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Critical role of the Rho-kinase pathway in TGF-β2-dependent collagen gel contraction by retinal pigment epithelial cells☆

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Abstract

Retinal pigment epithelial cells (RPEs) are thought to be one of the main components of fibrous membrane observed in eyes with proliferative vitreo-retinopathy. We investigated the signalling mechanisms of TGF-β2-dependent collagen gel contraction by RPEs.An in vitro type I collagen gel contraction assay was performed to evaluate the effect of TGF-β2 on gel contraction. The expression of alpha-smooth muscle actin (α-SMA) and the phosphorylation state of myosin light chain (MLC) were analyzed by Western blotting. The involvement of protein kinases such as p44/42 mitogen-activated protein kinase (MAPK), protein kinase C (PKC), p38 MAPK and phosphatidylinositol-3 kinase was investigated. The contribution of Rho-kinase and/or MLC-kinase was also evaluated using respective kinase inhibitors (Y27632, hydroxyfasudil and ML7). Additionally, RPEs were immunostained to examine whether the expression of α-SMA detected in our western blotting correlated to the stress fiber formation within the cells. TGF-β2 caused time (0–5 days)-and dose (0 10 ng ml^{-1})-dependent gel contraction associated with overexpression of α-SMA and phosphorylation of MLC ($p<0.01$, respectively). PKC inhibitor (GF109203X, 5 μM) and p38 MAPK inhibitor (SB203580, 10 μM) significantly attenuated TGF-β2-elicited gel contraction via partial downregulation of both α-SMA expression and MLC phosphorylation ($p<0.01$, respectively). The gel contraction was prominently inhibited in the presence of Y27632 (10 μM) or hydroxyfasudil (10 μM) with strong suppression of MLC phosphorylation but had no significant effect on α-SMA expression. Treatment with ML7, in contrast, resulted in a marginal inhibition of MLC phosphorylation and gel contraction. Finally, pretreatment of the cells with Y27632 or hydroxyfasudil prevented the formation of stress fiber within the cells.

These results indicate that TGF-β2-dependent myofibroblastic transdifferentiation and MLC phosphorylation by RPEs involve both PKC and p38 MAPK pathways at least in part. Myofibroblastic transdifferentiation of RPEs appears to be independent of the Rho-kinase pathway, and the presence of α-SMA does not necessarily reflect the contractile potential of a cell. While Rho-kinase inhibitors are incapable of preventing myofibroblastic transdifferentiation itself, this pathway could be one of the critical targets of cell-mediated contraction of the tissue containing fibrillar collagens by transdifferentiatied RPEs.

Keywords: retinal pigment epitelial cells; TGF-β2; rho-kinase; myosin light chani; myosin light chain kinase; gel contraction; proliferative vitreoretinopath; α-smooth uscle actin; myofibroblactic transdifferentiation; hydroxzyfasudil; Y27632; ML7

1. Introduction

Despite major advances in understanding the pathogenesis of various vitreo-retinal diseases and the development of vitreo-retinal surgeries, proliferative vitreo-retinopathy (PVR) is a serious problem in patients with retinal detachment, occasionally leading to severe vision loss or blindness (Pastor, 1998; Charteris, 2002). No established pharmacological treatment, however, is present to date. Accumulating evidence demonstrated that retinal pigment epithelial cells (RPEs) are one of the main components of fibrous epi-retinal membrane observed in eyes with various vitreo-retinal diseases including PVR (Machemer and Laqua, 1975; Kampik et al., 1981; Jerdan

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et al., 1989). Contraction of the epi-retinal membrane results in the progression of the diseased state, (Kampik et al., 1981) leading to a tractional retinal detachment which affects the prognosis of the diseased eyes.

On the other hand, high levels of cytokines including transforming growth factor (TGF)-β2 and platelet-derived growth factor (PDGF) have been found in the vitreous humor or epi-retinal membrane of patients with PVR, (Limb et al., 1991; Baudouin et al., 1993; Kauffmann et al., 1994; Charteris, 1998; Kon et al., 1999) suggesting a possible association of these cytokines with the development of vitreo-retinal pathology. TGF-β is one of the multifunctional cytokines known to modulate the proliferation and differentiation of many cell types, and is considered to bring about the increase of extracellular matrix production, resulting in the formation and contraction of proliferative epi-retinal membranes in PVR (Hunt et al., 1994; Choudhury et al., 1997; Carrington et al., 2000). Whilst recent evidence thus indicate that TGF-β2 is involved in the epi-retinal membrane contraction by RPEs, (Osusky et al., 1994) the intracellular signalling pathway still remains to be elucidated.

It is known that the contraction of smooth muscle cells is associated with the conformational change of actin-myosin interaction, and the phosphorylation of myosin light chain (MLC) is thought to be indispensable in this reorganization of the actin cytoskeleton (Bond and Somlyo, 1982). The phosphorylation state of MLC is thought to be regulated by various kinases and phosphatases such as protein kinase C (PKC), MLC kinase, MLC phosphatase, Rho-kinase and ZIP kinase (Frearson and Perry, 1975; Ikebe et al., 1985; Hartshorne et al., 1998; Ridley and Hall, 1992; Kimura et al., 1996; Niiro and Ikebe, 2001). Among them, Rho-kinase, which is a target protein of small GTPase Rho, increases the phosphorylation state of MLC both directly focused at Ser-19 residue and indirectly through inhibiting the MLC phosphatase activity by phosphorylating the myosin binding subunit (MBS) of the phosphatase (Amano et al., 1996). Rho-kinase is thought to be involved in the contractile activity of RPEs in vivo and in vitro, and α-SMA increases with RPE cell passages in vitro (Grisanti and Guidry, 1995; Kurosaka et al., 1996; Stocks et al., 2001; Zheng et al., 2004). However, the precise mechanisms responsible for the mediation of TGF-β2-elicited MLC phosphorylation and collagen gel contraction by RPEs are not fully understood. Additionally, no pharmacological treatment for PVR has been established to date.

Hydroxyfasudil, a metabolite of fasudil after oral administration, is a potent and selective inhibitor of Rho-kinase already in clinical use for cerebral vasospasm (Negoro et al., 1999). While the mechanisms of how hydroxyfasudil inhibits Rho-kinase activity are not completely clarified yet, its selective inhibitory effects on Rho-kinase activities have been demonstrated by in vitro kinase assay (Shimokawa et al., 1999). As reported, hydroxyfasudil is capable of inhibiting the Rho-kinase activity with minimal effects on other kinases such as PKC, MLC kinase and myotonic dystrophy kinase-related Cdc42-binding kinase beta (MRCKβ) at least up to the concentration of10 μM. Since the cell-mediated contraction of the tissue containing fibrillar collagens can be recognized at wound healing and often lead to the loss of organ functions, pharmacological treatment is desired in addition to the development of vitreo-retinal surgery (Wiedemann et al., 1984; Ryan, 1985; Weller, et al., 1990).

In the present study herein, we focused on the contribution of the Rho-and/or MLC-kinase pathways to TGF-β2-dependent MLC phosphorylation and collagen gel contraction by RPEs in vitro. We demonstrated the direct evidence of TGF-β2-dependent MLC phosphorylation by RPEs. The predominant contribution of the Rho-kinase-mediated pathway in TGF-β2-elicited MLC phosphorylation and collagen gel contraction by RPEs is also clarified. Certainly, hydroxyfasudil had no significant suppressive effect on the expression of α-SMA by RPEs, however it prominently prevented collagen gel contraction by transdifferentiated RPEs. These results indicate that through utilizing Rho-kinase inhibitors such as hydroxyfasudil the Rho-kinase pathway might be one of the therapeutic molecular targets for vitreo-retinal interface diseases including PVR.

2. Materials and methods

2.1. Materials

Recombinant human TGF-β2 was purchased from Sigma Japan. TGF-β2 was used as an active form by reconstitution of lyophilized powder with phosphate-buffered saline (PBS) containing 4 mM HCl. Goat polyclonal antibodies against myosin light chain (MLC) and phosphorylated-myosin light chain (p-MLC) were obtained from Santa Cruz (CA, USA). Mouse monoclonal antibody against α-SMA was obtained from Sigma Aldrich (CA, USA). A specific Rho-kinase inhibitor Y27632 and a typical MLC-kinase inhibitor ML7 were obtained from CalBiochem (CA, USA). Hydroxyfasudil, a potent and selective Rho-kinase inhibitor, was generously provided by Asahi Kasei Pharma Corporation (Tokyo, Japan). PKC inhibitor (GF109203X), p44/42 MAPK kinase inhibitor (PD98059), p38 MAPK inhibitor (SB203580) and phosphatidylinositol 3-kinase inhibitor (Wortmannin) were obtained from CalBiochem (CA, USA).

2.2. Cell culture

Bovine retinal pigment epithelial cells (RPEs) were isolated, cultured and characterized as previously described (Sakamoto, et al., 1994b). The RPEs were cultured on type I collagen-coated dishes in Dulbecco's Modified Eagle's Medium (DMEM [GIBCO, CA, USA]) supplemented with 10% heat-inactivated fetal bovine serum (FBS [GIBCO, CA, USA]), 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37 °C under 5% CO_2/95% air atmosphere. Cells sub-cultured in the same conditions and obtained between passages 3 and 6 were used for the following experiments.

2.3. Collagen gel contraction assay

The contraction assay was performed as we previously described, (Noda et al., 2004) with some modifications. RPEs were collected by the treatment of cultures with trypsin-EDTA for 3 min, washed with DMEM, and re-suspended in DMEM at a density of 2.2×10^6 cells ml^{-1}. Type I collagen (Koken Co., Ltd, Tokyo, Japan), a reconstitution buffer, RPEs suspension, and distilled water were mixed on ice at a ratio of 7:1:1:1:1 (final concentration of type I collagen gel, 1.9 mg ml^{-1}; final cell density, 2×10^5 cells ml^{-1}. The resultant mixture (0.5 ml) was added to a 24-multiwell plate (Nunc, Roskilde, Denmark), and the formation of collagen gel was induced by incubation at 37 °C under 5% CO_2 for 60 min). After gelatinization, 0.5 ml of DMEM containing 1% calf serum (CS) was added to each well. The gels were freed from the walls of the culture wells with a micro-spatula 24 hr after gelatinization, and used for the experiments. The diameter of the collagen gel was measured with a ruler at indicated time points after stimulation. For quantitative purposes, contraction data is presented as the reduction in diameter of the collagen gels.

2.4. Counting the viable cell number in collagen gels

The viable cell number in the collagen gels was counted to exclude the effect of cell growth or cytotoxicity on the collagen gel contraction or its inhibition. After 5 days' treatment, the medium was removed and 0.5 ml of serum-free DMEM containing 3 mg ml^{-1} collagenase (Worthington collagenase type II) was added to each well. They were incubated at 37 °C until the collagen gels were dissolved. The RPEs were sedimented and the supernatant was removed. The viable cell number was counted with a haemocytometer after trypan blue staining.

2.5. Expression of α-SMA

To evaluate the myofibroblastic transdifferentiation of RPEs, we investigated the expression level of α-smooth muscle actin by RPEs embedded in type I collagen gels as we previously described (Hirayama et al., 2004). The cells embedded in the collagen gels were collected after collagenase treatment as described above and then lysed in 1× Laemmli buffer. Total cell lysates were subjected to 12% SDS-PAGE and transferred to nitrocellulose filters. After blocking with 3% skim milk, the blots were incubated overnight at 4 °C with an antibody against α-SMA (1:2000). After washing, the membranes were incubated with horseradish peroxidase-labeled goat anti-mouse IgG (Bio-Rad, Richmond, Calif.) (1:4000) for 1 hr at room temperature. Visualization was performed using the Amersham ECL detection system per manufacturer's instructions. To normalize the loading differences, the membranes were deprobed and re-blotted with GAPDH (Santa Cruz Biotech). The signal density was measured and analyzed using analysis software, and all graphs are illustrated as α-SMA/GAPDH ratio versus each control.

2.6. Phosphorylation state of myosin light chain

Subconfluent RPEs seeded on type I collagen coated plates were starved in DMEM supplemented with 1% CS for 24 hr. The cells were then stimulated with 3 ng ml^{-1} of TGF-β2 (0.1 nM) for the indicated time. The RPEs were washed once in cold 1× PBS and lysed in 1× Laemmli buffer (50 mmol l^{-1} Tris, pH6.8, 2% SDS, 10% glycerol) containing protease inhibitors (1 mmol l^{-1} phenylmethfluorideylsulfonyl [PMSF], 2 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 1 mmol l^{-1} NaF, 0.5 mmol l^{-1} Na_3VO_4). The extraction was subjected to 15% SDS–PAGE and transferred to nitrocellulose filters (New England Biolabs, Beverly, MA, USA). After blocking with 3% skim milk, the blots were incubated overnight at 4 °C with an antibody against p-MLC (1:1000). After washing, the membranes were incubated with horseradish peroxidase-labeled rabbit anti-goat IgG (Bio-Rad, Richmond, Calif.) (1:4000) for 1 hr at room temperature. Visualization was performed using the Amersham enhanced chemiluminescence (ECL) detection system per manufacturer's instructions. Lane loading differences were normalized by re-blotting the membranes with an antibody against MLC (1:1000). The signal density was measured, analyzed using analysis software (NIH image, version 1.55) and demonstrated by the ratio of pMLC/MLC.

2.7. Estimation of the influences of Y27632, ML7 and hydroxyfasudil on the stress fiber formation elicited by TGF-β2

After cultivation on type I collagen-coated chamber slides, cells were starved in DMEM supplemented with 1% calf serum, pretreated with Y27632, ML7 or hydroxyfasudil, and stimulated with TGF-β2 (3 ng ml^{-1}) for 5 days. Then, they were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 0.2% bovine serum albumin, and immunostained with monoclonal anti α-SMA. Secondary antibody was Cy5-conjugated goat anti-mouse antibody (Chemicon International Inc.). Additionally, nucleuses were stained with DAPI (Dojindo, Tokyo, Japan). The distribution of the stress fiber in each cell was photographed and analyzed by microscopy (OLYMPUS, IX71-23FL/DIC-SP).

2.8. Statistical analysis

The experimental data is expressed as means ± SD. Statistical significance was assumed when $p<0.05$ using the Student t-test in normally distributed populations.

3. Results

3.1. TGF-β2 enhances collagen gel contraction by RPEs

First, we examined the dose-and time-dependent collagen gel contraction by RPEs in the presence of TGF-β2. The control gels showed no apparent contraction up to 5 days investigated, whereas TGF-β2 significantly shortened

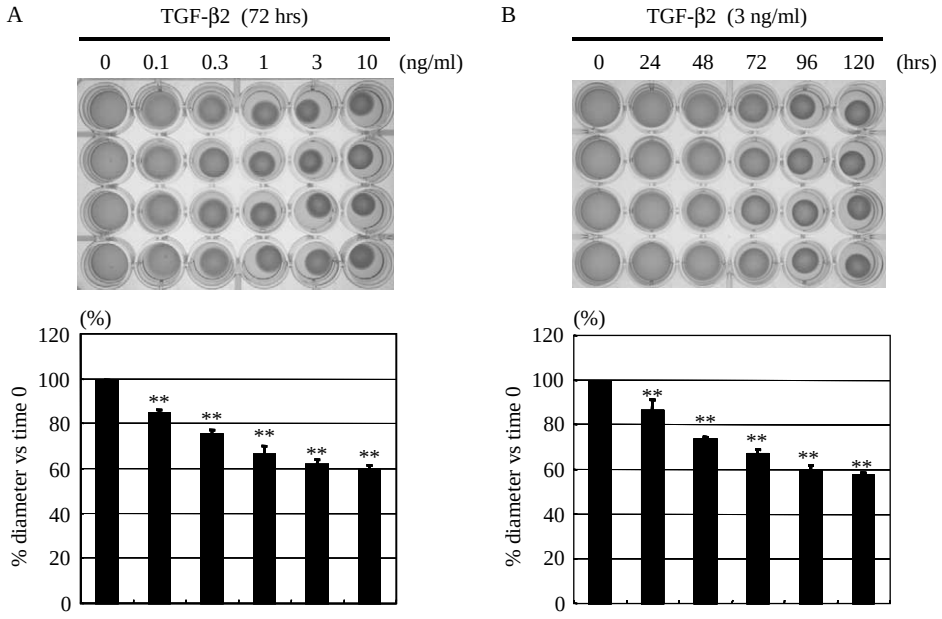

Fig. 1. Collagen gel contraction by TGF-β2. Three dimensional Type I collagen gels containing RPEs were prepared in a 24-multiwell plate. Dose (A)-and time (B)-dependent gel contraction by TGF-β2 was examined ($n=4$). Representative photographs are shown (top left/right). The gel diameter was measured and presented as percent of diameter as compared with time 0 (bottom left/right) (**: $p<0.01$ vs dose 0 or time 0 each).

the collagen gel diameter in a dose-dependent manner, initially significant at a concentration of 0.1 ng ml^{-1} (Fig. 1(A)). After 72 hrs' treatment with 10 ng ml^{-1} of TGF-β2, the collagen gel diameter was shortened by up to 59.6% ($p<0.01$) as compared with time 0. In addition, TGF-β2-induced collagen gel contraction was in a time-dependent manner at least up to 5 days. In the presence of 3 ng ml^{-1} of TGF-β2, the gels contracted by up to 57.5% ($p<0.01$) as compared with time 0 after 5 days cultivation (Fig. 1(B)).

3.2. Expression of α-SMA and phosphorylation state of MLC

Cellular expression of α-SMA implicates myofibroblastic transdifferentiation for non-muscle cells. We thus investigated the myofibroblastic transdifferentiation of RPEs embedded in type I-collagen gels. While the expression level of α-SMA by RPEs was scanty in control cells, TGF-β2 prominently induced the expression of α-SMA in a time-dependent manner initially significant after 1 day cultivation

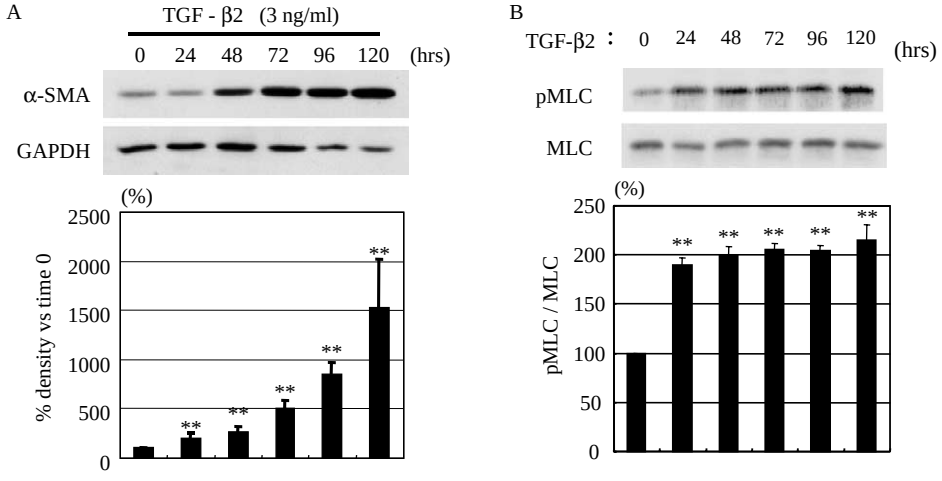

Fig. 2. Expression of α-SMA and MLC phosphorylation. (A) Collagen gels containing RPEs were cultivated in the absence or presence of TGF-β2 (3 ng ml^{-1}) for 1–5 days ($n=4$). The cells were retrieved after the gels were dissolved with collagenase. Total cell lysates were isolated and the same amount of protein was subjected to Western blotting to analyze the expression of α-SMA. A representative Western blot is demonstrated from four independent experiments. The signal density was measured, analyzed using analysis software and demonstrated as percent of density as compared with time 0 (**: $p<0.01$ vs time 0). (B) Starved RPEs were cultivated in the absence or presence of TGF-β2 (3 ng ml^{-1}) for indicated periods. Total cell lysates were subjected to Western blotting to analyze the phosphorylation state of MLC (pMLC). Lane loading differences were normalized by re-blotting the membranes with an antibody against MLC. The signal density was measured, analyzed using analysis software, and demonstrated by the ratio of pMLC/MLC ($n=3$, **: $p<0.01$ vs time 0).

(Fig. 2(A)). Cellular contraction needs both myofibroblastic transdifferentiation and MLC phosphorylation for non muscle cells. We therefore evaluated the effect of TGF-β2 on the phosphorylation state of MLC in RPEs. As shown in Fig. 2(B), TGF-β2 apparently increased the ratio of phosphorylated MLC vs total MLC initially significant after 1 day cultivation (181.6% vs time 0, $p<0.01$), and this elevation of the ratio was maintained at least up to 120 hr (218.6% vs time 0, $p<0.01$).

3.3. Involvement of protein kinases in TGF-β2-induced gel contraction

To investigate the involvement of various kinases in TGF-β2-dependent gel contraction, myofibroblastic transdifferentiation and MLC phosphorylation, the cells were pretreated with the kinase inhibitors as indicated and then stimulated with TGF-β2. Both PKC and p38 MAPK inhibitors partially but significantly abrogated TGF-β2-elicited collagen gel contraction (Fig. 3(A) top and middle). Treatment with kinase inhibitors such as PD98059 or Wortmannin resulted in no inhibitory effect. To exclude the effect of kinase inhibitors on the cell viability, the numbers of viable cells in collagen gels were counted after 5 days' treatment. However, there was no significant difference between respective conditions, indicating that the inhibitory effect of the kinase inhibitors on the gel contraction was not due to the toxic effect on the RPEs (Fig. 3(A) bottom).

The involvement of various kinases in the myofibroblastic transdifferentiation of RPEs and the phosphorylation state of MLC was evaluated. Both GF109203X (5 μM) and SB203580 (10 μM) partially but significantly attenuated TGF-β2-elicited overexpression of α-SMA (Fig. 3(B), $p<0.01$, respectively) and the ratio of phosphorylated MLC vs total MLC (Fig. 3(C), $p<0.01$, respectively). However, PD98059 (10 μM) and Wortmannin (100 nM) revealed no significant inhibitory effect both on α-SMA expression and MLC phosphorylation.

3.4. Predominant contribution of Rho-kinase pathway in TGF-β2-induced gel contraction

While the collagen gel's diameter was significantly shortened by the treatment with TGF-β2 (3 ng ml^{-1}) in agreement with the previous data as shown in Fig. 1, Y27632 attenuated collagen gel contraction in a dose-dependent manner (data not shown), and almost complete inhibition was attained at a concentration of 10 μM ($p<0.01$, Fig. 4(A)). On the other hand, it was revealed that ML7 had minimal inhibitory effect on the gel contraction at a concentration of 10 μM (114.8% vs TGF-β2 alone; $p<0.05$). However, not only ML7 at a concentration of 10 μM (115.3% vs TGF-β2 alone; not significant) but also Y27632 at a concentration of 10 μM had no significant effect on the expression of α-SMA (85.02% vs TGF-β2 alone; not significant) (Fig. 4(B)).

3.5. Predominancy of Rho-kinase pathway in TGF-β2-dependent MLC phosphorylation

We next examined the intracellular signalling pathway of TGF-β2-dependent MLC phosphorylation using Y27632 or ML7 at a concentration of 10 μM with pretreatment for 30 min. In accordance with the time points at which the cytokines significantly stimulated MLC phosphorylation as described above, the stimulation periods with TGF-β2 were 24 hr. As shown in Fig. 4(C), while the inhibitory effect by ML7 was marginal as compared with the control ($p<0.05$), Y27632 prominently attenuated the phosphorylation state of MLC both of vehicle-treated and TGF-β2-treated RPEs to the extent that was 5.2% ($p<0.01$) and 11.5% ($p<0.01$) of the vehicle alone, respectively.

3.6. Inhibitory effect of hydroxyfasudil on TGF-β2-elicited gel contraction, expression of α-SMA and MLC phosphorylation

Hydroxyfasudil attenuated the gel contraction in a concentration-dependent manner, showing initial significance at a concentration of 0.3 μM and strong suppression at a concentration of 10 μM ($p<0.01$) (Fig. 5(A), top and middle). Hydroxyfasudil showed no significant effects on cell viability in three dimensional collagen gels at least up to the concentration of 10 μM (Fig. 5(A), bottom). The treatment of collagen gels with TGF-β2 did not affect cell number either (data not shown), suggesting that collagen gel contraction by TGF-β2 and its inhibition by hydroxyfasudil are assumed to be independent of cell proliferation and cytotoxicity. Western blot analysis was performed to estimate the effect of hydroxyfasudil on the expression of α-SMA induced by TGF-β2. As shown in Fig. 5(B), TGF-β2-elicited overexpression of α-SMA was not suppressed by the treatment with hydroxyfasudil. As shown in Fig. 5(C), however, 10 μM of hydroxyfasudil prominently inhibited the phosphorylation state of MLC in RPEs both under the absence (8.40% vs control, $p<0.01$) and presence of TGF-β2 (8.14% vs TGF-β2 alone, $p<0.01$).

3.7. Rho-kinase inhibitors prevented stress fiber formation

RPEs were immunostained to examine whether the expression of α-SMA detected in our western blotting correlated to the stress fiber formation within the cells. There was no significant difference between cells pretreated with vehicle, Y27632, ML7 and hydroxyfasudil in the absence of TGF-β2. However, after stimulation with TGF-β2 for 5 days, stress fiber formation was augmented within the cells pretreated with vehicle or ML7. Meanwhile, Y27632 or hydroxyfasudil potently blocked the assembly of α-SMA in the cells (Fig. 6).

4. Discussion

In the present study herein, we first confirmed that TGF-β2 could enhance three-dimensional collagen gel contraction by cultured RPEs in agreement with the previous report

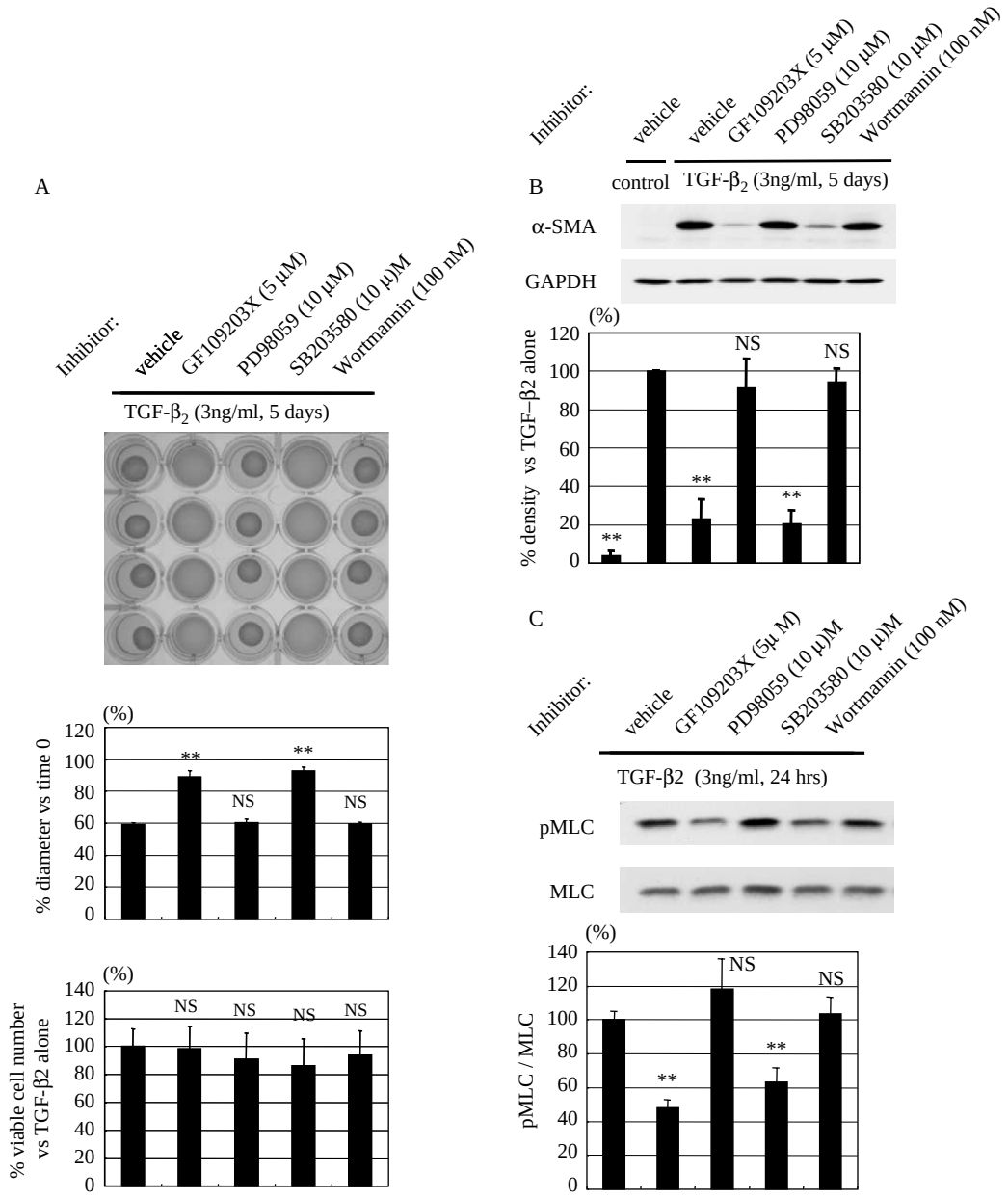

Fig. 3. Effects of various kinase inhibitors on TGF-β2-elicited gel contraction, cell viability, expression of α-SMA and MLC phosphorylation. (A) Collagen gels containing RPEs were pretreated with the various inhibitors indicated and then stimulated with TGF-β2 (3 ng ml^{-1}) for 5 days. A representative photograph after 5 days is demonstrated (top). The gel diameter was measured and presented as percent of diameter as compared with time 0 (middle). The gels were dissolved using collagenase and the viable cell number was counted with a haemocytometer after trypan blue staining. Data was demonstrated as percent of viable cell number as compared with TGF-β2 alone ($n=4$) (NS: not significant, **: $p<0.01$ vs TGF-β2 alone each). (B) Collagen gels containing RPEs were prepared as described before, pretreated with various kinase inhibitors for 30 min, stimulated with 3 ng ml^{-1} of TGF-β2 for 5 days. Total cell lysates were then obtained and subjected to Western blotting to analyze the expression of α-SMA as described before (NS: not significant, **: $p<0.01$ vs TGF-β2 alone each). (C) Starved RPEs were treated with 3 ng ml^{-1} of TGF-β2 for 24 hr in the presence of various kinase inhibitors as indicated. Total cell lysates were subjected to Western blot analysis using an antibody against phosphorylated-MLC (p-MLC). Lane loading differences were normalized by re-blotting the membranes with an antibody against MLC. A representative Western blot is demonstrated ($n=4$) (NS: not significant, **: $p<0.01$ vs TGF-β2 alone each).

(Sakamoto, et al., 1994a). We further demonstrated the direct evidence of TGF-β2-dependent MLC phosphorylation in RPEs in addition to the myofibroblastic transdifferentiation. As demonstrated, PKC and p38MAPK pathways seem to be involved in the expression of α-SMA, indicating myofibroblastic transdiferentiation of RPEs, and the phosphorylation state of MLC. However, highly selective or specific inhibitors to them, such as GF109203X or SB203580, have not been safety checked and used as drugs for clinical treatment yet. We also clarified that the Rho/Rho-kinase pathway is the predominant pathway in the contraction mechanism induced by TGF-β2 after myofibroblastic transdifferentiation of RPEs, indicating the pharmacological therapeutic potential of hydroxyfasudil, a potent and selective inhibitor of Rho-kinase already in clinical use, for the treatment of vitreo-retinal interface diseases.

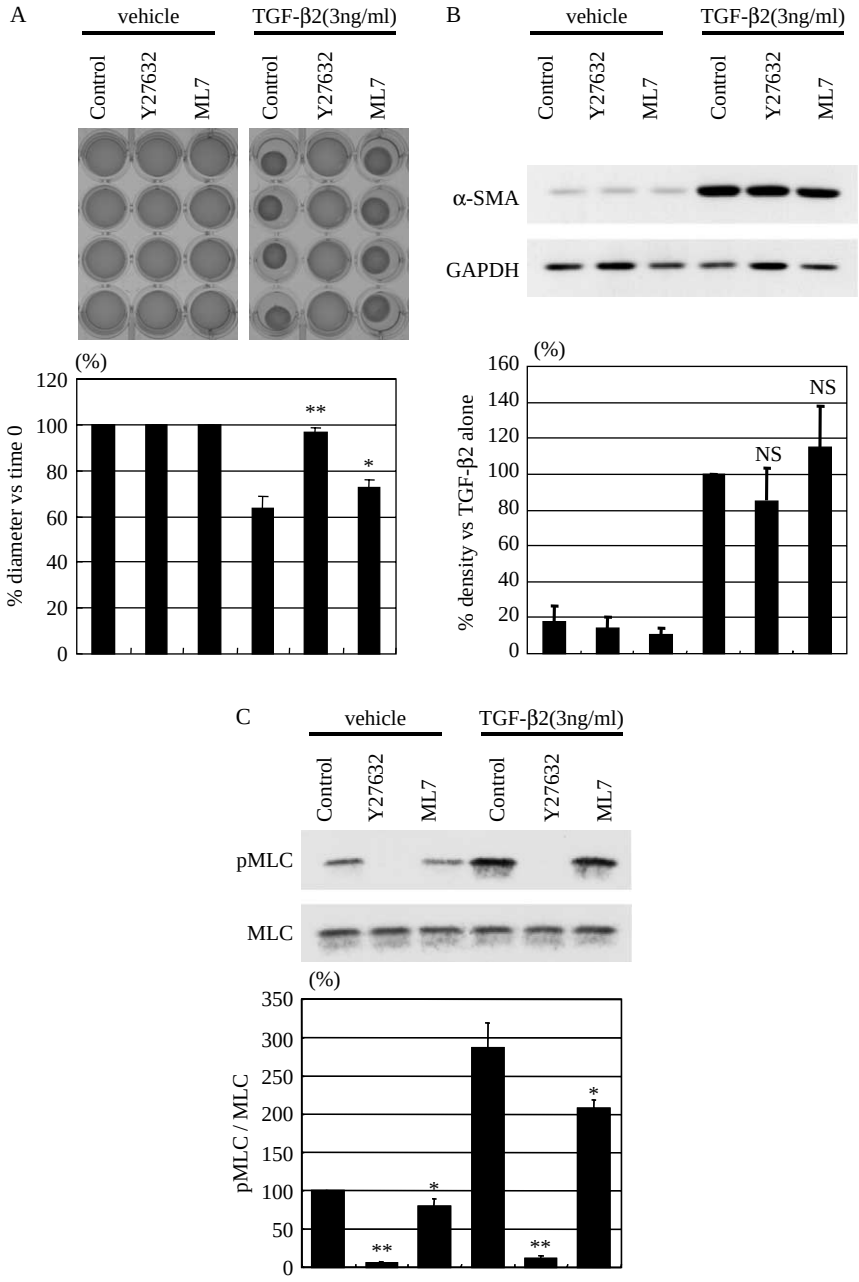

Fig. 4. Predominant role of the Rho-kinase pathway in TGF-β2-elicited gel contraction and MLC phosphorylation. (A) Collagen gels containing RPEs were pretreated with 10 μM of Y27632 or 10 μM of ML7 for 30 min and then stimulated with TGF-β2 (3 ng ml^{-1}) for 5 days ($n=4$). Representative photographs are shown (top). The gel diameter was measured and presented as percent of diameter as compared with time 0 (bottom) (NS: not significant, **: $p<0.01$ vs TGF-β2 alone each). (B) The gels were dissolved using collagenase and total cell lysates were subjected to Western blotting. The expression of α-SMA was analyzed as described in the materials and methods ($n=3$, NS: not significant vs TGF-β2 alone). (C) Starved RPEs were pretreated with Rho-kinase inhibitor (Y27632, 10 μM) or MLC-kinase inhibitor (ML7, 10 μM) and then stimulated with 3 ng/ml of TGF-β2 (0.1 nM) for 24 hr. Total cell lysates were subjected to Western blot analysis using an antibody against phosphorylated-MLC (p-MLC). Lane loading differences were normalized by re-blotting the membranes with an antibody against MLC. A representative Western blot is demonstrated (top). The signal density was quantified using analysis software, and demonstrated by the ratio of pMLC/MLC from three independent experiments (bottom) ($n=3$, *: $p<0.05$, **: $p<0.01$ vs each control).

The main cause of failure after retinal reattachment surgery is PVR, which is characterized by the migration and proliferation of several kinds of cell types including RPEs, retinal glial cells and others organizing into an epi-retinal membrane (Machemer and Laqua, 1975; Kampik et al., 1981; Jerdan et al., 1989; Vinores et al., 1990a,b). The contractile fibro-cellular membranes extend on the retinal surface and vitreous base, which exacerbate the diseased state.

Recently, elevated levels of cytokines have been immunohistochemically confirmed in the PVR membrane and measured in the vitreous of patients with PVR, suggesting their possible association with the pathogenesis (Limb et al.,

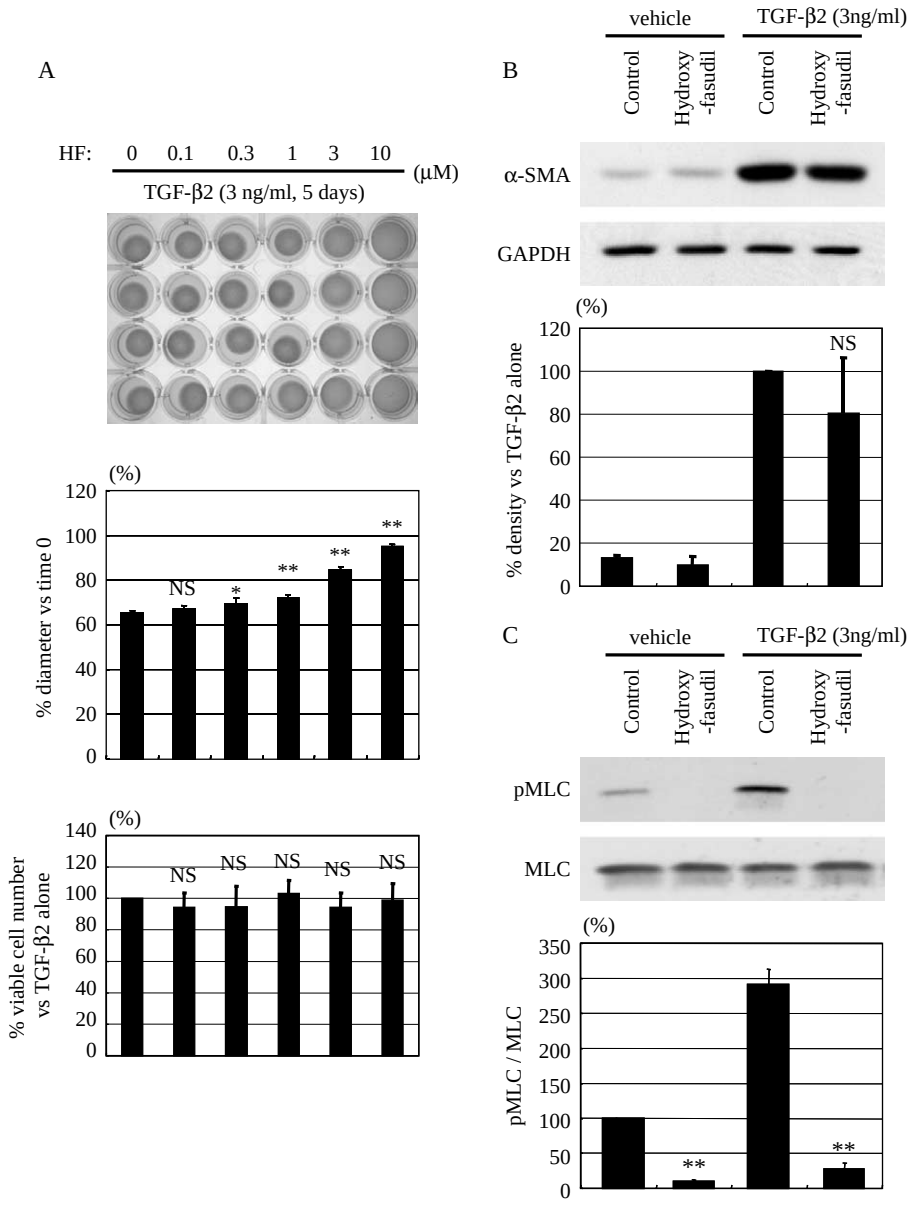

Fig. 5. Effect of hydroxyfasudil on gel contraction, cell viability, expression of α-SMA and MLC phosphorylation. (A) Collagen gels containing RPEs were treated with 3 ng ml^{-1} of TGF-β2 for 5 days in the presence of various concentrations of hydroxyfasudil (HF) ($n=4$). A representative photograph after 5 days is demonstrated (top). The gel diameter was measured and presented as percent of diameter as compared with time 0 (middle). The gels were dissolved using collagenase and the viable cell number was counted with a haemocytometer after trypan blue staining. Data was demonstrated as percent of viable cell number as compared with TGF-β2 alone (bottom, $n=4$) (NS: not significant, *: $p<0.05$, **: $p<0.01$ vs TGF-β2 alone). (B) The gels were dissolved using collagenase and total cell lysates were subjected to Western blotting. The expression of α-SMA was analyzed as described in the materials and methods ($n=3$, NS: not significant vs TGF-β2 alone). (C) Starved RPEs were treated with the vehicle or 3 ng ml^{-1} of TGF-β2 for 24 hr. Total cell lysates were subjected to Western blot analysis using an antibody against phosphorylated-MLC (p-MLC). Lane loading differences were normalized by re-blotting the membranes with an antibody against MLC. A representative Western blot is demonstrated. The signal density was quantified using analysis software, and demonstrated by the ratio of pMLC/MLC from three independent experiments ($n=3$, **: $p<0.01$ vs control/vehicle).

1991; Kauffmann et al., 1994; Charteris, 1998; Kon et al., 1999). TGF-β2 is a multifunctional cytokine supposed to be one of the key regulators of various vitreo-retinal diseases. While it is well recognized that the contraction of the epi-retinal membrane results in the progression of the diseased state, (Kampik et al., 1981) it is not fully understood how TGF-β2 affects the membrane in this process.

In vascular smooth muscle cells (VSMC), phosphorylation of MLC is a critical step in the reconstitution of actin stress fibers, and is known to be mediated through several pathways (Hartshorne et al., 1998). First, the Rho/Rho-kinase pathway, which enhances the phosphorylation state of MLC both directly, focused at Ser-19 residue, and/or indirectly by inactivating the MLC phosphatase through the phosphorylation of the myosin binding subunit (Kimura et al., 1996). Secondly, the MLC kinase pathway, which directly phosphorylates MLC at the same site as the Rho-kinase does (Frearson and Perry, 1975; Barany and Barany, 1977). Furthermore, ZIP kinase is

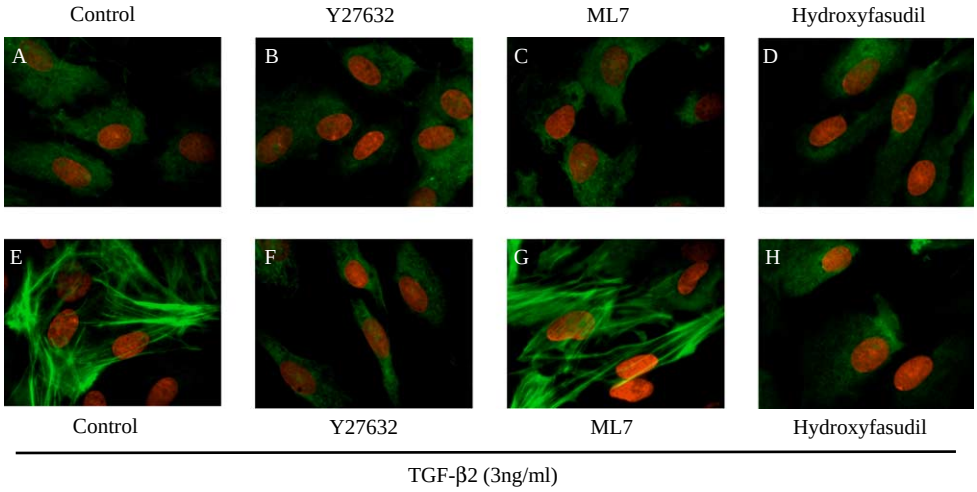

Fig. 6. Effect of protein kinase inhibitors on TGF-β2-induced stress fiber formation in BRPEs. Cells were pretreated with vehicle, Y27632 (10 μM), ML7 (10 μM) or hydroxyfasudil (10 μM) for 30 min, respectively, and cultivated in the absence (A, B, C, D) or presence (E, F, G, H) of TGF-β2 (3 ng ml^{-1}) for 5 days. The cells were fixed and immunostained for α-smooth muscle actin, and illustrated with representative photographs.

also involved in the phosphorylation of MLC at both Ser-19 and Thr-18 residues with the same rate constant (Niiro and Ikebe, 2001).

In our experiments, we focused on the contribution of the Rho-and/or MLC-kinase pathways in TGF-β2-elicited MLC phosphorylation and collagen gel contraction by RPEs. We clearly demonstrated that TGF-β2-elicited RPEs-containing gel contraction was closely associated with the phosphorylation status of MLC, which is predominantly mediated through the Rho-kinase pathway. However, we cannot completely exclude the involvement of other pathways such as the ZIP kinase. Direct examination such as kinase assay might clarify these possibilities.

Furthermore, it is known that the signalling mechanisms leading to the cellular contraction involve other kinases such as PKC, p44/p42 MAP kinase, p38 MAP kinase and PI3 kinase depending on the ligands (Sakamoto, et al., 1994a; Bogatkevich et al., 2001; Zolk et al., 2004; Iwabu et al., 2004; Magne et al., 2001; Yu et al., 2003; Grondin et al., 1991). However, the precise mechanisms responsible for the mediation of TGF-β2-elicited MLC phosphorylation and collagen gel contraction by RPEs are not well established. Our data clarified that PKC and p38 MAPK pathways mediate TGF-β2-elicited collagen gel contraction by RPEs through the induction of both α-SMA expression and MLC phosphorylation at least in part.

While the inhibitory effect of Rho-kinase inhibitors (Y27632 and hydroxyfasudil) on the expression of α-SMA was not significant as compared with PKC and p38 MAPK inhibitors, its inhibitory effect on the gel contraction was as strong as the latter. According to a recent report, the expression of α-SMA was attenuated by Y27632 in cultured rabbit RPE cells (Zheng et al., 2004). In this study, neither Y27632 nor hydroxyfasudil significantly suppressed the expression of α-SMA elicited by TGF-β2 (Fig. 4(B) and 5(B)) at least up to the concentration of 10 μM, however the same cardinality almost completely inhibited the phosphorylation state of MLC in RPEs we used as shown in Fig. 4(C) anc 5(C). The discrepancy between these results might be due to the difference of species or conditions of primarily isolated cells. Additionally, RPEs were immunostained to examine whether the expression of α-SMA detected in our western blotting correlated to the stress fiber formation within the cells. Pretreatment of the cells with Y27632 or hydroxyfasudil prevented the formation of actin stress fiber within the cells as shown in Fig. 6, however these agents did not significantly affect the expression of α-SMA itself as mentioned above. The phosphorylation state of myosin light chain thus appears to be of importance for stress fiber formation in addition to the expression of α-SMA, and the presence of α-SMA does not necessarily reflect the contractile potential of a cell.

In accordance with the result of our examination and recent reports, the Rho-kinase is considered to be located upstream of MLC phosphatase and independent of the PKC/CPI-17 pathway (Somlyo, et al., 2000; Wettschureck and Offermanns, 2002). TGF-β2 seems to induce phosphorylation state of MLC through PKC, p38MAPK and Rho-kinase separately, and leads to the expression of α-SMA not via the Rho-kinase pathway but the PKC and p38MAPK signalling pathways. It has also been suggested that p44/42MAPK might upregulate MLCK activity (Morrison et al., 1996; Nguyen et al., 1999). It is thus reasonable that PD98059, a selective MEK1 inhibitor, could not significantly suppress TGF-β2-elicited MLC phosphorylation, which lies downstream of the Rho-kinase pathway in our study (Fig. 3(C)).

In so far as suppressing both myofibroblastic transdifferentiation and MLC phosphorylation, a highly selective or specific inhibitor to PKC or p38MAPK might be expected to have a protective effect on the initiation and the progression of PVR. However, it is reported that PKC inhibition might have a negligible effect on contraction in vivo condition (Somlyo, et al., 1998). Moreover, there is no such reagent available for clinical use at present. Therefore, although the Rho/Rho-kinase pathway might not be concerned with cellular

transdifferentiation, it could be a possible therapeutic target for the treatment of vitreo-retinal interface diseases through the inhibition of the actomyosin hyper contraction.

Under these conditions hydroxyfasudil, a potent and selective inhibitor of Rho-kinase, is already in clinical use for the treatment of cerebral vasospasm (Ono-Saito et al., 1999; Seto et al., 1999). If hydroxyfasudil has pharmacological therapeutic potential for certain vitreo-retinal diseases however, it may also be of great benefit to patients confronted by blindness. Hydroxyfasudil dose-dependently (0.03–10 μM) suppressed MLC phosphorylation in RPEs (data not shown) in agreement with previous reports demonstrating the selective inhibition of Rho-kinase by hydroxyfasudil in vitro (Uehata et al., 1997; Suzuki et al., 1999; Shimokawa et al., 1999). As shown in Fig. 5(A), hydroxyfasudil also significantly inhibited the collagen gel contraction induced by TGF-β2. A corresponding concentration was found to almost completely inhibit MLC phosphorylation without affecting cell viability in our experiments.

Needless to say, an ideal treatment would protect the transdifferentiation itself but there is no such drug available for clinical use at present. As described above, while both myofibroblastic transdifferentiation and MLC phosphorylation are indispensable for providing the contractile force, selective inhibition of the Rho-kinase pathway might have therapeutic benefit for the treatment or inhibition of various vitreo-retinal interface diseases including PVR. However, pharmacological treatments sometimes lead to undesired side effects in vivo. Therefore, further examination in vivo is necessary to evaluate its therapeutic potential and its safety for clinical use in the area of intraocular diseases.

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