Involvement of SA channels in orienting response of cultured endothelial cells to cyclic stretch

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Naruse, Keiji, Takako Yamada, and Masahiro Sokabe. Involvement of SA channels in orienting response of cultured endothelial cells to cyclic stretch. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H1532–H1538, 1998.—The present work was designed to elucidate the involvement of Ca2+-permeable stretch-activated (SA) channels in the orienting response of endothelial cells to uniaxial cyclic stretch. Endothelial cells from human umbilical vein were cultured on an elastic silicone membrane and subjected to uniaxial cyclic stretch (120% in length, 1 Hz). The cells started to change their morphology 15 min after the onset of stretch, and >90% of the cells oriented perpendicularly to the stretch axis after 2 h. Associated with the orienting response, cell elongation proceeded with a slower rate. Both of the orientating and elongating responses were largely inhibited by the removal of external Ca2+ or by Gd3+, a potent blocker for the SA channel, but not by nifedipine. Intracellular Ca2+ concentration ([Ca2+]i) transiently increased in response to uniaxial stretch, and the basal [Ca2+]i gradually increased during cyclic stretch. This [Ca2+]i response was inhibited by the removal of extracellular Ca2+ or by the addition of Gd3+. These results suggest that stretch-dependent Ca2+ influx through SA channels is essential in the stretch-dependent cell orientation and elongation.

uniaxial cyclic stretch; cell morphology; gadolinium; calcium mobilization

ENDOTHELIAL CELLS in vivo show spindleshape and align their longitude along a running vessel, whereas they orient randomly when cultured in a dish. It has been suggested that the physical environment surrounding endothelial cells is important to form their characteristic two-dimensionally polarized shape in vivo (7, 22). Of particular interest are vectorial forces such as shear stress by blood flow and circumferential tension by periodic vessel expansion. To test this hypothesis, several experimental systems have been developed by which controlled shear stress or tension could be applied to cultured endothelial cells (2, 5, 13, 25). When endothelial cells were cultured under laminar flow exposed to shear stress, endothelial cells oriented their longitude parallel to the flow direction (22). Electrophysiology revealed that shear stress hyperpolarized endothelial cells by increasing K+ permeability (23). To simulate periodic circumferential stretch in the vessel wall, endothelial cells are cultured on an elastic membrane, and uniaxial cyclic stretch is applied to the membrane. Shirinsky et al. (25) reported that endothelial cells subjected to such an artificial stretch orient their longitude perpendicularly to the stretch axis as in vivo. This change in orientation was accompanied by the reorganization of actin filaments (stress fibers) and removal of myosin from actin filaments. These results suggest that reorganization of cytoskeletal structures is essential for the orienting response of endothelial cells to cyclic stretch.

The molecular mechanisms of converting physical forces to biochemical signals remain unclear. In particular, the molecular entity of mechanotransducers has been the greatest missing link. Several membrane proteins have been nominated as candidates for mechanotransducers (4, 27). Among them only stretch-activated (SA) channels (11) have been identified by using the patch-clamp technique. It was reported that porcine vascular endothelial cells possess a cation-selective SA channel that can permeate calcium ions (18). Our previous study also demonstrated in human umbilical endothelial cells that intracellular Ca2+ increased in response to mechanical stretch via Ca2+-permeable SA channel activation (21). These observations suggest that the cyclic stretch of endothelial cells activates SA channels and increases intracellular Ca2+ concentration ([Ca2+]i), which might trigger morphologically changes in endothelial cells.

The aim of the present study was to test a hypothesis of whether the activation of SA channels by cyclic stretch is essential for the stretch-dependent orienting response of endothelial cells.

MATERIALS AND METHODS

Cell preparation. Endothelial cells were prepared from a human umbilical cord vein as described previously (9). In brief, a human umbilical cord was aseptically removed from placenta immediately after a birth. The vein was washed with phosphate-buffered saline (in mM: 137 NaCl, 8.10 Na2HPO4, 2.68 KCl, 1.47 KH2PO4; pH 7.40), followed by a treatment with 0.2% trypsin for 10 min. The perfusate was centrifuged at 1,500 rpm for 10 min, and the resulting cells were washed with phosphate-buffered saline, plated in 35-mm plastic dishes, and maintained in Humedia-EG2 (Kurabo, Osaka, J apan). The cells used in this study were within three to four passages.

Application of cyclic stretch. Endothelial cells were removed from the dish with 0.01% EDTA-0.02% trypsin and transferred onto a 4-cm2 silicon chamber coated with 50 µg/ml fibronectin at a density of 5 × 104 cells/cm2. The silicon chamber had a 200-µm thick transparent bottom, and the side wall was 400 µm thick to prevent its bottom center from narrowing (Fig. 1A). The silicon chamber was attached to a stretching apparatus that was driven by a computer-controlled stepping motor (Fig. 1B). After cells were allowed to attach to the chamber bottom for several hours, uniaxial sinusoidal stretch (120% peak to peak, at 1 Hz) was applied at 37°C, 5% CO2. Using this system, we could apply quantitative and uniform stretch to most of the cells, and lateral thinning did not exceed 1% at 120% stretch.

Measurement of cell orientation. Phase-contrast images of endothelial cells were photographed using a ×20 objective
Measurement of \([\text{Ca}^{2+}]\). Endothelial cells on silicon membranes were incubated with 5 \(\mu\text{M}\) fura 2-acetoxymethyl ester (Molecular Probes, Eugene, OR) for 45 min and for another 30 min in a solution containing (in mM) 140 NaCl, 5 KCl, 2 CaCl\(_2\), 10 glucose, and 10 N-2-hydroxyethylpiperazine-N\(^\prime\)-2-ethanesulfonic acid, pH 7.40 (standard external solution, SES) as described previously (10, 21). \([\text{Ca}^{2+}]\) was measured by the fura 2 method using a fluorescence microscope (M1000; Inter Dec., Osaka, Japan) with a \(\times 20\) objective (Nikon, Fluor 20). The excitation wavelengths were set 340 and 380 nm at 200-ms intervals controlled by a computer, and the emission was detected at 510 nm by a photomultiplier. Fluorescence ratio \((R)\) was calculated from the following equation: \(R = (F_{340} - B_{340})/(F_{380} - B_{380})\), where \(F_{340}\) and \(F_{380}\) are intensities at 510 nm excited at 340 and 380 nm, respectively, and \(B_{340}\) and \(B_{380}\) are corresponding background autofluorescence values. To measure \([\text{Ca}^{2+}]\), the chamber was stopped for 1 s at 0, 10, 30, and 60 min after the start of stretch. We determined \([\text{Ca}^{2+}]\) by using a series of fura 2 solution ranging from 10 nM to 1 \(\mu\text{M}\). Although absolute values of \([\text{Ca}^{2+}]\) could be calculated based on the dissociation constant of fura 2 for \(\text{Ca}^{2+}\) binding, the constant in cytosol is different from that measured in the absence of protein. Thus we used a ratio in this study. The area of interest was \(\sim 5 \times 10^4\) \(\mu\text{m}^2\), and the number of the cells was \(\sim 60\).

Solutions. In most experiments, the contents of an extracellular solution were SES unless otherwise mentioned.

Chemicals. Gadolinium III chloride hexahydrate was purchased from Aldrich Chemical, and on arrival, it was dissolved in distilled water at 1 M and stored at \(-80^\circ\text{C}\). Because Gd\(^{3+}\) is unstable, the concentrated Gd\(^{3+}\) was first diluted at the concentration of 10 mM in distilled water and then diluted at desired concentrations in SES just before use. Human plasma fibronectin was purchased from Ko-ken (Tokyo, Japan). Other chemicals used were of special grade.

RESULTS

Morphological changes induced by cyclic stretch. Application of sinusoidal cyclic stretch (120% peak to peak, at 1 Hz) induced significant morphological changes in the cells particularly in their orientation. Before cyclic stretch was applied, the cells showed no particular orientation (Fig. 2A). The cells began to orient perpendicularly to the stretch axis within 30 min (Fig. 2B), and the percentage of oriented cells increased with time (Fig. 2C, 60 min). After 120 min most of the cells aligned almost perfectly transversely across the stretch axis (Fig. 2D). Histograms in Fig. 3 show time courses of the distribution of cell orientation under various conditions. Without stretch, cells showed little changes in their morphology and no tendency to exhibit particular orientation at any time point (Fig. 3, A-E). In contrast, as in Fig. 3, F-J, cells under cyclic stretch showed clear indication of alignment around 90\(^\circ\) as early as 30 min after the onset of stretch. We investigated stretch and frequency dependence on the orientation. We could observe orienting responses of the cells as low as 5\% stretch, which was the minimum resolution to control the stretch in our system. When cells were subjected to 5\% stretch, the morphological response of the cells could be visible around 60 min after the stretch onset, and after several hours most cells oriented perpendicularly to the stretch axis. The rate of the orienting response increased as the percentage of stretch increased. We could not test the effect of \(>20\%\) stretch because of mechanical damages (e.g., peeling off) to the cells. As for the frequency dependence, the orienting response could be observed at lower frequency \(<1\text{ Hz}\). For example, at 0.1 Hz the orienting response was initially observed after 2–3 h of cyclic stretch, and the most of the cells were perfectly trans-
versely oriented after 5–6 h. Effects of intrinsic surface feature of the silicon membrane or fibronectin on the orienting response could be eliminated because the cells cultured on substrata, which was earlier undergoing the same cyclic stretch, did not show any particular orientation.

Effects of external Ca$^{2+}$ and Gd$^{3+}$ on cell alignment. We have reported that stretching cultured endothelial cells increased [Ca$^{2+}$], via SA channel activation (21). The major evidence was that the stretch-induced [Ca$^{2+}$] increase was dependent on extracellular Ca$^{2+}$ and blocked by Gd$^{3+}$, a potent blocker of SA channels, whereas blockade of L-type Ca$^{2+}$ channels by nifedipine had no effect on the stretch-induced [Ca$^{2+}$], increase. We carried out similar pharmacology to check the possibility of SA channel involvement in the orienting response of endothelial cells to cyclic stretch. When cells were subjected to cyclic stretch in nominally Ca$^{2+}$-free media, the orienting response was almost completely inhibited as shown in Figs. 2E and 4, F-G. Next, we investigated the effect of Gd$^{3+}$. It has been reported that 1–100 µM Gd$^{3+}$ inhibited SA channels (28) and stretch-induced Ca$^{2+}$ entry in endothelial cells (21). When applied to the external media, this concentration range of Gd$^{3+}$ inhibited the orienting response in a dose-dependent manner and almost completely inhibited the response at 20 µM (Figs. 2F and 4, A-E). On the other hand, classic Ca$^{2+}$-channel blockers like nifedipine (10 µM) and verapamil (10 µM) did not block the response. The simplest and straightforward interpretation of these results may be that the orienting response of endothelial cells to cyclic stretch requires [Ca$^{2+}$] increase mediated by SA channel activation.

Increase in [Ca$^{2+}$] in response to cyclic stretch. To ensure that the cyclic stretch of the endothelial cells activates SA channels, [Ca$^{2+}$], was measured by using the fluorescent Ca$^{2+}$ indicator fura 2. As shown in Fig. 5A, a single stretch could elicit a transient increase in [Ca$^{2+}$]. The basal [Ca$^{2+}$] level was gradually increased with time in response to cyclic stretch (Fig. 5B), although a transient [Ca$^{2+}$] evoked by a single stretch after the cell alignment at 60 min was not significantly different from what was evoked before cyclic stretch. This increase in [Ca$^{2+}$], was inhibited by the removal of extracellular Ca$^{2+}$ or an application of 20 µM Gd$^{3+}$ (Fig. 5C). These results strongly suggested that cyclic stretch activated the SA channels that could mediate Ca$^{2+}$ influx in endothelial cells.

Orienting response involves elongation of cells. The morphological changes of endothelial cells induced by cyclic stretch involve not only the orienting response but also elongation of the cells. To estimate the magnitude of the elongation, we employed a ratio of lengths between long and short axes of the cells. Under static conditions slight change in this value was observed over a few hours, whereas it increased nearly linearly
with time when subjected to cyclic stretch (Fig. 6). In nominally Ca\textsuperscript{2+}-free media or in the presence of 20 µM Gd\textsuperscript{3+}, the cell elongation by cyclic stretch was largely inhibited. These results are fairly parallel with those of orienting response, although the elongation proceeded slower than the orienting response. Thus the orienting response could be restated as a formation of a new two-dimensional polarity of the cell but not as a rotation of the cell axis. A closer look at the time course of morphological changes of individual cells that had a polarity parallel with stretch axis revealed that cells first became more rounded than their initial shape and gradually extended processes perpendicularly to the stretch axis (Fig. 2). It is likely that the orienting response consists of at least two sequential steps; the first step is a banishment of the initial orientation and the second one is a formation of a new orientation perpendicularly to stretch axis. Finally, these responses occurred in both confluent and subconfluent cells without significant difference.

**DISCUSSION**

SA channel is a key molecule in stretch-induced [Ca\textsuperscript{2+}] increase. We demonstrated that the stretch-induced morphological response of endothelial cells
was dependent on external Ca\(^{2+}\) and blocked by Gd\(^{3+}\). This result strongly suggests that the response requires [Ca\(^{2+}\)]\(_{i}\) increases arising mainly from Ca\(^{2+}\) influx mediated by SA channels. Actually, as described in RESULTS, cyclic stretch increased the [Ca\(^{2+}\)]\(_{i}\) level, which was inhibited by Gd\(^{3+}\). We will discuss here the molecular mechanism of the stretch-induced [Ca\(^{2+}\)]\(_{i}\) increase that includes at least two steps: mechanotransduction and [Ca\(^{2+}\)]\(_{i}\) mobilization.

Several membrane proteins have been nominated as candidates for mechanotransducers, i.e., Na\(^{+}/H^{+}\) exchanger (15), adenylate cyclase (27), phospholipase C (4), and SA channels (11, 18). We performed pharmacological studies to investigate which molecule is responsible for the stretch-induced orienting response of endothelial cells. First, amiloride (10 µM), a blocker for Na\(^{+}/H^{+}\) exchanger (8), was tested, which gave no effect on the response. This is consistent with our previous observation that replacement of external Na\(^{+}\) with Li\(^{+}\) to inhibit Na\(^{+}/Ca^{2+}\) and Na\(^{+}/H^{+}\) exchangers had no effect on the stretch-induced [Ca\(^{2+}\)]\(_{i}\) mobilization in endothelial cells (21). Treatments of cells with either pertussis toxin (100 ng/ml for 16 h) to block G protein-coupled adenylate cyclase (26) or psi-tectorigenin (10 µg/ml) to inhibit phosphatidylinositol turnover following an inactivation of phospholipase C (14) did not block the orienting response. In contrast, the potent SA channel blocker Gd\(^{3+}\) (100 µM) almost completely

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**Fig. 4.** Histograms showing distribution of cell orientation in presence of 20 µM Gd\(^{3+}\) (A-E) and in nominally Ca\(^{2+}\)-free media (F-I). In both cases, cells do not show any specific orientation during cyclic stretch at any time point (A and F, 0 min; B and G, 30 min; C and H, 60 min; D and I, 120 min; E and J, 180 min). More than 200 cells from five independent experiments make up 100%.
blocked the orienting response, indicating that SA channels but not ion exchangers and G protein-coupled receptors are responsible for the stretch-induced orienting response of endothelial cells.

Although Gd3+ currently is known as the most potent SA channel blocker (28), it also blocks voltage-dependent Ca2+ channels (3). It is possible that a certain voltage-dependent Ca2+ channel is activated by stretch, resulting in [Ca2+]i increase. In smooth muscle, a voltage-dependent L-type Ca2+ channel is reported to possess mechanosensitivity (17). Therefore, we investigated the possible involvement of voltage-dependent Ca2+ channels by using classic Ca2+ channel blockers such as nifedipine (10 µM) and verapamil (10 µM); both of them had no effect on the orienting response. In addition, we observed no [Ca2+]i increase during membrane depolarization by external high K+ solution (unpublished data). These observations are in harmony with the previous studies suggesting that endothelial cells do not possess voltage-dependent Ca2+ channels (6, 16). In conclusion, the stretch-induced [Ca2+]i mobilization responsible for the orienting response in endothelial cells seems to be mediated by Ca2+-permeable SA channels that exist on the cell membrane of endothelial cells (11, 21). We also reported that Ca2+ released from intracellular Ca2+ stores via ryanodine receptors partially contributed to the stretch-induced [Ca2+]i increase (21), but ryanodine (100 nM) had no effect on the orienting response.

Possible mechanism involved in processes following [Ca2+]i increase. The morphological changes of endothelial cells induced by uniaxial cyclic stretch must include spatially polarized chemical reactions. It is apparent that only a uniform [Ca2+]i increase over the cell is not enough to induce such a polarized morphological response. In fact, uniform [Ca2+]i increases induced by a treatment with Ca2+ ionophore such as ionomycin, which was measured by using fura 2 fluorescent ratiometry, did not induce any visible polarized morphological changes in endothelial cells without uniaxial cyclic stretch (data not shown). The orienting response must include some mechanisms that convert the spatial polarity of uniaxial stretch into polarized cellular structures. One possible mechanism leading such a polarity may be a polarized increase in [Ca2+]i, as observed in the cell migration of neutrophil (12). In our case, a spatially polarized Ca2+ increase by heterogeneously activated SA channels would be one of the possible mechanisms. Although we could not succeed in detecting such a spatially heterogeneous [Ca2+]i increase during uniaxial cyclic stretch, detailed measurements are in progress.

Shear vs. stretch. It has been known in endothelial cells that fluid shear stress also induces morphological changes similar to those by stretch (7, 22). Cultured endothelial cells subjected to shear stress change their morphology from a round shape to a spindlelike shape.
and then align their long axes parallel to flow direction (22). In addition, shear stress induces development of stress fibers along the long axis and [Ca2+]i increases as observed in the endothelial cells exposed to cyclic stretch. It is likely that similar mechanisms are underlying between the shear stress- and cyclic stretch-induced morphological responses in endothelial cells. However, there is at least one significant difference between the effects of shear stress and cyclic stretch. It has been recently reported that shear stress-induced cell alignment was insensitive to Gd3+ (19), whereas the stretch-induced alignment was strongly inhibited by Gd3+ as presented in this study. The simplest interpretation of this discrepancy may be that the receptor for shear stress is different from that for stretch. Although the receptor for shear stress has not been identified, shear stress hyperpolarized the endothelial cells through the activation of a certain K+ channel, which does not seem to be a Ca2+-activated type (23). Thus the authors suggested that this hyperpolarization induces a [Ca2+]i increase by an acceleration of Ca2+ influx via background leak Ca2+ channels. In harmony with this hypothesis, a significant difference was observed in the mode of [Ca2+]i increase. Depletion of external Ca2+ diminished the plateau component but not the peak component of the shear-induced Ca2+ transient (2), whereas the same treatment induced an opposite result in the stretch-induced Ca2+ transient (21). It is suggested that the peak component arises from Ca2+ release from internal Ca2+ stores in the shear-induced Ca2+ transient and that in stretch-induced Ca2+ transients the peak component arises from the Ca2+ influx via Ca2+-permeable SA channels. Detailed comparison between the two averts further elucidation of molecular mechanisms following the mechanically induced [Ca2+]i increase.

Wetank Drs. Shibata and Narita for providing umbilical cord. We also thank Dr. K. Murase, M. Takahashi, H. Asano, and M. Inoue for excellent technical assistance. This work was supported by a grant-in-aid from the Ministry of Education Science and Culture of Japan (K. Naruse and M. Sokabe), the Science and Technology Agency of Japan (M. Sokabe), and by Grants-in-Aid for Scientific Research (K. Naruse). Address reprint requests to K. Naruse.

Received 13 January 1997; accepted in final form 9 January 1998.

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