Extracellular calcium-sensing receptor mediated signalling is involved in human vascular smooth muscle cell proliferation and apoptosis

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Abstract. Calcium-sensing receptor (CaSR) plays key role in vascular calcification in patients with chronic kidney disease (CKD). We investigated the role of CaSR in regulating smooth muscle cell (SMC) proliferation and apoptosis. Incubation with 300μM neomycin (CaSR agonist) resulted in 7.5-fold ($p<0.05$) increase in ERK1,2 phosphorylation. It was reduced ($p<0.01$) by 10μM PD98059 (MEK1 inhibitor), indicating that CaSR agonist-induced effects were mediated via MEK1/ERK1,2 pathway. ERK1,2 phosphorylation was abolished by 5μM U73122 (PLC inhibitor), indicating that PLC signalling was crucial for MEK1/ERK1,2 activation. Confirming PLC activation, inositol triphosphate (IP3) production was increased by neomycin/gentamycin ($p<0.05$) and reduced by U73122. To confirm that ERK1,2 and PLC signalling were mediated via CaSR, HAoSMC were transfected with CaSR siRNA. CaSR knockdown resulted in lower ERK1,2 neomycin response and IP3 production ($p<0.01$). Neomycin increased HAoSMC proliferation >3-fold, which was reduced in CaSR knockdown cells ($p<0.01$) and further inhibited by PD98059 and U73122 ($p<0.05$). Apoptosis was not affected by neomycin treatment. U73122 produced 3.5-fold increase in HAoSMC apoptosis, which was further increased by CaSR knockdown (5-fold, $p<0.05$). In conclusion, stimulation of CaSR leads to activation of MEK1/ERK1,2 and PLC pathways and up-regulation of cell proliferation. CaSR-mediated PLC activation is important for SMC survival and protection against apoptosis.

Key words: calcium-sensing receptor, vascular smooth muscle cell, ERK1,2, PLC, calcification, chronic kidney disease

Introduction

The extracellular CaSR is a cell surface protein belonging to the family C of the superfamily of seven-transmembrane receptors, also known as G protein-coupled receptors [1]. It consists of three structural domains – a large extracellular domain in the N-terminal portion of the receptor, a seven-transmembrane region that anchors it in the plasma membrane and an intracellular carboxyl terminal tail with regulatory protein kinase phosphorylation sites [2]. The CaSR was initially cloned and characterised in bovine parathyroid cells where it responds to changes in serum calcium concentration by regulating the synthesis and secretion of parathyroid hormone [3].

Binding of extracellular Ca^{2+} or other CaSR agonists to the extracellular domain of the receptor triggers a number of intracellular signalling systems including activation of Gq proteins, stimulation of phospholipase C leading to the generation of second messengers (diacylglycerol and IP3) and intracellular Ca^{2+} release, inhibition of adenylate cyclase activity resulting in suppression of intracellular cyclic AMP, activation of PKC and MAPK – p38, JNK/SAPK and MEK1/ERK1,2 [4]. Therefore, it is not surprising that CaSR is involved in the regulation of such diverse processes as hormone secretion, gene expression, ion channel activity, modulation of inflammation, proliferation, differentiation and apoptosis and [4,5,6]. The CaSR is present mainly in tissues involved in systemic calcium homeostasis – parathyroid, thyroid, kidney, bone and gastrointestinal tract [7]. However, a number of recent studies have shown that the receptor is widely expressed in cells and tissues, which are not directly involved in systemic calcium homeostasis. In the cardiovascular system, CaSR has been identified in rat ventricular cardiomyocytes [8,9], endothelial cells from rat mesenteric and porcine coronary arteries [10], human aortic endothelial cells [11].

Recent evidence also suggests that a functional CaSR is expressed in vascular SMC. Wonneberger *et al.* [12] and Ohanian *et al.* [13] demonstrated the expression of a functional CaSR involved in the regulation of myogenic tone in rat subcutaneous arteries and in the gerbil spiral modiolar artery. Ziegelstein *et al.* [11] reported the presence of CaSR in human aortic endothelial cells where it mediated nitric oxide production. Smajilovic *et al.* [14] detected CaSR mRNA and protein in rat aortic vascular SMC and showed that extracellular Ca^{2+} stimulated cell proliferation, most likely through the MEK1/ERK1,2 pathway. Our group has demonstrated the expression of a functional CaSR in HAoSMC [15]. Importantly, the receptor was capable of mediating specific agonist-induced activation of MEK1/ERK1,2 signalling pathway. The CaSR is also present in human SMC in renal and epigastric arteries from ESRD patients where its expression tended to decline in patients with progressive arterial calcification [15].

Accelerated medial arterial calcification represents a major cause of the high cardiovascular mortality in patients with ESRD [16,17]. However, little is known about the molecular mechanisms of vascular calcification in these patients. Important risk factors include elevated levels of circulating phosphate and calcium in the environment of chronic inflammation [18,19]. These factors were also shown to increase SMC calcification in ESRD patients [20] and *in vitro* [21]. Vascular calcification itself is an active, regulated process similar to that of osteogenesis in bone with SMC undergoing transformation to an osteoblast-like phenotype and forming calcified nodules [22,23,24]. Chattopadhyay *et al.* [25] recently reported that CaSR stimulated proliferation in rat calvarial osteoblasts. It is possible that stimulation of the CaSR can accelerate vascular SMC transformation into osteoblast-like cells followed by their proliferation and progressive calcification. Reynolds *et al.* [19] showed that this process involved vesicle-mediated calcification of SMC associated with apoptosis. Moreover, blocking apoptosis with pan-apoptosis inhibitor ZVAD could inhibit vascular calcification and associated loss of SMC in CKD patients [26]. Another approach to inhibit

vascular calcification includes stimulation of CaSR expressed on arterial SMC with calcimimetics, which were shown to reduce nodule formation and mineral deposition by vascular SMC [27]. These data suggests that intracellular signalling pathways and cellular functions regulated by the CaSR in vascular SMC are closely involved in the initiation and progression of vascular calcification. Consequently, pharmacological modulation of SMC proliferation and apoptosis could prove to be beneficial in the treatment of vascular calcification in CKD patients.

Therefore, this project examined CaSR downstream intracellular signalling pathways and investigated the potential role of CaSR in regulating SMC proliferation and apoptosis with regard to vascular calcification in CKD patients. Here we demonstrate that agonist-induced stimulation of the CaSR results in activation of the MEK1/ERK1,2 and PLC-IP3 pathways and up-regulation of proliferation in HAoSMC independently of PKC and PI3K signalling. In addition, CaSR-mediated PLC-IP3 activation is important for SMC survival and protection against apoptosis.

Materials and Methods

Cell culture and tissue samples

HAoSMC were purchased from PromoCell (Germany) and maintained in SMC growth medium 2 containing 5% foetal calf serum (FCS), 0.5 ng/ml epidermal growth factor, 2.0 ng/ml basic fibroblast growth factor and 5µg/ml insulin (PromoCell). The cells were grown in 5% CO_2 at 37°C in medium renewed every 3 days. Confluent cells were detached by trypsin/EDTA and sub-cultured with a split ratio 1:2. HAoSMC were used between 2 and 5 passages.

Inhibition of CaSR expression by specific siRNA

To examine the functional role of CaSR in SMC, we used siRNA to knock down the level of CaSR expression (Santa Cruz Biotechnology). The CaSR siRNA represents a pool of 3 target specific 20-25 nucleotide siRNAs designed to inhibit gene expression. The transfection of siRNA specifically targeted to the CaSR into HAoSMC was performed using lipofectamine (Invitogen) according to the manufacturer's protocol, as described previously [28]. Briefly, lipofectamine and siRNAs were diluted into OptiMEM medium (Invitrogen). Diluted lipofectamine lipids were mixed with diluted siRNAs and incubated for 30 minutes at room temperature for complex formation. Mixtures were further diluted in OptiMEM and added to each well so that the final concentration of siRNAs was 40 nM. Control siRNA (siRNA-A, Santa Cruz) and lipofectamine alone were used as negative controls. The effectiveness of transfection (CaSR knockdown) was monitored by Western blot.

Western blot analysis

HAoSMC were treated with specified inhibitors and agonists and then harvested. Briefly, the cells were washed three times with cold PBS, scraped and solubilised in 60 μl of cold RIPA lysis buffer (Upstate) with freshly added protease inhibitor cocktail and phosphatase inhibitor cocktails 1 and 2 (Sigma). Cell debris was pelleted by microcentrifugation at 10,000g for 10 minutes at 4°C. Aliquots of cell lysates containing 15 μg protein were separated by SDS-PAGE and Western blotted with the indicated antibodies. Densitometry was performed using Image J Analysis software using blots from three independent experiments with final results normalised using loading controls (smooth muscle α-actin or total ERK1,2).

Analysis of CaSR protein expression

HAoSMC lysates were solubilised on ice using modified lysis buffer containing 0.25 M Tris-HCl (pH 7.8), 0.5% Igepal, 5 mM DTT and freshly added protease inhibitor cocktail

(Sigma). The lysates were then separated by 7% SDS-PAGE and Western blotted with a polyclonal anti-human CaSR antibody (1:500) (Binding Site, UK) and anti-human smooth muscle α-actin antibody (Sigma), as previously described [15].

ERK1,2 phosphorylation

HAoSMC were plated at 2×10^5 cells/well of a 6-well plate and grown overnight in complete medium. The following day the medium was changed and cells were rested overnight in serum-free D-MEM:F-12(1:1) medium (Invitrogen) containing 0.2% BSA. Cells were rinsed in PBS for 5 min before equilibration for 20 min in experimental buffer containing 20 mM HEPES (pH 7.4), 125 mM NaCl, 4mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 5.5 mM glucose [14]. HAoSMC were then stimulated with CaSR agonists (300 μM neomycin or gentamycin for 15 min) and calcium itself (3 mM, 5 min) . Where indicated, the cells were pre-treated for 5 min with 10 μM PD98059 (specific MEK1 inhibitor), 5 μM U73122 (PLC inhibitor), 250 nmol calphostin C (PKC inhibitor) and 10 μM Ly294002 (PI3K inhibitor). All inhibitors were purchased from Calbiochem. After incubation the cells were lysed on ice in the RIPA lysis buffer, separated by 10% SDS-PAGE and Western blotted with anti-phospho-ERK1,2 (New England Biolabs). Protein concentration in the samples was measured using DC BioRad protein assay to ensure equal protein loading. To confirm that ERK protein levels were not altered by the experimental treatment, the samples were also immunoblotted with anti-total ERK1,2.

IP3 assay

IP3 production was assayed using D-*myo*-inositol 1,4,5-triphosphate (^3H) Biotrak assay system (Amersham Biosciences) following the manufacturer's protocol. Briefly, HAoSMC were plated in 6-well plates (2×10^5 cells/well) and grown in complete medium for 24 hours. After transfection with control siRNA-A or CaSR siRNA the cells were rested overnight in

serum-free D-MEM:F-12(1:1) medium with 0.2% BSA and incubated with 300 μM neomycin/gentamycin for 15 min in the presence of 10 mM LiCl to prevent IP3 degradation. Where indicated, the cells were pre-incubated for 5 min with 5 μM U73122. After stimulation HAoSMC were incubated for 20 min with 0.2 volume ice-cold 20% perchloric acid to extract IP3. Supernatants were neutralised with ice-cold 10 M KOH and assayed using D-*myo*-inositol (^{3}H) 1,4,5-triphosphate.

Cell proliferation Biotrak colorimetric assay

HAoSMC proliferation was measured using cell proliferation Biotrak ELISA system (Amersham Biosciences) according to the manufacturer's protocol. The assay is based on BrdU incorporation into the DNA of proliferating cells. Briefly, the cells were plated at 5000 cells/well of a 96-well plate and grown in complete medium for 24 hours. Cells were then transfected with control siRNA-A or CaSR siRNA as described above. After resting overnight in serum-free D-MEM medium with 0.2% BSA, HAoSMC were incubated with CaSR agonists and specific inhibitors for 24 hours in the presence of BrdU. Following the treatment, HAoSMC were fixed, incubated with peroxidase-labelled anti-BrdU antibody for 60 minutes and treated with substrate solution for 10 minutes. The resulting colour reaction was stopped and read at 450 nm in a microtitre plate spectrophotometer. The absorbance values correlate directly to the amount of DNA synthesis and therefore to the number of proliferating cells in culture.

Apoptosis assay

For apoptosis experiments, HAoSMC were plated at 2×10^5 cells/well of a 6-well plate and grown overnight in complete medium. The next day the cells were transfected with CaSR specific siRNA. After resting cells overnight in serum-free D-MEM:F-12(1:1) with 0.2% BSA, HAoSMC were incubated in serum-free medium with CaSR agonists and specific

inhibitors for 24 hours. Cells were harvested using trypsin/EDTA, washed in PBS and pelleted. The cell pellets were then resuspended in 1 ml ice cold PBS and 2 ml cold absolute methanol and incubated on ice for 30 minutes. Permeabilised cells were washed in PBS and stained with 0.5 ml propidium iodide (PI) solution (100 μg/ml PI in PBS) plus 0.5 ml RNAse solution (2 mg/ml RNAse in PBS) for 30 minutes at room temperature in the dark. The samples were analysed by flow cytometry within one hour with apoptotic cells observed in the subdiploid area of DNA histogram.

Flow cytometry

Flow cytometry was performed using a FACScan flow cytometer with CELLQuest software (Becton Dickinson) and the 488nm argon laser. Typically, 10,000 cells were collected from each sample. All data were acquired in a list mode with three parameters checked – forward scatter (FSC), side scatter (SSC) and fluorescence channel 2 (FL2).

Statistical analysis

All experiments were performed at least three times and the results were expressed as the mean ± SD. Statistical analysis was performed using descriptive statistics, two-tailed paired t-test and one-way ANOVA followed by Tukey's multiple comparison test. P values <0.05 were considered as statistically significant.

Results

ERK1,2 phosphorylation in HAoSMC treated with CaSR agonists and signalling inhibitors

Having shown previously that CaSR agonists can induce activation of MEK1/ERK1,2 signalling pathway [15], we investigated possible involvement of other CaSR mediated intracellular pathways in ERK1,2 activation in HAoSMC. Treatment of HAoSMC with 300

μM neomycin resulted in a significant (7.5-fold, $p<0.05$) up-regulation of ERK1,2 phosphorylation (Figure 1). Importantly, neomycin-induced ERK1,2 activation was dramatically reduced ($p<0.01$) after pre-incubation with PD98059 (a MEK1 inhibitor), confirming that the observed effect was specific. Interestingly, pre-treatment of HAoSMC with U73122 almost completely abolished ERK1,2 phosphorylation suggesting that PLC signalling was important for MEK1/ERK1,2 activation. Notably, the levels of ERK1,2 phosphorylation in cell cultures treated with inhibitors alone were comparable to those in control untreated cells. Pre-incubation with calphostin C and Ly294002 (PKC and PI3K inhibitors respectively) did not alter neomycin-induced ERK1,2 activation (Figure 1). Treatment with gentamycin induced responses similar to those observed in neomycin-treated cultures (data not shown). It is important to note that the described changes in ERK1,2 phosphorylation were not caused by altered total ERK1,2 expression.

ERK1,2 phosphorylation in CaSR siRNA transfected HAoSMC treated with CaSR agonists and signalling inhibitors

To confirm that the CaSR agonist-induced ERK1,2 activation was mediated via CaSR and to further investigate the role of MEK1 and PLC inhibitors, experiments were performed in HAoSMC in which the CaSR had been knocked-down by specific siRNA, as previously described [15]. Western blot analysis confirmed that CaSR expression was reduced by >80% ($p<0.05$). Notably, no changes were observed in their smooth muscle α-actin expression (data not shown).

Transfected cells were treated with 300 μM neomycin for 15 min. As in previous experiments, we observed a significant ($p<0.01$) up-regulation of ERK1,2 activation in HAoSMC transfected with control siRNA-A (Figure 2A). Pre-incubation with PD98059 and U73122 resulted in a marked inhibition of phospho-ERK1,2 bringing it to the levels similar to those in control cultures. In HAoSMC transfected with CaSR siRNA response to neomycin

was significantly lower ($p<0.01$) than in control siRNA-A transfected cells. Similarly, we observed a dramatic reduction in ERK1,2 activation in cells treated with either PD98059 or U73122 and neomycin, when compared to the same treatment of control siRNA-A cells (Figure 2A). Responses following gentamycin treatment (300 μM, 15 min) were similar (data not shown). Incubation with calcium (3 mM, 5 min) resulted in a significant ($p<0.01$) up-regulation of ERK1,2 phosphorylation in control siRNA-A cells (Figure 2B). In CaSR siRNA transfected cells, ERK1,2 activation in response to calcium was reduced ($p<0.05$) when compared to control siRNA-A cells (Figure 2B), although this reduction was somewhat less pronounced than in neomycin treated cultures. These data indicate that in HAoSMC activation of MEK1/ERK1,2 pathway induced by CaSR agonists is indeed mediated via CaSR with PLC signalling playing a central role.

IP3 production in CaSR siRNA transfected HAoSMC

To further verify PLC activation, we investigated IP3 accumulation in HAoSMC using D-*myo*-inositol 1,4,5-triphosphate (^{3}H) Biotrak assay. We observed a dramatic increase in IP3 production in control siRNA-A transfected cells treated with neomycin, which was significantly reduced in the presence of U73122, specific PLC inhibitor ($p<0.05$, Figure 3). Importantly, up-regulation of IP3 production was almost completely abrogated in CaSR siRNA transfected cells with IP3 concentration being significantly lower than in neomycin treated control cells ($p<0.05$, Figure 3). Gentamycin induced effects (300 μM, 15 min) were similar and significant although less pronounced than those of neomycin (data not shown). The results confirm that PLC pathway activation in HAoSMC is strongly linked to the CaSR activation.

Cell proliferation in CaSR siRNA transfected HAoSMC

The MEK1/ERK1,2 pathway is crucial for the regulation of cell proliferation and survival, therefore, we examined HAoSMC proliferation following 24 hour incubation with CaSR agonists and specific inhibitors in the presence of BrdU. Neomycin increased cell proliferation almost 3-fold ($p<0.001$) in control transfected cells (Figure 4). Addition of PD98059 or U73122 markedly ($p<0.01$) reduced the observed up-regulation of cell proliferation. In CaSR siRNA transfected cells neomycin did not increase cell proliferation compared to neomycin treated control siRNA-A cultures ($p<0.01$) (Figure 4). Incubation with PD98059 or U73122 alone did not affect cell proliferation in either cell type. In addition, cell proliferation was unchanged in control siRNA-A or CaSR siRNA transfected cells treated with calphostin C or Ly294002 (data not shown). These results are consistent with our ERK1,2 phosphorylation data and confirm the importance of CaSR mediated MEK1/ERK1,2 and PLC signalling pathways in the regulation of HAoSMC proliferation.

Apoptosis in CaSR siRNA transfected HAoSMC

Apoptosis was measured in parallel with cell proliferation after CaSR siRNA transfected cells were incubated in serum-free medium with neomycin/gentamycin and specific inhibitors for 24 hours. Apoptosis was unaltered in neomycin treated control siRNA-A or CaSR siRNA cultures (Figure 5). Incubation with PD98059 either alone or with neomycin also did not alter the number of apoptotic cells. However, incubation with U73122 resulted in a dramatic ($p<0.01$) up-regulation of apoptotic cell death, which was not altered by co-incubation with neomycin. The increase in apoptosis was significantly ($p<0.05$) greater in CaSR siRNA transfected cells. Pre-incubation of HAoSMC with calphostin C or Ly294002 did not alter the level of apoptosis in neomycin treated cultures (data not shown). Figure 6 shows several representative DNA histograms of PI stained HAoSMC with apoptotic cells visualised in the subdiploid area of the histogram. It demonstrates a dramatic increase in apoptosis in U73122 treated cells particularly in cultures transfected with CaSR siRNA. The data suggest that PLC

signalling in general and CaSR mediated PLC activation in particular are important for survival of HAoSMC.

Discussion

Growing experimental evidence suggests that a number of important intracellular signalling pathways and cell functions mediated by the CaSR in vascular SMC are closely involved in the regulation of vascular calcification in patients with CKD. Therefore, modulation of CaSR-mediated SMC functions can offer potential benefits in the clinical management of these patients. ERK represent one of such pathways. These kinases belong to the larger family of mitogen-activated protein kinases (MAPK) – serine/threonine kinases that play a major role in cell proliferation, differentiation, apoptosis, inflammation, peptide secretion and ion channel activity [7,29]. A number of studies have demonstrated that agonist-induced stimulation of CaSR leads to ERK1,2 activation in various cell types, including parathyroid and CaSR-transfected HEK293 cells [30], osteoblasts [31], kidney tubular cells [32], cardiomyocytes [8] and aortic SMC [14,15].

Our group has reported previously a consistent and significant up-regulation of ERK1,2 phosphorylation in HAoSMC treated with CaSR agonists – calcium, neomycin and gentamycin [15]. Therefore, we decided to further examine the potential contribution of other CaSR-mediated signalling systems in ERK1,2 activation.

It is generally acknowledged that there is a considerable "cross-talk" between cell signalling pathways including MAPK system [33]. Using rat aortic SMC and specific signalling inhibitors, Cho et *al.* [34] showed that ERK1,2 phosphorylation in response to glycated LDL involved activation of MEK1, PLC and PKC. In contrast, Ward *et al.* [32] demonstrated that in opossum kidney cells neomycin-induced ERK1,2 activation was mediated via MEK1, PIP2-PLC and PI3K, whereas two separate PKC inhibitors had no significant effect on ERK phosphorylation, indicating that neomycin effect was PKC-

independent. In this study we have demonstrated that phospho-ERK1,2 up-regulation induced by neomycin, gentamycin and calcium was markedly reduced in the presence of PD98059, a MEK1 inhibitor, indicating that their effects were mediated via the classic MEK1/ERK1,2 pathway. In addition, ERK1,2 phosphorylation was almost completely abolished after pre-incubation with U73122 (specific PLC inhibitor), indicating that PLC signalling was crucial for MEK1/ERK1,2 activation in HAoSMC. However, treatment of HAoSMC with specific PI3K and PKC inhibitors failed to significantly alter the ERK response to neomycin or gentamycin, suggesting that PI3K and PKC were not directly involved in CaSR agonist-induced ERK1,2 activation in HAoSMC.

It is known that neomycin and gentamycin are strongly charged at physiological pH and cannot cross the plasma membrane [32,35]. Therefore, the regulatory effects of these antibiotics on ERK1,2 can only be exerted via membrane-bound CaSR. To further confirm that the observed ERK activation was indeed mediated by the CaSR, we used siRNA technology to knock-down CaSR expression by >80% in HAoSMC (CaSR-KD). In CaSR-KD cells neomycin, gentamycin and calcium-induced ERK1,2 phosphorylation was significantly lower. Similarly, a reduction in ERK1,2 activation after treatment with MEK1 and PLC inhibitors was more pronounced in CaSR-KD cells. These data confirmed that in HAoSMC ERK1,2 phosphorylation induced by neomycin, gentamycin and calcium was mediated by the CaSR. It involved activation of MEK1 and PLC, but was independent of PKC and PI3K.

The central role of PLC in CaSR-mediated signalling was first identified by Brown *et al.* [3] and Kifor *et al.* [36] who reported activation of phospholipases A2, C and D and accumulation of IP3 in bovine parathyroid and CaSR-transfected HEK293 cells in response to CaSR stimulation. They showed that CaSR-induced PLC activation was likely to be mediated through $G_{alphaQ/11}$ subunit as IP3 accumulation was not inhibited by treatment with the pertussis toxin. Moreover, CaSR interaction with $G_{alphaQ/11}$ can trigger activation of PKC, which in turn modulates activity of the receptor [37]. Likewise, stimulation of rat ventricular

cardiomyocytes with extracellular calcium and gadolinium has been shown to increase IP3 production and intracellular calcium [9]. Importantly, this could be blocked by the IP3-specific PLC inhibitor U73122, suggesting that CaSR was linked to the PLC pathway. Up-regulation of IP3 production in rat cardiomyocytes in response to CaSR agonist stimulation (calcium and AMG073 calcimimetic) was also shown by Tfelt-Hansen *et al.* [8]. They found that calcium-induced increase in IP3 accumulation was attenuated in cells transfected with dominant negative CaSR confirming its direct involvement. Our observation of increased IP3 production in HAoSMC in response to CaSR agonists (neomycin and gentamycin) further confirmed PLC pathway activation. The failure of neomycin to induce IP3 production in CaSR-KD cells clearly demonstrated that PLC activation in HAoSMC was mediated via CaSR.

CaSR is involved in the regulation of cell cycle progression with proliferative response to the CaSR depending on the cell type. In pancreatic carcinoma cells, keratinocytes and colonic crypt cells, CaSR activation induced growth arrest, while in fibroblasts, osteoblasts, myeloma and astrocytoma it stimulated cell proliferation [reviewed by Smajilovic and Tfelt-Hansen, 7]. In this study we have revealed a marked increase in HAoSMC proliferation after treatment with neomycin, which was significantly reduced in the presence of specific MEK1 and PLC inhibitors. The observed up-regulation of cell proliferation was significantly attenuated in the CaSR-KD cells confirming the direct involvement of the receptor. A similar response to aminoglycosides was observed in opossum kidney and in CaSR-transfected HEK293 cells with proliferation being substantially reduced after incubation with PI3K and PLC inhibitors [38]. Smajilovic *et al.* [14] also showed that extracellular Ca^{2+} and neomycin induced DNA synthesis and proliferation of rat aortic SMC, which seemed to be mediated via MEK1/ERK pathway. However, there was no significant effect on IP3 levels and introduction of dominant negative CaSR did not change DNA synthesis or IP3 production in response to extracellular Ca^{2+}, indicating that CaSR is not coupled to the PLC/IP3 pathway in rat aortic SMC. The

authors also suggested that there may be other signalling mechanisms and additional calcium-sensing receptors involved in calcium-induced proliferation of SMC [14]. Interestingly, Wellendorph and Brauner-Osborne [39] recently cloned and sequenced GPRC6A, a novel family C G-protein-coupled receptor with a significant homology to the human CaSR. In another recent report Tfelt-Hansen *et al.* [8] demonstrated CaSR-mediated activation of PLC/IP3 signalling concomitant with ERK1,2 phosphorylation in neonatal cardiomyocytes. Interestingly, they found that extracellular Ca^{2+} induced a biphasic response in DNA synthesis with no effect on cell number. 3mM Ca^{2+} significantly increased DNA synthesis, while 6 mM Ca^{2+} reduced it. On the contrary, the calcimimetic AMG073 inhibited DNA synthesis at all calcium levels. It is possible that such differential regulation of cell proliferation by CaSR was due to modulation of the receptor activity, involvement of different signalling pathways and duration of ERK1,2 activation. Overall, our findings were in agreement with the ERK1,2 activation data further demonstrating the importance of MEK1/ERK1,2 and PLC signalling in CaSR-mediated SMC proliferation.

Lin *et al.* [40] demonstrated that extracellular Ca^{2+} can modulate apoptosis via the CaSR. They showed that CaSR stimulation with extracellular Ca^{2+} and neomycin protected against apoptosis induced by Sindbis virus, c-Myc overexpression or serum deprivation. Decreases in intracellular Ca^{2+} have been shown to precede apoptosis in several models, including cytokine and growth factor withdrawal, and they suggested that CaSR-mediated PLC activation leading to the generation of IP3 and moderate increase in intracellular Ca^{2+} maintain its levels to a point optimal for viability [40]. We examined whether agonist-induced activation of CaSR could modulate apoptosis and survival in HAoSMC. Our experiments did not reveal any significant changes in apoptosis in neomycin-treated cultures. However, incubation with PLC inhibitor (U73122) resulted in a dramatic increase in HAoSMC apoptosis. Notably, the increase in apoptosis was significantly higher in CaSR-KD cells, indicating that CaSR-mediated PLC activation was responsible for survival of HAoSMC and their resistance to pro-

apoptotic stimuli. Another potential anti-apoptotic mechanism linked to the CaSR is activation of PI3K and one of its downstream targets, AKT kinase [41]. However, in our study treatment with specific inhibitors of PI3K (Ly294002) and PKC (calphostin C) had no effect on the level of apoptosis in HAoSMC, suggesting that in HAoSMC these kinases are not directly involved in the regulation of SMC apoptosis. Recent studies that used human SMC [42], porcine SMC [43] and rat ventricular cardiomyocytes [44] showed that CaSR can mediate phosphorylation of p38 and JNK kinases and induce apoptotic cell death via activation of cytochrome c-caspase-9 signalling pathway. Overall, apoptosis seems to be simultaneously controlled by a number of factors and signalling pathways and the cell fate eventually depends on which factor (pro- or anti-apoptotic) will prevail.

In conclusion, we have demonstrated that in HAoSMC agonist-induced stimulation of the CaSR leads to activation of the MEK1/ERK1,2 and PLC-IP3 pathways and up-regulation of cell proliferation independently of PKC and PI3K signalling. In addition, CaSR-mediated PLC-IP3 activation is important for SMC survival and protection against apoptosis.

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Figure legends

Figure 1

Phospho-ERK1,2 expression in HAoSMC treated with neomycin and signalling inhibitors. HAoSMC in 6-well plates were incubated overnight in serum-free medium and treated with 300 μM neomycin for 15 min. Where indicated, the cells were pre-incubated for 5 min with 10 μM PD98059, 5 μM U73122, 250 nM calphostin C and 10 μM Ly-294002. Cell lysates were separated by 10% SDS-PAGE and Western blotted with anti-phospho-ERK1,2 and anti-total ERK1,2. Densitometry was performed using Image J Analysis software. The data are presented as mean ± SD (n=3) with antigen expression shown as standardised fold increase/decrease from control. ** $p<0.01$ compared to control untreated cells; ×× $p<0.01$ compared to cells treated with neomycin.

Figure 2

Effect of siRNA on CaSR expression and ERK1,2 activation in HAoSMC. HAoSMC were transfected with control siRNA-A and CaSR siRNA as described previously. After transfection HAoSMC were treated with 300 μM neomycin for 15 min (A) or 3 mM calcium for 5 min (B). Where specified, the cells were pre-incubated for 5 min with 10 μM PD98059 or 5 μM U73122. Cell lysates were Western blotted with anti-phospho-ERK1,2 and anti-total ERK1,2. Densitometry was performed using Image J Analysis software. The data are presented as mean ± SD (n=3) with protein expression shown as standardised fold increase/decrease from control (untreated cells). A, ** $p<0.01$ compared to control (untreated) cells transfected with control siRNA-A; * $p<0.05$ compared to control cells transfected with CaSR siRNA; ×× $p<0.01$ compared to neomycin treated cells transfected with control siRNA-A; ^ $p<0.05$, ^^ $p<0.01$ compared to neomycin treated cells transfected with CaSR siRNA. B, ** $p<0.01$ compared to corresponding control cells transfected with control siRNA-A or

CaSR siRNA; ×× p<0.01 compared to calcium treated cells transfected with control siRNA-A; ^^ p<0.01 compared to calcium treated cells transfected with CaSR siRNA.

Figure 3

IP3 production in HAoSMC transfected with CaSR siRNA and treated with neomycin and U73122. HAoSMC were transfected with control siRNA-A or CaSR siRNA as described previously. Transfected cells were treated with 300 μM neomycin for 15 min in the presence of 10 mM LiCl. Where indicated, the cells were pre-incubated for 5 min with 5 μM U73122. IP3 production was measured using D-*myo*-inositol 1,4,5-triphosphate (^{3}H) Biotrak assay using manufacturer's protocol. The data are presented as mean ± SD of three independent experiments. * p<0.05 compared to cells transfected with control siRNA-A; × p<0.05 compared to neomycin treated cells transfected with control siRNA-A.

Figure 4

Cell proliferation in HAoSMC transfected with CaSR siRNA and treated with neomycin and signalling inhibitors. HAoSMC were transfected with control siRNA-A or CaSR siRNA as described previously. Transfected cells were treated with 300 μM neomycin for 24 hours in the presence of BrdU. Where indicated, the cells were pre-incubated for 5 min with 10 μM PD98059 or 5 μM U73122. Cell proliferation was measured using Biotrak colorimetric assay as described above. The data are presented as mean ± SD of three independent experiments. *** p<0.001 compared to control cells transfected with control siRNA-A; ×× p<0.01, ××× p<0.001 compared to neomycin treated cells transfected with control siRNA-A; ^ p<0.05 compared to neomycin treated cells transfected with CaSR siRNA.

Figure 5

Apoptosis in HAoSMC transfected with CaSR siRNA and treated with neomycin and signalling inhibitors. HAoSMC were transfected with control siRNA-A or CaSR siRNA as described previously. Transfected cells were incubated with neomycin and signalling inhibitors for 24 hours. After treatment the cells were fixed, stained with PI and analysed by flow cytometry with apoptotic cells visualised in the subdiploid area of DNA histogram. The data are presented as mean ± SD of three independent experiments. * $p<0.05$ compared to control cells transfected with control siRNA-A; ** $p<0.01$ compared to control cells transfected with CaSR siRNA; × $p<0.05$ compared to neomycin and U73122 treated cells transfected with control siRNA-A; ^ $p<0.05$ compared to U73122 treated cells transfected with control siRNA-A.

Figure 6

DNA histograms of PI stained HAoSMC treated with neomycin and U73122. Apoptotic cells are shown in the subdiploid area of DNA histograms. A, B, C – cells transfected with control siRNA-A; D, E, F, - cells transfected with CaSR siRNA; A and D – control untreated cells; B and E – neomycin and U73122 treated cells; C and F – U73122 treated cells.