Evidence in Favor of a Calcium-Sensing Receptor in Arterial Endothelial Cells

Studies With Calindol and Calhex 231

Arthur H. Weston, Mais Absi, Donald T. Ward, Jacqueline Ohanian, Robert H. Dodd, Philippe Dauban, Christophe Petrel, Martial Ruat, Gillian Edwards

Abstract—Small increases in extracellular Ca\textsuperscript{2+} dilate isolated blood vessels. In the present study, the possibility that a vascular, extracellular Ca\textsuperscript{2+}-sensing receptor (CaSR) could mediate these vasodilator actions was investigated. Novel ligands that interact with the CaSR were used in microelectrode recordings from rat isolated mesenteric and porcine coronary arteries. The major findings were that (1) raising extracellular Ca\textsuperscript{2+} or adding calindol, a CaSR agonist, produced concentration-dependent hyperpolarizations of vascular myocytes, actions attenuated by Calhex 231, a negative allosteric modulator of CaSR. (2) Calindol-induced hyperpolarizations were inhibited by the intermediate conductance, Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} (IK\textsubscript{Ca}) channel inhibitors, TRAM-34, and TRAM-39. (3) The effects of calindol were not observed in the absence of endothelium. (4) CaSR mRNA and protein were present in rat mesenteric arteries and in porcine coronary artery endothelial cells. (5) CaSR and IK\textsubscript{Ca} proteins were restricted to caveolin-poor membrane fractions. We conclude that activation of vascular endothelial CaSRs opens endothelial cell IK\textsubscript{Ca} channels with subsequent myocyte hyperpolarization. The endothelial cell CaSR may have a physiological role in the control of arterial blood pressure. (Circ Res. 2005;97:391-398.)

Key Words: endothelium ■ calcium-sensing receptor ■ potassium channel ■ calindol ■ Calhex 231

Small increases in the extracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}])\textsubscript{o} from physiological levels relax isolated blood vessels.\textsuperscript{4} Although this phenomenon is partially endothelium-dependent,\textsuperscript{2} the detailed mechanisms underlying Ca\textsuperscript{2+}-induced vasodilation remain unknown. Nevertheless, the long-term blood pressure-lowering effects of increasing dietary calcium in low-renin hypertensive patients is now recognized.\textsuperscript{3}

In both vascular myocytes and in perivascular nerves, there is evidence of an extracellular Ca\textsuperscript{2+}-sensing receptor (CaSR)\textsuperscript{4-6} that is G protein–coupled and activated by millimolar concentrations of [Ca\textsuperscript{2+}]\textsubscript{o}.\textsuperscript{7} The resultant stimulation of a phosphatidyl inositol-specific phospholipase C releases Ca\textsuperscript{2+} from inositol trisphosphate-sensitive stores although other intracellular pathways have also been described.\textsuperscript{8} The CaSR is present in organs involved in Ca\textsuperscript{2+} homeostasis such as the parathyroid and kidney although important roles for the receptor in the intestine and placenta are also indicated.\textsuperscript{7,9}

CaSR activation in vascular myocytes should produce a contractile effect as reported by Wonneberger et al\textsuperscript{5} in the gerbil spiral modiolar artery. However, in most arteries, raising [Ca\textsuperscript{2+}]\textsubscript{o}, above the narrow range (1.1 to 1.3mmol/L) in which it is maintained in mammalian serum produces vasorelaxation.\textsuperscript{6,10} Because this phenomenon still occurred following endothelium removal but was reduced after perivascular nerve destruction, it was concluded that activation of a neuronal CaSR induced the release of a neuronally-derived hyperpolarizing factor,\textsuperscript{6,10} although such an agent has never been identified.

In a recent study, Edwards et al\textsuperscript{11} showed that myocyte hyperpolarizations, generated following activation of endothelial cell small and intermediate conductance Ca\textsuperscript{2+}-sensitive channels (SK\textsubscript{Ca} and IK\textsubscript{Ca}, respectively), were affected by small changes in [Ca\textsuperscript{2+}]\textsubscript{o}. The present study was initiated to investigate the possible involvement of the CaSR in these responses. Using a variety of techniques, together with novel ligands known to activate and to inhibit the CaSR, we now report strong evidence in favor of this possibility and suggest that an endothelial cell CaSR may also have a role in the control of vascular tone.

Materials and Methods

Animals

Mesenteric artery branches (second and third order; \(\approx 150 \text{ to } 250 \mu \text{m diameter}\)) were dissected from male Sprague-Dawley rats (body weight 250 to 300 g) previously euthanized by stunning and cervical

Original received March 8, 2005; revision received July 8, 2005; accepted July 12, 2005.
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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/01.RES.0000178787.59594.a0
dislocation in compliance with Schedule 1 of the UK Animals (Scientific Procedures) Act 1986. Left anterior descending coronary arteries were dissected from pig hearts (obtained from the local abattoir and transported to the laboratory in ice-cold Krebs solution).

**Microelectrode Experiments**

Intact vessels were pinned to the Sylgard base of a 10 mL heated bath and superfused (10 mL/min), at 37°C, with Krebs solution (pH 7.5; which unless otherwise stated comprised [mmol/L]: NaCl 118, KCl 3.4, CaCl₂ 1.0, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25, glucose 11) containing 300 μmol/L N⁵-nitro-L-arginine and 10 μmol/L indo- methacin and gassed with 95% O₂/5% CO₂. Smooth muscle cells were impaled from the adventitial side using microelectrodes filled with 3 mol/L KCl (resistance 40 to 80 mol/L) as previously described. In some experiments, artery segments were deendothelialized by exposing the lumen to deionized water for 20 s; endothelial cell loss was confirmed by the lack of response to 10 μmol/L acetylcholine.

**Immunohistochemistry**

Cryosections (9 μm) were prepared and immunostained as previously described but with an additional step (boiling for 7 minutes in 40% sucrose (900 g/mL) containing 300 μmol/L N⁵-nitro-L-arginine and 10 μmol/L indo- methacin, levcromakalim (SmithKline Beecham), N⁵-nitro-L-arginine, phenylephrine hydrochloride. TRAM-39 (2-(2-chlorophenyl)-2,2- diphenylacetoneitrile) and TRAM-34 (1-[2-chlorophenyl]diphenylmethyll- H₄-pyrazole) were gifts from Dr H. Wulff (UC Davis School of Medicine, Calif). Calindol, S-calindol, and Calhex 231 were prepared as previously described. Unless otherwise stated, all compounds were obtained from Sigma-Aldrich.

**Data Analysis**

Values are given as mean±SE; n indicates number of tested cells or arteries from individual animals. Statistical analysis was performed using 1-way analysis of variance followed by a Tukey-Kramer multiple comparison test or a Student t test, as appropriate; P<0.05 was considered significant.

**Results**

**Pharmacological Evidence for a CaSR in Mesenteric Artery**

Under control conditions ([Ca²⁺], 1 mmol/L), myocyte resting membrane potential in endothelium-intact segments was −53.5±0.2 mV (n=20). Calindol (a positive allosteric modulator of CaSR) produced a concentration-dependent hyperpolarization (100 nmol/L, 4.0±0.4 mV, n=6; 300 nmol/L, 12.1±0.4 mV, n=11; 1 μmol/L, 16.9±0.6 mV, n=8; see Figure 1) whereas the S-enantiomer of calindol (S-calindol18; 1 μmol/L, n=4) was without effect (Figure 1). Under similar conditions, 10 μmol/L acetylcholine produced a hyperpolarization of 20.9±0.2 mV (n=4). After endothelium removal, confirmed by the loss of the response to 10 μmol/L acetylcholine, 1 μmol/L calindol produced a small depolarization (1.1±0.3 mV; see Figure 1b, n=4). At the end of each experiment, 10 μmol/L levcromakalim (added to confirm microelectrode penetration) induced a hyperpolarization of 24.5±1.3 mV (n=4).

**Inhibition of Responses to Calindol: Comparison With 1-EBIO**

In some cells, activation of the CaSR increases [Ca²⁺]ᵢ and such an effect in the endothelium should activate calcium-sensitive K⁺ channels. To investigate this possibility, the effects of calindol were compared with those of the IKᵦᵦ activator, 1-EBIO.

In the presence of a functional endothelium, the hyperpolarization produced by 1-EBIO (600 μmol/L; 17.3±0.6 mV, n=8) was similar to that produced by 1 μmol/L calindol (Figure 2a). Responses to 1-EBIO and calindol were each abolished by the IKᵦᵦ inhibitor TRAM-3921 (10 μmol/L) which alone depolarized the membrane by 3.7±0.4 mV (n=4). In contrast, in the presence of 10 μmol/L TRAM-39, the hyperpolarization to 10 μmol/L acetylcholine (21.4±0.5
mV, n=4), was similar to that produced by acetylcholine alone (20.9±0.2 mV, n=4).

In endothelium-intact artery segments, 1 μmol/L Calhex 231, a negative allosteric modulator of CaSR, depolarized the myocytes by 3.4±0.2 mV (Figure 2b; n=4). The hyperpolarization to 300 nmol/L calindol was significantly reduced in the subsequent presence of 1 μmol/L Calhex 231 (from 12.4±0.8 mV to 4.6±0.7 mV, n=4, P<0.001). In contrast, the hyperpolarization to 600 μmol/L 1-EBIO was not inhibited by Calhex 231. Indeed, because of the depolarizing effect of Calhex 231, the hyperpolarizations generated by 600 μmol/L 1-EBIO were slightly larger (P<0.05) in the presence (20.0±0.5 mV, n=4) than in the absence of 1 μmol/L Calhex 231 (17.7±0.2 mV, n=4; Figure 2b). A submaximally-effective concentration of 1-EBIO (300 μmol/L) which hyperpolarized the membrane by 11.9±0.9 mV (similar to that produced by 300 nmol/L calindol, see above) was also unaffected by 1 μmol/L Calhex 231. Indeed, because of the depolarizing effect of Calhex 231, the hyperpolarizations generated by 600 μmol/L 1-EBIO were slightly larger (P<0.05) in the presence (20.0±0.5 mV, n=4) than in the absence of 1 μmol/L Calhex 231 (17.7±0.2 mV, n=4; Figure 2b). A submaximally-effective concentration of 1-EBIO (300 μmol/L) which hyperpolarized the membrane by 11.9±0.9 mV (similar to that produced by 300 nmol/L calindol, see above) was also unaffected by 1 μmol/L Calhex 231.
231. Thus, 300 μmol/L 1-EBIO hyperpolarized the membrane to –64.5 ± 0.8 mV in the absence and to –63.0 ± 0.8 mV in the presence of 1 μmol/L Calhex 231 (n = 4; P > 0.05, Student paired t-test).

These results show that calindol, like 1-EBIO, opens IKCa channels in vascular endothelial cells. The finding that Calhex 231 inhibited calindol but not 1-EBIO, suggests that Calhex 231 is not an IKCa inhibitor but is consistent with an action at a CaSR site that overlaps with that of calindol.22

Effects of Extracellular Calcium
An axiomatic feature of the CaSR is that its activation is sensitive to fluctuations in [Ca2+]o. Thus, it was important to investigate the effect of such changes on those vascular myocyte responses that hypothetically resulted from endothelial cell CaSR activation.

The myocyte resting membrane potential in endothelium-intact artery segments was slightly greater when [Ca2+]o was 3 mmol/L (–54.4 ± 0.1 mV, n = 5) than when it was 0.3 mmol/L (–51.6 ± 0.3 mV, n = 9; P < 0.001; Tukey-Kramer test; Figure 3) although there was no such difference when the [Ca2+]o was 1 or 3 mmol/L. With 0.3 mmol/L [Ca2+]o, there was a trend for Calhex 231 to produce a small myocyte depolarization, although this was not significant (membrane potential –51.3 ± 0.4 mV in the absence and –49.8 ± 0.4 mV in the presence of 1 μmol/L Calhex 231, n = 4). In the continued presence of 1 μmol/L Calhex 231, increasing [Ca2+]o from 0.3 to 3 mmol/L had no effect (membrane potential –50.5 ± 0.4 mV, n = 4).

The magnitude of the calindol-induced myocyte hyperpolarizations was also influenced by the [Ca2+]o, (P < 0.001, 2-way ANOVA; Figure 3). Thus, the calindol threshold concentration was 100 nmol/L at 0.3 mmol/L [Ca2+]o, and 30 nmol/L when [Ca2+]o was 3 mmol/L. (Figure 3b). The mean calindol EC50 concentration was 268 nmol/L when the [Ca2+]o was 0.3 mmol/L and 82 nmol/L when [Ca2+]o was 3 mmol/L. (log EC50 values for calindol were –6.57 ± 0.06 mol/L and –7.09 ± 0.05 mol/L at 0.3 mmol/L and 3.0 mmol/L [Ca2+]o, respectively; paired Student t test, P < 0.01; n = 4).

Effect of Phenylephrine on Responses to Calindol
Vascular myocyte hyperpolarization is usually associated with relaxation23 and thus calindol-induced hyperpolarizations should relax precontracted arteries. However, using a wire myograph, no such effects of calindol were observed in mesenteric artery segments precontracted with phenylephrine (1 to 3 μmol/L).

To determine whether the extracellular K+ cloud associated with phenylephrine-induced contractions24,25 inhibited the action of calindol, we tested whether, as with other endothelium-dependent hyperpolarizations,26 any such inhibitory effect could be reversed by iberiotoxin (a selective inhibitor of the large-conductance Ca2+-sensitive K+ channel, BKCa27). Phenylephrine (1 μmol/L) depolarized the smooth muscle by 12.9 ± 0.7 mV (n = 4) and in its presence the responses to 1-EBIO and calindol were markedly reduced in comparison to those before phenylephrine addition (Figure 4a and 4b). In the continued presence of phenylephrine, iberiotoxin produced a further depolarization (7.5 ± 1.2 mV, n = 4) and the hyperpolarizing effects of 1-EBIO and calindol were partially restored (Figure 4).

Pharmacological Evidence for a CaSR in Porcine Coronary Artery Endothelium
Myocyte resting membrane potential of endothelium-intact porcine coronary artery segments with 1 mmol/L [Ca2+]o, was
Calindol (R-enantiomer, 300 nmol/L) produced a hyperpolarization of 7.5 ± 0.6 mV (n = 4) whereas S-calindol (300 nmol/L, n = 4) was without effect (Figure 5). Calhex 231 (1 μmol/L), which alone produced a small but significant membrane depolarization (1.5 ± 0.5 mV, n = 4; P < 0.001, Students paired t-test), inhibited the response to 300 nmol/L calindol but not that to 300 μmol/L 1-EBIO (Figure 5). The ability of the IKCa inhibitor TRAM-3421 to modify the response to calindol was tested in coronary arteries from 2 pig hearts and in each the hyperpolarization to 1 μmol/L calindol (initially 14.6 mV and 19.4 mV) was almost abolished by 10 μmol/L TRAM-34 (reduced to 2.1 mV and 2.3 mV, respectively). In the absence (n = 4) of the endothelium, confirmed by the lack of myocyte hyperpolarization to 100 nmol/L substance P, calindol (1 μmol/L) produced a small depolarization of 2.1 ± 0.3 mV (n = 4).

Evidence of Endothelial Cell CaSR Protein
Amplicons of the anticipated size (283 bp) were produced in PCR reactions on RNA extracted from rat kidney and mesenteric artery and from porcine coronary artery endothelial cells (Figure 6a) and kidney (not shown). Over the region that could be accurately analyzed (165 nucleotides), the pig sequence shared 89% identity with nucleotides 1730 to 1894 of the rat CaSR sequence (GenBank/EMBL accession #U20289; Figure 7). No product was obtained from porcine coronary arteries after endothelium removal (not shown).

Using an anti-CaSR antibody, bands of appropriate size (~150 kDa) were obtained in Western blots performed on samples prepared from rat mesenteric and porcine coronary arteries and from porcine coronary artery endothelial cells (Figure 6b). In mesenteric artery cryostat sections, pronounced CaSR immunoreactivity was observed in both endothelial and adventitial layers, whereas only faint staining of the smooth muscle layer was obtained (Figure 6c and 6d). No immunostaining was observed in sections incubated with the secondary antibody alone (Figure 6e).

Localization of CaSR and IKCa
One explanation for the finding that CaSR stimulation activates only IKCa (and not SKCa) channels could be that the CaSR and the IKCa channel are colocalized within the endothelium. Membrane fractions were thus prepared from rat mesenteric arteries and subjected to sucrose density gradient analysis. CaSR and IK1 (IKCa α-subunit) proteins separated in the same ‘noncaveolin’ fractions whereas SK3 protein (which forms endothelial SKCa channels) was in the fraction rich in caveolin-1 (Figure 6f and 6g).
Discussion

Several mechanisms have been proposed to explain the vasorelaxation produced by small increases in [Ca\(^{2+}\)], and one of these involves a CaSR. In the absence of pharmacological tools, a combination of RT-PCR and immunohistochemistry was used in an attempt to identify the presence of CaSR in the rat mesenteric artery in which Ca\(^{2+}\)-induced relaxation occurs. However, no CaSR mRNA was identified in whole-artery extracts. This fact, together with the observation that the CaSR antibody labeled the perivascular nerves, led to the conclusion that a vascular CaSR was present but neuronally-located. This was supported by later studies in which in vivo destruction of sensory nerves (using capsaicin) reduced the relaxant effects of raising [Ca\(^{2+}\)].

In the present investigation, however, CaSR mRNA and protein were detected in rat mesenteric arteries although the small size of the vessels prevented determination of the cellular location. However, immunohistochemical staining of mesenteric artery sections for CaSR protein indicated the presence of CaSR in the rat mesenteric artery in which Ca\(^{2+}\)-induced relaxation occurs. However, no CaSR mRNA was identified in whole-artery extracts. This fact, together with the observation that the CaSR antibody labeled the perivascular nerves, led to the conclusion that a vascular CaSR was present but neuronally-located. This was supported by later studies in which in vivo destruction of sensory nerves (using capsaicin) reduced the relaxant effects of raising [Ca\(^{2+}\)].

In the present investigation, however, CaSR mRNA and protein were detected in rat mesenteric arteries although the small size of the vessels prevented determination of the cellular location. However, immunohistochemical staining of mesenteric artery sections for CaSR protein indicated its distribution in the endothelium (but not the myocytes) with additional immunoreactivity in the adventitial layer. Similarly, samples prepared from porcine coronary arteries indicated the presence of CaSR mRNA and protein in the endothelium and its absence from the myocytes.

Porcine coronary artery endothelial cell scrapes generated a pronounced band of immunoreactivity with an appropriate molecular mass (~150 kDa). This was similar to that previously described by Ohanian and coworkers in rat subcutaneous arteries (159 kDa) using a monoclonal antibody raised to the same amino acid sequence. The single 150 kDa band observed in the present study in endothelium-intact rat mesenteric arteries and in porcine coronary artery endothelial cells, SK3 immunoreactivity was associated with the caveolin-1-rich fraction (C) whereas IK1 and CaSR were predominantly associated with the less buoyant (NC2) of the 2 noncaveolin (NC) fractions. F1 indicates upper fraction (5% sucrose).

**Figure 6.** Immunolocalization and identification of calcium-sensing receptor (CaSR) protein and mRNA. a, Amplicons of the anticipated size (283 bp) were produced by RT-PCR amplification of RNA obtained from rat mesenteric artery (rma) and kidney (rk) and from porcine coronary artery endothelial cells (pca E). b, Western blot analysis of homogenates obtained from HEK293 cells transfected with CaSR (HEK), rat mesenteric and porcine coronary arteries (pca), and porcine coronary artery endothelial cell samples (each 55 µg protein) using mouse monoclonal anti-CaSR antibodies. c and d, Same transverse section of rat mesenteric artery without (c) or with (d) DAPI staining of nuclei (blue). Immunoreactivity to the anti-CaSR antibody (red) was observed in the single layer of endothelial cells which is separated from the multiple layers of myocytes by the internal elastic lamina (green autofluorescence). The external elastic lamina defines the inner limit of the adventitial layer in which strong immunoreactivity to the antibody was also observed. e, Section of artery incubated with DAPI and secondary, but not primary, antibody. In (c through e), the horizontal scale bar represents 100 μm. After density gradient separation of membrane fractions from (f) rat mesenteric artery and (g) porcine coronary artery endothelial cells, SK3 immunoreactivity was associated with the caveolin-1-rich fraction (C) whereas IK1 and CaSR were predominantly associated with the less buoyant (NC2) of the 2 noncaveolin (NC) fractions. F1 indicates upper fraction (5% sucrose).

**Figure 7.** Sequence alignments of pig and rat (GenBank/EMBL database accession #U20289) CaSR nucleotides indicating 89% identity over the 165 nucleotide region studied.
Pharmacological Approach to the Localization of CaSR in Vascular Endothelial Cells

The first CaSR agonists were phenylalkylamines and some of their effects, especially at high concentrations, may have arisen from L-type Ca\(^{2+}\) channel inhibition. Because ion channels are inhibited to a similar extent by \(R-\) and \(S-\)enantiomers of calcimimetics, Nemeth\(^{31}\) has suggested that any agonist stereoselective effects should be used to indicate that a CaSR rather than an ion channel is involved in the response to a calcimimetic. In the present study, rat mesenteric and porcine coronary artery smooth muscle hyperpolarization was induced by 100 mmol/L calindol (\(R-\)enantiomer) whereas a 10-fold higher concentration of \(S-\)calindol was without effect. This is consistent with the relative potencies of these \(R-\) and \(S-\)enantiomers on CaSRs heterologously expressed in CHO cells.\(^{18}\) The effects of calindol on myocyte membrane potential were lost after endothelium removal, suggesting that perivascular nerves were not involved. Furthermore, Calhex 231 substantially reduced the hyperpolarization to 300 mmol/L calindol. Previous studies have demonstrated cooperation between CaSR agonists and [Ca\(^{2+}\)]\(_o\) in the activation of the CaSR.\(^{18,22,32}\) Thus, the finding of the present study that the hyperpolarization concentration-effect curve to calindol was shifted leftwards by increasing [Ca\(^{2+}\)]\(_o\), further supports a role for CaSRs in the observed endothelium-dependent myocyte hyperpolarizations induced by calindol. Indeed, elevation of [Ca\(^{2+}\)]\(_o\), alone (from 0.3 to 3 mmol/L) produced a Calhex 231-sensitive hyperpolarization, consistent with the activation of CaSR by [Ca\(^{2+}\)]\(_o\). Furthermore, in the presence of a quasi-physiological concentration of [Ca\(^{2+}\)]\(_o\), (1 mmol/L), Calhex 231 depolarized the myocytes, suggesting that, under physiological conditions, the CaSR is partially activated in rat mesenteric artery endothelial cells.

Evidence That the CaSR Is Linked to the Opening of IK\(_{Ca}\) Channels

Ye et al\(^{33}\) recently showed that the calcimimetic NPS R-467 activated a 140 pS K\(^+\) channel in U87 astrocytoma cells. In the present study, the hyperpolarizing actions of calindol were inhibited by the specific IK\(_{Ca}\) blockers TRAM-34 and TRAM-39 in conditions that also abolished the hyperpolarizations generated by the IK\(_{Ca}\) activator 1-EBIO. These findings suggest that the CaSR and IK\(_{Ca}\) channels could be closely associated in a plasmalemmal microdomain. However neither was present in caveolin-enriched fractions (although SK\(_{Ca}\) channels, that were not activated by CaSR, were), suggesting that CaSRs and IK\(_{Ca}\) channels interact at a non-caveolin site at the plasma membrane.

With 1 mmol/L Ca\(^{2+}\) in the Krebs solution, the depolarization induced by Calhex 231 was not significantly different from that produced by inhibition of IK\(_{Ca}\) using TRAM derivatives. This may indicate that the CaSR is basally activated, providing low-level activation of IK\(_{Ca}\) even under basal conditions and in the absence of agonists. Collectively, these electrophysiological data also suggest close coupling of the CaSR with endothelial IK\(_{Ca}\) channels.

Endothelium-dependent hyperpolarization of the rat mesenteric artery smooth muscle occurs predominantly by two mechanisms both of which result from the opening of endothelial IK\(_{Ca}\) and SK\(_{Ca}\) channels.\(^{34,35}\) Endothelial cell hyperpolarization may be transferred to the myocytes via myo-endothelial gap junctions or the effluxing K\(^+\) may activate myocyte Na\(^+\)/K\(^+\)/ATPases and inwardly-rectifying K\(^+\) channels. Both mechanisms are likely to contribute to the mesenteric artery myocyte hyperpolarization observed when CaSR stimulation opened endothelial cell IK\(_{Ca}\) channels.

In the experiments that led to the conclusion that the location of the rat mesenteric artery CaSR responsible for the vascular smooth muscle relaxation was on the neurons,\(^{6,36}\) the Krebs solution contained HEPES (20 to 50 mmol/L) which may have inhibited gap junction coupling.\(^{37}\) In addition, the spasmoden used would have raised intracellular [Ca\(^{2+}\)] and depolarized the myocytes. The associated extracellular “K\(^+\) cloud” generated by K\(^+\) efflux from myocyte K\(_{Ca}\) channels saturates the mechanisms normally stimulated by endothelial cell-derived K\(^+\).\(^{24}\) Thus, under their conditions, Bukoski and coworkers\(^{6,36}\) had probably dissected out any neuronal component of the CaSR-induced relaxant response. However, in the present study, calindol-induced smooth muscle hyperpolarization was abolished following endothelial cell denudation. Therefore, contrary to previous conclusions,\(^{6,38}\) it seems unlikely that any CaSR-induced relaxation results from the release of a neuron-derived hyperpolarizing factor.

Effect of Calindol on Vascular Tone

Paradoxically, activation of the CaSR did not relax phenylephrine-precontracted vessels. However, as previously described, this \(\alpha\)-1 adrenoceptor agonist probably generated K\(^+\) clouds by stimulating the opening of myocyte BK\(_{Ca}\) channels, thus preventing the hyperpolarizing action of K\(^+\) effluxing from endothelial cell K\(_{Ca}\) channels.\(^{38}\) This conclusion is supported by the relatively small calindol-induced hyperpolarizations in the presence of phenylephrine that were enhanced by subsequent BK\(_{Ca}\) blockade using iberiotoxin. Nevertheless, a recent investigation has demonstrated a relaxant effect of Ca\(^{2+}\) and Mg\(^{2+}\) on basal vasoconstriction in rat mesenteric arteries.\(^{29}\) Because both Ca\(^{2+}\) and Mg\(^{2+}\) activate CaSRs, their effect on basal tone, together with the favorable long-term effects of increasing dietary Ca\(^{2+}\) levels on blood pressure in low-renin hypertension in man,\(^{3,39}\) suggest that Ca\(^{2+}\), acting at the CaSR, may contribute to the setting of basal blood pressure levels.

Conclusions

Collectively, these findings strongly suggest that CaSR is present on the vascular endothelium of both rat and pig arteries. Furthermore, CaSR activation is linked to the opening of endothelial IK\(_{Ca}\) channels resulting in myocyte hyperpolarization, an action that may be facilitated by the close association of CaSR and IK\(_{Ca}\) proteins in the plasmalemma. In view of the increasing clinical use of calcimimetics like cinacalcet\(^{40}\) in diseases of the parathyroid gland, a greater understanding of the CaSR and its role in blood pressure control is essential.

Acknowledgments

G.E. and A.H.W. are funded by the British Heart Foundation (project grant no. PG/05/010/18272), M.A. by Aleppo University, Syria, and
C.P., in part, by a fellowship from the Association pour la Recherche sur le Cancer.

References


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_Circ Res._ 2005;97:391-398; originally published online July 21, 2005;
doi: 10.1161/01.RES.0000178787.59594.a0

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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