Evidence for a functional calcium-sensing receptor that modulates myogenic tone in rat subcutaneous small arteries

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Ohanian, Jacqueline, Kelly M. Gatfield, Donald T. Ward, and Vasken Ohanian. Evidence for a functional calcium-sensing receptor that modulates myogenic tone in rat subcutaneous small arteries. Am J Physiol Heart Circ Physiol 288: H1756–H1762, 2005. First published December 2, 2004; doi:10.1152/ajpheart.00739.2004.—Myogenic tone of small arteries is dependent on the presence of extracellular calcium (Ca2+), and, recently, a receptor that senses changes in Ca2+, the calcium-sensing receptor (CaR), has been detected in vascular tissue. We investigated whether the CaR is involved in the regulation of myogenic tone in rat subcutaneous small arteries. Immunoblot analysis using a monoclonal antibody against the CaR demonstrated its presence in rat subcutaneous arteries. To determine whether the CaR was functionally active, segments of artery (<250 μm internal diameter) mounted in a pressure myograph with an intraluminal pressure of 70 mm Hg were studied after the development of myogenic tone. Increasing Ca2+o concentration ([Ca2+]o) cumulatively from 0.5 to 10 mM induced an initial constriction (0.5–2 mM) followed by dilation (42 ± 5% loss of tone). The dose-dependent dilation was mimicked by other known CaR agonists including magnesium (1–10 mM) and the aminoglycosides neomycin (0.003–10 mM) and kanamycin (0.003–3 mM). PKC activation with the phorbol ester phorbol-12,13-dibutyrate (20 nM) inhibited the dilation induced by high [Ca2+]o, or neomycin, whereas inhibition of PKC with GF109203X (10 μM) increased the responses to Ca2+o or neomycin, consistent with the role of PKC as a negative regulator of the CaR. We conclude that rat subcutaneous arteries express a functionally active CaR that may be involved in the modulation of myogenic tone and hence the regulation of peripheral vascular resistance.

vessel smooth muscle; magnesium; aminoglycosides; contractility; peripheral vascular resistance

The extracellular calcium-sensing receptor (CaR) is a G protein-coupled receptor capable of sensing changes in extracellular calcium (Ca2+o) concentration ([Ca2+]o). Primarily, the CaR regulates calcium homeostasis by suppressing parathyroid hormone secretion and renal Ca2+ reabsorption. In addition, the CaR is also expressed in tissues and cell types outside the calcium homeostatic system including neural and cardiovascular tissues [reviewed by Brown and Macleod (8)]. However, the functional significance of the receptor in these noncalcium homeostatic tissues remains unclear.

Calcium is an important regulator of contractility. Elevated intracellular calcium (Ca2+cyt) levels stimulate smooth muscle contraction (17), and Ca2+o is required for the maintenance of myogenic tone (14), the intrinsic contractile activity of arterial wall smooth muscle that represents an important determinant of vascular resistance. The response to Ca2+o varies between artery type with some tissues showing contraction and others relaxation (3, 22, 35, 49–51), but, in either case, these studies demonstrate that arteries can sense changes in [Ca2+]o. In this regard, CaR expression has been reported in the perivascular sensory nerves of rat small mesenteric (10), basilar, renal, and coronary arteries (43). Increasing [Ca2+]o (1.25–5 mM) induced a concentration-dependent relaxation of rat small mesenteric arteries precontracted with norepinephrine that was dependent on intact perivascular nerves (10, 34, 44) and associated with the release of a neurally derived relaxing factor, possibly the cannabinoid anandamide (21).

The CaR also responds to other divergent cations such as extracellular Mg2+ (Mg2+o). Clinically, an Mg2+ infusion is a useful treatment in acute hypertensive conditions such as eclamptic fit, and yet its mechanism of action is poorly understood. One possible explanation is that the increase in Mg2+o concentration ([Mg2+]o) could be detected by the perivascular CaR with a resulting vasorelaxation. In addition, the CaR can also be stimulated by aminoglycoside antibiotics (AGAs) with a rank order of potency of neomycin > gentamicin > kanamycin (7, 30). Again, these polycationic drugs have also been shown to exhibit vasorelaxant effects (15). In each of these studies, the effects of the CaR agonists on vasorelaxation were studied using preconstricted vessels lacking internal pressure and without myogenic tone. Therefore, the primary aim of the present study was to investigate whether changes in [Ca2+]o, or treatment with other CaR agonists modulate myogenic tone in rat small subcutaneous arteries.

The CaR acts pleiotropically via Gq, Gi, and G12/13 proteins [reviewed by Ward (45)] to elicit various intracellular signaling pathways including activation of MAPKs (1, 13, 26, 31). Feedback regulation of signaling through the CaR is believed to occur and to be mediated via direct phosphorylation of the receptor by PKC (2, 24). Consequently, we tested the effects of specific inhibitors of these intracellular signaling pathways to clarify the intracellular pathways by which Ca2+o and other CaR agonists act in small arteries.

Materials and methods

Preparation of small arteries. Adult Sprague-Dawley rats (200–300 g) were used for all experiments. All procedures performed were carried out by trained personnel in accordance with institutional guidelines and the United Kingdom Animals (Scientific Procedures) Act of 1986. Animals were killed by stunning and cervical dislocation; ventral skin flaps were removed, and subcutaneous small arteries of internal diameter (ID) <300 μm, supplied by the axillary artery, were dissected in ice-cold physiological salt solution (PSS). Segments

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of the artery <250 μm ID without side branches were taken for contractile studies as detailed below.

**Immunodetection.** Tissue extracts were prepared as previously described (36, 37). Briefly, small arteries were homogenized in ice-cold homogenization buffer [0.15 M NaCl, 50 mM Tris (pH 7.2), 1% deoxycholate, 1% Triton-X-100, 0.1% SDS, 5% glycerol, and 1 mM dithiothreitol] containing protease and phosphatase inhibitors (Complete Mini-Tab, Roche). The homogenates were placed on a rotary mixer for 30 min at 4°C and centrifuged at 12,000 g for 5 min at 4°C. The supernatant was removed, an aliquot was taken for protein estimation (Bio-Rad), Laemmli sample buffer was added to the remainder, and the samples were heated to 95°C and stored at -20°C.

Tissue extracts (20 μg protein) were subjected to SDS-PAGE on 10% polyacrylamide gels as previously described (37). The resolved proteins were electrophoretically transferred to nitrocellulose membranes, blocked in 5% Teleostean gelatin-0.1% Tween-Tris-buffered saline and incubated with monoclonal CaR primary antibody. Signals were developed by horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence kit (Pierce) according to the manufacturer’s instructions.

**Small artery contraction.** Small artery contractile responses were determined by pressure myography. Segments of the small artery (<250 μm ID) were cannulated and mounted in a Living Systems pressure myograph as described previously (23, 36, 46). Arteries were mounted in a small fixed volume (3 ml) arteriograph, in which temperature is controlled with a heating element and the organ bath is gassed directly. After equilibration for 45 min at 37°C and 20-mmHg intraluminal pressure, the intraluminal pressure was raised to 70 mmHg, and the vessel was left to stabilize and develop myogenic tone for a minimum of 15 min. Arterial segments that did not develop myogenic tone were discarded. Criteria in this study for myogenic tone were as follows: constriction of >15% of maximum diameter in Ca2+-free PSS and distension followed by constriction to a step change in intraluminal pressure from 20 to 70 mmHg. HEPES-PSS was used for all incubations, and inhibitors and agonists were administered directly to the myograph chamber. Vessel diameter was continuously monitored using a Video Dimension Analyser (Living Systems). Data were collected and analyzed using a computerized data-acquisition system (Windaq). Intraluminal diameter was used as an index of the contractile response. To study the effect of Ca2+, vessels were mounted in the myograph in 0.5 mM CaCl2 and allowed to develop myogenic tone as described (36, 46). At the end of all experiments, endothelial integrity was tested by the addition of 1 μM acetylcholine; vessels that did not show substantial endothelium-dependent relaxation were not included in the study, and maximum vessel diameter was recorded in the presence of calcium-free HEPES-PSS.

**Solution and drugs.** PSS contained (in mM) 119 NaCl, 4.7 KCl, 25 NaHCO3, 1.17 MgSO4·7H2O, 1.18 KH2PO4, 0.026 K2EDTA, 5.5 glucose, and 1.6 CaCl2·2H2O; pH 7.4. HEPES-PSS contained (in mM) 119 NaCl, 4.7 KCl, 5 NaHCO3, 1.17 MgSO4·7H2O, 1.18 KH2PO4, 0.026 K2EDTA, 5.5 glucose, 1.6 CaCl2·2H2O, and 20 HEPES; pH 7.4, gassed with 5% CO2 in air. Stock solutions of the inhibitor and phorbol esters were prepared in DMSO as follows: PD98059 (15 mM), SB203580 and SB202470 (1 mM), GF109203X (10 mM), and PDBu and 4αPDD (20 μM). Stock solutions were diluted 1:1,000 in HEPES-PSS to achieve the correct final concentration; 0.1% DMSO in HEPES-PSS was used as a vehicle control. All other drugs were prepared in deionized distilled water.

**Data analysis.** All data were analyzed using GraphPad Prism software. Comparisons between two groups were analyzed by Student’s paired t-test. Repeated-measures ANOVA with Dunnett’s multiple-comparison test was used for comparisons between multiple groups. P < 0.05 was considered statistically significant.

**Materials.** SB203580, SB202474, PD98059, GF109203X, PDBu, and 4αPDD were purchased from Calbiochem. Neomycin, kanamycin, and all other chemicals, standards, and salts were obtained from Sigma Chemical. For SDS-PAGE, Protogel [30% (wt/vol) acrylamide] was from National Diagnostics, the Supersignal Chemiluminescent kit was from Pierce, and NitroPlus nitrocellulose membrane was purchased from Genmic Research Instruments. Monoclonal CaR antibody (1:5,000 dilution, raised to amino acids 214 –235 of the extracellular domain of the human parathyroid CaR) was supplied by NPS Pharmaceuticals (Salt Lake City, UT) and Drs. Allen Spiegel and Paul Goldsmith (Metabolic Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health).

**RESULTS**

**Immunodetection of CaR in rat subcutaneous artery.** Immunoblotting of rat subcutaneous artery homogenates with monoclonal CaR antibody revealed a single immunoreactive band at ~159 kDa (Fig. 1). This antibody also detected two bands of ~145 and 168 kDa in rat kidney homogenate (Fig. 1), corresponding to the glycosylated monomeric form of CaR expressed in renal tissue (47).

**Effect of increasing [Ca2+]o on contractility.** To test the effects of increasing [Ca2+]o, on contractility, segments of subcutaneous artery were mounted on canulae in a pressure myograph with an intraluminal pressure of 70 mmHg, under which conditions they developed myogenic tone. Once a stable level of tone was present, [Ca2+]o was increased cumulatively from 0.5 to 10 mM. A biphasic response was observed (Fig. 2A) such that increasing [Ca2+]o from 0.5 to 2 mM induced a small (~10%) vasodilation followed by progressive vasoconstriction due to a 3 to 10 mM. At the highest level of [Ca2+]o, tested, there was 42 ± 5% (n = 8) loss of myogenic tone. The effect was reversible as shown by the immediate return to basal lumen diameter on washout of 10 mM and replacement with...
1.6 mM [Ca$^{2+}$]$_o$ (Fig. 2A, top), demonstrating that the relaxation response to increasing [Ca$^{2+}$]$_o$ was not due to nonspecific loss of myogenic tone by the arterial preparation.

**Effect of Mg$^{2+}$ and aminoglycosides on contractility.** In addition to Ca$^{2+}$, the CaR is activated by other cations such as magnesium and aminoglycoside antibiotics (6, 7, 30, 32). Increasing [Mg$^{2+}$]$_o$ from 1.17 to 10 mM induced relaxation of subcutaneous arteries in a similar manner to Ca$^{2+}$ (Fig. 2B). At the highest level of [Mg$^{2+}$]$_o$ tested, there was a 44/4% ($\pm$ 3) loss of myogenic tone. Vessel segments also relaxed in a concentration-dependent manner to neomycin (3/2–10 mM) and kanamycin over the range of 3/2–3 mM (Fig. 3, A and B). At the highest concentration of neomycin tested, there was a 75/21% ($\pm$ 4) loss of myogenic tone and with kanamycin a 57/4% ($\pm$ 5) loss of myogenic tone. For all treatments, vessel diameter returned to basal levels after washout (data not shown).

**Regulation of vascular CaR by PKC.** Phosphorylation of the CaR by PKC inhibits its activity and is thought to be an important regulator of this receptor (2, 8, 18, 24). To investigate whether PKC was involved in the response to Ca$^{2+}$ or neomycin, arteries were stimulated with 10 mM Ca$^{2+}$ or 100 nM neomycin before and after incubation with phorbol ester or PKC inhibitor. Time control experiments in the presence of 0.1% DMSO showed that reproducible repetitive Ca$^{2+}$ and neomycin responses could be obtained (Table 1). However, although vasodilation to 10 mM Ca$^{2+}$ in the absence of any treatment was always observed, there was variation in the magnitude of the response (Fig. 4 and Table 1).

As there was no significant difference in vessel size or the level of tone between the treatment groups, this variability in the magnitude of response most probably reflects differences in sensitivity between batches of animals. Incubation of vessels with 20 mM PDBu (a PKC activator) inhibited the vasodilator response to 10 mM [Ca$^{2+}$]$_o$ and 100 nM neomycin (Fig. 4A). 4aPDD, a negative control for PDBu, had no effect on either Ca$^{2+}$- or neomycin-induced relaxation (Fig. 4B). Additionally, 10 μM GF109203X, a PKC inhibitor (29), potentiated the...
vasodilation induced by 10 mM [Ca\(^{2+}\)]\(_o\) and 100 \(\mu\)M neomycin (Fig. 4C). Accordingly, in agreement with data in other cell types (2, 24), PKC appears to be a negative regulator of the CaR in vascular tissue. It should be noted that the concentration of PDBu employed here (20 nM) did not affect basal myogenic tone (data not shown).

**MAPKs and the vascular CaR.** CaR activation results in MAPK activation in multiple cell types [reviewed by Ward (45)], and ERK1/2 and p38 MAPK have been implicated in CaR-stimulated parathyroid hormone-related peptide secretion (28, 42). We have demonstrated that in small arteries, ERK1/2 and p38 MAPKs are activated by G protein-coupled receptor agonists and are involved in the regulation of contractility (36, 46). Accordingly, we investigated whether these MAPKs were involved in the CaR agonist-stimulated vasodilation in rat subcutaneous arteries. Inhibition of ERK1/2 with 15 \(\mu\)M PD98059 (46) had no effect on either 10 mM [Ca\(^{2+}\)]\(_o\) or 100 \(\mu\)M neomycin-induced vasodilation (Table 1). Similarly, inhibition of p38 MAPK with 1 \(\mu\)M SB203580 (36) did not affect the response to either treatment (Table 1). These data show that neither ERK1/2 nor p38 MAPK is likely to couple CaR agonist responses to vasodilation in vascular tissue.

### DISCUSSION

Our data show that rat subcutaneous arteries respond to changes in [Ca\(^{2+}\)]\(_o\) from 0.5 to 10 mM with a biphasic response: constriction from 0.5 to 2 mM followed by relaxation at higher concentrations. Furthermore, these arteries contain a CaR and relax to magnesium and aminoglycoside antibiotics at concentrations known to stimulate the CaR, suggesting that the alteration in myogenic tone in response to [Ca\(^{2+}\)]\(_o\) could be mediated at least in part by the CaR. Increases in [Ca\(^{2+}\)]\(_o\) have long been known to induce relaxation of isolated smooth muscle preparations (3, 5, 49, 50). This apparently paradoxical effect of elevated [Ca\(^{2+}\)]\(_o\) has been attributed to its ability to depress membrane permeability or excitability (for a review, see Ref. 4). However, the recent discovery of the CaR in vascular tissues raises the possibility that such a receptor could play a role also in modulating contractility in response to changes in [Ca\(^{2+}\)]\(_o\).

The CaR has been found in a wide range of rat small arteries: mesenteric, basilar, renal, coronary (10, 43), and here in subcutaneous vessels, where we detected a 159-kDa CaR-reactive protein consistent with that observed in the rat kidney (47). Immunohistochemistry demonstrated that in mesenteric, basilar, renal, and coronary arteries, the receptor was localized to perivascular nerves (10, 43), although the expression in coronary arteries was low compared with the other arteries. Increasing [Ca\(^{2+}\)]\(_o\) from 1.5 to 5 mM caused a concentration-dependent relaxation of these arteries except for the coronaries, which showed very little response (43), suggesting that Ca\(^{2+}\)\(_o\) was activating a neuronally mediated vasodilatory response possibly through the CaR. Further studies with rat mesenteric arteries demonstrated that this most probably was the case (10, 34, 44) and that the neuronally released vasodilator substance was a cannibinoid (21). However, these studies were all performed using arteries preconstricted by agonists and under isometric conditions. In addition, the relative effects of other CaR agonists were not fully

### Table 1. Effect of inhibition of ERK1/2 and p38 MAPK on the vasodilator response to calcium and neomycin in rat subcutaneous arteries

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0.1% DMSO</th>
<th>15 (\mu)M PD98059</th>
<th>1 (\mu)M SB203580</th>
<th>1 (\mu)M SB202474</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM calcium</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Inhibitor + 10 mM calcium</td>
<td>6.4 (\pm) 1.7</td>
<td>12.6 (\pm) 1.95</td>
<td>18.3 (\pm) 2.04</td>
<td>23.8 (\pm) 2.08</td>
</tr>
<tr>
<td>100 (\mu)M Neomycin</td>
<td>6.5 (\pm) 1.2</td>
<td>7.4 (\pm) 1.59</td>
<td>5.4 (\pm) 0.34</td>
<td>6.2 (\pm) 0.45</td>
</tr>
<tr>
<td>Inhibitor + 100 (\mu)M neomycin</td>
<td>6.6 (\pm) 1.0</td>
<td>9.6 (\pm) 1.69</td>
<td>6.0 (\pm) 0.2</td>
<td>5.9 (\pm) 0.3</td>
</tr>
</tbody>
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Data are means \(\pm\) SE expressed as % changes from control; \(n\), no. of animals. Segments of small arteries were mounted in a pressure myograph, lumen diameter was monitored continuously, and inhibitors were administered as described in MATERIALS AND METHODS.
METHODS.

or 100 lumen diameter was monitored continuously as described in MATERIALS AND METH-ODS. Segments of small arteries were mounted in a pressure myograph, and the effect of PKC activation and inhibition on CaR-stimulated vasodilation was investigated. In our study, we used arteries under isobaric conditions with myogenic tone, a preparation that overcomes the need for pharmacological induction of constriction and more closely mimics physiological conditions.

To further investigate whether the vasodilation of rat subcutaneous arteries induced by Ca\textsuperscript{2+} was most likely due to CaR activation, we examined the effects of other known CaR agonists, including high [Mg\textsuperscript{2+}] and neomycin (6, 32, 48). These agents induced concentration-dependent relaxation similar to that observed with Ca\textsuperscript{2+}. Furthermore, in agreement with other studies (6, 7, 30), the aminoglycoside antibiotic neomycin was more potent a vasodilator than the divalent cations. That Mg\textsuperscript{2+} appears a more potent relaxant than Ca\textsuperscript{2+} is in contrast to the rank order of these cations as CaR agonists (6); however, this could result from the complexity of the response to Ca\textsuperscript{2+}. That is, whereas Mg\textsuperscript{2+} exerts a purely relaxant effect even at low concentrations, the effect of Ca\textsuperscript{2+} is biphasic, with constriction observed over the low range and relaxation observed at higher concentrations. We believe that the constriction component is unlikely to be CaR mediated and thus for Ca\textsuperscript{2+}, the putative CaR-induced relaxation is superimposed on top of an unrelated mechanism of Cu\textsuperscript{2+}-mediated constriction.

Activation of PKC with phorbol ester decreased the response to Ca\textsuperscript{2+} and neomycin, whereas PKC inhibition increased the response. Given that phosphorylation of the CaR by PKC is thought to decrease its sensitivity to [Ca\textsuperscript{2+}], (2, 24), these data further support a role for the CaR in Ca\textsuperscript{2+}-induced loss of myogenic tone. However, an alternative explanation could be that activation of PKC may have increased the sensitivity of the myofilaments to calcium (40), which would oppose the vasodilator response to CaR activation. However, activation or inhibition of PKC did not alter basal myogenic tone suggesting that in rat subcutaneous arteries, PKC does not play a major role in the regulation of tone.

Myogenic reactivity is an intrinsic property of smooth muscle cells where in response to stretch, the cells constrict (25). In arteries, this property results in a decrease in vessel diameter in response to increased intraluminal pressure and is an important regulator of peripheral vascular resistance (27). While myogenic tone is an intrinsic property of smooth muscle cells, the level of tone in an artery in vivo will reflect not only intraluminal pressure but also the balance between vasoconstrictors and vasodilators released by nerves and endothelial cells within the artery in response to the local environment. As all of these factors play a role in setting peripheral vascular resistance and hence blood pressure, it is important to understand the mechanisms by which changes in local environment may affect the level of tone. [Ca\textsuperscript{2+}] is normally maintained between 1 and 2 mM in mammals, and, at this level, the CaR is held in a partially activated state, which is normally maintained between 1 and 2 mM in mammals, and, at this level, the CaR is held in a partially activated state, which allows the receptor to sense small changes of [Ca\textsuperscript{2+}] against the relatively high basal concentrations (Ref. 7; for a review, see Ref. 8). Therefore, it is possible that CaRs within the artery wall could respond to changes in local calcium levels. However, the circumstances under which such changes might occur in the vasculature are not clear. It has been shown that stimulation of cells with calcium-mobilizing agonists that raise Ca\textsuperscript{2+} concentration results in extrusion of calcium into the extracellular space by plasma membrane ATPase (11, 12, 33). Recently, Hofer et al. (19, 20) showed that calcium extruded from agonist-stimulated “donor”...
cells could act in a paracrine fashion, stimulating CaRs on adjacent cells when diffusion from the interstitial space was restricted. This raises the possibility that a physiological role for vascular CaRs could be as a negative feedback system, such that stimulation of smooth muscle with vasoconstrictors that increase Ca\(^{2+}\) concentration could result in a localized increase in [Ca\(^{2+}\)]o of sufficient magnitude to activate CaRs inducing a vasodilator response to counterbalance the initial stimulus. However, it is also possible that at low [Ca\(^{2+}\)]o, increases in extracellular calcium could lead to increased myogenic tone driven by an influx of extracellular calcium, which could lower [Ca\(^{2+}\)]o in the vicinity of the CaR so inactivating it and further enhancing tone. Indeed, such a mechanism may underlie the small increase in myogenic tone that we observed over the range of 0.5–3 mM [Ca\(^{2+}\)]o. Further rigorous studies are required to investigate this possibility. It should also be noted that while the primary agonist of the CaR is Ca\(^{2+}\), other potential endogenous agonists of the receptor include polyamines such as spermine and spermidine (39). Thus CaR-mediated vasodilation may occur physiologically, even in the absence of changes in [Ca\(^{2+}\)]o. To prove conclusively whether the CaR is involved in the physiological modulation of myogenic tone, it will be necessary to employ the CaR-agonizing “calcilytic” agents and to study the effect of high [Ca\(^{2+}\)]o on arteries from transgenic mice lacking the CaR gene.

In conclusion, there is extensive clinical and epidemiological evidence of a link between the diverent cations Ca\(^{2+}\) and Mg\(^{2+}\) and the relief of certain hypertensive states (16, 38, 41); however, the mechanism(s) of action of these ions has remained unexplained. Following the initial studies by Bukoski et al. (10) (reviewed in Ref. 9) demonstrating Ca\(^{2+}\)-induced vasorelaxation of preconstricted arteries, in which they proposed a role for the CaR, we here demonstrate for the first time that increased [Ca\(^{2+}\)]o, [Mg\(^{2+}\)]o, and other CaR agonists inhibit myogenic tone in vessels studied at physiological intraluminal pressure.

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