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# THROMBIN-STIMULATED PHOSPHORYLATION OF MYOSIN LIGHT CHAIN AND ITS POSSIBLE INVOLVEMENT IN ENDOTHELIN-1 SECRETION FROM PORCINE AORTIC ENDOTHELIAL CELLS

KAZUHIRO KITAZUMI and KENJI TASAKA*

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Okayama University, Okayama 700, Japan

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**Abstract**—Thrombin-stimulated secretion of endothelin-1 (ET-1) from porcine aortic endothelial cells was inhibited in the presence of 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8), trifluoperazine and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7). 1-(5-Chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine (ML-9) also prevented the thrombin-stimulated secretion of ET-1 but it enhanced the accumulation of ET-1 in the endothelial cells. When the endothelial cells were treated with thrombin, the phosphorylation of a 20-kDa protein which was identified as myosin light chain (MLC) was detected. Phosphorylation was augmented in a time-dependent manner. As in the case of ET-1 secretion, MLC phosphorylation was prevented by TMB-8, trifluoperazine, W-7 and ML-9 at the same concentrations which were effective in inhibiting the ET-1 secretion. The site of phosphorylation of MLC was identified as a serine residue. Parallel to the phosphorylation of MLC, thrombin increased the amounts of the 43- and 200-kDa proteins in the Triton-insoluble fraction; these proteins were identified as actin and myosin heavy chain, respectively. These results suggest that the MLC phosphorylation elicited by MLC kinase may facilitate the formation of filamentous myosin and actin which are probably involved in ET-1 secretion, possibly in the transport of ET-1-containing vesicles in thrombin-stimulated endothelial cells.

Endothelin-1 (ET-1†), a vasoactive peptide consisting of 21 amino acid residues, is synthesized by and secreted from endothelial cells [1]. The ET-1 secretion from endothelial cells is known to be stimulated by several substances such as thrombin, transforming growth factor $\beta$, arg-vasopressin and angiotensin II [2–5]. However, the mechanism of ET-1 secretion is not yet clear, and it is assumed that receptor-mediated intracellular $Ca^{2+}$ mobilization and activation of $Ca^{2+}$/phospholipid-dependent protein kinase (protein kinase C) are associated with ET-1 synthesis and/or secretion [2, 6]. On the other hand, it has been shown that ET-1 is generated from a 39-amino acid intermediate, big ET-1, through cleavage between $Trp^{21}$-$Val^{22}$; a specific protease, endothelin-converting enzyme, cleaves at the $Trp^{21}$-$Val^{22}$ bond to yield ET-1(1–21) and ET-1(22–39) [1, 7]. The latter is located in the porcine endothelial cells [1, 7]. Nakamura *et al.* [8] reported recently that immunoreactive ET-1 is localized mainly in the rough endoplasmic reticulum, Golgi cisternae, Golgi vesicles and small vesicles beneath the cell membrane of the endothelial cells. It has been shown previously that the microtubular systems play an important role in ET-1 secretion from endothelial cells, especially in the transport of ET-1 [5]. The present study investigated whether or not the phosphorylation of myosin light chain (MLC) by $Ca^{2+}$-calmodulin-dependent protein kinase (MLC kinase) is involved in ET-1 secretion from porcine aortic endothelial cells.

* Corresponding author. Tel. (81) 862-52-1111 Ext. 979; FAX (81) 862-55-7456.

† Abbreviations: ET-1, endothelin-1; IR-ET-1, immunoreactive ET-1; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide; ML-9, 1-(5-chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine; MLC, myosin light chain; protein kinase C, $Ca^{2+}$/phospholipid-dependent protein kinase; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; EGTA, ethyleneglycol-bis($\beta$-aminoethyl ether) *N*,*N*,*N*′,*N*′,-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; MHC, myosin heavy chain.

## MATERIALS AND METHODS

*Cell culture.* The endothelial cells were harvested by scraping the intimal surface of porcine aorta with a scalpel blade, and were grown in Dulbecco's modified Eagle's medium (DMEM, Nissui, Japan) supplemented with 10% fetal calf serum, 100 U/mL penicillin (Meiji, Japan) and 100 $\mu$g/mL streptomycin (Meiji) in a humidified atmosphere of 5% $CO_2$/95% air as described previously [5]. After the cells had been grown to confluence in 24-well plates (Corning), the cells were washed twice with 300 $\mu$L of serum-free DMEM and incubated for 6 hr in 300 $\mu$L of the same medium containing the test compounds. To measure the intracellular ET-1 content, the endothelial cells incubated for 6 hr in 6-well plates containing 1.5 mL of DMEM in the presence and in the absence of test compounds were rinsed three times with 2 mL of ice-cold solubilizing buffer

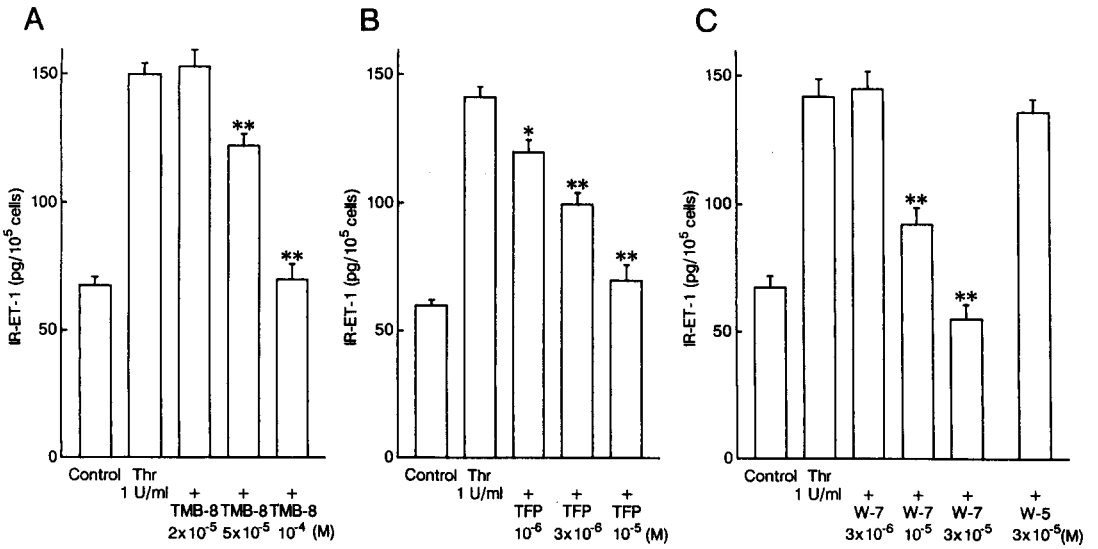


Fig. 1. Effects of TMB-8 (A), trifluoperazine (TFP) (B) and W-7 (C) on IR-ET-1 secretion from the porcine aortic endothelial cells induced by thrombin (Thr). The endothelial cells were incubated with thrombin in the presence of TMB-8, TFP or W-7 for 6 hr. Each column represents the mean ± SEM of 4–6 experiments. * and ** significantly different from the thrombin-treated group at $P < 0.05$ and $P < 0.01$, respectively.

consisting of 50 mM phosphate buffer (pH 7.4), 50 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.3% bovine serum albumin (BSA) and 0.1% $NaN_3$ for 1 hr and the insoluble materials were removed by centrifugation at 105,000 *g* for 30 min at 4°. The ET-1 content of the culture medium and the ET-1 remaining inside the cell were determined by radioimmunoassay using a specific antiserum for ET-1 (Peptide Institute, Osaka, Japan) and [$^{125}$I]ET-1 (Amersham, U.K.) as a tracer as described previously [5]. The cross-reactivity of the antibody to big ET-1 (1–39) and big ET-1 (22–39) was determined by comparison of the standard curves in radioimmunoassay, and it became apparent that the cross-reactivity was less than 0.1% in each case.

*Protein phosphorylation of cytoskeletal elements.* The endothelial cells were washed three times with 500 μL of phosphate-free DMEM plus 10 mM HEPES and incubated at 37° for 1 hr in 300 μL of the same medium containing [$^{32}$P]orthophosphoric acid (0.2 mCi/mL, Dupont). The cells were then washed three times with phosphate-free DMEM plus 10 mM HEPES and preincubated for 30 min in 300 μL of the same medium containing the test compounds. Thereafter, thrombin was added and the incubation was continued for various periods of time (15–180 sec).

SDS–PAGE analysis of the whole cells was carried out according to the method of Laemmli [9]. Incubations were stopped by removal of the medium and 100 μL of sample buffer (20 mM Tris, pH 6.8, 20% glycerol, 1% β-mercaptoethanol and 1% SDS kept at 95°) were added. The samples were transferred to microcentrifuge tubes, boiled for 5 min and subjected to electrophoresis. Cytoskeletal elements were extracted from the endothelial cells, either stimulated by thrombin or not, by treating the cells for 5 min with 100 μL of ice-cold extraction buffer consisting of 1% Triton X-100, 5 mM EGTA, 20 mM NaF, 25 mM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.001% leupeptin and 20 mM Tris, pH 7.4, and collecting the insoluble fraction by centrifugation at 16,000 *g* for 10 min at 4°. The insoluble fraction was solubilized in 10 μL of twice concentrated sample buffer and boiled for 10 min. Triton soluble fraction was treated with an equal volume of the twice concentrated sample buffer and boiled for 5 min. Samples were subjected to SDS–PAGE.

SDS–PAGE was carried out in 10 or 10–20% polyacrylamide gradient gels (Daiichi, Japan) using an electrode buffer contained 50 mM Tris, 373 mM glycine, pH 8.3, and 0.1% SDS. The gels were stained with Coomassie brilliant blue, dried and exposed to Hyperfilm-MP (Amersham) at −80°. Autoradiographs were scanned with a laser densitometer (Molecular Dynamics). The extent of $^{32}$P incorporation into a specific protein was indicated as percentage of the control value measured in the absence of thrombin.

*Immunoprecipitation of myosin light chain.* The endothelial cells incorporated with [$^{32}$P]orthophosphoric acid were incubated with or without thrombin at 1 U/mL for 30 sec at 37°. After removal of the medium, the cells were lysed in 100 μL of radioimmunoprecipitation assay buffer consisting of 25 mM Tris, pH 8.8, 250 mM NaCl, 100 mM sodium pyrophosphate, 50 mM NaF, 5 mM EGTA, 5 mM EDTA, 2 mM $Na_3VO_4$, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 0.001% leupeptin and 1 mM PMSF for 30 min at 0°. The extract was incubated with 50 μL of anti-mouse IgM Sepharose

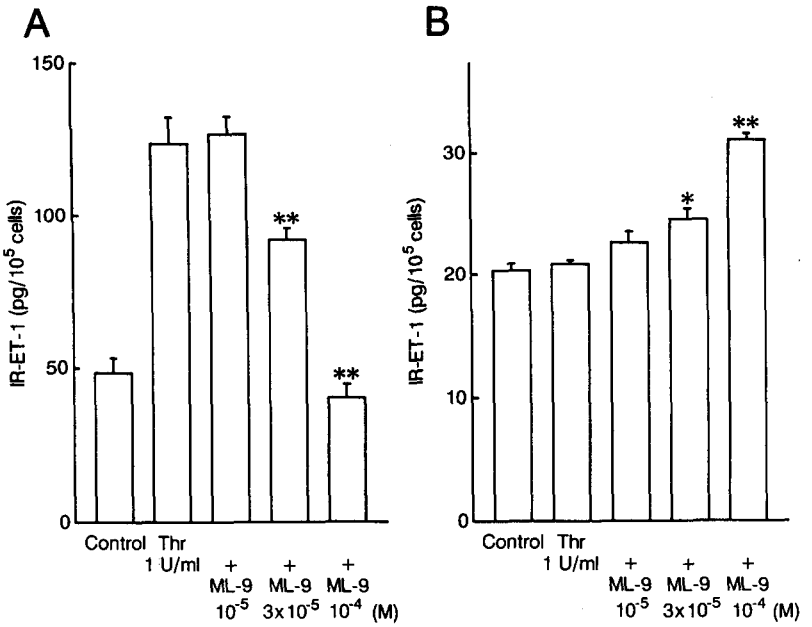


Fig. 2. Effects of ML-9 on IR-ET-1 secretion induced by thrombin (Thr) (A) and the intracellular IR-ET-1 contents of porcine aortic endothelial cells (B). The endothelial cells were incubated with thrombin in the presence of ML-9 for 6 hr. Each column represents the mean ± SEM of 4–6 experiments. * and ** significantly different from the thrombin-treated group at $P < 0.05$ and $P < 0.01$, respectively.

4B (Zymed, San Francisco) for 15 min and centrifuged at 16,000 *g* for 5 min, and the supernatant was incubated with 25 μL of monoclonal antibody against MLC (BioMakor, Israel) for 1 hr at 0°. The sample was then incubated with 50 μL of anti-mouse IgM Sepharose 4B for 1 hr. The immunoprecipitate was centrifuged at 16,000 *g* for 5 min at 4° and the pellet was washed twice with radioimmunoprecipitation assay buffer and once with phosphate-buffered saline. The pellet was boiled in sample buffer and subjected to SDS–PAGE.

*Phosphoamino acid analysis.* After SDS–PAGE of the $^{32}$P-phosphorylated cytoskeletal elements of the endothelial cells, the band of MLC was cut out from the gel with a razor blade, homogenized and extracted with 500 μL of 50 mM phosphate buffer (pH 7.4) containing 0.1% SDS and 1 mM PMSF at 37° overnight. The mixture was centrifuged at 16,000 *g* for 5 min at 4° and the supernatant was mixed with 10 μL of 10 mg/mL BSA and 125 μL of 100% trichloroacetic acid at 4° for 60 min. The precipitate was collected by centrifugation at 16,000 *g* for 10 min at 4°, washed three times with acetone and dried *in vacuo*. The residue was hydrolysed in 100 μL of 6 N HCl for 2 hr at 110° under $N_2$ phase and dried *in vacuo*. The hydrolysate was dissolved in distilled water, applied onto a cellulose TLC plate (Merck, Darmstadt, Germany) and subjected to electrophoresis at 600 V for 1.5 hr in a mixed solution consisting of acetic acid:pyridine:$H_2O$ (50:5:945). Standard phosphoamino acids (3 μL, 0.5 mg/mL), i.e. phosphoserine and phosphothreonine, were detected by ninhydrin reagent. The $^{32}$P-labeled amino acid was detected by autoradiography.

*Western blot analysis of actin and myosin heavy chain (MHC).* The SDS-solubilized extract from the endothelial cells was separated by SDS–PAGE and blotted to polyvinylidene-difluoride protein sequencing membrane (Bio-Rad, Richmond, CA, U.S.A.) by a semi-dry electroblotter (Sartorius). The membrane was washed three times with phosphate-buffered saline containing 0.1% Tween 20 and then incubated overnight with 3% skim milk (Difco) at 1% BSA at 4°. The membrane was incubated with anti-actin (dilution 1:25, Biomedical Technologies Inc.) or anti-MHC (dilution 1:50, Biomedical Technologies Inc.) for 2 hr at 37°. After the membrane had been thoroughly washed, incubation was continued with biotinylated goat anti-rabbit IgG (Vector). The blot was detected with avidin-biotinylated horseradish peroxidase complex (Vectastain elite ABC kits, Vector).

*Chemicals and statistical analysis.* Thrombin (from bovine plasma), TMB-8, trifluoperazine, W-7 and W-5 were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). ML-9 was obtained from the Seikagaku Co. (Japan). Calphostin C was provided by the Kyowa Hakko Kogyo Co. (Japan). Other chemicals used were all reagent grade and were purchased from commercial sources. The data were expressed as means ± SEM. Statistical estimation was conducted using analysis of variance and Dunnett's test.

## RESULTS

The effects of the intracellular $Ca^{2+}$ blocker (TMB-8) and calmodulin inhibitors (trifluoperazine

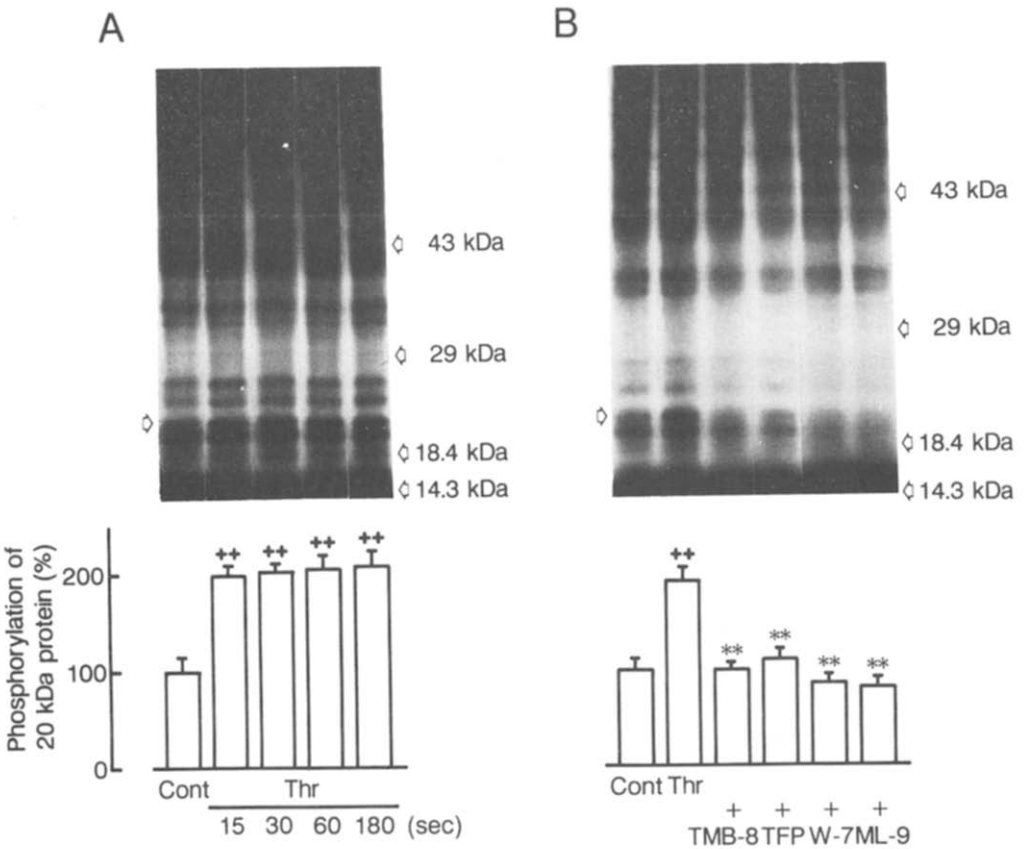


Fig. 3. Time-course of a 20-kDa protein phosphorylation stimulated by thrombin (A) and the effects of TMB-8, trifluoperazine, W-7 and ML-9 on the thrombin-stimulated phosphorylation of 20-kDa protein in the porcine aortic endothelial cells (B). The $^{32}P$-incorporated endothelial cells were incubated with thrombin (1 U/mL) for 15, 30, 60 or 180 sec (A) and 60 sec after preincubation with test drugs for 30 min (B). The phosphorylation of 20-kDa protein was evaluated by densitometric scanning of the autoradiographs. Under the autoradiographs, the amount of phosphorylation was depicted in columns. Each column represents the mean ± SEM of 4–5 experiments. ++ Significantly different from the control group at $P < 0.01$. ** Significantly different from the thrombin-treated group at $P < 0.01$. Cont, control; Thr, thrombin 1 U/mL; TMB-8, TMB-8 $10^{-4}$ M; TFP, trifluoperazine $10^{-5}$ M; W-7, W-7 $3 \times 10^{-5}$ M; ML-9, ML-9 $10^{-4}$ M.

and W-7) on the thrombin-induced secretion of immunoreactive ET-1 (IR-ET-1) from porcine aortic endothelial cells are shown in Fig. 1A, B and C. When the endothelial cells were treated with thrombin at a fixed concentration of 1 U/mL for 6 hr, the level of IR-ET-1 secretion was about twice as high as in the controls. When BSA alone was added at a concentration of 0.5 μg/mL, which is equivalent to the protein concentration of the thrombin solution, no increase in IR-ET-1 secretion was observed. In addition, it was confirmed that thrombin-induced IR-ET-1 secretion was not influenced in the presence of BSA at a concentration of 0.5 μg/mL. In the presence of TMB-8 at concentrations ranging from $2 \times 10^{-5}$ to $10^{-4}$ M, the IR-ET-1 secretion induced by thrombin (1 U/mL) was inhibited dose dependently and significantly. When trifluoperazine and W-7 were added to the medium at concentrations ranging from $10^{-6}$ to $10^{-5}$ M or from $3 \times 10^{-6}$ to $3 \times 10^{-5}$ M simultaneously with thrombin (1 U/mL), the amount of IR-ET-1 released into the medium decreased significantly and dose dependently. However, when the cells were treated with W-5, IR-ET-1 secretion was not inhibited even at a concentration of $3 \times 10^{-5}$ M.

Treatment with ML-9, a MLC kinase inhibitor, at concentrations ranging from $10^{-5}$ to $10^{-4}$ M, elicited dose-dependent and significant inhibition of the IR-ET-1 secretion caused by thrombin (Fig. 2A). Also, when the IR-ET-1 content of the cells was measured under the same conditions, the amount of IR-ET-1 increased in a dose-dependent fashion after treatment with ML-9 (Fig. 2B). When the endothelial cells were exposed to calphostin C, a specific inhibitor of protein kinase C [10], there was no inhibition of thrombin-induced IR-ET-1 secretion, even at a concentration of $10^{-5}$ M (data not shown). The basal secretion of IR-ET-1 from non-treated endothelial cells was also moderately but significantly inhibited

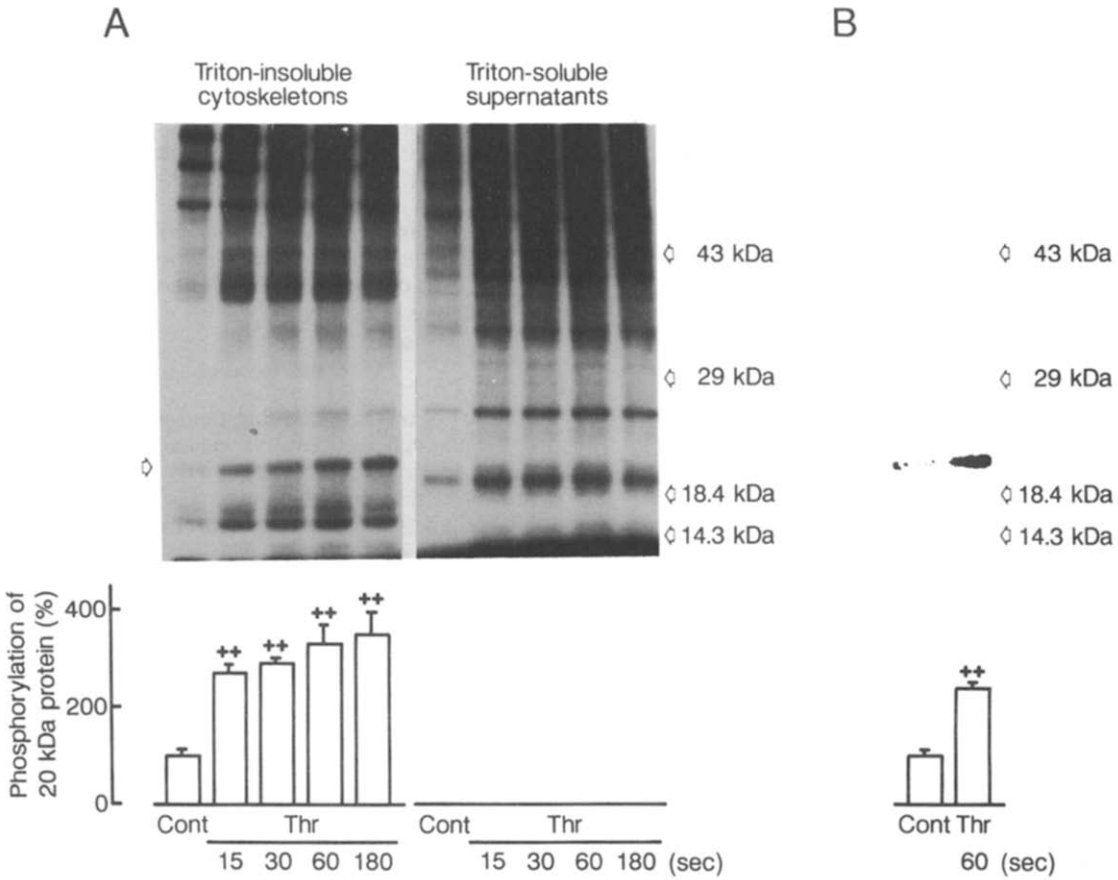


Fig. 4. Time-course of a 20-kDa protein phosphorylation stimulated by thrombin in Triton-insoluble cytoskeletal elements of the endothelial cells (A) and immunoprecipitation of MLC (B). The $^{32}P$-labeled endothelial cells were incubated with thrombin (1 U/mL) for 15, 30, 60 or 180 sec (A) and 60 sec (B). The phosphorylation of 20-kDa protein was estimated by a densitometric scanning of the autoradiographs. Each column represents the mean ± SEM of 4–5 experiments. ++ Significantly different from the control group at $P < 0.01$. Cont, control; Thr, thrombin 1 U/mL.

by TMB-8, trifluoperazine, W-7 and ML-9 at the same concentration ranges that inhibited thrombin treated cells and the percentages of inhibition were 38.5 ± 2.2% (TMB-8; $10^{-4}$ M, $P < 0.01$, $N = 5$), 23.2 ± 1.1% (trifluoperazine; $10^{-5}$ M, $P < 0.01$, $N = 5$), 35.9 ± 3.8% (W-7; $3 \times 10^{-5}$ M, $P < 0.01$, $N = 5$) and 27.3 ± 1.8% (ML-9; $10^{-4}$ M, $P < 0.01$, $N = 5$), respectively. Inhibition in each case occurred to a lesser extent than in thrombin-treated endothelial cells.

Since the involvement of $Ca^{2+}$-calmodulin and MLC kinase was assumed in thrombin-induced ET-1 secretion, the effect of thrombin on protein phosphorylation in the endothelial cells was studied. Figure 3A shows the pattern of protein phosphorylation when the endothelial cells were stimulated with thrombin for various periods of time. Treatment of the endothelial cells with thrombin caused a time-dependent and significant phosphorylation of a protein having an apparent molecular mass of 20 kDa compared to that of the control. The phosphorylation of the 20-kDa protein took place within the first 15 sec of incubation and rose to 201.3 ± 8.5, 203.1 ± 6.3, 206.3 ± 9.9 and 207.3 ± 14.0% of the untreated control at 15, 30, 60 and 180 sec, respectively ($N = 4$–5). When the endothelial cells were treated with various concentrations of thrombin for 60 sec, the phosphorylation of this protein rose to 116.6 ± 0.3, 164.8 ± 2.0 and 203.8 ± 4.2% of the control at concentrations of 0.1, 0.3 and 1 U/mL, respectively ($N = 4$). A significant increase in phosphorylation above the control level was observed at a concentration of 0.3 U/mL ($P < 0.01$). As shown in Fig. 3B, protein phosphorylation was significantly inhibited by TMB-8, trifluoperazine, W-7 and ML-9 at concentrations of $10^{-4}$, $10^{-5}$, $3 \times 10^{-5}$ and $10^{-4}$ M, respectively. However, calphostin C was not effective in inhibiting the thrombin-induced phosphorylation at a concentration of $10^{-5}$ M (data not shown).

To determine whether or not the 20-kDa phosphorylated protein is contained in cytoskeletal elements, SDS–PAGE of the Triton-insoluble cytoskeletons and Triton-soluble supernatants from control and thrombin-stimulated endothelial cells

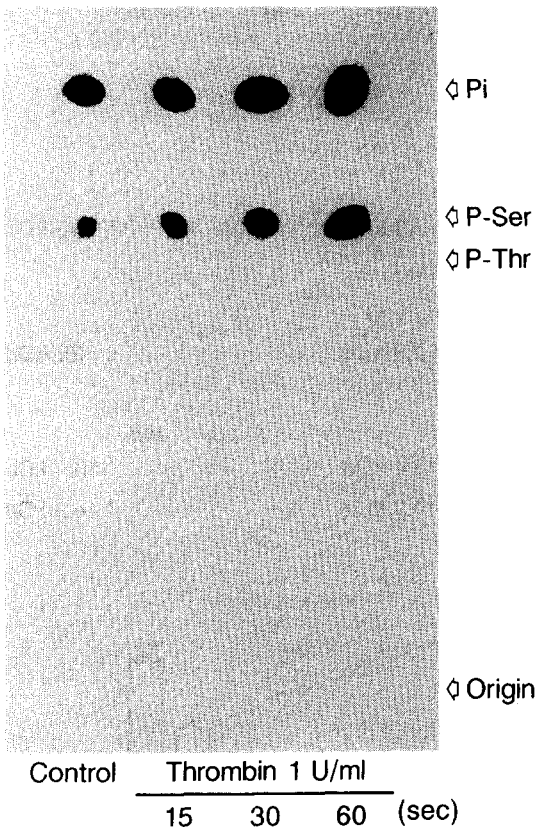


Fig. 5. Phosphoamino acid analysis of MLC in the thrombin-stimulated endothelial cells. The $^{32}P$-labeled endothelial cells were incubated with thrombin (1 U/mL) for 15, 30 and 60 sec. P-Ser, phosphoserine; P-Thr, phosphothreonine.

was carried out. As shown in Fig. 4A, a 20-kDa phosphorylated protein was clearly detected in Triton-insoluble fractions, and phosphorylation of this protein was time-dependently and significantly enhanced as seen in the case of the whole cells. However, the degree of phosphorylation of the 65-kDa protein (considered as a reference) in the Triton-insoluble fractions was almost consistent (100% in non-treated control, $101.0 \pm 1.9\%$ at 15 sec, $98.8 \pm 3.3\%$ at 30 sec, $98.1 \pm 4.7\%$ at 60 sec and $98.9 \pm 3.5\%$ at 180 sec, $N = 4$–5). Furthermore, it became apparent that the 20-kDa phosphorylated protein was definitely immunoprecipitated by monoclonal antibody against MLC (Fig. 4B). Phosphoamino acid analysis of the 20-kDa phosphorylated protein revealed that serine is, indeed, the only phosphoamino acid it contains (Fig. 5). It is possible to assume that the phosphorylation of MLC may regulate myosin filament assembly in the endothelial cells. Therefore, we carried out an experiment to examine changes in the amounts of polymerized cytoskeletal proteins, such as myosin and actin, using SDS–PAGE of Triton-insoluble fractions in endothelial cells. Figure 6A shows a comparison of the cytoskeletal proteins in the Triton-insoluble residues obtained from control and thrombin-activated endothelial cells. The four most prevalent proteins in the Triton-insoluble residue of the control endothelial cells had apparent molecular masses of 43, 56, 200 and 250 kDa. When the endothelial cells were stimulated with thrombin, the amounts of 43-, 200- and 250-kDa protein all increased in a time-dependent fashion in the Triton-insoluble residue. The 43- and 200-kDa proteins were identified as actin and myosin heavy chain (MHC), respectively, as clearly shown in Western blot analysis (Fig. 6B).

## DISCUSSION

It has been suggested that the secretion of ET-1 from endothelial cells can be regulated by intracellular $Ca^{2+}$ concentration [2, 6]. We have reported previously that thrombin induced an increase in the intracellular $Ca^{2+}$ concentration of endothelial cells prior to ET-1 secretion [5]. The present experiments demonstrated that the thrombin-induced secretion of ET-1 from the endothelial cells was inhibited by an intracellular $Ca^{2+}$ blocker TMB-8 [11] as well as by calmodulin inhibitors, such as trifluoperazine and W-7 [12, 13]. At the concentrations employed in the present experiment, TMB-8 was effective in inhibiting inositol 1,4,5-trisphosphate-induced $^{45}Ca$ release from the endoplasmic reticulum [14]. In a previous study, $IC_{50}$ values of trifluoperazine and W-7 for calmodulin-dependent phosphodiesterase activity were approximately $10^{-5}$ and $10^{-4}$ M [15], respectively; concentrations which are almost identical to those employed in the present experiment. These results suggest that the secretion of ET-1 from the endothelial cells might be regulated by intracellular $Ca^{2+}$ released from the $Ca^{2+}$ store and by $Ca^{2+}$–calmodulin complex. Furthermore, in the endothelial cells treated with thrombin simultaneously with ML-9, a MLC kinase inhibitor [16], ET-1 secretion decreased, while the intracellular content of ET-1 increased. It has been shown that ML-9 binds at or near the ATP binding site at the active center of the kinase, and this may result in inhibition of the catalytic activity of MLC kinase [16, 17]. It is well known that an application of ML-9 at concentrations ranging from $10^{-5}$ to $3 \times 10^{-4}$ M induces the relaxation of vascular strips via its direct inhibitory effect on MLC kinase [17]. Furthermore, it has been reported that ML-9 also inhibits the catalytic activities of other protein kinases, such as protein kinase C and cAMP-dependent protein kinase, with $K_i$ values of at least an order of magnitude higher than that for MLC kinase [16, 17]. However, calphostin C, a specific inhibitor of protein kinase C ($IC_{50}$, 50 nM) [10], KT5720, a specific inhibitor of cAMP-dependent protein kinase ($K_i$, 60 nM) [18], and KT5822, a specific inhibitor of cGMP-dependent protein kinase ($K_i$, 2.4 nM) [18], were not effective in inhibiting the thrombin-induced secretion of ET-1 from endothelial cells even at concentrations of $10^{-5}$, $10^{-6}$ and $10^{-6}$ M, respectively (data not shown). These findings seem to indicate that the inhibitory effect of ML-9 on the thrombin-induced secretion of ET-1 may be attributed to its effect on MLC kinase. It has been shown that MLC

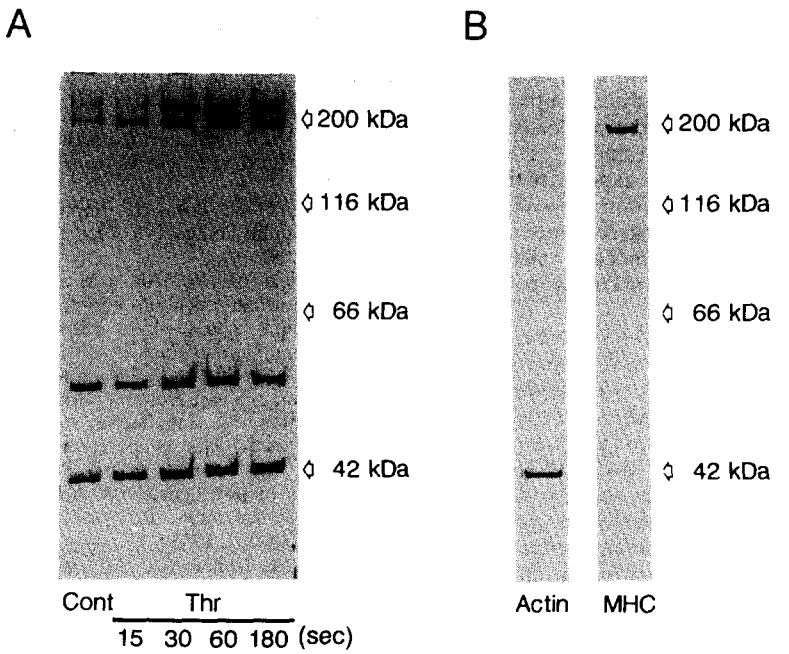


Fig. 6. SDS–PAGE analysis of Triton-insoluble cytoskeletons in the thrombin-stimulated endothelial cells. (A) Sequential detections and (B) immunological identification of an actin and myosin heavy chain (MHC). The endothelial cells were incubated with thrombin (1 U/mL) for 15, 30, 60 or 180 sec. Cont, control; Thr, thrombin 1 U/mL.

kinase, activated by the $Ca^{2+}$–calmodulin complex, catalyses the phosphorylation of the 20-kDa MLC, which may trigger alteration of the cell shape and granule secretion in non-muscle cells such as platelets and rat pheochromocytoma PC12h cells [19–21]. Indeed, in the endothelial cells thrombin induced the phosphorylation of a 20-kDa protein which was found in Triton-insoluble fractions (Fig. 4A) indicating that a 20-kDa protein is one of the cytoskeletal elements. Moreover, this protein was immunoprecipitated with monoclonal anti-MLC antibody (Fig. 4B). This clearly indicates that the 20-kDa protein is actually MLC. The phosphorylation of MLC was inhibited by TMB-8, trifluoperazine, W-7 and ML-9, at concentrations ranging from $10^{-5}$ to $10^{-4}$ M, which are also effective in inhibiting ET-1 secretion. This may indicate that MLC phosphorylation is involved in ET-1 secretion.

It has been reported that MLC is phosphorylated not only by MLC kinase but also by protein kinase C [22–25]. However, when phosphoamino acid analysis was carried out, serine was found to be the only phosphoamino acid in phosphorylated MLC as shown in Fig. 5. The site of phosphorylation of MLC for MLC kinase is usually serine 19 [22]. However, another phosphorylation site of MLC, i.e. threonine 18, has been identified; phosphorylation of MLC occurs at this site only in the presence of a high concentration of MLC kinase [23]. It has been emphasized that serine 19 is readily phosphorylated preferentially to threonine 18 [22]. This indicates that the phosphorylation of MLC induced by thrombin in the endothelial cells is catalysed by MLC kinase. Actually, a specific inhibitor of protein kinase C, calphostin C [10], was ineffective not only in the case of the thrombin-induced ET-1 secretion but also the phosphorylation of MLC. The $IC_{50}$ value of calphostin C in inhibiting protein kinase C is 50 nM [10]. The concentration of calphostin C employed in the present experiment was $10^{-5}$ M; this concentration is 200 times higher than the $IC_{50}$ [10].

In addition, it was demonstrated that the amount of both Triton-insoluble actin and MHC increased during the stimulation of endothelial cells with thrombin. The filamentous actin in non-muscle cells is known to be resistant to Triton solubilization, while unpolymerized actin is Triton soluble [26].

Following electron microscopy studies, it has been suggested that when myosin is dephosphorylated in non-muscle cells, each myosin tail folds back to adhere to a "sticky patch" on the head and in this folded configuration myosin molecules are not able to assemble [27]. However, as a consequence of the phosphorylation of MLC, conformational changes in the myosin head may occur so as to expose its actin-binding site, and this allows the myosin molecule to assemble into a bipolar filament [27]. Scholey *et al.* [28] and Jennings *et al.* [29] also reported that MLC phosphorylation enables non-muscle myosin molecules to assemble filaments, and consequently, myosin may become resistant and insoluble to Triton. These findings suggest that thrombin causes the polymerization of myosin through the phosphorylation of MLC in the endothelial cells. Moreover, the phosphorylation of

MLC by MLC kinase causes an increase in actin-activated ATPase activity and, consequently, it stimulates myosin–actin filament interactions. Such an actin–myosin interaction plays an important role in regulating shape change and granule secretion in non-muscle cells [27]. Furthermore, it has been reported that when platelets are exposed to thrombin, the amount of polymerized actin increases during platelet activation [29, 30]. In accordance with this, the actin filament content evidently increased as shown in Fig. 6. This can be ascribed to increased actin–myosin interaction [29].

In the endothelial cells, ET-1 synthesized in the rough endoplasmic reticulum is transported to the Golgi complexes, packed into Golgi vesicles and secreted through the microtubular system [5, 8]. From these results, it is suggested that the thrombin-induced phosphorylation of MLC and subsequent formation of polymerized actin and myosin may be intimately involved in ET-1 secretion, probably in the transport of ET-1-containing vesicles in the endothelial cells in response to thrombin stimulation.

## REFERENCES


1. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K and Masaki T, A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332**: 411–415, 1988.
2. Emori T, Hirata Y, Ohta K, Shichiri M and Marumo F, Secretory mechanism of immunoreactive endothelin in cultured bovine endothelial cells. *Biochem Biophys Res Commun* **160**: 93–100, 1989.
3. Schini VB, Hendrickson H, Heublein DM, Burnett JC Jr and Vanhoutte PM, Thrombin enhances the release of endothelin from cultured porcine aortic endothelial cells. *Eur J Pharmacol* **165**: 333–334, 1989.
4. Kurihara H, Yoshizumi M, Sugiyama T, Takaku F, Yanagisawa M, Masaki T, Hamaoki M, Kato H and Yazaki Y, Transforming growth factor-$\beta$ stipulates the expression of endothelin mRNA by vascular endothelial cells. *Biochem Biophys Res Commun* **159**: 1435–1440, 1989.
5. Kitazumi K, Mio M and Tasaka K, Involvement of the microtubular system in the endothelin-1 secretion from porcine aortic endothelial cells. *Biochem Pharmacol* **42**: 1079–1085, 1991.
6. Yanagisawa M, Inoue A, Takuwa Y, Mitsui Y, Kobayashi M and Masaki T, The human pre-proendothelin-1 gene: Possible regulation by endothelial phosphoinositide turnover signaling. *J Cardiovasc Pharmacol* **13**(Suppl 5): S13–S17, 1989.
7. Sawamura T, Kimura S, Shinmi O, Sugita Y, Yamagisawa M and Masaki T, Analysis of endothelin related peptides in culture supernatant of porcine aortic endothelial cells: evidence for biosynthetic pathway endothelin-1. *Biochem Biophys Res Commun* **162**: 1287–1294, 1989.
8. Nakamura S, Naruse M, Naruse K, Demura H and Uemura H, Immunocytochemical localization of endothelin in cultured bovine endothelial cells. *Histochemistry* **94**: 475–477, 1990.
9. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
10. Kobayashi E, Nakano H, Morimoto M and Tamaoki T, Calphostin C (UCN-1028C), A novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun* **159**: 548–553, 1989.
11. Chiou CY and Malagodi MH, Studies on the mechanism of action of a new $Ca^{2+}$ antagonist, 8-(*N,N*-diethylamino)-octyl 3,4,5-trimethoxybenzoate hydrochloride in smooth and skeletal muscles. *Br J Pharmacol* **53**: 279–285, 1975.
12. Weiss B, Technique for measuring the interaction of drug with calmodulin. *Methods Enzymol* **102**: 171–184, 1983.
13. Hidaka H and Tanaka T, Naphthalenesulfonamides as calmodulin antagonists. *Methods Enzymol* **102**: 185–194, 1983.
14. Yoshii N, Mio M, Akagi M and Tasaka K, Role of endoplasmic reticulum, an intracellular $Ca^{2+}$ store, in histamine release from rat peritoneal mast cell. *Immunopharmacology* **21**: 13–22, 1991.
15. Mio M, Izushi K and Tasaka K, Substance P-induced histamine release from rat peritoneal mast cells and its inhibition by antiallergic agents and calmodulin inhibitors. *Immunopharmacology* **22**: 59–66, 1991.
16. Saitoh M, Naka M and Hidaka H, The modulatory role of myosin light chain phosphorylation in human platelet activation. *Biochem Biophys Res Commun* **140**: 280–287, 1986.
17. Saito M, Ishikawa T, Mitsushima S, Naka M and Hidaka H, Selective inhibition of catalytic activity of smooth muscle myosin light chain kinase. *J Biol Chem* **262**: 7796–7801, 1987.
18. Kase H, Iwahashi K, Nakanishi S, Matsuda Y, Yamada K, Takahashi M, Murakata C, Sato A and Kaneko M, K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem Biophys Res Commun* **142**: 436–440, 1987.
19. Daniel JL, Hormsen H and Adelstein RS, Thrombin-stimulated myosin phosphorylation in intact platelets and its possible involvement secretion. *Thromb Haemost* **38**: 984–989, 1977.
20. Haslam RJ and Lynham JA, Relationship between phosphorylation of blood platelet proteins and secretion of platelet granule constituents. I. Effects of different aggregating agents. *Biochem Biophys Res Commun* **77**: 714–722, 1977.
21. Nagatsu T, Suzuki H, Kiuchi K, Saitoh M and Hidaka H, Effects of myosin light-chain kinase inhibitor on catecholamine secretion from rat pheochromocytoma PC12h cells. *Biochem Biophys Res Commun* **143**: 1045–1048, 1987.
22. Ikebe M and Hartshorne DJ, Phosphorylation of smooth muscle myosin at two distinct sites by myosin light chain kinase. *J Biol Chem* **260**: 10027–10031, 1985.
23. Ikebe M, Hartshorne DJ and Elzinga M, Identification, phosphorylation, and dephosphorylation of a second site for myosin light chain kinase on the 20,000-dalton light chain of smooth muscle myosin. *J Biol Chem* **261**: 36–39, 1986.
24. Bengur AR, Robinson EA, Appella E and Sellers JR, Sequence of the sites phosphorylated by protein kinase C in the smooth muscle myosin light chain. *J Biol Chem* **262**: 7613–7617, 1987.
25. Ikebe M, Hartshorne DJ and Elzinga M, Phosphorylation of the 20,000-dalton light chain of smooth muscle myosin by the calcium-activated, phospholipid-dependent protein kinase. *J Biol Chem* **262**: 9569–9573, 1987.
26. Bray D and Thomas C, Unpolymerized actin in fibroblasts and brain. *J Mol Biol* **105**: 527–544, 1976.
27. Cooke R, Kirschner M, Klymkowsky M, Mooseker M, Pollard T, Rosenbaum J, Tilney L and Weber K, In: *Molecular Biology of the Cell, 2nd Edn* (Eds. Alberts B, Bray D, Lewis J, Raff M, Roberts K and Watson JD), pp. 613–680. Garland Publishing, New York, 1989.

28. Scholey JM, Taylor KA and Kendrick-Jones J, Regulation of non-muscle myosin assembly by calmodulin-dependent light chain kinase. *Nature* **287**: 233–235, 1980.
29. Jennings LK, Fox JEB, Edwards HH and Phillips DR, Changes in the cytoskeletal structure of human platelets following thrombin activation. *J Biol Chem* **256**: 6927–6932, 1981.
30. Phillips DR, Jennings LK and Edwards HH, Identification of membrane protein mediating the interaction of human platelets. *J Cell Biol* **86**: 77–86, 1980.