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The effect of a Rho kinase inhibitor Y-27632 on superoxide production, aggregation and adhesion in human polymorphonuclear leukocytes

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Abstract

We investigated the involvement of p160ROCK (a Rho-associated coiled coil-forming protein kinase), one of Rho kinases on superoxide anion production (O_2^- production), aggregation and adhesion of human polymorphonuclear leukocytes under physiological condition, using a selective p160ROCK inhibitor, (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide (Y-27632). Y-27632 inhibited the O_2^- production stimulated by phorbol-12-myristate-13-acetate (PMA) in a dose-dependent manner. Stauroprorine blocked the PMA-induced O_2^- production while wortmannin did not. Y-27632 also inhibited the O_2^- production by guanosine 5′-*O*-(3-thiotriphosphate) ($GTP_\gamma S$) 100 μM. *N*-formyl-Met-Leu-Phe (fMLP)-induced O_2^- production was not influenced by Y-27632, but was inhibited by wortmannin. The enhanced O_2^- production by Ca-ionophore A23817 and thapsigargin was not inhibited by Y-27632. Y-27632 did not change the basal intracellular Ca^{2+} concentration nor its elevation stimulated by fMLP. Polymorphonuclear leukocytes aggregation induced by PMA was dose-dependently decreased by Y-27632 while their aggregation stimulated by fMLP was enhanced by the agent. Polymorphonuclear leukocytes adhesion induced by PMA or fMLP was not influenced by Y-27632.

These results suggest that p160ROCK is involved in the PMA-induced O_2^- production and aggregation in human polymorphonuclear leukocytes. This kinase might locate in downstream of protein kinase C in polymorphonuclear leukocytes.

Keywords: Polymorphonuclear leukocyte; Rho kinase; p160ROCK; Superoxide production; Aggregation

1. Introduction

The important role of phagocytic cells, including polymorphonuclear leukocytes, is to protect against invading bacteria by various functions, such as phagocyte, respiratory burst (O_2^- production), chemotaxis, aggregation and adhesion. The signal pathways of these functions have been examined using several stimulants [tumor necrosis factor-α (TNF-α), Ca-ionophore A23817, *N*-formyl-Met-Leu-Phe (fMLP) and phorbol-12-myristate-13-acetate (PMA)] (Bergstrand et al., 1992; Jacobson et al., 1995). These studies showed that an activation of phospholipase C mediated by GTP-binding protein is involved in the signal transduction system of O_2^- production stimulated by fMLP, a classical chemoattractant. The activated phospholipase C produces phosphatidylinositol 1,4,5-triphosphate (PI_3) and diacylglycerol, which, in turn, lead to a transient rise in intracellular [Ca^{2+}] and activation of protein kinase C (Azuma et al., 1993). On the other hand, PMA-induced O_2^- production is induced by its direct effect on protein kinase C (Nishizuka, 1984). The O_2^- production is finally caused by the activation of NADPH oxidase, consisting of membrane b_{558} and cytosolic factors (Maridonneau Parini et al., 1986; Ohsaka et al., 1988). However, the mechanism involved in the activation of NADPH oxidase is not fully understood (Babior, 1999). Recent studies revealed the role of small GTP-binding protein, especially Rho family (Rho, Rac and Cdc42) in the functions of phagocytic cells including neutrophils. For example, Rac is involved in the control of oxidase activation while Rho regulates chemo-

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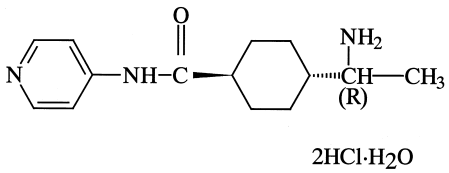

Fig. 1. Structure of Y-27632.

taxis of these cells. In addition, there is a potential cross-talk between Rac and Rho (Dagher et al., 1995; Hall, 1998). As a target of Rho, a 160-kDa serine/threonine kinase called a Rho-associated coiled coil-forming protein kinase (p160ROCK or ROCK I) was isolated from platelets (Ishizaki et al., 1996). Recently, the involvement of Rho kinase in the Ca^{2+}-sensitaizaton mechanism of smooth muscle is reported using a novel and specific p160ROCK inhibitor, (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)-cyclohexanecarboxamide (Y-27632) (Fig. 1) (Hirata et al., 1992; Uehata et al., 1997; Fu et al., 1998). Data concerning a role of p160ROCK in the functions of neutrophils are limited. It has been shown that p160ROCK is expressed in human neutrophils and is important for motile functions of these cells (Niggli, 1999). However, its role in other functions of neutrophils remains to be determined.

In this study, we investigated the role of p160ROCK in human polymorphonuclear leukocytes functions, such as O_2^- production, aggregation and adhesion using Y-27632.

2. Materials and methods

2.1. Reagents

PMA, 4-α-PMA, fMLP, Ca-ionophore A23817, guanosine 5′-*O*-(3-thiotriphosphate) ($GTP_\gamma S$), bis-indolylmaleimide I hydrochloride (GF109203X) were obtained from Sigma (St. Louis, MO, USA). Staurosporine, wortmannin, thapsigargin were purchased from Wako (Osaka, Japan). Y-27632 was a kind gift from Yoshitomi Pharmaceutical (Osaka, Japan). 1-[2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid pentaacetoxymethyl ester (fura-2/AM) was purchased from Molecular Probes (Eugene, OR, USA). PMA, 4-α-PMA, staurosporine, wortmannin, Ca-ionophore A23817, thapsigargin were dissolved just before use in 0.1% dimethyl sulfoxide in phosphate buffer solution (PBS) and other reagents were directly diluted with PBS.

2.2. Preparation of polymorphonuclear leukocytes

Twelve male healthy subjects participated in this study after they gave informed consent to the protocol. Heparinized blood samples were taken from forearm vein after overnight fast. Mixed leukocyte suspension was obtained after sedimentation in the presence of 1% dextran in saline and gradient centrifugation with Lymphoprep (Nycomed Pharma, Oslo, Norway). Purified polymorphonuclear leukocyte suspension (cell viability > 95%) was obtained by hypotonic lysis at 4°C and washing twice in PBS. Polymorphonuclear leukocytes were resuspended in Hanks' balanced salt solution (Nissui Pharmaceutical, Tokyo, Japan) with 0.1% human serum albumin at the concentration of 6×10^6 cells/ml.

2.3. Assay for O_2^- production

O_2^- production by polymorphonuclear leukocytes was determined by the superoxide dismutase-inhibitable cytochrome *c* reduction method with a minor modification (Yuo et al., 1989). The following materials were placed on fetal bovine serum-coated wells at a total volume of 200 μl/well; (1) antagonist or its control buffer, (2) polymorphonuclear leukocytes suspension containing 3×10^5 cells for 10 min, (3) stimulant or its control buffer, (4) superoxide dismutase or its control buffer, (5) cytochrome *c* (12 mg/ml). After incubation for 60 min at 37°C, O_2^- production was determined by measuring the absorbance at 540 and 550 nm in a spectrophotometer (Beckman DU-640, Fullerton, CA, USA). The assay was performed in duplicate. Y-27632 at 10 μM did not influence basal O_2^- production nor cell counts by staining trypan blue solution.

2.4. Activation by $GTP_\gamma s$ with permeabilization

The cell suspension (6×10^6 cells/ml) in the pulse cuvette was incubated at 37°C with an inhibitor (or control buffer) for 10 min. Thereafter, 100 μM $GTP_\gamma S$ was added to the suspension in the pulse cuvette cooled on ice, which was pulsed once with Electro Cell Manipulator (cell-porator™, BRL, Gaithersbarg, USA) (300 V, 330 μF). Immediately, the suspension was transferred into an assay cuvette and its O_2^- production was measured.

2.5. Measurement of intracellular $[Ca^{2+}]$

Polymorphonuclear leukocytes were attached to a glass coverslip using Cell-Tak® (Becton Dickinson Labware, Bedford, MA, USA) and 10 μM of fura-2/AM was loaded for 30 min at 37°C. Thereafter, the cells were washed and resuspended in Hanks' balanced salt solution containing 1 mM Ca^{2+}. Intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) was measured at 37°C by a microscopic fluorescence photometry system (OSP-3, Olympus, Tokyo, Japan). It takes at least 10 ms to obtain one fluorescence ratio $R = F_{340}/F_{380}$.

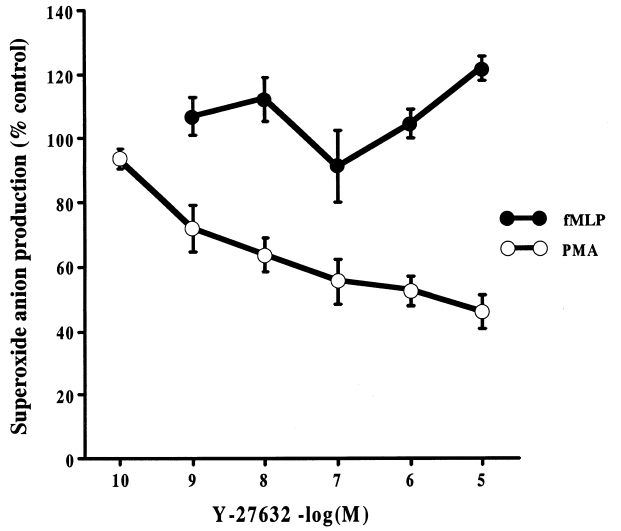

Fig. 2. Effect of Y-27632 concentration on superoxide anion production stimulated by PMA (100 ng/ml) and fMLP(100 nM). Mean ± S.E.M. $n = 5–12$ in each point.

We employed the following equation for $[Ca^{2+}]_i$ as described by Hanaoka et al. (1993):

$$[Ca^{2+}]_i = K_d(F_{min}/F_{max})(R - R_{min})/(R_{max} - R),$$

where R_{min} is the ratio at zero Ca^{2+} in the bathing fluid and R_{max} is the ratio at saturated Ca^{2+} concentration. F_{min} and F_{max} are the fluorescence at 380 nM at zero Ca^{2+} and at Ca^{2+} saturation in the bath, respectively. K_d is the dissociation constant for Ca^{2+} of fura-2 (224 nM).

2.6. Assay for polymorphonuclear leukocytes aggregation

Polymorphonuclear leukocytes aggregation was determined by an aggregometer (multichannel platelet aggregation analyzer, Mebanix Model PAT-2A, Tokyo, Japan) using polymorphonuclear leukocytes suspension containing 1.2×10^6 cells, and stimulant (PMA or fMLP) or its control buffer at a total volume of 200 μl.

2.7. Assay for polymorphonuclear leukocytes adherence

After the assay for O_2^- production, each well was washed twice in PBS 200 μl. After each well was dried, adhered polymorphonuclear leukocytes to fetal bovine serum-coated wells was determined as protein content by Lowry method. The assay was performed in duplicate.

2.8. Statistical analysis

Results were expressed as the mean ± S.E.M. Data were analyzed by analysis of variance and paired *t*-test as appropriate using SAS (SAS Institute, Cary, NC, USA).

3. Results

3.1. Effect of Y-27632 on PMA- and fMLP-induced superoxide production in human polymorphonuclear leukocytes

We investigated whether Y-27632 affects O_2^- production induced by fMLP, a chemoattractant, and PMA, a well-known protein kinase C activator, in human polymorphonuclear leukocytes. Under the present condition, O_2^- production induced by PMA was 4–7 nmol/3×10^5 cells/h, which was fourfold higher than that of fMLP activation. We also observed that the α-isomer of PMA, an inactive compound, induces weak O_2^- production (less than 0.1 nmol/3×10^5 cells/h). A Rho kinase inhibitor, Y-27632, inhibited O_2^- production stimulated by PMA in a dose-dependent manner up to 10 μM while this inhibition seemed to be saturated at this concentration (Fig. 2, Table 1). In contrast to PMA, O_2^- production induced by fMLP was not significantly influenced (Fig. 2, Table 1).

Table 1 shows the degree of inhibition using two other antagonistic agents on this response. Staurosporine, a non-selective protein kinase C inhibitor, dose-dependently inhibited O_2^- production by PMA (64.6 ± 10.2% control at 10 nM, 20.6 ± 3.3% control at 100 nM) while this agent did not block significantly the response by fMLP at 100 nM. Another protein kinase C inhibitor, GF109203X, exerted similar effects against O_2^- production induced by PMA (46.8 ± 8.3% control at 100 nM, $n = 4$) and by fMLP (98.8 ± 2.2% control at 100 nM, $n = 4$). However, wortmannin, a PI_3 kinase inhibitor, suppressed O_2^- production by fMLP, but not by PMA at 100 nM. Similar data were obtained by another PI_3 kinase inhibitor, LY29004 (data not shown).

Table 1
Effects of antagonistic agents on PMA- and fMLP-induced O_2^- production in human polymorphonuclear leukocytes

Stimulant	O_2^- production (% control)		
	Y-27632 (100 nM)	Staurosprine (100 nM)	Wortmannin (100 nM)
PMA	55.5 ± 7.1 ($n = 10$)[a]	20.6 ± 3.3 ($n = 8$)[a]	97.0 ± 6.0 ($n = 8$)
fMLP	91.3 ± 11.2 ($n = 5$)	84.5 ± 4.2 ($n = 6$)	20.4 ± 4.3 ($n = 6$)[a]

Polymorphonuclear leukocytes were preincubated for 10 min with or without antagonistic agent. Subsequently, PMA (100 ng/ml) or fMLP (100 nM) was added and the incubation continued for further 60 min at 37°C. Mean ± S.E.M.

[a] $P < 0.01$ vs. control.

3.2. $GTP_\gamma S$-induced superoxide production in human polymorphonuclear leukocytes

$GTP_\gamma S$ induces O_2^- production through the activation of protein kinase C and phospholipase C in electropermeabilized human neutrophils (Tamura et al., 1999). O_2^- production induced by $GTP_\gamma S$ (100 μM) was 2.0 ± 0.4 nmol/3×10^5 cells/h ($n = 5$). Y-27632 (1 μM) caused about 35% inhibition of this O_2^- production.

3.3. Effect of Y-27632 on O_2^- production induced by Ca-ionophore A23817 and thapsigargin

Under the identical condition, we next examined the effect of antagonistic agents on O_2^- production by Ca-ionophore A23817, which increases $[Ca^{2+}]_i$ from extracellular source (Nishizuka, 1984) and thapsigargin, which increases $[Ca^{2+}]_i$ from intracellular storage. Enhancement of O_2^- production was large for A23817 (0.8–1.5 nmol/3×10^5 cells/h) and small for thapsigargin (0.2–0.5 nmol/3×10^5 cells/h) (without stimulant, < 0.1 nmol/3×10^5 cells/h). The O_2^- production by A23817 or thapsigargin was not suppressed by Y-23762 (1 μM).

3.4. Intracellular calcium in polymorphonuclear leukocytes elicited by fMLP with or without Y-27632

We examined whether Y-27632 influences the intracellular Ca^{2+} concentration in polymorphonuclear leukocytes with and without fMLP. Y-27632 10 μM per se did not change the $[Ca^{2+}]_i$ in polymorphonuclear leukocytes ($n = 3$). In addition, the agent (10 μM) did not disturb the transient rise in $[Ca^{2+}]_i$ induced by fMLP (200 nM) ($n = 3$).

3.5. Effect of Y-27632 on polymorphonuclear leukocytes aggregation

Representative tracings of effect of Y-27632 (10 μM) on PMA (100 ng/ml)- and fMLP (100 nM)-induced polymorphonuclear leukocytes aggregation were shown in Fig. 3a. Y-27632 significantly inhibited polymorphonuclear leukocytes aggregation by PMA while the agent significantly enhanced it by fMLP (Fig. 3b). Y-27632 (1 μM) did not show significant inhibition against polymorphonuclear leukocytes aggregation by PMA (94.4 ± 7.1% control, $n = 5$) or by fMLP (100.6 ± 1.5% control, $n = 3$).

4. Effect of Y-27632 on polymorphonuclear leukocytes adhesion

We measured polymorphonuclear leukocytes adherence to fetal bovine serum-coated wells as an index of adhesion after O_2^- production assay. Around 10 μg protein/well was detected without stimulant or Y-27632. No difference was observed in this parameter using each dose of Y-27632 (9–10 μg/well, $n = 5$–12). Protein content was around

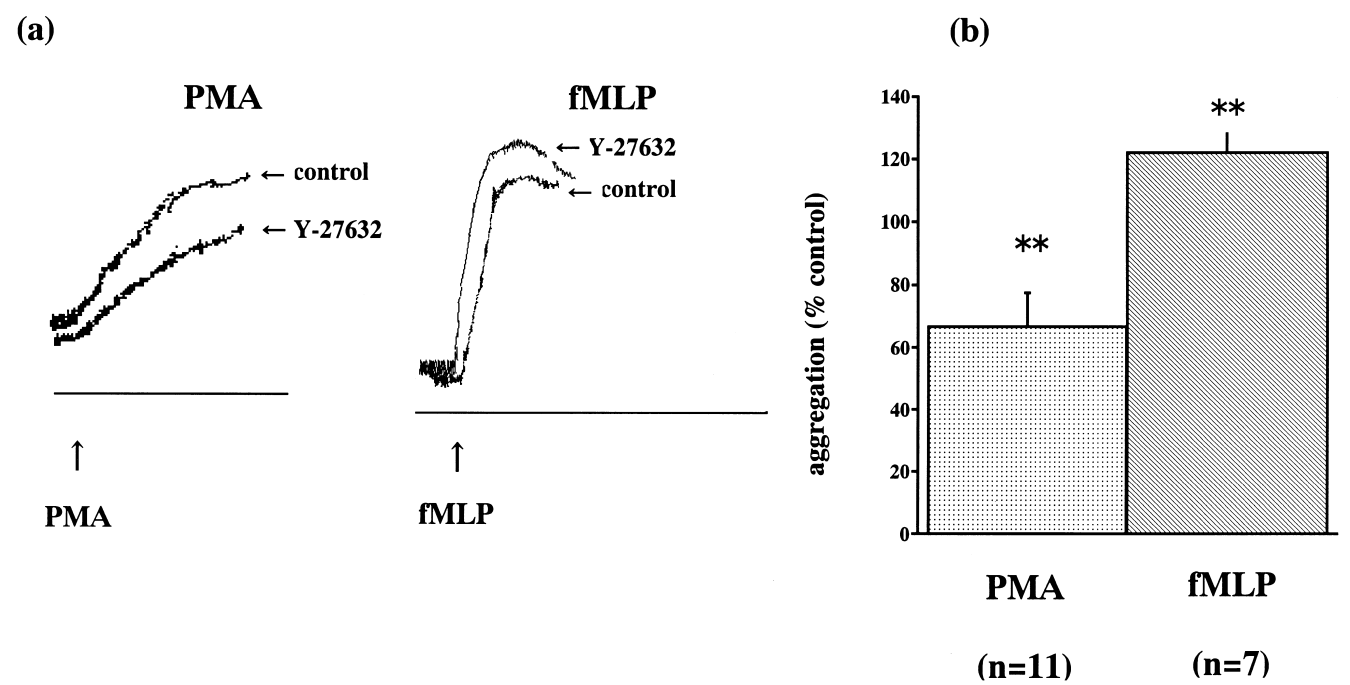

Fig. 3. (a) Representative tracing of effect of Y-27632 (10 μM) on PMA (100 ng/ml)- and fMLP (100 nM)-induced aggregation. (b) Summary of effect of 10 μM Y-27632 on PMA- and fMLP-induced aggregation. Mean ± S.E.M. $^{**}P < 0.01$ vs. control.

50 μg/well stimulated by PMA and around 15 μg/well by fMLP. Y-27632 did not influence adhesion of polymorphonuclear leukocytes stimulated by PMA or fMLP up to 10 μM of each stimulant.

5. Discussion

Superoxide anion (O_2^-) contributes to the defense mechanism of polymorphonuclear leukocytes against microorganisms, but the regulatory pathways of its production is not fully evaluated. Recently, p160ROCK, one of Rho kinases, was shown to exist in human neutrophils and to play a major role in their motile functions (Niggli, 1999). These findings led us to speculate that p160ROCK is also involved in the polymorphonuclear leukocytes O_2^- production. To address this issue, we used a novel and specific p160ROCK inhibitor, Y-27632, as an antagonistic agent in this study.

As expected, staurosporine, a nonselective protein kinase C inhibitor, decreased the polymorphonuclear leukocytes O_2^- production by PMA, a protein kinase C activator, while wortmannin, a PI_3 kinase inhibitor, did not influence it. Y-27632 decreased the PMA-induced O_2^- production in a dose-dependent manner in this study. Y-27632 is highly selective to p160ROCK, and its affinity for this kinase is more than 2000 times higher than that for conventional protein kinase C (Uehata et al., 1997). Therefore, we think that Y-27632 did not inhibit protein kinase C, but p160ROCK downstream of protein kinase C. As protein kinase C is also involved in the polymorphonuclear leukocytes O_2^- production stimulated by $GTP_\gamma S$ (Lowe et al., 1996; Tamura et al., 1999), Y-27632 might inhibit p160ROCK downstream of protein kinase C and subsequent O_2^- production observed in this study.

Various components, such as PI_3 kinase, protein kinase C, phospholipase C, phospholipase D and tyrosine kinase, are involved in the polymorphonuclear leukocytes O_2^- production induced by fMLP (Tamura et al., 1999). In this study, wortmannin significantly inhibited and staurosporine slightly inhibited this response. These data indicate that the role of PI_3 kinase in the fMLP-stimulated O_2^- production is large while that of protein kinase C is negligible.

It is well known that the elevation of $[Ca^{2+}]_i$ causes the polymorphonuclear leukocytes O_2^- production, which was confirmed using Ca-ionophore A23817 and thapsigargin in this study. The present study also showed that Y-27632 does not influence such the response by Ca-ionophore and thapsigargin, which indicates that p160ROCK is not involved in the elevated $[Ca^{2+}]_i$-related polymorphonuclear leukocytes O_2^- production. Previous studies have shown that $[Ca^{2+}]_i$ regulates the time of onset, but not the amount of O_2^- production by PMA (Bei et al., 1998; Hu et al., 1999). In the present study, basal $[Ca^{2+}]_i$ concentration and its elevation induced by fMLP did not be influenced by Y-27632. Thus, it remains possible that p160ROCK plays some role in Ca-sensitive function in polymorphonuclear leukocytes like smooth muscle (Hirata et al., 1992; Somlyo and Somlyo, 1994).

Previous studies have demonstrated that p160ROCK downstream of protein kinase C plays a role in the aggregation process of platelet and lymphocyte induced by PMA (Morii et al., 1992; Tominaga et al., 1993). In this study, the PMA-stimulated polymorphonuclear leukocytes aggregation was dose-dependently inhibited by Y-27632 [insignificantly by 1 μM (near to the 50% inhibition for O_2^- production) and significantly by 10 μM]. These findings are compatible with the hypothesis that p160ROCK downstream of protein kinase C is also involved in the aggregation process of the PMA-stimulated polymorphonuclear leukocytes aggregation. On the other hand, polymorphonuclear leukocytes aggregation induced by fMLP was unexpectedly enhanced by Y-27632 10 μM under the present condition. Further studies are needed to evaluate this interesting phenomenon. Finally, the present study indicates that a role of p160ROCK in the adhesion of polymorphonuclear leukocytes induced by PMA or fMLP is small, if any.

Based on the present data, we think that Y-27632 inhibits p160ROCK downstream of protein kinase C in polymorphonuclear leukocytes and consequently decreases their O_2^- production and aggregation by PMA. It is believed that Rho regulates chemotaxis of phagocytic cells while Rac is involved in the control of oxidase activation of these cells (Quinn et al., 1993; Freeman et al., 1996). In addition, this is a cross-talk between Rho and Rac (Dagher et al., 1995; Hall, 1998). Therefore, it remains possible that Y-27632 inhibits Rho kinase activity, which, in turn, influences Rac and subsequent neutrophil functions. Moreover, recent studies have shown that the activation of neutrophil phospholipase D by protein kinase C and $GTP_\gamma S$ is regulated by Rho family (Bowman et al., 1993; Lopez et al., 1995). The possibility that Y-27632 blocks the phospholipase D pathway cannot be ruled out.

For the purpose of clinical implications, the effects of Y-27632 are now extensively examined. For example, the agent is shown to lower blood pressure in spontaneously hypertensive rats (Uehata et al., 1997) and to prevent the transcellular invasion of malignant cells (Itoh et al., 1999; Sahai et al., 1999). Recently, we observed that the enhanced O_2^- production by polymorphonuclear leukocytes in diabetic patients contributes to the development of diabetic triopathy (Ohmori et al., 2000). Thus, if Y-27632 actually inhibits polymorphonuclear leukocytes O_2^- production in vivo, the agent might be a promising drug for the prevention of diabetic triopathy.

In summary, Rho kinase inhibitor Y-27632 inhibited the response of polymorphonuclear leukocytes (O_2^- production and aggregation) by PMA. These results suggest that p160ROCK is involved in the pathway of these polymorphonuclear leukocytes functions.

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