Calcification is associated with loss of functional calcium-sensing receptor in vascular smooth muscle cells

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Aims Vascular calcification (VC) is highly correlated with increased morbidity and mortality in advanced chronic kidney disease (CKD) patients. Allosteric modulation of the calcium-sensing receptor (CaR) by calcimimetics inhibits VC in animal models of advanced CKD. Here, we investigated the expression of the CaR in the vasculature and tested the ability of calcimimetics to prevent vascular smooth muscle cell (VSMC) calcification in vitro.

Methods and results Immunohistochemical staining demonstrated that CaR protein is present in VSMC in normal, non-calcified human arteries. In contrast, low levels of CaR immunoreactivity were detected in atherosclerotic, calcified arteries. Immunofluorescence and immunoblotting revealed that CaR protein was also expressed by human and bovine VSMC in vitro. Acute stimulation of VSMC with increased Ca2+ stimulated extracellular signal-regulated kinase (ERK1/2) phosphorylation, suggesting that the VSMC CaR is functional. VSMC CaR expression decreased when these cells deposited a mineralized matrix or following 24 h incubation in mineralization medium with increased (i.e. 1.8 or 2.5 mM) Ca2+. Culturing VSMC in mineralization medium containing 1.8 and 2.5 mM Ca2+ or with the membrane-impermeant CaR agonist Gd3+ enhanced mineral deposition compared with that observed in 1.2 mM Ca2+. Over-expression of dominant-negative (R185Q) CaR enhanced, whereas the calcimimetic R-568 attenuated, VSMC mineral deposition.

Conclusion These results demonstrate that: (i) VSMCs express a functional CaR; (ii) a reduction in CaR expression is associated with increased mineralization in vivo and in vitro; (iii) calcimimetics decrease mineral deposition by VSMC. These data suggest that calcimimetics may inhibit the development of VC in CKD patients.

Keywords
Calcification; Extracellular calcium-sensing receptor, CaR; Vascular smooth muscle cells; Secondary hyperparathyroidism; Calcimimetics

1. Introduction
Atherosclerosis and vascular calcification (VC) are major complications of advanced chronic kidney disease (CKD). Localized within the thickened intima and media of blood vessel walls, calcification can increase the risk of plaque rupture,1 increase pulse-wave velocity and left ventricular hypertrophy,2 and is associated with a 20–30-fold increase in cardiovascular mortality.3 It is well established that VC is an active, cell-regulated process, with many similarities to bone formation.1,4,5 Indeed, recent studies have shown that vascular smooth muscle cells (VSMC) can lose the expression of α-smooth muscle actin, and begin to express osteogenic markers and deposit a mineralized bone-like matrix.1,4–7 However, the trigger(s) for the phenotypic transdifferentiation of VSMC into osteoblast-like cells in CKD patients is still only partly understood.

The initial observations that hyperphosphataemia and high calcium–phosphorus product (Ca×Pi) are independently associated with increased cardiovascular risk and mortality in haemodialysis patients8 suggested that phosphorus could induce VSMC transdifferentiation. Indeed, there is evidence...
that hyperphosphataemia compatible with levels seen in CKD patients induces VSMC phenotypic changes and calcification in vitro,\textsuperscript{9,10} effects which appear to be mediated by Pit-1 and osterix.\textsuperscript{11,12} However, experiments performed using serum from uremic patients show that calcification occurs irrespectively of phosphate levels,\textsuperscript{7,13,14} suggesting that other factors in addition to hyperphosphataemia are required to induce mineralization in the vasculature.

Disordered calcium homeostasis has also been associated with increased cardiovascular-related deaths.\textsuperscript{15} Furthermore, randomized controlled trials have demonstrated that patients treated with calcium-based phosphate binders exhibit accelerated arterial calcification compared with patients treated with calcium-free phosphate binders (i.e. sevelamer hydrochloride).\textsuperscript{16,17} The extracellular calcium-sensing receptor, CaR, plays a key role in the regulation of extracellular calcium homeostasis.\textsuperscript{18,19} Cinacalcet, a potent and selective type II calcimimetic, is an allosteric modulator of the CaR which has been approved for treatment of hyperparathyroidism secondary to renal failure. Studies in these patients have shown that cinacalcet controls parathyroid hormone (PTH) secretion and reduces Ca\textsuperscript{2+}, serum Ca, and serum Pi levels.\textsuperscript{20,21} Interestingly, recent evidence suggests that calcimimetic therapy might also induce cardiovascular events in rat models of secondary hyperparathyroidism and renal failure\textsuperscript{22–24} and in subjects with advanced CKD.\textsuperscript{25} However, whether the CaR plays a role in regulating VC and whether calcimimetics exert their effects directly by acting on VSMC, or indirectly via suppression of PTH secretion, is unknown. Indeed, the presence of the CaR in VSMC is controversial; two groups have reported that CaR protein and mRNA are expressed by VSMC in vitro,\textsuperscript{26,27} whereas two other groups failed to detect CaR transcripts in these cells.\textsuperscript{28,29} Therefore, this study aimed to: (i) determine whether the CaR is expressed in normal and calcified human arteries, (ii) establish whether the CaR regulates mineral deposition by VSMC in vitro, and (iii) examine whether calcimimetics reduce calcification by directly targeting a CaR in VSMC.

2. Methods

Detailed experimental protocols are in the online supplement.

2.1 Reagents

Primary antibodies were: mouse anti-CaR monoclonal antibody (MA1-934; Affinity BioReagents, Cambridge, UK), rabbit anti-CaR polyclonal antibody raised against amino acids 214–230 of rat kidney CaR,\textsuperscript{30} rabbit anti-phospho-p44/42 mitogen-activated protein kinase monoclonal antibody (4377; New England Biolabs, Hitchin, UK), rabbit anti-pan extracellular signal-regulated kinase (ERK) polyclonal antibody (V1141; Promega, Southampton, UK). Secondary antibodies were conjugated to horseradish peroxidase or fluorescein isothiocyanate (FITC, DakoCytomation, Glostrup, Denmark). Unless otherwise stated, other reagents were from Sigma (Poole, UK).

2.2 Immunohistochemistry

Atheromatous tibial artery specimens from 11 lower limb amputations and normal internal mammary artery (IMA) specimens from six patients were used for detection of the CaR by immunohistochemistry. Approval from the Local Research Ethics Committee was granted for human tissue use, and the procedures used were in accordance with institutional guidelines (COREC Ref No: 05/Q1402/17). The investigation conforms to the principles outlined in the Declaration of Helsinki.

2.3 Cell culture

VSMCs were plated at 1 × 10\textsuperscript{4} cells/cm\textsuperscript{2} and routinely maintained in Dulbecco’s Modified Eagle Medium (DMEM) containing either 5% foetal calf serum (FCS) (5% FCS-DMEM) or 10% FCS (10% FCS-DMEM) and either 1.2 mM Ca\textsuperscript{2+} or 1.8 mM Ca\textsuperscript{2+}, until confluency. At this time, cells were incubated in medium containing 1.2, 1.8 or 2.5 mM Ca\textsuperscript{2+} or 10 or 50 μM Gd\textsuperscript{3+}, in the presence or absence of 5 mM β-glycerophosphate\textsuperscript{31} and/or the calcimimetic R-568 (1 or 10 nM). Full details of culture conditions used in each experiment are specified in the relevant figure legends. Experiments were performed at least three times with triplicate cultures used in every experiment, unless stated otherwise.

2.4 Adenoviral infection

VSMCs were infected at 80% confluency (day 4) using an empty vector recombinant adenovirus (RAd) (Ad5/BglII; Control virus) or a dominant-negative CaR RAd (pacAd5CMV/dnCaR), encoding the full-length human CaR with a R185Q mutation\textsuperscript{22} using multiplicity of infections = 150, as described.\textsuperscript{31} Cells were incubated in medium containing 1.8 mM Ca\textsuperscript{2+} and 5 mM β-glycerophosphate 48 h later and over-expression of the transgene was maintained by re-infection on day 8.

2.5 Immunofluorescence

Immunofluorescence staining was performed using anti-CaR monoclonal antibody and rabbit anti-mouse FITC-conjugated immunoglobulins (IgG). Controls included pre-incubating the antibody with an excess of immunizing peptide, and incubating cells with mouse IgG. Nuclei were detected using 4’,6-diamidino-2-phenylindole.

2.6 Extracellular signal-regulated kinase activation assay

VSMCs were incubated in 5% FCS-DMEM until ~80% confluent, and ERK activation in response to Ca\textsuperscript{2+} (0.5 or 5 mM) was assessed.\textsuperscript{30}

2.7 Immunoblotting

Microsomal membranes were isolated by differential centrifugation\textsuperscript{30} and whole cell lysates were prepared using lysis buffer.\textsuperscript{31} Samples (10–20 μg) were analysed for CaR, p-ERK (phosphorylated ERK), and total ERK expression by immunoblotting.\textsuperscript{30}

2.8 Mineralization assays

Mineralization assays were performed as described.\textsuperscript{31} Data are shown as mean ± SEM of at least three experiments performed in triplicate. Groups were compared using one-way ANOVA; P < 0.05 was considered statistically significant.

3. Results

3.1 Calcium-sensing receptor is expressed by vascular smooth muscle cells in vivo and in vitro

A recent study has shown that CaR mRNA and protein are expressed in arteries from normal subjects and that levels are lower in patients with end-stage renal disease.\textsuperscript{26} Furthermore, immunohistochemical staining demonstrated that low CaR expression was detected at sites of calcification in an epigastric artery from one patient.\textsuperscript{26} Therefore, we used two different anti-CaR antibodies to examine CaR expression in a panel of normal and calcified atheromatous human arteries. These studies demonstrate that high levels...
of CaR immunoreactivity are present in VSMC in the media of normal arteries (Figure 1A, iii). In marked contrast, only weak immunoreactivity was detected in cells adjacent to calcified regions of atherosclerotic tibial arteries (Figure 1B, iii). The specificity of the antibody was confirmed by the lack of staining detected when the antibody was pre-incubated with the immunizing peptide prior to immunostaining (Figure 1A, ii). In addition, no staining was observed in mouse IgG Controls (Figure 1B, ii). These results were a consistent finding in six IMA and 11 human tibial artery specimens examined.

Next, we examined the expression of CaR by both human and bovine VSMC. In these studies, we used primary cells which had been isolated and maintained in the presence of physiological concentrations of Ca\(^{2+}\) (1.2 mM), rather than the 1.8 mM Ca\(^{2+}\) which is present in regular culture medium and which had been used previously by other groups. Immunofluorescence staining demonstrated that bovine (Figure 2A, i) and human (see Supplementary material online, Figure S1) VSMC express the CaR. No immunostaining was observed when the antibody was pre-incubated with the immunizing peptide (Figure 2A, ii). Immunoblotting of microsomal membrane preparations and whole cell lysates using two different CaR antibodies also confirmed the presence of the CaR in both bovine and human VSMC (Figure 2B and C). CaR immunoreactivity in VSMC was comparable with that observed in human embryonic kidney (HEK293) cells induced to stably express the human CaR and with that observed previously.\(^{30}\) The specificity of the CaR antibody was confirmed by the absence of immunoreactive bands when the antibody was incubated with the immunizing peptide prior to immunoblotting (Figure 2B).

Stimulation of the CaR activates the ERK1/2.\(^{26,27,30}\) Therefore, to investigate whether the VSMC CaR is functional, we measured ERK1/2 phosphorylation levels as a consequence of CaR activation. Immunoblotting using anti-phosphoERK and anti-pan ERK antibodies demonstrated that ERK phosphorylation was significantly increased when VSMCs were incubated for 5 min with 5 mM Ca\(^{2+}\) when compared with 0.5 mM Ca\(^{2+}\) alone (Figure 2D), demonstrating that acute exposure to CaR agonists stimulates ERK phosphorylation in VSMC.

### 3.2 Expression of the calcium-sensing receptor is down-regulated as vascular smooth muscle cells deposit a mineralized matrix

When maintained to post-confluence in the absence of β-glycerophosphate, some populations of VSMC form multicellular nodules, the matrix of which becomes mineralized.\(^4\) Therefore, to determine whether the expression of the CaR is modulated during the growth and osteogenic differentiation of VSMC independently of increased levels of phosphate, cells were incubated in growth medium containing 1.2 mM Ca\(^{2+}\) and cell lysates were harvested on days 4 (confluence), 11 (nodules beginning to form), 19 (large multicellular nodules formed), and 38 (nodules had become mineralized) and analysed for CaR expression (Figure 3). Immunoblotting shows that CaR protein expression decreases as VSMC deposit a mineralized matrix (Figure 3B, upper panel), consistent with the immunohistochemical findings in human tissue. Equal protein loading in each track was confirmed by staining with India ink (Figure 3B, lower panel).

### 3.3 Increasing extracellular Ca\(^{2+}\) concentration or exposure to Gd\(^{3+}\) down-regulates calcium-sensing receptor expression and enhances mineralization

To determine whether short-term treatment with CaR agonists modulates CaR expression, confluent cells were incubated with concentrations of Ca\(^{2+}\) mimicking physiologic serum-free ionized calcium levels (i.e., 1.2 mM) and calcium concentrations similar to those observed during stage 5 CKD (1.8 and 2.5 mM).\(^{33}\) These studies were also conducted in the presence and absence of β-glycerophosphate, which has previously been shown to induce VSMC calcification following long-term treatment.\(^{14,28,31}\) Cell lysates were prepared after 24 h and analysed for CaR protein expression by immunoblotting (Figure 4A). Mineralization was not detected in any of the cultures at this time point (data not shown). The results demonstrate that incubating VSMC in the presence of 1.8 and 2.5 mM Ca\(^{2+}\) decreases CaR expression compared with that observed in 1.2 mM Ca\(^{2+}\) (Figure 4A). Interestingly, the decrease in CaR expression was not observed if β-glycerophosphate was omitted from the culture medium during this 24 h period (see Supplementary material online, Figure S2).

To determine whether chronic incubation with CaR agonists induces the deposition of a mineralized matrix by VSMCs, confluent cells were incubated with 1.2, 1.8 or 2.5 mM Ca\(^{2+}\) in the presence of 5 mM β-glycerophosphate for 10 days, and mineral deposition was assessed by alizarin red staining (Figure 4B, i–iii) and by measuring \(^{45}\)Ca incorporation into the cell layer (Figure 4C). In addition, to discriminate between true CaR-mediated effects and possible effects of calcium ions entering the cell through ion channels, the membrane-impermeant CaR agonist Gd\(^{3+}\) was used at 10 and 50 μM, in the presence of 1.2 mM Ca\(^{2+}\) (Figure 4B, iv and v). These results demonstrate that both Ca\(^{2+}\) and Gd\(^{3+}\) significantly increase mineral deposition in a dose-dependent manner (P < 0.001; n ≥ 3) by VSMC (Figure 4B and C).
3.4 Over-expression of dominant-negative calcium-sensing receptor enhances mineral deposition by vascular smooth muscle cells

To determine whether a functional CaR regulates matrix mineralization, VSMCs were induced to over-express a dominant-negative version of this receptor (dnCaR) using RAds (pacAd5CMV/dnCaR) and were then incubated in medium containing 1.8 mM Ca$^{2+}$ and 5 mM β-glycerophosphate. An empty vector RAd (Ad5/BglII) was used as a Control. This approach was chosen in preference to siRNA as the R185Q construct exerts its dominant-negative effect by dimerizing with wild-type CaR thereby reducing its functionality. Furthermore, high levels of transgene expression, and hence non-functional CaR, can be maintained using RAds throughout.

**Figure 2** Calcium-sensing receptor (CaR) is expressed by vascular smooth muscle cells (VSMC) in vitro and is functional. (A) Bovine VSMC were cultured in 5% FCS-DMEM containing 1.2 mM Ca$^{2+}$ and incubated with (i) CaR monoclonal antibody and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies, or (ii) CaR monoclonal antibody pre-incubated with immunizing peptide and FITC-conjugated secondary antibodies (Control) as detailed in Methods. Nuclei were stained blue with 4',6-diamidino-2-phenylindole. Bar: 50 μm. (B) Microsomal membranes were prepared from bovine VSMC (BVSMC) cultured to ~90% confluence in 5% FCS-DMEM containing 1.2 mM Ca$^{2+}$, separated by SDS–PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and analysed by immunoblotting using anti-CaR monoclonal antibody (left panel) or anti-CaR monoclonal antibody pre-incubated with the immunizing peptide (right panel). Cell extracts prepared from HEK293 stably transfected with the human CaR were used as a positive Control (CaR-HEK). (C) Cell lysates prepared from bovine and human VSMC cultured as previously mentioned were analysed by immunoblotting using anti-CaR monoclonal antibody. The migration positions of molecular weight markers are also shown. (D) VSMCs were cultured in 5% FCS-DMEM containing 1.2 mM Ca$^{2+}$ until ~80% confluency when they were incubated with serum-free DMEM containing 0.5 mM Ca$^{2+}$ or 5 mM Ca$^{2+}$ for 5 min at 37°C, lysed and analysed by Immunoblotting for phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2) and total ERK. Densitometric analysis of the data is also shown (*P < 0.05).
Figure 3 Expression of the calcium-sensing receptor (CaR) during nodule formation. Bovine vascular smooth muscle cells (VSMC) were cultured in 5% FCS-DMEM containing 1.2 mM Ca\(^{2+}\). On days 4, 11, 19, and 38, cells were lysed and analysed by immunoblotting using an anti-CaR monoclonal antibody. (A) Phase contrast micrographs of VSMC at each time point. Bar: 500 \(\mu\)m. (B) Western blot analysis of CaR in cell lysates collected on days 4, 11, 19 and 38 (upper panel). India ink staining shows protein loading (lower panel). Results are representative of two independent experiments.

3.5 Calcimimetic R-568 suppresses vascular smooth muscle cell mineralization

To further examine the effects of the CaR on VC, cells were incubated in the presence of the calcimimetic R-568 and the effects on mineralization determined. In these experiments, confluent VSMCs were incubated in medium containing 1.8 mM Ca\(^{2+}\) and 5 mM \(\beta\)-glycerophosphate in the presence of R-568 at concentrations that specifically target the CaR (i.e. 1 and 10 nM) for 12 days. Vehicle was added to Control cultures. Mineralization was assessed by alizarin red staining (Figure 5C) and by measuring \(^{45}\)Ca incorporation (Figure 5D). The results show that 1 and 10 nM R-568 significantly diminishes mineral deposition by VSMCs (\(P < 0.001, n = 3\)). Intriguingly, when higher concentrations of R-568 were used, the response was more variable. Furthermore, if the duration of the experiments was extended, some mineral deposition was detected in the R-568-treated cells (data not shown), indicating that calcimimetics delay the onset of calcification, rather than inhibiting it completely.

4. Discussion

Advanced CKD is commonly associated with increased risk of VC, which in turn leads to a substantial increase in cardiovascular morbidity and mortality. We now demonstrate for the first time that loss of CaR expression or function is associated with a significant increase in VC, and that calcimimetics can delay mineralization in vitro.

Previous studies have identified CaR in a wide range of rat small arteries and subcutaneous vessels, where the receptor was localized mainly to perivascular nerves or endothelium. However, the detection of CaR in VSMC is more controversial. Several groups report CaR protein and mRNA expression in VSMC, whereas others report absence of its transcripts or the presence of a receptor which is functionally related to, but molecularly distinct from, CaR. In this study, we demonstrate the expression of CaR protein in human arteries and in human and bovine VSMCs cultured in the presence of normal physiological concentrations of Ca\(^{2+}\) (1.2 mM) in vitro. Strikingly, we also demonstrate for the first time that CaR expression is markedly reduced in calcified areas of atherosclerotic tibial arteries, and in mineralized VSMC. The reduction in CaR expression in mineralized cultures of VSMC occurs in the absence of factors known to induce mineralization, namely supraphysiological levels of Ca\(^{2+}\) and/or phosphate, and is markedly accelerated when experimental conditions are manipulated to mimic those observed in hypercalcaemic CKD patients by increasing Ca\(^{2+}\) levels to 1.8–2.5 mM and by adding 5 mM \(\beta\)-glycerophosphate to the culture medium. Interestingly, as little as 24 h exposure to 1.8–2.5 mM Ca\(^{2+}\) induces loss of CaR expression in the presence of \(\beta\)-glycerophosphate whereas no down-regulation was detected in its absence. Notably, this down-regulation in CaR expression occurred before any increase in mineralization was noted in the cultures. Thus, culture conditions used to mimic pathological, advanced CKD-like events (i.e. standard DMEM medium containing 1.8 mM Ca\(^{2+}\) and/or phosphate) could induce down-regulation of CaR expression when compared with physiological Ca\(^{2+}\) and Pi levels which, therefore, could account for the inconsistent detection of CaR expression in VSMCs as in previous studies.

Changes in CaR expression levels have been previously reported in other tissues as the consequence of dietary manipulations in Pi or plasma PTH levels. However, to our knowledge this is the first demonstration that Ca\(^{2+}\) decreases CaR expression. This result is particularly relevant in the vasculature as it suggests that conditions associated with advanced CKD (e.g. hypercalcaemia and hyperphosphataemia) exacerbate loss of CaR by VSMC and induce mineralization, thereby increasing the risk of cardiovascular morbidity and mortality.

Our study confirms that the VSMC CaR is biologically active, as demonstrated by phosphorylation of ERK1/2 following receptor activation by Ca\(^{2+}\). Other authors have shown ERK1/2 activation in VSMC in the presence of high (3–5 mM) Ca\(^{2+}\) or in response to known CaR agonists including the aminoglycoside antibiotics gentamicin and neomycin. In this latter study, ERK1/2 activation was prevented by the MEK1 inhibitor, PD-98059 and by CaR knockdown with siRNA. Together these results demonstrate that the VSMC CaR exhibits a pharmacological profile.
overlapping that described for native tissues, such as the parathyroid and the kidney.

The current study also shows that incubation of VSMC with the orthosteric CaR agonists, Ca\(^{2+}\) or Gd\(^{3+}\), significantly induces the deposition of a mineralized matrix by these cells. These results are consistent with previous studies demonstrating that high extracellular Ca\(^{2+}\) induces matrix mineralization by VSMC in the presence of Pi.\(^{10,40}\) Importantly, the demonstration herein that matrix mineralization is also induced by the membrane-impermeant CaR agonist Gd\(^{3+}\) is novel and suggests that the effects on mineralization are likely to be mediated by the VSMC CaR rather than by channel-mediated Ca\(^{2+}\) entry. Interestingly, previous studies have shown that, like Ca\(^{2+}\), Gd\(^{3+}\) stimulates the transcription of matrix Gla protein by rat VSMC.\(^{29}\) Whether CaR agonists also induce the expression or activation of other osteogenic markers by these cells, such as Cbfa-1, alkaline phosphatase, and osteocalcin is an intriguing possibility which will be examined in future studies.

The demonstration that CaR expression decreases in vivo at sites of calcification in human arteries and in vitro as VSMC mineralize, and that Ca\(^{2+}\) is a potent
regulator of the VSMC CaR, strongly suggest that a functional VSMC CaR is directly involved in preventing mineral deposition by these cells. This hypothesis is supported by our experiments using dominant-negative CaR, in which a significant increase in mineralization was demonstrated in dnCaR-infected VSMCs compared with empty vector-infected controls. Since the R185Q construct exerts its dominant-negative effects by dimerizing with wild-type CaR, rather than by suppressing its expression, these results strongly suggest that a functional CaR is necessary to maintain a VSMC phenotype and/or to keep mineralization suppressed.

Figure 5  Over-expression of dominant negative calcium-sensing receptor (dnCaR) enhances, whereas the calcimimetic R-568 attenuates mineralization. (A, B) Vascular smooth muscle cells (VSMCs) were cultured in 10% FCS-DMEM containing 1.8 mM Ca$^{2+}$ until 80% confluence when they were infected with dnCaR recombinant adenovirus (pacAdSCMV/dnCaR) or empty vector (Ad5/BglII) recombinant adenovirus using MOI (multiplicity of infection) = 150. After 48 h, 5 mM β-glycerophosphate was added to the culture medium and cells were re-infected after 4 days to maintain high transgene expression. VSMCs were stained with alizarin red 5 days after the addition of β-glycerophosphate to the medium. Data are representative of three separate experiments with triplicate flasks in each experiment. (A) Phase-contrast micrographs of cells treated with (i) Control virus (Ad5/BglII) or (ii) pacAdSCMV/dnCaR and stained with alizarin red (bar: 500 μm). (B) Quantification of mineralization was performed by eluting alizarin red stain and normalizing per mg protein (*P < 0.01). (C, D) VSMCs were cultured in 5% FCS-DMEM containing 1.2 mM Ca$^{2+}$. At confluence, the medium was changed to 5% FCS-DMEM containing 1.8 mM Ca$^{2+}$ and 5 mM β-glycerophosphate and either 0 (vehicle control), 1 or 10 nM R-568. On day 12, mineralization was assessed by alizarin red staining and by measuring $^{45}$Ca incorporation. (C) Phase-contrast micrographs of cells treated with (i) vehicle, (ii) 1 nM R-568, and (iii) 10 nM R-568 and stained with alizarin red (bar: 500 μm). (D) $^{45}$Ca accumulation by VSMCs cultured as in (C). Radioactivity incorporated into the cell layer from day 10 to day 12 was quantified and is shown as mean ± SEM of a representative experiment performed in triplicate (*P < 0.001).
Importantly, from a clinical standpoint, our study also demonstrates that the calcimimetic R-568 significantly delays mineral deposition by VSMC, which is consistent with the decreased VC detected in calcitriol-treated uremic rats treated with calcimimetics.22–24 There is strong evidence that CaR41–43 and calcimimetics bind at different sites on the CaR,41–43 suggesting that the effects observed in our study are mediated by the CaR and are not due to competition between these two CaR agonists. Indeed, Huang and Breitwieser44 recently demonstrated that calcimimetics stabilize and increase cell surface expression of the CaR in HEK293 cells induced to stably express this receptor. Therefore, we hypothesize that R-568 delays mineralization by increasing CaR expression at the plasma membrane. Further evidence in support of this hypothesis is provided by studies demonstrating that calcimimetics directly increase CaR expression in the parathyroid glands in rat models of secondary hyperparathyroidism45 and in the intima of sham-operated and uremic rats24 compared with controls. This upregulation/stabilization of CaR at the plasma membrane could, in turn, activate protective, anti-osteo-genic mechanisms, suppressing osteo-genic transdifferentiation thereby maintaining a smooth muscle cell-like phenotype; this possibility will be examined in future studies. Of course, we cannot rule out the possibility that calcimimetics sensitize the CaR to Ca2+, which, in turn, could help maintain a smooth muscle-like phenotype and prevent mineralization. Indeed, cinacalcet suppresses PTH release by human parathyroid cells with pathologically reduced CaR levels.46 Finally, orthosteric and allosteric agonists of the CaR can activate different signalling pathways downstream of CaR activation.47,48 One or more of these possibilities could co-exist and we are currently actively investigating the mechanism(s) of action of calcimimetics in VSMC.

In conclusion, our study demonstrates that the CaR is expressed by VSMC and that conditions associated with advanced CKD, such as hypercalcaemia and hyperphosphataemia, induce the loss of functional CaR molecules in VSMCs and this loss appears to drive mineral deposition by VSMCs. By achieving the disease treatment targets set by the National Kidney Foundation (NKF-KDOQI™), calcimimetics may, therefore, lower cardiovascular risks and risks associated with CKD9 and our studies suggest that that one way calcimimetics may do this is by directly targeting the VSMC CaR. Interestingly, recent reports have also suggested that calcimimetics can have beneficial effects on calciphylaxis in rodents24 and in humans.30 Therefore, the use of calcimimetics in advanced CKD patients, and perhaps in human subjects with other types of ectopic calcification, is an exciting possibility which warrants further investigation.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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