Uni-axial cyclic stretch induces c-src activation and translocation in human endothelial cells via SA channel activation

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Abstract The kinase activity of c-src increased and peaked at 15 min after an application of uni-axial cyclic stretch in HUVECs followed by a translocation of c-src to Triton-insoluble fraction. Suppression of c-src by an antisense *S*-oligodeoxynucleotide inhibited the stretch-induced tyrosine phosphorylation and morphological changes. The stretch-induced increase in c-src activity was inhibited by FK506, a specific inhibitor for calcineurin, by Gd^{3+}, a blocker for stretch activated channels, and by the extracellular Ca^{2+} depletion suggesting the involvement of SA channels. These results strongly suggest c-src plays an important role in the downstream of SA channel activation followed by the morphological changes.

Key words: Ca^{2+}; Gd^{3+}; Calcineurin; FK506; ECV304

1. Introduction

Vascular endothelial cells composing the inner lining of blood vessels show a spindle-like shape and align their longitude along the vessel, whereas cells in culture show a polygonal shape with random orientation. These morphological features in situ are considered to be created by physical forces from blood, such as the hydrodynamic shear stress from blood flow [1] and the periodic stretch due to wall distention [2]. Particularly, the effects of pulsative stretch have been extensively investigated using a variety of in-vitro simulation systems [3–8]; however, the intracellular signaling mechanism regulating the stretch-dependent morphological changes has not been well elucidated.

Previous studies indicated that tyrosine phosphorylation of focal proteins are critical for the stretch-induced morphological changes [7,9]. Treatment with tyrosine kinase inhibitors, such as genistein and herbimycin A, blocked the stretch-induced morphological changes. Moreover, treatment with antisense *S*-oligodeoxynucleotide (S-ODN) against $pp125^{FAK}$ also suppressed the morphological changes [7]. However, few studies have been directed at verifying protein tyrosine kinases and their coupling with mechanoreceptor signaling.

As a possible candidate for the mechanotransducer in HUVECs, we have been nominating a Ca^{2+}-permeable SA channel [6,7,10]. Existence of an SA channel has been demonstrated in vascular endothelial cells by patch clamp technique [10,11]. In addition, our previous studies indicated that stretching endothelial cells increased intracellular Ca^{2+} concentration via SA channel activation [10] and that Ca^{2+} influx via SA channels was critical for the stretch-induced morphological changes [6].

Several lines of evidence implicated that Ca^{2+}-dependent tyrosine phosphorylation pathway is playing an important role in intracellular signal transduction [12,13]. In cultured epidermal keratinocytes, a rapid Ca^{2+} mobilization was proposed to stimulate the phosphatase activity of calcineurin resulting in c-src activation [12]. In human platelets, addition of EDTA to chelate extracellular Ca^{2+} reduced the protein tyrosine phosphorylation induced by thrombin, suggesting that platelet tyrosine kinase activity is controlled by extracellular Ca^{2+} [13].

Thus, these data led us to further investigate whether c-src is activated upon cyclic stretch and whether Ca^{2+} influx through SA channel is critical for the activation of tyrosine kinase, c-src, followed by tyrosine phosphorylation of focal proteins and stretch-induced morphological changes.

In the present study, we demonstrated that cyclic stretch to cultured endothelial cells activates c-src via SA channel activation followed by phosphorylation of focal proteins, resulting in morphological changes.

2. Materials and methods

2.1. Cell culture

Endothelial cells were prepared from human umbilical cord vein as described previously [14]. In brief, human umbilical cord was aseptically removed from placenta just after birth. The vein was washed with phosphate buffered saline (PBS, 137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.40), followed by treatment with 0.2% trypsin for 10 min. The perfusate was centrifuged at 1500 rpm for 10 min. The resulting cells were washed with PBS again, then plated in 35-mm dishes and maintained in EGM-UV2 (Kurabo, Osaka, Japan). The cells used were within 2–4 passages.

ECV304 (JCRB 0744, Japan Health Science Research Resources Bank) is an immortalized HUVEC, established by Takahashi et al. [15], which has similar characteristics to HUVEC [16]. ECV304 were maintained in DMEM supplemented with 10% fetal calf serum and 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 250 μg/mg amphotericin B. Cells were incubated in a tissue culture incubator at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

2.2. Stretch apparatus

The cells were stretched as previously described [5–8]. Briefly, cells were removed from the dish with 0.01% EDTA/0.02% trypsin and transferred onto a silicone chamber coated with 50 μg/ml fibronectin. One end of the chamber is firmly attached to a fixed frame, while the other end is held on a movable frame. The movable frame is connected to a motor driven shaft. The amplitude and the frequency of stretch were controlled by a programmable microcomputer. The silicone membrane was uniformly stretched over the whole membrane area and lateral thinning did not exceed 1% at 20% stretch.

2.3. Immunoblotting

After the application of cyclic stretch, the cells were washed with

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ice-cold PBS containing 0.5 mM Na_3VO_4 and were lysed with a sample buffer 2× (62 mM Tris, pH 6.8, 2% SDS, 1% 2-mercaptoethanol, 0.003% bromophenol blue, 5% glycerol). Total cell lysate from equivalent cell numbers were separated by 12.5% SDS-PAGE. The proteins were transferred electrophoretically onto polyvinylidene fluoride (PVDF) membrane (Immobile P, Millipore Crop, Bedford, MA, USA). The membranes were blocked with 3% ovalbumin in PBS, the membranes were subsequently probed with monoclonal antibodies in PBS containing 3% ovalbumin for 1 h. The antibody-antigen complexes were detected by using horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000 dilution). Immunoreactivity was determined using the ECL chemiluminescence reaction (Amersham, Arlington Heights, IL, USA), according to the instructions provided by the manufacturer and was measured using densitometry.

2.4. Immunoprecipitation and in vitro kinase assay

For immunoprecipitation, the cells were washed with ice-cold PBS and were lysed by buffer A (10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% DOC, 1% SDS, pH 7.40). Cell lysates were centrifuged at 16000×*g* for 20 min at 4°C twice and 50 μl of protein A-Sepharose-anti-mouse IgG were added to pre-clear the lysate. One μl of monoclonal anti-c-src were added and the samples were incubated at 4°C for 1 h. Then 30 μl of protein A-Sepharose-anti-mouse IgG were added, and samples were incubated at 4°C for 1 h with gentle agitation. The resulting immunoprecipitates were washed five times with buffer, A and three times with kinase buffer (10 mM Tris-HCl, pH 7.40, 5 mM $MnCl_2$). The kinase reaction was started with the addition of 5 μCi of [^{32}P]ATP, 1 μl ATP, and acid-treated enolase. The reaction was terminated after 10 min with the addition of an equal volume of 2× SDS-sample buffer and the samples were boiled for 5 min. The samples were separated on 12.5% SDS-PAGE and the autoradiogram was analyzed by BAS1500 radioactivity imaging system (Fuji Photo Film, Tokyo, Japan).

2.5. Anti-sense S-oligodeoxynucleotide treatment

The anti-sense sequence against human c-src (accession number X59932; 5′-GTC GGG GGC TGC TGT CTT-3′) and the sense sequence (3′-CAG CCC CCG ACG ACA GAA-5′) were designed using the Genetyx (Softwear Development, Tokyo, Japan) so that they were not engaged in snapback structures and not potentially exposed to the stem structure of the mRNA. The designed sequences have relatively low homology to any cDNA sequences found in EMBL and GenBank databases. The S-ODNs were synthesized by RIKAKEN, Nagoya, Japan. The cells were removed with 0.01% EDTA, 0.02% trypsin and were suspended at a density of 1×10^6 cells/ml in PBS. 100 ng of S-ODN was added to 400 μl of cell suspension and the suspension was transferred to an electroporation chamber, and were square-electroporated at 75 V, 40 ms, 1 pulse using Electro Square Porator-T820 (BTX, San Diego, CA, USA). The efficiency was greatest when cell viability was 70%. Then the S-ODN treated cells were transferred onto a 4-cm^2 silicone chamber, incubated for 24 h.

2.6. Reagents

Mouse anti-c-src monoclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit anti-mouse IgG and goat anti-rabbit IgG were obtained from Sigma (St. Louis, MO, USA). Herbimycin A and genistein were purchased from Wako (Tokyo, Japan). Gadolinium(III) chloride hexahydrate was purchased from Aldrich (Milwaukee, MI, USA). Human plasma fibronectin was purified according to Regnault et al. [17]. FK506 was a kind gift from Fujisawa Pharmaceutical [18]. Other chemicals used were of special grade.

3. Results

3.1. Cyclic stretch activated c-src

As we demonstrated that cyclic stretch-induced tyrosine phosphorylation of focal proteins of HUVECs, we investigated cyclic stretch-induce c-src kinase activation using an in vitro kinase assay. After being subjected to 15–60 min of cyclic stretch (20%, 1 Hz), the cells were lysed and were immunoprecipitated with anti-c-src antibody. The immunoprecipitates were subjected to an in vitro kinase assay using enolase as a substrate followed by SDS-PAGE and autoradiography. The autoradiogram of phosphorylated enolase at 40 kDa shows an increase in kinase activity of c-src (Fig. 1A, upper panel). Immunoblot probed with anti-src monoclonal antibody (Fig. 1A, lower panel) shows that an equal amount of the protein was loaded in each lane. The activity of c-src peaked at 15 min (3.2-fold increase, $n=5$) and decreased gradually (Fig. 1B). The autophosphorylation of c-src of HUVECs was so faint that we could not detect a band at 60 kDa corresponding to c-src. In the immortalized HUVEC cell line (ECV304 cells), cyclic stretch also induced c-src kinase activation, which were detected as autophosphorylation and enolase phosphorylation (data not shown).

3.2. Cyclic stretch induced translocation of c-src

As c-src requires membrane localization for their normal function in intracellular signaling, the subcellular distribution of c-src was determined by immunoblotting using anti-c-src antibody. The amount of c-src in the cytoskeletal fraction gradually increased in the course of stretch (Fig. 2A). Localization of c-src in HUVECs was identified by immunostaining. In control static cells, no particular structure could be

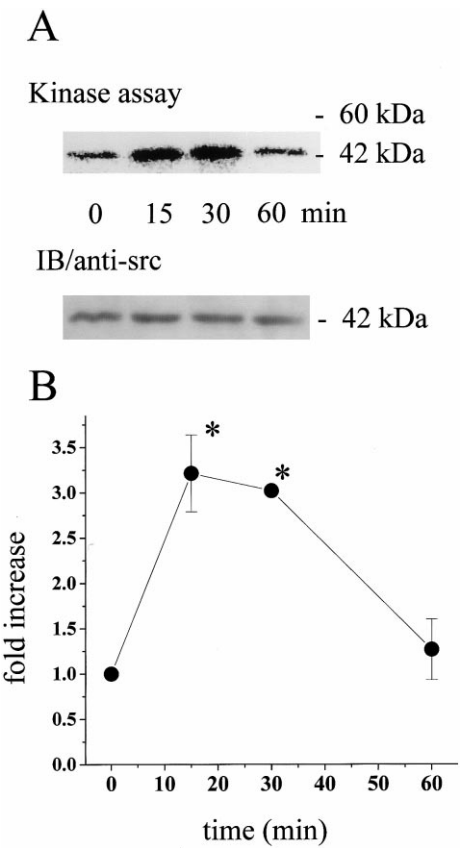

Fig. 1. Stretch-induced kinase activation of c-src. A: Upper panel: Autoradiogram of in vitro kinase assay of c-src using acid-treated enolase as a substrate. The phosphorylation of enolase increased and peaked at 15 min. The autophosphorylation of c-src could not be observed in HUVECs at 60 kDa. The autoradiogram is representative of 4 experiments. Lower panel: Immunoblot probed with anti-src monoclonal antibody shows that an equal amount of the protein was loaded in each lane. B: Time course of the kinase activation of c-src in response to cyclic stretch. Asterisks denote those values that are significantly different ($P<0.01$) from control (0 min).

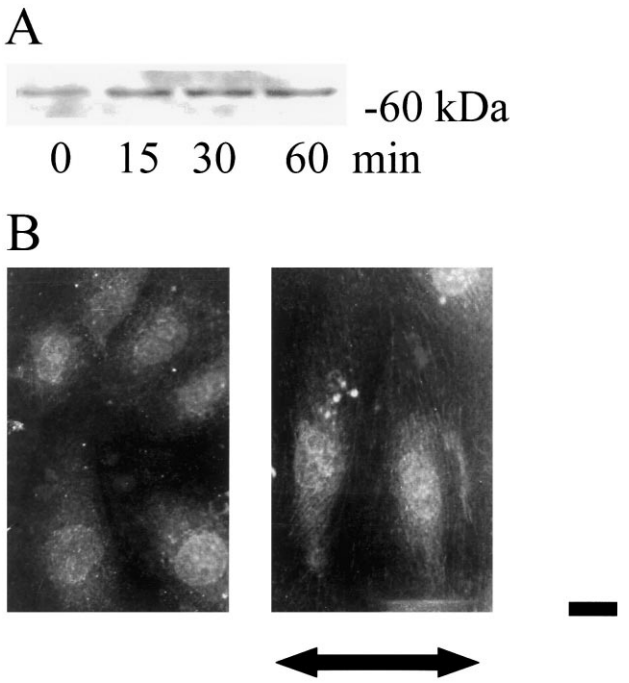

Fig. 2. A: Translocation of c-src to the cytoskeletal fraction in response to cyclic stretch. After the application of cyclic stretch (1 Hz, 20% in length) for 20 min, the cytoskeletal fraction was immunoblotted with anti-src monoclonal antibody. The amount of c-src in the cytoskeletal fraction increased after the application of stretch. The immunoblot is a representative of 5 experiments. B: Immunostaining study of c-src in response to cyclic stretch. In control static cells, no particular structure could be observed (left). After the application of cyclic stretch (1 Hz, 20% in length) for 20 min, the cells aligned perpendicularly to the stretch direction and the staining could be observed as patches along the cytoskeletal structures (right). The bar at the bottom indicates 10 μm. A double-headed arrow indicates the direction of the stretch axis.

observed (Fig. 2B, left), whereas in stretched cells (20 min) the cells aligned perpendicular to the stretch direction and the staining could be observed as patches along cytoskeletal structures (Fig. 2B, right).

3.3. Antisense c-src suppressed stretch-induced tyrosine phosphorylation

To confirm that the activated c-src really tyrosine-phosphorylate cellular proteins, we employed antisense *S*-oligodeoxynucleotides (ODNs) against c-src to reduce specifically the expression of c-src. Synthesized antisense S-ODNs against c-src including the first Met (atg) was electroporated into HUVECs and the cells were cultured for 24 h. The sense S-ODN against c-src was used as a control. Immunoblot using anti-c-src monoclonal antibody revealed that in the antisense S-ODN treated cells the amount of c-src was decreased within 24 h, whereas in the sense S-ODN treated cells, the amount of c-src was unchanged (Fig. 3A, upper panel). The effect of antisense S-ODN was dose- and time-dependent. For further experiments, the cells were electroporated at the concentration of 20 μM S-ODN and were cultured for 24 h before being subjected to stretch. As an internal control, the amount of actin was accessed by immunoblot with anti-actin monoclonal antibody (Fig. 3A, lower panel). The amount of actin was not significantly changed with the S-ODN treatment. In the sense S-ODN treated cells, the level of tyrosine phosphorylation increased in response to cyclic stretch as previously reported, whereas in the antisense S-ODN treated cells the level of tyrosine phosphorylation slightly increased but the increase was much less as compared with the sense S-ODN treated cells (Fig. 3B, upper panel). The effects of the ODNs on the morphological changes of HUVECs in response to mechanical stretch were examined. In the sense S-ODN treated cells, morphological changes of the cells were very similar to those in control cells. In the cells treated with the antisense S-ODN, the cells had almost no morphological changes. These results clearly indicate that c-src may play an important role in stretch-induced morphological changes.

3.4. Activation of c-src was regulated by the SA channel

It is of interest to investigate the mechanism(s) by which c-src is activated under cyclic stretch, as previous studies from our laboratory [6,10] and others [19] demonstrated that mechanical stretch activated Ca^{2+}-permeable SA channels leading to an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

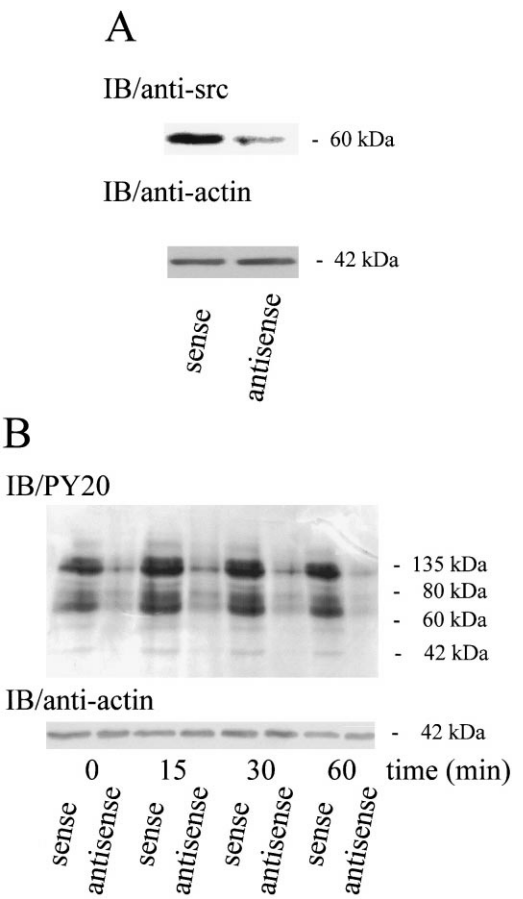

Fig. 3. Effect of the antisense S-ODN against c-src. A: Upper panel: The treatment of HUVEC with the antisense S-ODN against c-src for 24 h depleted the amount of c-src protein, whereas that with the sense S-ODN did not. Lower panel: S-ODN treatment had no effect in the expression of actin as an internal control. B: Upper panel: The level of tyrosine phosphorylation increased in time dependent manner in response to cyclic stretch in the sense S-ODN treated cells, whereas the level of tyrosine phosphorylation was much lower in the antisense S-ODN treated cells. Lower panel: Immunoblot probed with anti-src monoclonal antibody shows that an equal amout of the protein was loaded in each lane. The immunoblot is representative of 5 experiments.

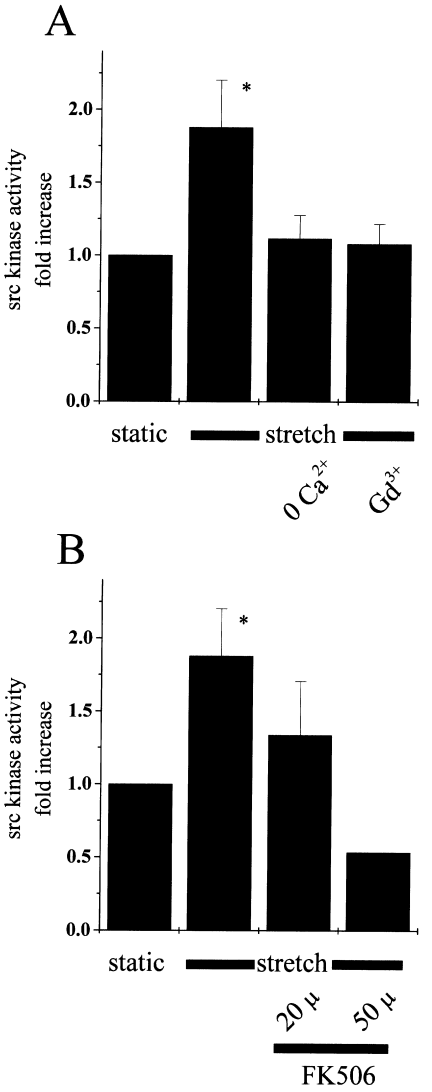

Fig. 4. A: The involvement of SA channel in the regulation of c-src kinase activity. HUVEC was cyclically stretched for 30 min in the absence of extracellular Ca^{2+} or after addition of 10 μM Gd^{3+}. The kinase activity of c-src increased in normal control, whereas it was inhibited by the removal of extracellular Ca^{2+} or by the addition of 10 μM Gd^{3+}. B: The effects of FK506 on the stretch induced activation of c-src. HUVEC was cyclically stretched for 30 min in the presence or absence of FK506. The cell lysate was subjected to the in vitro kinase assay. The increase in the kinase activity of c-src by cyclic stretch was inhibited in dose-dependent manner (20, 50 μM). Asterisks denote those values are significantly different ($P < 0.01$) from control values.

and that the SA channel-dependent $[Ca^{2+}]_i$ increase was indispensable for the stretch-induced morphological changes in endothelial cells. In view of the involvement of Ca^{2+} through the SA channel in the stretch-dependent morphogenesis in HUVECs, we examined the effects of Gd^{3+}, SA channel blocker [20], extracellular Ca^{2+} chelation (EGTA), and intracellular Ca^{2+} chelation (BAPTA/AM) on the kinase activity of c-src. The application of Gd^{3+} (10–50 μM) inhibited the increase in the c-src kinase activity dose-dependently and the removal of extracellular Ca^{2+} by EGTA or intracellular Ca^{2+} by 5 μM BAPTA/AM also inhibited the kinase activity (Fig. 4A). These results strongly suggest that Ca^{2+} influx through SA channels is critical for the activation of c-src.

3.5. Involvement of Ca^{2+}-dependent phosphatase in the activation of c-src

It is well known that the dephosphorylation of tyrosine-phosphorylated Tyr(P)-527 is essential for the activation of c-src [21]. Several papers reported that this is due to an activation of intracellular PTPases, including Ca^{2+}-dependent phosphatase. Thus, we investigated the involvement of calcineurin, Ca^{2+}/calmodulin-dependent protein phosphatase (PPIIB), in this system. FK506, one of the immunosuppressants, is known to inhibit calcineurin specifically [22]. In the presence of FK506, the increase in kinase activity of c-src induced by stretch was almost completely inhibited at 50 μM and partially inhibited at 20 μM (Fig. 4B). The morphological change induced by stretch was also inhibited by treatment with FK506 (data not shown).

4. Discussion

In the present study, we first demonstrated that unidirectional cyclic stretch induced the activation and translocation of c-src in HUVECs. Furthermore, the activation of c-src kinase is indispensable for the stretch-induced morphological change, such as alignment and elongation perpendicular to the stretch axis.

Several lines of evidence suggest that these stretch-induced morphological changes contain complex signaling mechanisms. First, the morphological change process involves protein tyrosine phosphorylation of focal adhesion proteins. Stretching the cells increased the phosphotyrosine contents in the focal proteins, and tyrosine kinase inhibitors such as herbimycin A and genistein blocked the stretch-induced morphological changes [7]. Second, suppression of $pp125^{FAK}$ by an antisense S-ODN blocked the morphological changes [7]. Third, the morphological changes are likely to be triggered by SA channels. Depletion of extracellular Ca^{2+} and an application of Gd^{3+}, an inhibitor of SA channel, inhibited the morphological changes, thus the Ca^{2+} influx through SA channels is critical for the morphological changes [6]. These observations led us to further investigate which tyrosine kinase(s) are responsible for the stretch-induced protein tyrosine phosphorylation and morphological changes. Among the tyrosine kinases, we first examined the activation of c-src because $pp125^{FAK}$ is known to be a good substrate of c-src and a recent study reported the activation of c-src kinase in lung cells to strain [23]. As shown in Fig. 1, cyclic stretch induced rapid increase in kinase activity, which may be regulated by intracellular Ca^{2+}. Other members of the src kinase family (fyn, lyn) were examined, however, we could not detect any changes in their kinase activity (data not shown).

Activation of c-src is induced by the dephosphorylation of tyrosine-phosphorylated Tyr(P)-527 [21]. Although the regulation mechanism of the kinase activity is still under investigation, it is reported that c-src kinase (CSK) constitutively phosphorylates Tyr(P)-527 of c-src to inactivate the c-src kinase activity and that the dephosphorylation of Tyr(P)-527 by phosphatase increases the activity [24]. In our study, a Ca^{2+}-dependent phosphatase is one of the favorable candidates since the stretch-dependent protein tyrosine phosphorylation was demonstrated to be $[Ca^{2+}]_i$-dependent [11]. Calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase (PP2B) belongs to the family of Ser/Thr specific enzymes [25] but is also active on phosphotyrosine residues [26]. Thus, it is pos-

sible to think that changes in $[Ca^{2+}]_i$ regulate the kinase activity through calcineurin activation. To confirm this, we employed FK506, a specific inhibitor of calcineurin. FK506 is reported to bind FK506 binding protein (FKBP) and the complex inhibits the activity of calcineurin [22]. Actually, we confirmed the existence of FKBP and calcineurin in HUVECs by immunoblotting experiments (data not shown). In our data (Fig. 4B), inhibition of the Ca^{2+} influx or the application of FK506 suppressed c-src kinase activity.

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