2+}]_o$-induced subcortical Ca^{2+} wave has been shown to regulate several Ca^{2+}-dependent signaling proteins residing in or translocating to the plasma membrane, e.g. NO synthase and PLA_2 [34]. Therefore, it is conceivable that changes in $[Ca^{2+}]_o$ modulate CMEC activity, thus controlling the Ca^{2+}-dependent synthesis of NO and prostacyclin, which in turn govern both cardiac blood flow and adjacent cardiomyocytes [10]. The scenario described above is strongly supported by two pieces of evidence: (1) within the interstitial fluid of the beating heart Ca^{2+} levels undergo dramatic fluctuations due to the opening of voltage-gated Ca^{2+} channels [35], and (2) extracellular Ca^{2+} levels may fluctuate near the CMEC surface as a consequence of intracellular Ca^{2+} signaling [7]. Indeed, Ca^{2+} release from stimulated cells results in an increase in $[Ca^{2+}]_o$ due to the extrusion of Ca^{2+} across the plasma membrane [2]. Such an extracellular Ca^{2+} signal could in turn stimulate CaSR on neighboring cells or on the same cells, thus conferring an oscillatory pattern to the intracellular Ca^{2+} mobilization [7]. Therefore, CaSR activation might contribute to the repetitive Ca^{2+} transients brought about by UTP and epidermal growth factor in CMEC [12, 13].

CaSR on the coronary microvasculature might also serve as a physiological target for spermine, a naturally occurring polyamine which plays an important role in cell growth, differentiation and replication in the heart [36]. Plasma levels of spermine are extremely variable, ranging from the nanomolar to the micromolar range [37], and the most frequently reported value for spermine content in rat heart is around 400 nmol/g [38]. However, local levels of spermine may be much higher than the circulating concentration, as reported in the nervous system, where presynaptically released spermine achieves high millimolar levels within synaptic clefts [39]. Moreover, an increase in spermine content has been associated with cardiac hypertrophy, as observed in the ventricular tissue of spontaneously hypertensive rats, whose spermine content is 5-fold higher than in normotensive rats [40].

In conclusion, we have provided evidence that CMEC express a functional CaSR. In particular, the interaction between CaSR and NCX leads to a sub-plasmalemmal $[Ca^{2+}]_i$ increase which may not be resolved when the bulk cytosolic Ca^{2+} is monitored. This observation could explain the apparent lack of response of CaSR upon exposure of some endothelial cells to high $[Ca^{2+}]_o$. In CMEC, CaSR might be involved in the endothelial control of cardiac function [10] under physiological and pathophysiological conditions. At present, an investigation aiming to assess whether extracellular Ca^{2+} elevation is able to induce NO production in CMEC, both under physiological and under Na^+-free conditions, is underway.

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