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Protamine augments stretch induced calcium increase in vascular endothelium

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1 Human umbilical vein endothelial cells cultured on a transparent silicone chamber were subjected to a short stretch pulse (*ca.* 1 s, 5–25% stretch) of their substrate and following increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured by fluorescence intensity ratiometry using fura-2.

2 In response to mechanical stretch, the cells in HEPES buffered saline exhibited a Ca^{2+} transient in a dose dependent way. The response was completely dependent on external Ca^{2+} and inhibited by gadolinium (Gd^{3+}), suggesting that it was mediated by the activation of a stretch activated cation channel (SACatC).

3 Interestingly, the stretch induced Ca^{2+} transient was significantly augmented in the presence of basic polypeptide, protamine. This augmented Ca^{2+} response was inhibited neither by Gd^{3+} nor by the deprivation of external Ca^{2+}, indicating that the SACatC is not responsible for this phenomenon.

4 In contrast, this augmentation was inhibited by depletion of intracellular Ca^{2+} stores with thapsigargin or by the pretreatment with phospholipase inhibitors such as U73122 and manoalide.

5 These results suggest the presence of a metabotropic mechanoreceptor distinct from the SACatC in vascular endothelium. This augmented $[Ca^{2+}]_i$ increase may contribute to the vasodilating response induced by protamine during heparin neutralization in cardiac surgery.
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Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; HBS, HEPES buffered saline; HUVEC, human umbilical vein endothelial cell; PLC, phospholipase C; SACatC, stretch activated cation channel

Introduction

Vascular endothelial cells lining the innermost vascular wall are exposed to haemodynamic forces such as shear stress by blood flow, normal stress by blood pressure, and pulsatile stretch by vessel expansion. In response to these haemodynamic forces vascular endothelial cells show a variety of reactions including releases of vasoactive substances (Sumpio & Banes, 1988; Sumpio & Widmann, 1990), gene expression (Chien *et al*., 1998), and morphological changes (Shirinsky *et al*., 1989; Naruse *et al*., 1998). However, the molecular mechanisms by which the cells sense the mechanical forces remain largely unknown. A potential candidate for mechanoreceptors in endothelial cells is the stretch activated cation channel (SACatC) that is permeable to calcium ion (Ca^{2+}) (Lansman *et al*., 1987; Naruse & Sokabe, 1993; Kohler *et al*., 1998). Although it has recently been reported that the yeast MID1 and the *Drosophila nompC* genes encode SACatCs (Kanzaki *et al*., 1999; Walker *et al*., 2000), the molecular entity of the SACatC in vascular endothelium has not yet been identified.

We have reported that activation of SACatCs is responsible for intracellular Ca^{2+} ($[Ca^{2+}]_i$) increase to stretching the substrate in cultured human umbilical vein endothelial cells (HUVECs) (Naruse & Sokabe, 1993). However, the applied forces in the study were not uniaxial and therefore might not be physiological, because endothelial cells *in situ* are subjected to pulsatile uniaxial stretch. In the present study, a stretch chamber has been developed in order to measure Ca^{2+} mobilization in cells subjected to controlled uniaxial stretch. HUVECs were plated at the bottom of a rectangular transparent silicone chamber, which was stretched uniaxially by a computer-controlled stepping motor. The system was mounted on the stage of an inverted microscope and $[Ca^{2+}]_i$ measurements were carried out using the standard fura-2 method. The results reaffirmed our previous finding that Ca^{2+} mobilization to mechanical stretch in HUVECs requires Ca^{2+} influx *via* SACatCs when studied in HEPES buffered saline (HBS). Moreover, we demonstrate here that $[Ca^{2+}]_i$ response induced by mechanical stretch is significantly augmented in the presence of protamine. This sensitization of $[Ca^{2+}]_i$ increase to mechanical stimuli may contribute to hypotension caused by protamine administration during heparin neutralization in cardiac surgery (Horrow, 1985), since endothelial cells are known to secret several vasoactive substances including nitric oxide and prostacyclin *via* internal Ca^{2+} mobilization. Furthermore,

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pharmacological investigations revealed that the mechanisms of this augmentation were independent of SACatCs, suggesting the presence of a mechanoreceptor distinct from SACatCs in vascular endothelial cells.

Methods

Cell culture

HUVECs were prepared as described elsewhere (Naruse & Sokabe, 1993). In brief, HUVECs were removed from the umbilical vein lumen by 10-min trypsin incubation. Then the cells were pelleted and resuspended in HuMedia-EG2 (Kurabo, Osaka, Japan) and seeded in plastic flasks. Cells between passage 2 and 5 were used in this study. All experiments were performed with at least five different dispersions of cells from different umbilical cords.

Application of uniaxial stretch

HUVECs removed from the flasks with trypsin were plated on a silicone chamber coated with fibronectin. The chamber had a 100 μm-thick transparent bottom (7-mm length, 15-mm width, and 5-mm depth). One end of the chamber was clamped with a fixed metal frame, and the other end with a movable frame that was connected to a shaft driven by a computer controlled stepping motor. This apparatus was able to control the amplitude and the rate of stretch. In this study, the chamber was uniaxially stretched by 5–25% of the initial length at a rate of 0.1% m s^{-1}. Then the chamber was held for 1 s at the stretched position, and returned to the initial unstretched state at the same rate of the stretching phase. We confirmed that the bottom of the chamber was stretched uniformly over the area of interest.

Measurement of stretch induced $[Ca^{2+}]_i$ increase

$[Ca^{2+}]_i$ was measured by the fura-2 method using a fluorescence microscope (M1000; Inter Dec., Osaka, Japan) with a 20× objective (Fluor 20; Nikon, Tokyo, Japan), as described previously (Naruse & Sokabe, 1993). The excitation wavelengths were set 340 and 380 nm, and the emission was detected at 510 nm by a photomultiplier. Fluorescence intensity ratio (340/380) was calculated from the following equation: ratio (340/380) = (F340-B340)/(F380-B380), where F340 and F380 are intensities at 510 nm excited at 340 and 380 nm, respectively, and B340 and B380 are corresponding background fluorescence values. Because the fura-2 method has several intrinsic problems in the estimation of absolute $[Ca^{2+}]_i$ (Karaki, 1989; Ziche *et al*., 1993), we simply employed a ratio (340/380) in this study. The number of the cells in the area of interest was ~60. The experiments were undertaken at 37°C in HBS containing (in mM): NaCl 145, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, and HEPES 10, pH 7.40, otherwise indicated.

Statistical analysis

Results are expressed as mean ± s.e.mean. The statistical difference between two mean values was analysed by Wilcoxon test. For multiple comparisons, analyses were performed with *H*-test of Kruskal and Wallis to assess the presence of significant intergroup inhomogeneity followed by Mann–Whitney *U*-test for further comparisons. A $P<0.05$ was considered statistically significant.

Results

Effect of extracellular Ca^{2+} and Gd^{3+} on stretch-induced $[Ca^{2+}]_i$ increase in HUVECs in HBS

Endothelial cells cultured on an elastic silicone membrane were stretched 5–25% uniaxially for 1 s, and changes in $[Ca^{2+}]_i$ were measured. In response to mechanical stretch, rapid $[Ca^{2+}]_i$ increase followed by gradual decrease to the initial basal level was observed (Figure 1). However, exactly when in relation to stretch the peak of the rise in $[Ca^{2+}]_i$ happens was not determined, since the changes during stimulation could not be measured because the cells were out of focus during stretch. The $[Ca^{2+}]_i$ response increased as the degree of the stretch increased. The Ca^{2+} response was reproducible as clearly shown in the figure. Generally there are two mechanisms for intracellular Ca^{2+} mobilization: Ca^{2+} influx through Ca^{2+} permeable channels, and releases from intracellular Ca^{2+} stores. To test the first mechanism, extracellular Ca^{2+} was reduced to a nominally Ca^{2+} free level. Under this condition, the response was completely abolished (Figure 2). Readdition of extracellular Ca^{2+} recovered the response (data not shown). These results indicated that external Ca^{2+} and/or Ca^{2+} influx was essential to Ca^{2+} mobilization to mechanical stretch. The trivalent cation, Gd^{3+}, is known to inhibit stretch activated channels (Gustin *et al*., 1988; Yang & Sachs, 1989; Franco & Lansman, 1990; Kawakubo *et al*., 1999). In the presence of 10 μM Gd^{3+}, the response to mechanical stretch was significantly inhibited, however, the cells subjected to 15–25% stretch exerted small responses (Figure 3). One hundred μM Gd^{3+} completely inhibited the response (data not shown). It has been reported that a pressure modulated cation channel insensitive to 10 μM Gd^{3+} exists in native endothe-

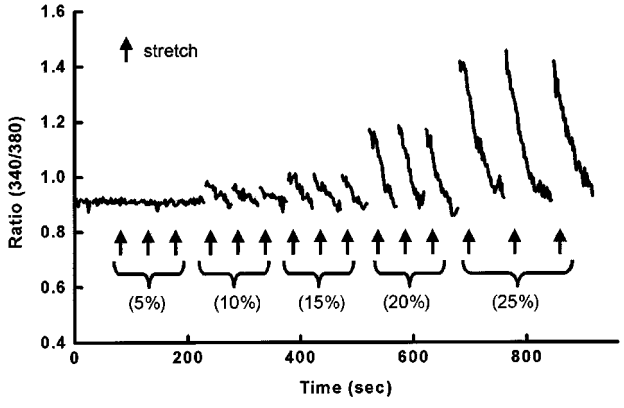

Figure 1 Typical tracing of Ca^{2+} increase in human umbilical vein endothelial cells subjected to uniaxial stretch pulses. Cells cultured on an elastic silicone membrane were stretched by 5–25% uniaxially for 1 s at the times indicated by arrows. Per cent stretch is shown in the parentheses. The response was reproducible and dependent on the extent of stretch. Extracellular solution was HEPES buffered saline.

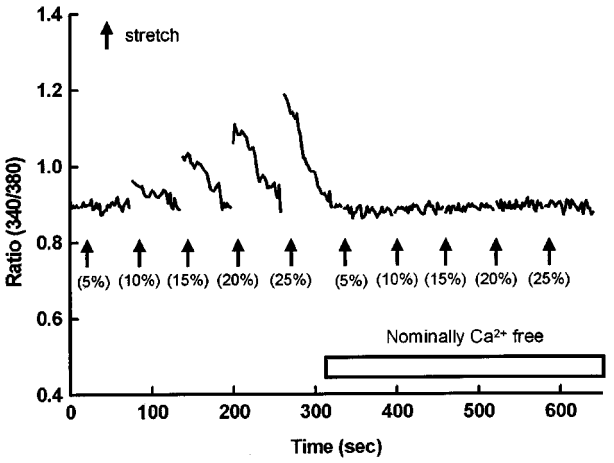

Figure 2 Effect of extracellular Ca^{2+} on the Ca^{2+} response to stretch. Human umbilical vein endothelial cells in Ca^{2+} containing HEPES buffered saline (HBS) were subjected to 5–25% stretch, then the solution was changed to nominally Ca^{2+}-free HBS as indicated by the open bar and the cells were subjected to the same series of mechanical stimuli. Cells in Ca^{2+}-free solution did not respond to stretch. Arrows indicate the onset of stretch and per cent stretch is shown in the parentheses. The trace was constructed from a typical set of data from six separate preparations.

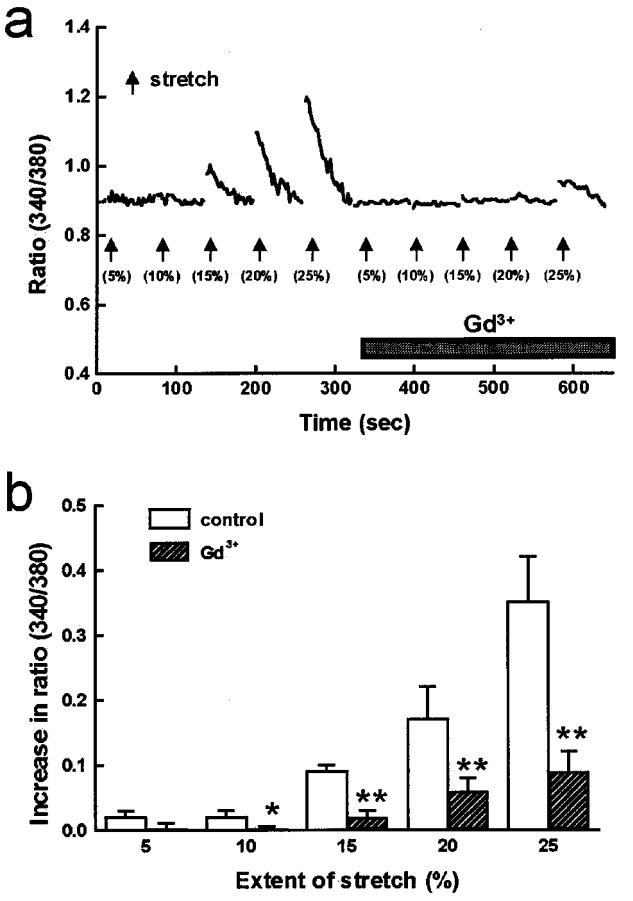

Figure 3 Effect of Gd^{3+} on the Ca^{2+} response to stretch. (a) Human umbilical vein endothelial cells were subjected to 5–25% stretch in the absence and the presence of 10 μM $GdCl_3$ (hatched bar), an inhibitor of SACatCs. The trace was constructed from a typical set of data from six separate preparations. (b) Each column indicates means ± s.e.mean of the peak value from six separate preparations. Control cells (open columns) displayed a dose-dependent Ca^{2+} response to stretch. In the presence of Gd^{3+} (hatched columns), the response was significantly inhibited. $^*P<0.05$, $^{**}P<0.01$ vs control.

lium (Marchenko & Sage, 1997). Such a channel may have contributed to the responses observed here. After perfusion with HBS without Gd^{3+}, the response was recovered (data not shown).

Effect of stretch on Mn^{2+} permeability

We investigated the changes in Mn^{2+} permeability caused by mechanical stretch of the cells. The entry of Mn^{2+} into fura-2 loaded cells is known to quench fura-2 fluorescence (Grynkiewicz *et al*., 1985). To see the Mn^{2+} entry into cytoplasm, extracellular Ca^{2+} was replaced with Mn^{2+}, and the changes in fluorescence intensity excited at 360 nm were measured (i.e., the isosbestic, Ca^{2+} insensitive wavelength of fura-2 fluorescence). Application of Mn^{2+} extracellulary caused gradual decrease in fluorescence, indicating that Mn^{2+} entered into the cells under unstretched condition. Furthermore, a mechanical stretch caused a sudden drop of the intensity of fura-2 fluorescence, suggesting a rapid entry of Mn^{2+} into the cytoplasm during mechanical stretch (Figure 4). Note that stretching the cells in the absence of Mn^{2+} did not change the intensity of fura-2 fluorescence at 360 nm, indicating that stretch did not cause leakage of intracellular dye. This and above results indicate that uniaxial stretch in HUVECs activates a Ca^{2+} and Mn^{2+} permeable pathway, which is highly likely a SACatC as evidenced in our previous study (Naruse & Sokabe, 1993). Thus it is strongly suggested that the Ca^{2+} mobilization induced by uniaxial stretch involves essentially the same mechanism as that of Ca^{2+} mobilization induced by uniform stretch (Naruse & Sokabe, 1993).

Augmentation of stretch induced $[Ca^{2+}]_i$ increase by protamine

When protamine (20 μg ml^{-1}) was present in extracellular solutions the stretch induced $[Ca^{2+}]_i$ response of HUVECs was strongly augmented (Figure 5). The concentration of protamine was comparable to clinical protamine dose of 1 mg kg^{-1} body weight used to reverse the anticoagulant effect of heparin. Application of protamine *per se* did not cause measurable Ca^{2+} mobilization in HUVECs. It is of interest whether this augmentation originated from the sensitization of the putative SACatC or an activation of different Ca^{2+} mobilizing system in HUVECs. To determine which mechanism is responsible, we carried out following experiments mainly concerning the origin of the augmented $[Ca^{2+}]_i$ increase by protamine.

First to test the contribution of extracellular Ca^{2+} to this phenomenon, extracellular Ca^{2+} was reduced to a nominally Ca^{2+} free level. Under this condition, the $[Ca^{2+}]_i$ increase in response to mechanical stretch in the presence of protamine was not abolished (Figure 6a). This indicated that external Ca^{2+} was not essential to Ca^{2+} mobilization by mechanical stretch in the presence of protamine. Actually, application of 10 μM Gd^{3+}, a SACatC blocker, under the same condition did not affect significantly the stretch induced Ca^{2+}

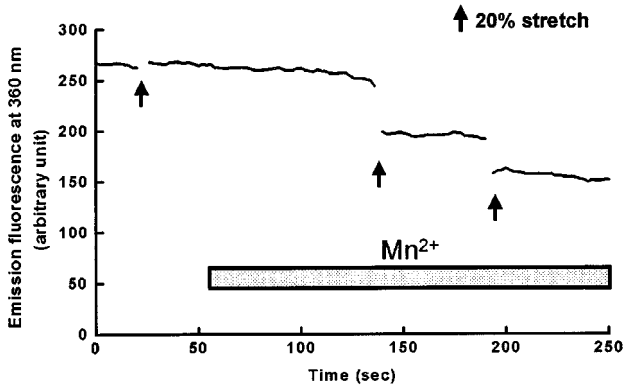

Figure 4 Effect of mechanical stretch on fura-2 fluorescence in the presence of Mn^{2+}. Fura-2 loaded human umbilical vein endothelial cells (HUVECs) were illuminated with excitation light at 360 nm, and fluorescence was measured at 510 nm. Arrows indicate the onset of 20% stretch. Cells were first stretched in Ca^{2+} containing HEPES buffered saline (HBS), and no change in fluorescence was observed. The extracellular solution was then changed to HBS containing 1 mM Mn^{2+} instead of Ca^{2+} as indicated by a dotted bar. HUVECs were subjected to a second and a third stretch, which caused a sudden drop of the intensity of fura-2 fluorescence, suggesting a rapid entry of Mn^{2+} into the cytoplasm in response to mechanical stretch. The same results were obtained with six separate preparations.

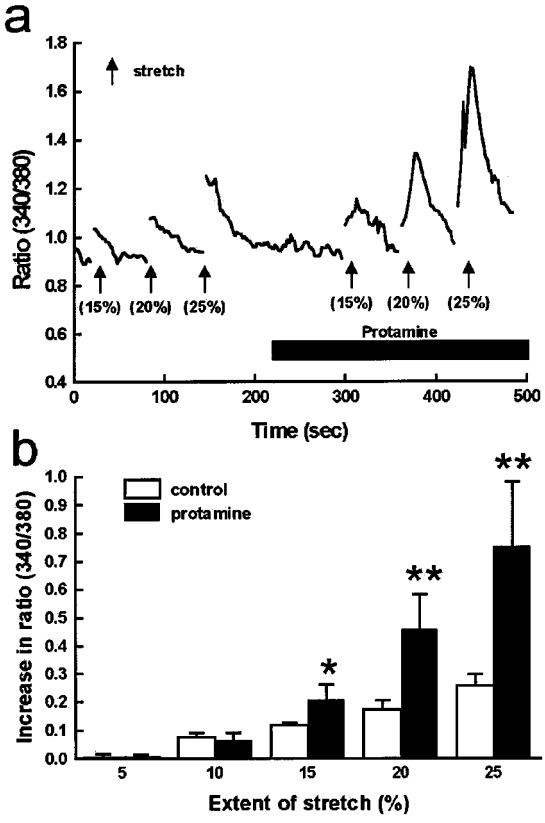

Figure 5 Effect of protamine on Ca^{2+} response to stretch. (a) Human umbilical vein endothelial cells were first stretched in standard HEPES buffered saline (HBS) and then in HBS containing 20 μg ml^{-1} protamine (solid bar). The trace was constructed from a typical set (control, protamine) of data from six separate preparations. (b) Each column indicates means$\pm$s.e.mean of the peak value from six separate preparations. Control cells (open bars) and cells with protamine (solid columns) display dose-dependent Ca^{2+} responses to stretch. The Ca^{2+} increases in response to 15–25% stretch were significantly augmented, while the baseline Ca^{2+} was not changed significantly by application of protamine (compare the levels in the trace just before 15% stretch in standard HBS and in protamine containing HBS). $^*P<0.05$, $^{**}P<0.01$ vs control.

mobilization (Figure 6b), indicating that SACatCs do not contribute to the protamine augmented Ca^{2+} response. Furthermore, the stretch induced $[Ca^{2+}]_i$ increases declined, when the cells were stimulated repetitively in the absence of extracellular Ca^{2+} (data not shown), suggesting the depletion of intracellular Ca^{2+} stores. To support this idea, the $[Ca^{2+}]_i$ reached its peak value 18 ± 5 s after the stimulus in the presence of protamine, while cells in a solution without protamine responded immediately after the stimulus followed by gradual decrease to the basal $[Ca^{2+}]_i$ as clearly seen in Figure 6a,b. These results suggest that the mechanism of $[Ca^{2+}]_i$ increase induced by stretch in the presence of protamine is different from that observed in HBS alone.

Therefore, we examined the involvement of Ca^{2+} releases from intracellular stores by depleting cells of stored Ca^{2+} with thapsigargin. The cells treated with thapsigargin (50 nM, 15 min) did not respond to mechanical stretch even in the presence of protamine when the extracellular Ca^{2+} was depleted (Figure 6c). Essentially the same results were obtained when the cells were depleted of stored Ca^{2+} with repetitive treatment with Ca^{2+} mobilizing agonists such as ATP and histamine in Ca^{2+} free solutions (data not shown). To examine whether protamine acts on extracellular Ca^{2+} dependent $[Ca^{2+}]_i$ increase in response to stretch, we also tested the effect of protamine in cells pretreated with thapsigargin in the presence of extracellular Ca^{2+}. Protamine did not augment the response and the time course of the response was similar to those observed in cells without protamine (Figure 7). We also tested the effect of protamine on mechanically induced $[Ca^{2+}]_i$ increase in the presence of Gd^{3+} in Ca^{2+} containing HBS. Protamine exerted essentially the same effect as observed in Ca^{2+} free solution (data not shown), suggesting that the effect of protamine is independent of SACatCs. These results suggest that Ca^{2+} mobilization in response to mechanical stretch in the presence of protamine is attributable to Ca^{2+} releases from intracellular stores.

We hypothesized that Ca^{2+} mobilization induced by mechanical stretch in the presence of protamine may utilize a common intracellular signalling pathway as a variety of agonists which release intracellular Ca^{2+} *via* activation of phospholipase C (PLC). Therefore, we examined the effect of phospholipase inhibitors on Ca^{2+} mobilization induced by stretch in the presence of protamine. Manoalide is a time- and temperature-dependent inhibitor of phospholipases (Diamond *et al*., 1994). Fura-2 loaded HUVECs incubated with 10 μM manoalide for 10 min were unresponsive to the Ca^{2+} mobilizing agonists such as ATP and histamine (data not shown). While manoalide treated cells responded to mechanical stretch in the presence of extracellular Ca^{2+}, they were almost unresponsive to mechanical stretch in the absence of extracellular Ca^{2+} irrespective of the presence of protamine (Figure 6d). We also tested the effect of U-73122 (10 μM, 15 min) on the stretch induced $[Ca^{2+}]_i$ response in the presence of protamine. U-73122 is a widely used PLC inhibitor (Bleasdale *et al*., 1990; Thompson *et al*., 1991), and its structure is different from that of manoalide. U-73122

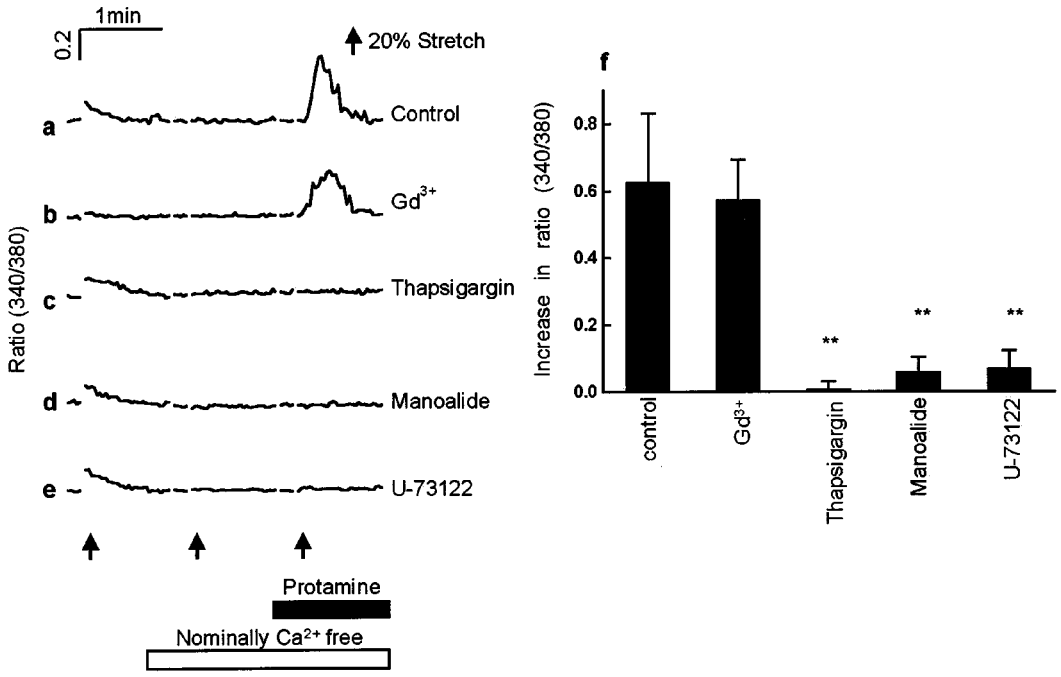

Figure 6 Effect of Ca^{2+}, Gd^{3+}, thapsigargin, manoalide, and, U-73122 on protamine sensitized Ca^{2+} mobilization to stretch. Human umbilical vein endothelial cells (HUVECs) were stretched by 20% at the times indicated by arrows in the bottom trace. Each stretch was made in three different solutions (standard HEPES buffered saline, HBS, Ca^{2+}-free HBS, Ca^{2+}-free HBS containing protamine). Open bar and solid bar indicate the period with nominally Ca^{2+}-free HBS and protamine (20 μg ml^{-1}), respectively. Typical traces are shown from 5–8 separate preparations. (a) Control: relatively small response is seen at the first stretch, while no response at the second stretch in Ca^{2+}-free HBS. In contrast, a huge but a relatively slow Ca^{2+} response can be seen at the last stretch in the presence of protamine. (b) Ten μM Gd^{3+} does not seem to have any effect on the Ca^{2+} response in the presence of protamine. (c) Thapsigargin treatment completely abolished the protamine sensitized Ca^{2+} response. (d), (e) The phospholipase inhibitors manoalide and U-73122 inhibited the protamine sensitized Ca^{2+} response. (f) Summary of Ca^{2+} increase to 20% stretch in HUVECs in Ca^{2+}-free HBS containing protamine. Cells treated with thapsigargin, manoalide, and, U-73122 displayed a significantly reduced response to stretch. HUVECs were preincubated with thapsigargin (50 nM) and U-73122 (10 μM) for 15 min, or manoalide (10 μM) for 10 min, while Gd^{3+} (10 μM) was added just before measurement. Data represent means$\pm$s.e.mean from 5–8 separate preparations. **$P<0.01$ vs control.

also exerted an inhibitory effect on Ca^{2+} mobilization induced by mechanical stretch in cells treated with protamine in the absence of extracellular Ca^{2+} (Figure 6e). These results indicated that a signalling pathway involving PLC activation is required for the protamine-sensitized response to the mechanical stretch. Figure 6f summarizes the results from the experiments concerning the mechanisms of protamine-sensitized Ca^{2+} mobilization.

Effects of other basic polypeptides on Ca^{2+} mobilization to stretch

Protamine is a polycationic peptide rich in basic amino acids such as arginine and lysine. We examined whether other polycationic peptides have similar effects on the Ca^{2+} mobilization to stretch. Twenty μg ml^{-1} of poly-L-arginine (MW 5000–15,000) exerted essentially the same effect as protamine on the stretch induced Ca^{2+} response in Ca^{2+} free solutions (Figure 8). Poly-L-lysine showed a similar effect to that of poly-L-arginine and protamine (data not shown). We also examined the effect of organic cations with smaller molecular weights such as L-arginine (1 mM), L-lysine (1 mM), neomycin (100 μM), and spermine (100 μM, 1 mM). These cations did not mimic the effect of protamine at the concentration tested (data not shown), indicating that the sensitization of the Ca^{2+} response to stretch caused by polycations may be specific to large molecular weight peptides.

Discussion

In a previous study, we observed a Ca^{2+} transient to stretch in cultured HUVECs using a cylinder-shaped chamber, the bottom of which was expanded uniformly with a negative pressure pulse (Naruse & Sokabe, 1993). In this study, we have developed a system by which we can apply controlled uniaxial stretch to endothelial cells, and observed essentially the same phenomena including its ionic mechanism that the Ca^{2+} response to mechanical stretch in HUVECs in HBS requires Ca^{2+} influx *via* SACatCs. Three lines of evidence support this as follows. First, Ca^{2+} mobilization to mechanical stretch in HUVECs in HBS is dependent on extracellular Ca^{2+} (Figure 2). Second, 10 μM Gd^{3+}, a blocker of stretch activated channels (Gustin *et al*., 1988; Franco & Lansman, 1990; Kohler *et al*., 1998; Kanzaki *et al*., 1999), inhibited stretch induced Ca^{2+} responses (Figure 3). Third, replacement of Ca^{2+} with Mn^{2+} induced quenching of fura-2

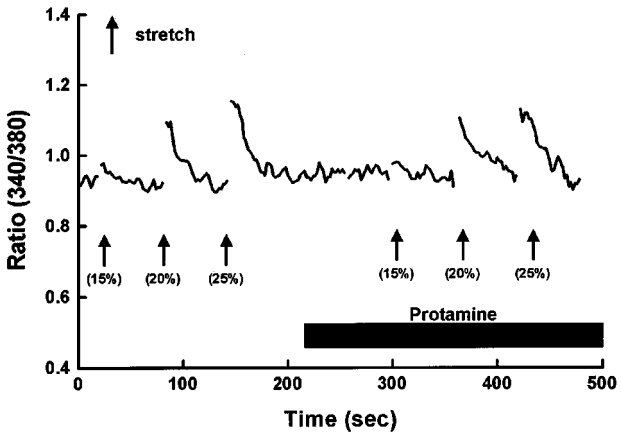

Figure 7 Effect of protamine on extracellular Ca^{2+} dependent $[Ca^{2+}]_i$ increase in response to stretch. Fura-2 loaded human umbilical vein endothelial cells were depleted with stored Ca^{2+} by pretreatment with 50 nM thapsigargin for 15 min, and then subjected to mechanical stretch. The cells were first stretched in standard HEPES buffered saline (HBS) and then in HBS containing 20 μg ml^{-1} protamine (solid bar). Arrows indicate the onset of stretch and per cent stretch is shown in the parentheses. The trace was constructed from a typical set (control, protamine) of data from six separate preparations. The extracellular solution was HEPES buffered saline containing 1 mM Ca^{2+}.

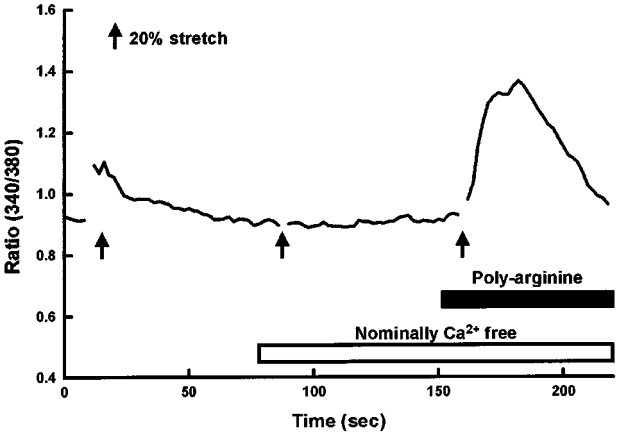

Figure 8 Effect of poly-L-arginine (PA) on Ca^{2+} mobilization to stretch. Human umbilical vein endothelial cells (HUVECs) were subjected to 20% stretch at the times indicated by arrows. Open bar and solid bar indicate the periods with nominally Ca^{2+}-free solution and PA (20 μg ml^{-1}) containing solution, respectively. Ca^{2+} response was observed in the presence of PA in nominally Ca^{2+}-free condition.

fluorescence when the cells were stretched (Figure 4), suggesting that the stretch causes influx of Mn^{2+}, which has been reported to be permeable for non-selective type of SACatC (Ohmori, 1985).

Furthermore, the $[Ca^{2+}]_i$ response to mechanical stretch in HBS was virtually unaffected by the pretreatment with PLC inhibitors as well as the depletion of intracellular Ca^{2+} stores with thapsigargin (Figure 6). These results are in contrast to the report by Diamond *et al.* (1994), who mentioned that mechanically induced $[Ca^{2+}]_i$ increase in endothelium is

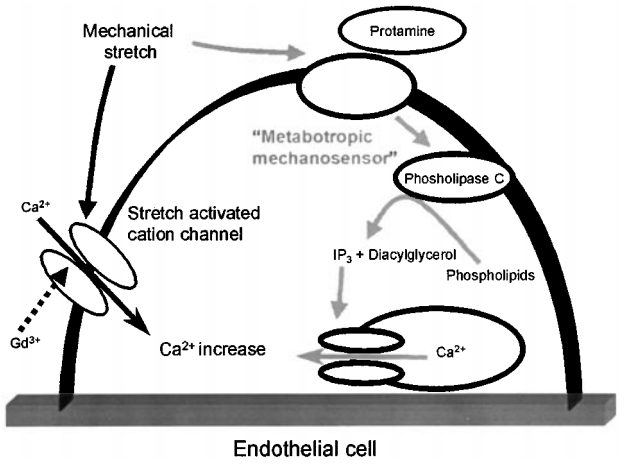

Figure 9 Hypothetical scheme for the augmented endothelial Ca^{2+} increase to stretch in the presence of protamine. Black arrows depict stretch-induced signalling pathways in standard HEPES buffered saline. Grey arrows mark the pathways leading to an enhanced Ca^{2+} increase in the presence of protamine. Note that protamine *per se* does not activate Ca^{2+} releases from intracellular stores.

dependent on Ca^{2+} mobilization from intracellular stores mediated by PLC activation. This discrepancy may be explained by the differences of experimental settings. They used bovine aortic endothelial cells in a solution containing serum albumin, and applied mechanical forces by micropipette prodding, while we used HUVECs in HBS without albumin, and applied mechanical forces by stretching the elastic substrate uniaxially.

We found that Ca^{2+} mobilization in response to stretch is augmented in the presence of protamine (Figure 5), and investigated the mechanisms of this phenomenon using pharmacological interventions. The protamine-augmented $[Ca^{2+}]_i$ increase by stretch was independent of extracellular Ca^{2+} (Figure 6a) and insensitive to Gd^{3+} (Figure 6b), indicating that this Ca^{2+} increase is caused by a mechanism distinct from the activation of SACatCs. The abolishment of the effect of protamine in cells treated with thapsigargin (Figures 6c and 7) clearly indicates that Ca^{2+} increase in response to stretch in the presence of protamine is dependent on Ca^{2+} releases from intracellular stores. Furthermore, pretreatment with two chemically distinct PLC inhibitors, manoalide and U-73122, inhibited the mechanically induced Ca^{2+} releases in the presence of protamine (Figure 6d,e). These results indicate that a signalling pathway including PLC activation is required for the protamine-sensitized Ca^{2+} response to mechanical stretch. It should be noted that administration of protamine *per se* does not seem to activate PLC, since application of protamine did not induce measurable Ca^{2+} increase in HUVECs unless the cells were stretched. Based on these observations, we can assume the presence of a 'metabotropic' type mechanoreceptor distinct from the ionotropic type receptors such as SACatCs in endothelial cells (Figure 9). This is not unnatural because many ligands including glutamate, purines, and acetylcholine are known to act on both metabotropic and ionotropic receptors. The mechanism by which these basic polypeptides enable the stretch-evoked Ca^{2+} releases remains to be studied in the future. One possible explanation is that polycations

with a large molecular size may bind to the negatively charged endothelial cell membrane and change its physical property. Another hypothesis is that as yet unidentified 'polycation sensing receptor' of which activation needs not only the binding of the polycation but also mechanical stretch, may be involved in the Ca^{2+} releases from the intracellular stores.

Vascular endothelium plays a critical role in regulating vascular tone through the secretion of vasoactive substances. For example, endothelial nitric oxide synthase is a Ca^{2+}-calmodulin-dependent enzyme and it is generally accepted that this enzyme is activated by increases in $[Ca^{2+}]_i$ elicited by activation of diverse G protein-coupled receptors in endothelial cells (Sase & Michel, 1997). Protamine, which is routinely used to neutralize heparin in cardiac surgery, causes hypotension following rapid administration (Horrow, 1985). It has been also demonstrated that this substance induces endothelium-dependent vasodilation (Pearson *et al*., 1992; Raikar *et al*., 1996). Other basic polypeptides such as poly-arginine, lysine, and ornithine can also influence the release of the endothelium-derived relaxing factor, nitric oxide (Ignarro *et al*., 1989). However, administration of protamine and poly-arginine *per se* did not increase $[Ca^{2+}]_i$ in HUVECs unless they were stretched (Figures 5 and 8). Since the endothelial cells *in situ* are constantly subjected to mechanical forces, it is possible that intravascular administration of basic polypeptides including protamine increases $[Ca^{2+}]_i$ in endothelial cells and results in hypotension *via* enhanced activation of nitric oxide synthase.

In conclusion, we have developed a system by which we can measure $[Ca^{2+}]_i$ of the cells under uniaxial stretch, and found that protamine augments stretch induced $[Ca^{2+}]_i$ increase in cultured vascular endothelium *via* a pathway that involves Ca^{2+} releases mediated by PLC activation. It is suggested that a metabotropic type mechanoreceptor as well as an ionotropic type receptor, SACatC, is expressed in vascular endothelium.

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