NQ-Y15 Inhibits the Calcium Mobilization by Elevation of Cyclic AMP in Rat Platelets

Tong-Shin CHANG,^a Ki-Seon LEE,^a Gwi-Yeop LEE,^a Sun-Duck JEON,^a Dhong-Su SO,^a Lee-Yong KHIL,^a Myung-Kiu CHUNG,^b and Chang-Kiu MOON^{*,a}

College of Pharmacy, Seoul National University,^a San 56–1 Shillim-Dong, Kwanak-Gu, Seoul 151–742, Korea and Department of Environmental Engineering, Sunmoon University,^b Asan-Si, Chung-Nam, 336–840, Korea.

Received September 7, 2000; accepted February 6, 2001

2-[(4-Cyanophenyl)amino]-3-chloro-1,4-naphthalenedione (NQ-Y15) is a dual action drug which acts as a thromboxane A₂ (TXA₂) synthase inhibitor and TXA₂/PGH₂ receptor antagonist. In the present study, we examined the effects of NQ-Y15 on Ca²⁺ mobilization, which is the common event in various types of platelet activation, in arachidonic acid (AA)-stimulated rat platelets. The elevation of cytosolic Ca²⁺ concentration ([Ca²⁺]_i) induced by AA was inhibited by NQ-Y15 in a concentration-dependent manner. This inhibition-effect of NQ-Y15 was found to be based on the suppression of the rise in [Ca²⁺]_i by the inhibition of both Ca²⁺ release from internal stores and Ca²⁺ influx from the extracellular space. Our successive trial was focused on the role of cyclic AMP (cAMP) in the action of NQ-Y15, because cAMP was reported to be increased by dual action drugs such as picotamide and to inhibit the increase in [Ca²⁺]_i. NQ-Y15 was confirmed to increase cAMP in AA-stimulated rat platelets. These results suggested that NQ-Y15 might inhibit the rise in [Ca²⁺]_i in AA-treated rat platelets by increasing cAMP, which is involved in the inhibition of platelet activation.

Key words 2-[(4-cyanophenyl)amino]-3-chloro-1,4-naphthalenedione; platelet; calcium mobilization; cyclic AMP

Thromboxane A₂ (TXA₂) is a powerful platelet agonist and its formation in stimulated platelets represents an important amplifying signal for platelet activation.¹⁾ TXA₂ exerts its effects on platelets by binding to TXA₂/PGH₂ (prostaglandin H₂) receptors,²⁾ followed by the activation of both phospholipase C (PLC) and protein kinase C (PKC), leading to an increase in cytosolic Ca²⁺ concentration ([Ca²⁺]_i).^{3,4)} Cytosolic Ca²⁺ plays important roles in the regulation of various physiological functions, including aggregation and granule secretion of platelets.⁵⁾

The inhibition of TXA₂ synthase alone cannot fully suppress the platelet activation because PGH₂ could be accumulated due to TXA₂ synthase inhibition and could act as a potent platelet stimulant by interacting with the platelet TXA₂/PGH₂ receptors.^{6,7)} Thus, it has been suggested that the TXA₂ synthase inhibitors and TXA₂/PGH₂ receptor antagonists may provide an antiplatelet regimen superior to those previously available.^{8–10)} Furthermore, the combinatorial use of TXA₂ synthase inhibitor and TXA₂/PGH₂ receptor antagonist deserves particular interest, because this can lead to the increase in cAMP^{9,10)} which serves as a second messenger to inhibit the increase in [Ca²⁺]_i in activated platelets.^{11,12)} An increased cAMP was observed in platelets only when arachidonic acid (AA)-stimulated platelets were pretreated with TXA₂ synthase inhibitor and TXA₂/PGH₂ receptor antagonist simultaneously; this increase, however, did not occur in platelets preincubated with only one of those two agents or in unstimulated platelets.^{9,10)}

Some drugs that act as both TXA₂ synthase inhibitors and TXA₂/PGH₂ receptor antagonists, also increased intraplatelet cAMP in AA-stimulated platelets.^{13,14)} 2-[(4-Cyanophenyl)amino]-3-chloro-1,4-naphthalenedione (NQ-Y15) exerts dual action as a TXA₂ synthase inhibitor and a TXA₂/PGH₂ receptor antagonist in rat platelets, as previously reported.¹⁵⁾ In the present study, we investigated whether NQ-Y15 increases cAMP in AA-stimulated platelets, leading to the inhibition of

Ca²⁺ mobilization.

MATERIALS AND METHODS

Materials NQ-Y15 was synthesized and characterized as described previously.¹⁶⁾ It was dissolved in dimethyl sulfoxide (DMSO) so that the final DMSO concentration in reaction mixtures never exceeded 0.5% (v/v). AA, bovine serum albumin (BSA), indomethacin, imidazole, DMSO, ethylene diamine tetraacetic acid (EDTA), ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and fura 2/AM were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Picotamide was purchased from Biomol (Plymouth Meeting, PA, U.S.A.). Radioimmuno assay (RIA) kits of cAMP were obtained from Amersham (Little Chalfont, Buckinghamshire, England). All other reagents were purchased from Sigma Chemical Co.

Preparation of Platelets Rat blood was taken from Sprague-Dawley female rats weighing 200–250 g. Rats were anesthetized with ethyl ether and blood was collected from the abdominal aorta and anticoagulated with sodium citrate (3.8%; 1 : 9, v/v). The blood was centrifuged at 120 **g** for 15 min. The supernatants were pooled and centrifuged at 600 **g** for 15 min at room temperature. The platelet pellets were washed with modified Tyrode-HEPES buffer (129 mM NaCl, 2.8 mM KCl, 8.9 mM NaHCO₃, 0.8 mM MgCl₂, 0.8 mM KH₂PO₄, 2 mM EGTA, 5.6 mM glucose, 10 mM HEPES, 0.35% BSA, pH 7.4) and centrifuged at 600 **g** for 15 min. This washing procedure was repeated twice and platelets were then gently resuspended in Tyrode-HEPES buffer (129 mM NaCl, 2.8 mM KCl, 8.9 mM NaHCO₃, 0.8 mM MgCl₂, 0.8 mM KH₂PO₄, 1 mM CaCl₂, 5.6 mM glucose, 10 mM HEPES, 0.35% BSA, pH 7.4). Platelet counts were determined using a model ZM Coulter Counter (Coulter Electronics, Hialeah, FL, U.S.A.).

Determination of Intracellular Ca²⁺ in Platelets The

* To whom correspondence should be addressed. e-mail: moonck@snu.ac.kr

$[Ca^{2+}]_i$ was determined in fura-2-loaded platelets using the method described by Pollock and Rink.¹⁷⁾ Briefly, platelets ($8 \times 10^8/\text{ml}$) were incubated with fura-2/AM ($3 \mu\text{M}$) for 45 min at 37°C and then centrifuged for 15 min at $800g$; the resultant pellet was washed with EDTA (1 mM)-containing Tyrode-HEPES buffer. After centrifugation, platelets were gently resuspended to a concentration of $2 \times 10^8/\text{ml}$ in $CaCl_2$ free Tyrode-HEPES buffer and kept at room temperature. Fluorescence was measured at the emission wavelength of 505 nm with the excitation wavelength switched continuously between 340 and 380 nm using a Shimadzu RF-5000 spectrofluorimeter, which was equipped with the thermostated (37°C) and magnetically stirred cuvette. The ratio of the fluorescence intensities at the two excitation wavelengths was used to determine $[Ca^{2+}]_i$.¹⁸⁾ Intracellular Ca^{2+} concentration was determined by calibration of the fura-2 signal. Platelets were lysed with 0.1% (v/v) Triton X-100 to determine the maximum fluorescence; 10 mM EGTA (pH 9.0) was added to determine the minimum fluorescence.

cAMP Assay Platelet suspension ($3 \times 10^8/\text{ml}$) was incubated with NQ-Y15 or its vehicle at 37°C for 8 min under shaking at 1200 rpm with a Thermomixer® (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany), and then 50 μM AA or an equal volume of saline was added. After 6 min-further incubation, the reaction was stopped by adding 10 mM EDTA followed immediately by boiling for 5 min as described by Karniguian *et al.*¹⁹⁾ Upon cooling to 4°C , precipitated protein was sedimented by centrifugation in an Eppendorf microcentrifuge (model 5413). The supernatant was used to determine cAMP content by RIA.

Statistical Analysis Results are expressed as mean $\pm$ S.E.M. Statistical significance was assessed using the Student's *t* test with $p < 0.05$ or $p < 0.01$.

RESULTS

Effects of NQ-Y15 on AA-Induced Ca^{2+} Release from Internal Stores As shown in Fig. 1A, platelets were incubated in the presence of 0.5 mM EGTA (that was the virtual absence of free Ca^{2+} in the incubation media) followed by the addition of 50 μM AA. AA caused a prompt increase in

$[Ca^{2+}]_i$ from 61.5 ± 3.2 to 95.6 ± 2.7 nM ($n=4$). This increase reflected Ca^{2+} release from internal stores.²⁰⁾ As shown in Fig. 1B, NQ-Y15 inhibited this Ca^{2+} release by 45.7% ($n=4$) at 30 μM .

Effects of NQ-Y15 on AA-Induced Ca^{2+} Influx To examine the effect of NQ-Y15 on Ca^{2+} influx, we measured the change in $[Ca^{2+}]_i$, excluding the increase in Ca^{2+} release from intracellular store. In platelets suspended in Tyrode-HEPES buffer containing 0.5 mM EGTA, 50 μM AA increased $[Ca^{2+}]_i$ from 62.8 ± 4.7 to 94.7 ± 53.2 nM ($n=4$) (Fig. 2A). This increase in $[Ca^{2+}]_i$ was caused by the release of Ca^{2+} from internal stores, which led to Ca^{2+} depletion in internal stores.²¹⁾ 12 minutes of stimulation with AA followed by the addition of 2 mM Ca^{2+} increased $[Ca^{2+}]_i$ to 584.0 ± 53.2 nM ($n=4$), which represented Ca^{2+} influx (Fig. 2A). This Ca^{2+} influx was substantially (by 79.9%) reduced by 30 μM NQ-Y15 (Fig. 2B).

Effect of NQ-Y15 on cAMP Level in AA-Stimulated Platelets The level of cAMP was found to be very low in resting platelets (6.4 ± 0.2 pmol/ 3×10^8 platelets). At the same condition, 80 μM PGI₂, an adenylate cyclase activator, increased the cAMP level up to 51.0 ± 1.1 pmol/ 3×10^8 . The

Fig. 1. Effect of NQ-Y15 on AA-Induced Ca^{2+} Release from Internal Stores in Fura-2-Loaded Platelets

To determine Ca^{2+} release from internal stores, we used fura-2-loaded platelets suspended in Tyrode-HEPES buffer containing 0.5 mM EGTA, excluding the extracellular Ca^{2+} . DMSO (0.1%, A) or NQ-Y15 (30 μM , B) was added to the reaction mixture at 8 min before AA (50 μM) stimulation. Traces are representative of 4 experiments.

Fig. 2. Effect of NQ-Y15 on AA-Induced Ca^{2+} Influx in Fura-2-Loaded Platelets

Ca^{2+} depletion from internal stores was induced by AA (50 μM) in the absence of external Ca^{2+} (0.5 mM EGTA). Ca^{2+} influx from medium was induced by Ca^{2+} repletion to 2.0 mM $CaCl_2$, at 12 min after AA stimulation. DMSO (0.1%) or NQ-Y15 (30 μM) was added to the reaction mixture at 8 min before applying excess Ca^{2+} . Traces are representative of 4 experiments.

Fig. 3. Effect of NQ-Y15 on cAMP Levels in Unstimulated Platelets (A) and AA-Stimulated Platelets (B)

(A) cAMP formation was assayed at 8 min of incubation of platelets with DMSO (0.5%, Basal), 30 μ M NQ-Y15 (NQ30), 50 μ M NQ-Y15 (NQ50) or 80 μ M PGI₂ (PGI₂) at 37 °C. (B) 50 μ M AA was added to the reaction mixture 8 min after the addition of vehicle, NQ-Y15 or picotamide. Five minutes after the addition of AA, cAMP formation was stopped by adding 10 mM EDTA and immediately boiling for 5 min. Values are means $\pm$ S.E.M. ($n=5$). Key: a) $p<0.01$ as compared to basal; b) $p<0.01$ compared to AA. Control: platelet suspension+vehicle of NQ-Y15 (DMSO 0.5%), AA control: control+AA (50 μ M), NQ30: platelet suspension+NQ-Y15 (30 μ M)+AA, NQ50: platelet suspension+NQ-Y15 (50 μ M)+AA, picotamide: platelet suspension+picotamide (1 mM)+AA.

cAMP level was not affected by NQ-Y15 at 30 and 50 μ M in unstimulated platelets. This means that NQ-Y15 has no direct effect on adenylate cyclase. By the stimulation of platelets with 50 μ M AA, the level of cAMP was lowered to 2.9 ± 0.2 pmol/ 3×10^8 platelets, which is about 60% of its level in resting platelets (Fig. 3B). This decrease was overcome by preincubation with picotamide, which has dual functions as a TXA₂ synthase inhibitor and a TXA₂/PGH₂ receptor antagonist.¹³⁾ As a consequence of the preincubation of AA stimulated platelets with 1 mM picotamide, the cAMP level rose to 7.1 ± 0.4 pmol/ 3×10^8 , which is higher than that in resting platelets. Likewise, NQ-Y15 (30 and 50 μ M) caused significant elevation of the cAMP level in AA-stimulated platelets (7.9 ± 0.4 and 9.5 ± 0.2 pmol/ 3×10^8 , respectively).

DISCUSSION

Exogenous AA-induced aggregation, granule release and the rise in $[Ca^{2+}]_i$ of platelets are due to the formation of TXA₂.^{3,4)} AA is metabolized to TXA₂ via two enzymatic steps, including PGH synthesis and TXA₂ synthesis. TXA₂ exerts its effects on platelets by binding to the TXA₂/PGH₂ receptor.²⁾ In platelets, the stimulation of TXA₂/PGH₂ receptors leads to the activation of G_q proteins, which in turn stimulates the phosphoinositide-specific PLC induced hydrolysis

of PIP₂ to IP₃ and diacylglycerol.²²⁾ IP₃ causes Ca²⁺ release from intracellular stores by binding to its intracellular receptor.²³⁾ It is generally accepted that platelets lack voltage-operated Ca²⁺ channels⁵⁾ and that various forms of receptor-mediated Ca²⁺ influx are activated by agonists. In our previous study, it was found that NQ-Y15 inhibits $[Ca^{2+}]_i$ elevation induced by AA.¹⁵⁾ This inhibition mechanism was investigated, referring to studies using U46619, a thromboxane mimic, in which both Ca²⁺ influx and its release from intracellular storage sites were found to contribute to the increase in $[Ca^{2+}]_i$.²⁴⁾ In the present study, we investigated whether NQ-Y15 acts preferentially on Ca²⁺ influx from outer spaces or on its release from internal stores. As shown in Figs. 1B and 2B, NQ-Y15 significantly inhibited Ca²⁺ mobilization in AA-stimulated platelets through its inhibitory effects on both Ca²⁺ release from intracellular stores and Ca²⁺ influx. Inhibitory effects of NQ-Y15 on Ca²⁺ mobilization in AA-stimulated platelets may be mainly due to the TXA₂/PGH₂ receptor blockade, which would decrease the formation of IP₃.

The increase in $[Ca^{2+}]_i$ and platelet activation were not able to be fully inhibited by only suppressing the production or action of thromboxane.^{9,10)} While TXA₂ synthase inhibitors or TXA₂/PGH₂ receptor antagonists alone do not affect the Ca²⁺ mobilization in AA stimulated platelets, dual acting drugs like picotamide, which act as TXA₂ synthase inhibitors and as TXA₂/PGH₂ receptor antagonists, showed profound inhibitory effects on the increase of $[Ca^{2+}]_i$ after stimulation with AA (Fig. 3B). It would be explained by the action of cAMP increasing during the metabolism of AA.¹³⁾ cAMP serves as a second messenger to inhibit the increase in $[Ca^{2+}]_i$.^{11,12)} Adenylate cyclase forms cAMP from ATP. The activity of adenylate cyclase is regulated by G protein; it is stimulated by G_s and inhibited by G_i. G_s is coupled to the receptors for the inhibitory PGs, such as PGD₂ and prostaglandin I₂ (PGI₂).²⁵⁾ It has been demonstrated that the TXA₂ receptor is also coupled, in addition to the G_q activating PLC, to G_{i2}, which inhibits adenylate cyclase.²⁶⁾ The combination of a TXA₂ synthase inhibitory activity with a TXA₂ receptor antagonistic action may lead to an increase in cAMP levels in AA-activated platelets.^{9,10)} To investigate whether the inhibitory effect of NQ-Y15 on Ca²⁺ mobilization was attributable to cAMP, we examined the effects of NQ-Y15 on the cAMP level in AA-stimulated platelets. As shown in Fig. 3B, NQ-Y15 increased the cAMP level in AA-stimulated platelets.

We found that NQ-Y15 enhanced the PGD₂ production in AA-stimulated platelets (data not shown). PGD₂ was known to inhibit aggregation induced by ADP or collagen in human platelets but not in rat platelets.²⁷⁾ Thus, we examined the effect of PGD₂ on Ca²⁺ mobilization induced by AA or ADP and found that PGD₂ does not inhibit the increase of $[Ca^{2+}]_i$ in rat platelets (data not shown). Vargaftig and Chignard (1975) reported that PGE₁ and PGF_{2 α} have anti-platelet aggregation-effects, but PGE₂ shows the inhibitory effect on platelet aggregation induced by AA at the concentrations of more than 2 μ M.²⁸⁾ Considering the fact that the amounts of prostaglandins, other than PGD₂, required to inhibit platelet activation are higher than those produced in the platelet, they might play a minor role in Ca²⁺ regulation. Sage and Heemskerk (1992) reported that thromboxane receptor stim-

ulation inhibits adenylyl cyclase and reduces cAMP-mediated inhibition of ADP-stimulated platelets.²⁹⁾ Their results imply that blockade of the TXA₂ receptor may activate adenylyl cyclase by inhibiting the stimuli induced by thromboxane of the basal level or by PGH₂ accumulated due to the inhibition of thromboxane synthesis. Judging from the previous reports and our experimental results, it was suggested that enhancement of cAMP might be due to both the adenylyl cyclase stimulation and the antagonism of the adenylyl cyclase inhibition by accumulated PGH₂ or residual TXA₂.^{9,10,30)} It was reconfirmed in this experiment that PGD₂ produced by TXA₂ inhibition could not directly increase [Ca²⁺]_i in rat platelets. Therefore, adenylyl cyclase activation may play a more prominent role in AA-stimulated rat platelets, and prostaglandins other than PGD₂ are probably responsible for the elevation of cAMP.

The increase in cAMP concentration leads to the reduced IP₃ formation,³¹⁾ which stimulates Ca²⁺ influx and Ca²⁺ release from intracellular storage sites.³²⁾ The elevation of cAMP concentration causes the activation of PKA, which in turn phosphorylates a number of substrates. Proteins phosphorylated by PKA include glycoprotein Ib β (a thrombin binding site), myosin light chain and actin binding protein (involved in platelet contraction), a 22 kDa protein regulating the activity of a PLC (possibly rap 1B).^{33,34)} These facts suggest that NQ-Y15 might increase the cAMP level in stimulated platelets, which acts as an important inhibitory signal for Ca²⁺ mobilization.

In summary, NQ-Y15 was found to inhibit the increase of [Ca²⁺]_i in AA-stimulated rat platelets through the inhibition of calcium release from intracellular storage sites and Ca²⁺ influx. And this inhibition of Ca²⁺ mobilization might be caused by the elevation of cAMP, which contributes to the inhibition of platelet aggregation.

Acknowledgements This study was supported by grants from the '96 Good Health R&D project, Ministry of Health & Welfare, R.O.K (HMP-96-D-5-1045) and supported in part by the year (1999—2000) BK21 project for Medicine, Dentistry and Pharmacy and Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University.

REFERENCES

- 1) FitzGerald G. A., *Am. J. Cardiol.*, **68**, 11B—15B (1991).
- 2) Parise L. V., Venton D. L., Le Breton G. C., *J. Pharmacol. Exp. Ther.*, **228**, 240—244 (1984).
- 3) Siess W., Siegel F. L., Lapetina E. G., *J. Biol. Chem.*, **258**, 11236—11242 (1983).
- 4) Brass L. F., Shaller C. C., Belmonte E. J., *J. Clin. Invest.*, **79**, 1269—1275 (1987).
- 5) Rink T. J., Sage S. O., *Annu. Rev. Physiol.*, **52**, 431—449 (1990).
- 6) Mayeux P. R., Morton H. E., Gillard J., Lord A., Morinelli T. A., Boehm A., Mais D. E., Halushka P. V., *Biochem. Biophys. Res. Commun.*, **157**, 733—739 (1988).
- 7) Gesele P., Deckmyn H., Nenci G. G., Vermynen J., *Trends. Pharmacol. Sci.*, **12**, 158—163 (1991).
- 8) Gesele P., Van Houtte E., Arnout J., Deckmyn H., Vermynen J., *Thromb. Haemost.*, **52**, 364 (1984).
- 9) Gesele P., Arnout J., Deckmyn H., Huybrechts E., Pieters G., Vermynen J., *J. Clin. Invest.*, **80**, 1435—1445 (1987).
- 10) Watts I. S., Wharton K. A., White B. P., Lumley P., *Br. J. Pharmacol.*, **102**, 497—505 (1991).
- 11) Feinstein M. B., Egan J. J., Sha'afi R. I., White J., *Biochem. Biophys. Res. Commun.*, **113**, 598—604 (1983).
- 12) Yamanishi J., Kawahara Y., Fukuzaki H., *Thromb. Res.*, **32**, 183—188 (1983).
- 13) Gesele P., Deckmyn H., Arnout J., Nenci G. G., Vermynen J., *Thromb. Haemost.*, **61**, 479—484 (1989).
- 14) Hoet B., Falcon C., De Reys S., Arnout J., Deckmyn H., Vermynen J., *Blood*, **75**, 646—653 (1990).
- 15) Chang T. S., Kim H. M., Lee K. S., Khil L. Y., Ma W. C., Ryu C. K., Moon C. K., *Biochem. Pharmacol.*, **54**, 259—268 (1997).
- 16) Ryu C. K., *Yakhak Hoeji*, **32**, 245—250 (1988).
- 17) Pollock W. K., Rink T. J., *Biochem. Biophys. Res. Commun.*, **139**, 308—314 (1986).
- 18) Grynkiewicz G., Poenie M., Tsien R. Y., *J. Biol. Chem.*, **260**, 3440—3450 (1985).
- 19) Karniguan A., Legrand Y. J., Caen J. P., *Prostaglandins*, **23**, 437—457 (1982).
- 20) Magocsi M., Sarkadi B., Kovacs T., Gardos G., *Biochim. Biophys. Acta*, **984**, 88—96 (1989).
- 21) Hashimoto Y., Ogihara A., Nakanishi S., Matsuda Y., Kurokawa K., Nonomura Y., *J. Biol. Chem.*, **267**, 17078—17081 (1992).
- 22) Baldassare J. J., Tarver A. P., Henderson P. A., Mackin W. M., Sahagan B., Fisher G. J., *Biochem. J.*, **291** (Pt 1), 235—240 (1993).
- 23) Berridge M. J., Irvine R. F., *Nature (London)*, **341**, 197—205 (1989).
- 24) Yun J. C., Ohman K. P., Gill J. R., Jr., Keiser H., *Can. J. Physiol. Pharmacol.*, **69**, 599—604 (1991).
- 25) Blockmans D., Deckmyn H., Vermynen J., *Blood. Rev.*, **9**, 143—156 (1995).
- 26) Ushikubi F., Nakamura K., Narumiya S., *Mol. Pharmacol.*, **46**, 808—816 (1994).
- 27) Smith J. B., Silver M. J., Carol M. Ingerman, James J. Kocsis, *Thromb. Res.*, **5**, 291—299 (1974).
- 28) Vargaftig B., Chignard M., *Agents Action*, **5**, 137—144.
- 29) Sage S. O., Heemskerk J. W., *FEBS Lett.*, **298**, 199—202 (1992).
- 30) Avdonin P. V., Svitina-Ulitina I. V., Leytin V. L., Tkachuk V. A., *Thromb. Res.*, **40**, 101—112 (1985).
- 31) Tohmatsu T., Hattori H., Nagao S., Ohki K., Nozawa Y., *Biochem. Biophys. Res. Commun.*, **134**, 868—875 (1986).
- 32) Kaser-Glanzmann R., Gerber E., Luscher E. F., *Biochim. Biophys. Acta*, **558**, 344—347 (1979).
- 33) Brass L. F., Hoxie J. A., Manning D. R., *Thromb. Haemost.*, **70**, 217—223 (1993).
- 34) Torti M., Lapetina E. G., *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 7796—7800 (1992).