Verapamil inhibits calcification and matrix vesicle activity of bovine vascular smooth muscle cells

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Calcium channel activity in vascular smooth muscle cells is a critical component during vascular calcification and formation of matrix vesicles. Here, we examined whether the blockade of L-type calcium channels inhibits these functions. Bovine vascular smooth muscle cells or rat aorta organ cultures were incubated in media known to promote calcification and treated with the L-type calcium channel inhibitors verapamil, nifedipine, or nimodipine. The phenylalkylamine, verapamil, significantly decreased calcification of the vascular smooth muscle cells and rat aorta, in a dose-dependent manner, whereas the dihydropyridines, nifedipine and nimodipine, had no effect. Furthermore, verapamil, but not nifedipine, significantly decreased the alkaline phosphatase activity of bovine vascular smooth muscle cells. Verapamil pretreatment of the cells also inhibited matrix vesicle alkaline phosphatase activity and reduced the ability of these matrix vesicles to subsequently calcify on a type I collagen extracellular matrix scaffold. As L-type channels are blocked by verapamil and dihydropyridines, we suggest that verapamil inhibits vascular smooth muscle mineralization and matrix vesicle activity by mechanisms other than the simple blockade of this calcium channel activity.

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Vascular calcification is a prominent finding in aging, diabetes, chronic kidney disease, and inflammatory diseases. The pathophysiology of vascular calcification is complex but appears to parallel the mechanism of normal mineralization, in which osteoblast/chondrocytes (or osteoblast-like vascular smooth muscle cells) produce cell outpockets containing calcium and phosphorus called matrix vesicles (MVs). On the basis of the phospholipid content, high alkaline phosphatase activity, and the presence of specific transporters and receptors, MVs are believed to originate from cells by 'pinching off' from the cell membrane.[1,2] These MVs bind to extracellular matrix proteins to initiate mineralization. MVs produced by mineralizing chondrocytes and calcifying vascular smooth muscle cells (VSMCs), unlike non-mineralizing MVs, have high alkaline phosphatase activity and contain annexins II, V, and VI, and actively take up Ca^{2+}.[3–5] The importance of the calcium influx is further shown by our studies demonstrating that the blockade of annexin calcium channel activity by K201 significantly decreased ALP activity and reduced the ability of the MVs to subsequently calcify on collagen.[5]

Intracellular Ca^{2+} is tightly regulated in vascular smooth muscle cells and calcium channels are the major route by which Ca^{2+} enters the smooth muscle cells.[6] Differentiated VSMCs express a large repertoire of ion channels, including voltage-dependent L-type Ca^{2+} channels.[7] These channels are critical in regulating VSMC differentiation, function, and synthesis of matrix components. L-type calcium channel blockers are a widely used group of agents for hypertension and cardiovascular disease and include phenylalkylamines (verapamil) and dihydropyridines (nifedipine and nimodipine), each of which have unique structures and differing binding sites on the channel. We have previously found that K201, an inhibitor of the annexin calcium channel, inhibited VSMC calcification. However, annexins and L-type calcium channels also have effects beyond changes in calcium influx. The objective of this study is to determine whether the prevention of intracellular calcium increase by blockade of L-type calcium channels may also inhibit calcification and MV formation and activity in VSMCs, which would provide insights into the pathogenesis of calcification.

RESULTS

Intracellular calcium $[Ca]_i$ changes in BVSMCs treated with verapamil or nifedipine

To confirm the presence of L-type calcium channels in control or calcified bovine vascular smooth muscle cells (BVSMCs), western blot was performed using anti L-type calcium channel (α1c) antibody (a gift from Dr Randall Duncan, University of Delaware) to determine the expression of L-type calcium channels. As shown in Figure 1a, there is expression of L-type calcium channel in BVSMCs. However, there is no significant difference in the expression of L-type calcium channel between control and calcified BVSMCs. To determine whether L-type calcium channel blockers can prevent the increase in $[Ca]_i$, BVSMCs were pretreated with or without the phenylalkylamine L-type calcium channel blocker verapamil (10 μmol/l) or the dihydropyridine L-type calcium channel blocker nifedipine (10 μmol/l) before loading with fura 4-AM. Cells were depolarized by 80 mmol/l K^+ (high K) buffer to induce increase in $[Ca]_i$. Intracellular calcium changes were examined by confocal microscopy and quantified (Figure 1b). There was an increase in $[Ca]_i$ in control BVSMCs in response to depolarization with high K (Figure 1b). However, both verapamil and nifedipine blocked the increase in $[Ca]_i$.

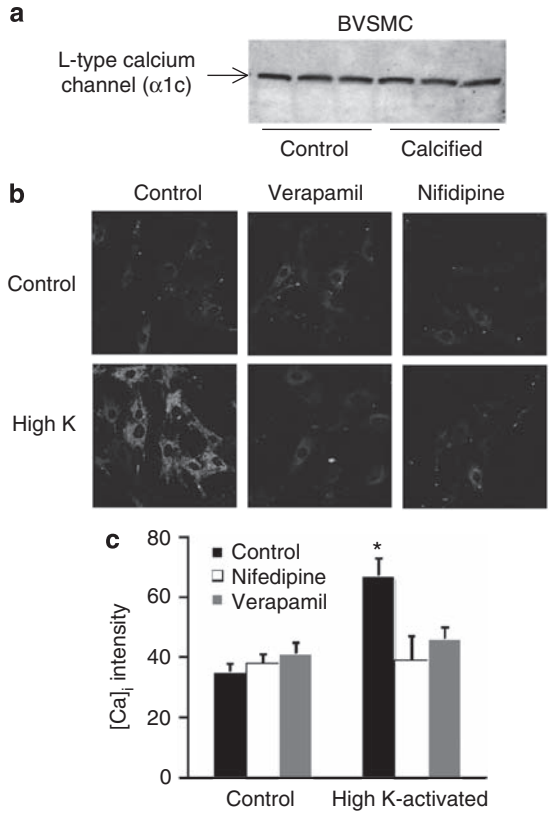

Figure 1 | The expression of L-type calcium channels and intracellular calcium $[Ca]_i$ changes in BVSMCs treated with verapamil or nifedipine. (**a**) To determine the expression of L-type calcium channels in BVSMCs, cell lysates were isolated from control or calcified BVSMCs and western blot performed using anti L-type calcium channel (α1c subunit). (**b**) To determine whether L-type calcium channel blockade can prevent the increase in $[Ca]_i$, BVSMCs were pretreated with or without phenylalkylamine L-type calcium channel blocker verapamil or dihydropyridine L-type calcium channel blocker nifedipine before loading with fura 4-AM. Cells were depolarized by 80 mmol/l K^+ (high K) buffer to induce increase in $[Ca]_i$. Intracellular calcium changes were examined by confocal microscopy and quantified. (**c**) Quantitative analysis of changes in $[Ca]_i$ in BVSMCs treated with verapamil or nifedipine. Data are shown as mean ± s.d. from three separate experiments (total $n = 9$). *$P < 0.05$, high K vs normal Ca^{2+} buffer. BVSMCs, bovine vascular smooth muscle cells.

The role of L-type calcium channel blockade in alkaline phosphatase and calcification in BVSMCs

To determine the role of L-type calcium channels in calcification and alkaline phosphatase (ALP) activity in BVSMCs, cells were incubated in DMEM in calcification media (with 10 mmol/l beta-glycerophosphate) in the presence or absence of various concentrations of the L-type calcium channel blockers verapamil (phenylalkylamine), nifedipine, or nimodipine (both dihydropyridine) for 7 days. Calcification was determined by HCL extraction. As shown in Figure 2, verapamil significantly decreased calcification in BVSMCs in a dose-dependent manner. However, neither nifedipine nor nimodipine had an effect on calcification in BVSMCs (Figure 2). Furthermore, verapamil, but not nifedipine, significantly decreased alkaline phosphatase activity in BVSMCs (Figure 3). These results suggest that only the phenylalkylamine/L-type calcium channel blocker verapamil prevented calcification and decreased ALP activity in BVSMCs.

Ex vivo effect of L-type calcium channel blockade on rat aorta calcification

We next investigated whether the observed effect of L-type calcium channel blockade on *in vitro* BVSMC calcification

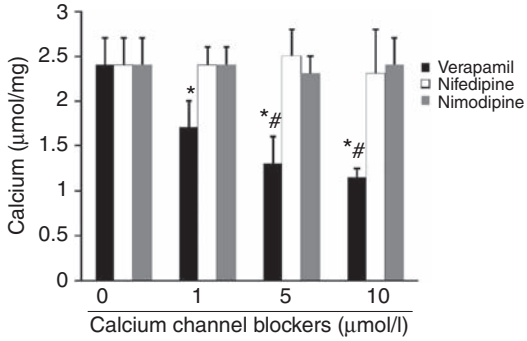

Figure 2 | The effect of L-type calcium channel blockade on calcification in BVSMCs. BVSMCs were incubated in calcification media (with 10 mmol/l β-glycerophosphate) in the presence or absence of various concentrations of the L-type calcium channel blockers verapamil (phenylalkylamine), nifedipine, or nimodipine (dihydropyridines) for 7 days. Calcification was determined by HCL extraction. The results showed that verapamil dose-dependently decreased calcification in BVSMCs. Neither nifedipine nor nimodipine had an effect in calcification in BVSMCs. Data are shown as mean ± s.d. from four separate experiments (total $n = 12$). *$P < 0.05$ vs no blocker, #$P < 0.05$, high dose vs 1 μmol/l blocker.

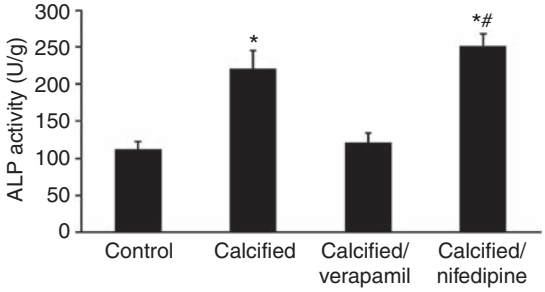

Figure 3 | The effect of L-type calcium channel blockade on alkaline phosphatase (ALP) activity in BVSMCs. BVSMCs were treated with control (no β-glycerophosphate) or calcification media (with β-glycerophosphate calcified) in the presence of 10 μmol/l of verapamil or nifedipine for 7 days and alkaline phosphatase activity was determined by colorimetric assay. The results showed that verapamil, but not nifedipine, significantly decreased ALP activity in BVSMCs. Data are shown as mean ± s.d. from four separate experiments ($n = 12$). *$P < 0.01$, control vs calcified, #$P < 0.05$, verapamil vs nifedipine, calcified.

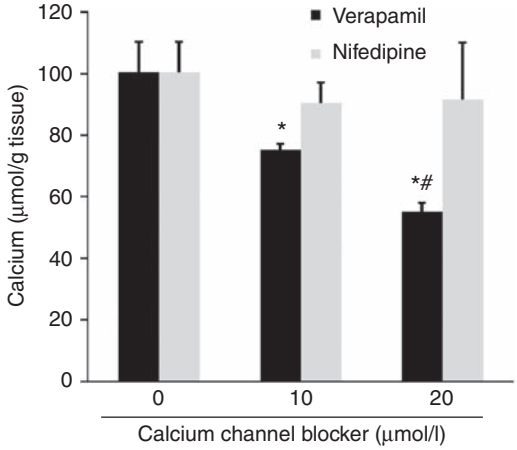

Figure 4 | *Ex vivo* effect of L-type calcium channel blockade on rat aorta calcification. Rat aortic rings were incubated immediately after harvest in DMEM with 7.5 units/ml of calf intestinal alkaline phosphatase and 3.8 mmol/l sodium phosphate (calcification medium) in the presence or absence of verapamil or nifedipine (0, 10 or 20 μmol/l) for 7 days. The calcium content was determined by HCL extraction and normalized by tissue weight. The results showed that verapamil dose-dependently inhibited rat aortic calcification, whereas nifedipine had no effect. Data are shown as mean ± s.d. ($n = 3$ from a total of three rats). *$P < 0.01$ vs no blocker, #$P < 0.05$, 20 μmol/l vs 10 μmol/l blocker.

could be reproduced *ex vivo* using an organ culture system. The aorta organ cultures have the benefit of including endothelial cells and vascular smooth muscle cells as well as other cell types, which may all contribute to aorta calcification. Aortas were calcified in an organ culture system by the addition of calf intestinal alkaline phosphatase and sodium phosphate to DMEM without serum (calcification media) in the presence or absence of increasing concentrations of verapamil or nifedipine. Viability of organ cultures at 7 days was assessed by the measurement of lactic dehydrogenase secretion into the medium to determine maximal tolerable concentration. The results showed that verapamil dose-dependently decreased calcium content in rat aorta ring cultures, whereas nifedipine had little effect on aorta calcification (Figure 4). These results suggest that, similar to an *in vitro* cell study, verapamil but not nifedipine significantly inhibited rat aorta calcification *ex vivo*.

The effects of ryanodine receptor antagonists and activators on calcification in BVSMCs

Studies in rabbit skeletal muscle cells have reported that verapamil, but not dihydropyridines, can also directly bind to the ryanodine receptor to inhibit ryanodine receptor-mediated release of calcium from the sarcoplasmic reticulum.[8] To determine whether ryanodine receptors are involved in the inhibition of calcification by verapamil in BVSMCs, cells were treated with calcification media in the presence or absence of various concentrations of the ryanodine receptor antagonists dantrolene and ryanodine, as well as ryanodine receptor activators caffeine and 4-chloro-m-cresol (CMC) for 7 days and the calcification determined. Our results showed that blocking or activating ryanodine receptors had no effect on calcification in BVSMCs (data not shown). These results suggest that ryanodine receptors are not involved in calcification.

Verapamil inhibited alkaline phosphatase activity in matrix vesicles (MVs) from calcified BVSMCs and reduced the ability of MVs to calcify collagen

We then asked if verapamil had similar effects to K201, an annexin calcium channel inhibitor, by determining whether there was an additive effect of verapamil with K201 on BVMSC calcification. There was no additive inhibitory effect on calcification (1.10 ± 0.15 μmol/mg, verapamil; 1.21 ± 0.20 μmol/mg, verapamil + K210), suggesting similar mechanisms of action. We have previously shown that K201 inhibited the activity of matrix vesicles in BVSMCs,[5] and therefore we determined whether verapamil also reduced MV activity. BVSMCs were incubated in DMEM in calcification media (with 10 mmol/l β-glycerophosphate) or non-calcifying media (no β-glycerophosphate) for 7 days in the presence or absence of verapamil, and MVs isolated by collagenase digestion from these cell cultures.[5] The addition of verapamil significantly decreased ALP activity in MVs isolated from calcified BVSMCs (calcified = 4165 ± 273 U/g protein; calcified + verapamil = 2460 ± 97 U/g protein, $P < 0.01$), but had no effect on ALP activity in MVs from control (non-calcified) BVSMCs (control = 607 ± 231 U/g protein; control + Y-27632 = 804 ± 347 U/g protein) (Figure 5).

To determine the role of verapamil in MV calcification on the extracellular matrix, BVSMCs were incubated in calcifying or non-calcifying conditions in the presence or absence of verapamil for 7 days and MVs were isolated, placed on type I collagen-coated dishes, and incubated with calcification media (10 mmol/l β-glycerophosphate) but no cells for 3 days for the MV—collagen calcification assay.[5] MVs

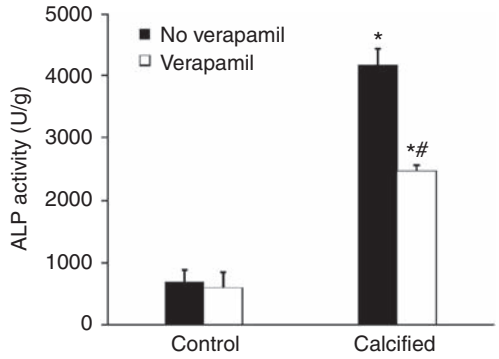

Figure 5 | The effect of verapamil on matrix vesicle (MV) activity. BVSMCs were incubated in control (no β-glycerophosphate) or calcification media (with 10 mmol/l β-glycerophosphate, calcified) in the presence or absence of 10 μmol/l verapamil for 7 days. MVs were isolated by collagenase digestion and alkaline phosphatase activity was determined and normalized by total MV protein content. Verapamil significantly decreased MV alkaline phosphatase activity from calcified BVSMCs but had no effect on control BVSMCs. Data are shown as mean ± s.d. from three separate experiments ($n = 9$). *$P < 0.05$, calcified vs control, same treatment; #$P < 0.05$, verapamil vs no verapamil.

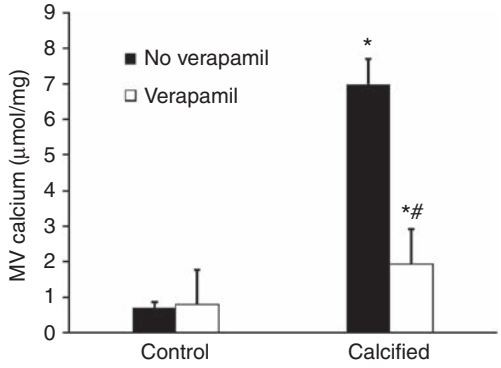

Figure 6 | The effect of verapamil on matrix vesicle collagen calcification. BVSMCs were incubated in control (no β-glycerophosphate) or calcification media (with 10 mmol/l β-glycerophosphate, calcified) in the presence or absence of 10 μmol/l verapamil for 7 days. MVs were isolated by collagenase digestion and added to type I collagen-coated coverslips in the presence of calcification media (with 10 mmol/l β-glycerophosphate) and incubated for 3 days. The calcium content was determined by HCL extraction. Verapamil significantly decreased the ability of MV to subsequently calcify on type I collagen. Data are shown as mean ± s.d. from three separate experiments ($n = 9$). *$P < 0.01$, calcified vs control, same treatment; #$P < 0.05$, verapamil vs no verapamil, calcified or control.

isolated from BVSMCs incubated with verapamil significantly impaired the ability of MVs to subsequently calcify collagen (Figure 6, *$P < 0.001$, calcified vs control; #$P < 0.01$, verapamil vs no verapamil). However, when verapamil was added to calcifying MVs incubated with type I collagen after the MVs were already formed, verapamil did not inhibit calcification (6.46 ± 0.85 μmol/mg, no verapamil; 6.26 ± 1.20 μmol/mg, verapamil). Furthermore, MV isolated from calcifying BVSMCs did not express L-type calcium channels by western blot (data not shown) despite the expression in BVSMCs (Figure 1). These results showed that verapamil, when added during mineralization, decreased MV alkaline phosphatase activity and inhibited the ability of matrix vesicles to mineralize in BVSMCs. However, the addition of verapamil to existing MVs had no effect. These data suggest that the phenylalkylamine L-type calcium channel blocker verapamil regulates BVSMC MV formation and their subsequent ability to calcify.

DISCUSSION

In this study, we have shown that bovine vascular smooth muscle cells express L-type calcium channels and that both the phenylalkylamine L-type calcium channel blocker verapamil and the dihydropyridine L-type calcium channel blocker nifedipine can block an increase in intracellular calcium induced by depolarization with 80 mmol/l K^+ solution. Verapamil also dose-dependently decreased calcification and alkaline phosphatase activity in BVSMCs, whereas nifedipine and nimodipine had no effect on calcification or alkaline phosphatase activity. Furthermore, *ex vivo* studies showed that verapamil, but not nifedipine, inhibited rat aorta calcification in a dose-dependent manner in the organ culture system. Verapamil also significantly decreased alkaline phosphatase activity in matrix vesicles isolated from calcifying BVSMCs and impaired the ability of MVs to subsequently calcify on collagen. The failure of the dihydropyridine L-type calcium channel blockers to inhibit calcification, despite similar changes in intracellular calcium, suggests that there are unique properties of verapamil beyond the simple blockade of the L-type calcium channel that is responsible for its inhibitory effect on VSMC calcification.

L-type Ca^{2+} channel blockers are efficacious and widely used drugs in the treatment of hypertension and cardiovascular disease.[9] L-type calcium channel blockers may affect many calcium-dependent events in the formation of atherosclerosis, such as the localized accumulation of collagen, elastin, and calcium, together with monocyte infiltration and smooth muscle proliferation and migration.[10] In a human study that evaluated calcification by CT imaging, nifedipine, but not amlodipine, plus a diuretic showed significant inhibition of coronary calcium progression over a 3-year period.[11] In rats, amlodipine prevented aortic medial elastocalcinosis, pulse wave velocity, and pulse pressure induced in a calcification model induced by inactivation of the matrix Gal protein with vitamin K.[12] An early study by Schraven *et al.*[13] showed that verapamil inhibited vitamin D3-induced aorta calcification by 15% in rats. However, our *ex vivo* and *in vitro* studies clearly indicated that only verapamil and not nifedipine had an inhibitory effect on VSMC calcification. In an *in vitro* study, amlodipine also had no effect on calcification in primary cultures of rat VSMCs incubated with high calcium media,[14] similar to our findings with other dihydropyridines. The differences in the effect of dihydropyridines given *in vivo* compared with *in vitro* or

ex vivo may be attributed to the presence of an endothelium *in vivo* or other systemic factors, which may itself affect vascular smooth muscle cells. In contrast, isolated VSMC cultures provide a mechanistic insight into the pathogenesis of medial calcification that can occur despite normal endothelium.[15,16]

Differentiated VSMCs express various ion channels, including voltage-dependent L-type calcium channels.[17,18] Most ion channels are not expressed in the multipotential cells that give rise to VSMCs, but rather appear during differentiation/maturation. Calcium channels are particularly important in VSMCs and are responsible for regulating VSMC contractility, proliferation, and cell death.[19] The expression of L-type Ca^{2+} channel α1 subunits, a functional Ca^{2+} channel, is highly coordinated with expression of the smooth muscle-specific proteins required for specialized smooth muscle cell functions.[7] Our data confirm the presence of these channels in BVSMCs, but despite similar effects on intracellular calcium influx, only verapamil but not dihydropyridines inhibited calcification and the differentiation marker alkaline phosphatase activity. These classes of calcium channel blockers are known to have physiological differences, in part because of differences in their binding sites of the L-type channel. Our findings that verapamil, but not nifedipine or nimodipine, inhibited calcification in BVSMCs despite similar effects on intracellular calcium influx suggest that additional mechanisms are involved.

To explore the mechanism by which verapamil, but not dihydropyridines, inhibits alkaline phosphatase and calcification in BVSMCs, we examined the role of the ryanodine receptor. Ryanodine receptors are intracellular Ca^{2+} channels that mediate the release of calcium from the sarcoplasmic reticulum, an essential step in muscle contraction.[20] In skeletal muscle, it is thought that activation occurs through a physical coupling to the L-type calcium channels. Ryanodine receptors in the sarcoplasmic reticulum control myocardial calcium homeostasis, which in turn regulates myocardial function,[21] and inhibition of ryanodine receptor activity was associated with myocardial calcification in the rat.[22] Verapamil can directly bind to the ryanodine receptor channel of sarcoplasmic reticulum and inhibit the calcium release in rabbit skeletal muscle, whereas dyhydropyridines do not.[8] However, our results showed that neither the inhibition nor the activation of ryanodine receptors had an effect on calcification in BVSMCs, showing that our observed effect of verapamil on calcification is not mediated by the inhibition of the ryanodine receptor.

To further examine the observed differences between verapamil and dihydropyridines, we examined the effect of verapamil on the formation and activity of MVs, the membrane outpockets that are believed to be the initiator of extracellular matrix calcification. We found that verapamil decreased MV activity and its ability to calcify on type I collagen. However, when verapamil was added to MVs isolated from calcifying cells after MV formation from cells not exposed to verapamil, there was no effect. In addition, MVs did not express L-type calcium channels. MVs are known to arise from cell membranes based on their lipid composition and expression of various proteins and receptors commonly expressed in cell membranes, including annexins.[23] We have previously shown that annexin calcium channel activity is critical for calcification in BVSMCs and that the blockade of annexin calcium channel activity by K201 also inhibited matrix vesicle activity and its ability to subsequently calcify on collagen.[5] Annexins also affect membrane structure and function, interacting with the hydrocarbon chains of membrane lipids. This results in alterations of phospholipid membrane domains with resultant diverse effects.[24] We have previously shown that K201, which alters annexin conformation enabling calcium entry into the cells, had similar effects as verapamil on calcification, alkaline phosphatase activity, and MV activity. Furthermore, there was also no additive effect of verapamil and K201 on BVSMC calcification, suggesting similar mechanisms of action. Verapamil, but not dihydropyridines, bind to multidrug-resistant membrane vesicles,[25] and this effect is felt secondary to the specific effect of verapamil on membrane lipid bilayers,[26] which subsequently affects membrane potential.[27] Thus, incubation of BVSMCs with verapamil may alter membrane lipid dynamics and thereby alter MV formation and subsequent BVSMC calcification, a hypothesis supported by our findings that verapamil added to MV after the formation had no effect.

In conclusion, our results showed that although L-type calcium channels are present in BVSMCs, only the phenylalkylamine L-type calcium channel blocker verapamil is involved in VSMC mineralization and MV activity and function. The failure of dihydropyridine L-type calcium channel blockers to exert a similar effect suggests that there are unique properties of verapamil beyond the simple blockade of the L-type calcium channel that is responsible for its inhibitory effect on VSMC calcification and matrix vesicle activity. Our data suggest that these effects are likely because of the alterations in the membrane structure, similar to the proposed mechanism by which annexins may be involved in calcification. However, further studies are required to confirm this.

MATERIALS AND METHODS

Cell culture

Bovine vascular smooth muscle cells (BVSMCs) were isolated from the descending thoracic aorta by the explant method as previously described.[28] The BVSMCs were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St Louis, MO, USA), with 10% FBS until confluent, at which time they were replated for specific experiments. Only cells between passages 2–8 were used in the experiments. To induce calcification, BVSMCs were treated with 10 mmol/l β-glycerophosphate, 10^{-7} mol/l insulin, and 50 μg/ml ascorbic acid in the presence of 15% serum.[28] Control or non-calcifying BVSMCs were cultured in identical conditions, but without the β-glycerophosphate. In some experiments, BVSMCs were also treated with various concentrations of calcium channel blockers: verapamil, nifedipine, diltiazem (Calbiochem, CA, USA).

BVSMCs were also treated with the ryanodine receptor antagonists dantrolene and ryanodine, and ryanodine receptor activator 4-chloro-M-cresol (CMC) (Calbiochem, CA, USA).

Intracellular Ca^{2+} measurement

Cells were loaded with 5 μmol/l fura 4-AM (Molecular Probes, Eugene, OR, USA), a fluorescent calcium indicator, in Hank's balanced saline solution (HBSS) for 30 min at 37 °C. Cells were rinsed and incubated for an additional 30 min with HBSS alone to allow for complete de-esterification of the fluorescent probe. Intracellular calcium changes were examined by confocal microscopy and quantified using Metamorph software.

Western blotting

Western blotting was performed as previously described.[28] The blots were incubated overnight at 4 °C with antibody against the L-type calcium channel (α1c subunit, 1:1000, a gift from Dr Randall Duncan, University of Delaware), followed by incubation with peroxidase-conjugated secondary antibody (1:5000 dilution), and immunodetection with the Enhanced Chemiluminescence Kit (Amersham, Piscataway, NJ, USA). The band intensity was analyzed by scanning densitometry (Quantity One, Bio-Rad, Richmond, CA, USA).

Matrix vesicle (MV) isolation

MVs were isolated by collagenase digestion as previously described.[5] Cells were incubated with crude collagenase (500 U/ml, type IA, Sigma) in a solution of 0.25 mol/l sucrose, 0.12 mol/l NaCl, 0.01 mol/l KCL, and 0.02 mol/l Tris buffer, pH 7.45, at 37 °C for 3 h. The digests were centrifuged at 800 *g* and 30,000 *g*, respectively, to remove cell debris and microsomes. The supernatant was centrifuged at 250,000 *g* to pellet the MV followed by resuspension in TBS (pH 7.6) with 0.25 mol/l sucrose. The MV amount was determined by protein concentration (Bio-Rad).

Calcium deposition

After incubation in calcification or control media (with and without β-glycerophosphate), BVSMCs were decalcified with 0.6 N HCl for 24 h. The calcium content of HCl supernatants was determined colorimetrically by the *o*-cresolphthalein complex one method (Calcium kit; Pointe Scientific, Canton, MI, USA) and normalized to protein content as previously described.[28]

Alkaline phosphatase activity

Alkaline phosphatase activity was measured using *p*-nitrophenyl substrate supplied in an alkaline phosphatase assay kit (Pointe Scientific) and normalized by protein content.[28]

MV-collagen calcification assay

MV-collagen calcification was determined as we previously described.[5] Briefly, glass coverslips were coated with type I collagen (Sigma) in a 0.01% solution in 0.1 mol/l acetic acid at room temperature for 4 h, which yields approximately 8–10 $\mu g/cm^2$ coating. Matrix vesicles isolated as above were added in equal concentrations (10 μg per dish) to type I collagen-coated coverslips in the presence of calcification media (DMEM with 15% FBS and 10 mmol/l β-glycerophosphate) to yield an acellular MV-collagen culture and incubated at 37 °C for 72 h. To determine the magnitude of calcification of this MV-collagen culture, the media were removed and the MV–ECM complex on the coverslips incubated in 0.6 N HCl for 24 h. The calcium content of HCL supernatants was determined colorimetrically by the *o*-cresolphthalein complex one method (Calcium kit; Pointe Scientific).

Organ culture

Segments of rat thoracic aorta measuring 3 cm were harvested from male Sprague–Dawley rats and gently cleared of surrounding tissues. Aortic segments were incubated in DMEM (Gibco, Long Island, NY, USA) containing 1X penicillin/streptomycin and without serum at 37 °C in a humidified 5% CO_2/95% air atmosphere. To induce calcification, 7.5 units/ml of calf intestinal alkaline phosphatase was added to DMEM and the phosphate ion concentration was increased to 3.8 mmol/l by the addition of sodium phosphate (calcification medium) as per the methods of O'Neil and colleagues.[29] Verapamil or nifedipine (10, 20 and 50 μmol/l) was pre-equilibrated in medium and then added to the culture medium. Culture medium was changed every 2–3 days. After 7 days, aortic specimens were rinsed in normal saline, minced, and decalcified in 300 μl of 0.6 N HCL for 72 h with gentle agitation. The calcium content of HCL supernatants was determined colorimetrically as described above and normalized by tissue weight. Viability of aorta organ cultures was determined by lactic dehydrogenase secretion into the medium using CytoTox-One Homogeneous Membrane Integrity Assay (Promega, Madison, WI, USA). When incubated with 50 μmol/l verapamil or nifedipine, there was a marked increase in lactic dehydrogenase indicating decreased viability of organ cultures, and therefore we only evaluated results with 0, 10, or 20 μmol/l verapamil or nifedipine.

DISCLOSURE

All the authors declared no competing interests.

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