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Thromboxane A_2-mediated shape change: independent of Gq-phospholipase C-Ca^{2+} pathway in rabbit platelets

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1 Thromboxane A_2 (TXA_2) receptor-mediated signal transduction was investigated in washed rabbit platelets to clarify the mechanisms of induction of shape change and aggregation.

2 The TXA_2 agonist, U46619 (1 nM to 10 μM) caused shape change and aggregation in a concentration-dependent manner. A forty-times higher concentration of U46619 was needed for aggregation (EC_{50} of 0.58 μM) than shape change (EC_{50} of 0.013 μM). The aggregation occurred only when external 1 mM Ca^{2+} was present, but the shape change could occur in the absence of Ca^{2+}.

3 SQ29548 at 30 nM and GR32191B at 0.3 μM (TXA_2 receptor antagonists) competitively inhibited U46619-induced shape change and aggregation with similar potency, showing that both aggregation and shape change induced by U46619 were TXA_2 receptor-mediated events. However, ONO NT-126 at 1 nM, another TXA_2 receptor antagonist, inhibited U46619-induced aggregation much more potently than the shape change, suggesting the possible existence of TXA_2 receptor subtypes.

4 ONO NT-126 (2 nM to 3 μM) by itself caused a shape change without aggregation in a concentration-dependent manner, independent of external Ca^{2+}. Therefore, ONO NT-126 is a partial agonist at the TXA_2 receptor in rabbit platelets.

5 U46619 (10 nM to 10 μM) increased internal Ca^{2+} concentration ($[Ca^{2+}]_i$) and activated phosphoinositide (PI) hydrolysis in a concentration-dependent manner with a similar concentration-dependency.

6 U46619 (3 nM to 10 μM) also activated GTPase concentration-dependently in the membranes derived from platelets. U46619-induced activation of GTPase was partly inhibited by treatment of membranes with QL, an antibody against $G_{q/11}$.

7 The EC_{50} values of U46619 in Ca^{2+} mobilization (0.15 μM), PI hydrolysis (0.20 μM) and increase in GTPase activity (0.12 μM) were similar, but different from the EC_{50} value in shape change (0.013 μM), suggesting that activation of TXA_2 receptors might cause shape change via an unknown mechanism.

8 U46619-induced shape change was unaffected by W-7 (30 μM), a calmodulin antagonist or ML-7 (30 μM), a myosin light-chain kinase inhibitor, indicating that an increase in $[Ca^{2+}]_i$ might not be involved in the shape change. In fact, U46619 (10 nM) could cause shape change without affecting $[Ca^{2+}]_i$ level, determined by simultaneous recordings.

9 [^{3}H]-SQ29548 and [^{3}H]-U46619 bound to platelets at a single site with a K_d value of 14.88 nM and B_{max} of 106.1 fmol/10^8 platelets and a K_d value of 129.8 nM and B_{max} of 170.4 fmol/10^8 platelets, respectively. The inhibitory constant K_i value for U46619 as an inhibitor of ^{3}H-ligand binding was similar to the EC_{50} value of U46619 in GTPase activity, phosphoinositide hydrolysis and Ca^{2+} mobilization, but significantly different ($P<0.001$ by Student's t test) from the effect on shape change.

10 Neither U46619 nor ONO NT-126 affected the adenosine 3',5'-cyclic monophosphate (cyclic AMP) level in the presence or absence of external Ca^{2+} and/or isobutyl methylxanthine.

11 The results indicate that TXA_2 receptor stimulation causes phospholipase C activation and increase in $[Ca^{2+}]_i$ via a G protein of the $G_{q/11}$ family leading to aggregation in the presence of external Ca^{2+}, and that shape change induced by TXA_2 receptor stimulation might occur without involvement of the G_q-phospholipase C-Ca^{2+} pathway.

Keywords: Thromboxane A_2; platelet shape change; platelet aggregation; Ca^{2+}; phospholipase C; GTPase; G_q; thromboxane A_2 receptor; U46619; ONO NT-126

Introduction

Thromboxane A_2 (TXA_2) is a potent activator of platelets and a constrictor of vascular and respiratory smooth muscle (Hamberg *et al.*, 1975). TXA_2 and its precursor, prostaglandin H_2 (PGH_2), cause platelet activation including shape change, secretion and aggregation. It has been demonstrated that TXA_2/PGH_2 interacts with the TXA_2 receptor, causing activation of phospholipase C (PLC) (Baldassare *et al.*, 1993), which hydrolyzes phosphoinositide (PI) to produce two second messengers: inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DG) (Siess *et al.*, 1985; Brass *et al.*, 1987). IP_3 raises internal Ca^{2+} concentration ($[Ca^{2+}]_i$) level by releasing Ca^{2+} ions from the dense-tubular-system, a Ca^{2+} store in platelets (Berridge & Irvine, 1984). DG activates protein kinase C in the presence of Ca^{2+} ions and acidic phospholipids (Nishizuka, 1984). It has been reported that the TXA_2 receptor belongs to a receptor family coupled to the guanine nucleotide binding protein (G protein) (Hirata *et al.*, 1991). Shenker *et al.* (1991) reported that an anti-$G_{q/11}$ antibody could inhibit TXA_2 receptor-stimulated GTPase activation in human platelet membrane, suggesting that the G_q family couples with TXA_2

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receptor and acts as a transducer from TXA_2 receptor activation to PLC activation. Recently, a reconstitution study demonstrated that TXA_2 receptor-stimulated PI hydrolysis might be caused by stimulation of PLC-β isoforms through $G_{q/11}$ activation (Baldassare *et al.*, 1993).

Several pharmacological studies have suggested the existence of TXA_2 receptor subtypes including tissue differences (Lefer *et al.*, 1980; Mais *et al.*, 1985; Furci *et al.*, 1991). However, species differences in TXA_2 receptors may confuse the issue of the existence of different TXA_2 receptor subtypes (Narumiya *et al.*, 1986; Dorn, 1991). Platelet TXA_2 receptors have also been well characterized by binding studies and by the study of the rank order potency of TXA_2 receptor antagonists. Dorn & Dejesus (1991) reported the presence of two receptors possessing different affinities for the ligand [^{125}I]-[1S-(1α,2β(5Z),3α-(1E,3S),4α)]-7-[3-(3-hydroxy-4-(4'-iodophenoxy)-1-butenyl)-7-oxa-bicyclo[2.2.1]heptan-2-yl]-5-heptenoic acid ([^{125}I]-BOP) in human platelets, but [^{125}I]-9,11-dimethylmethano-11,12-methano-16-(3-iodo-4-hydroxyphenyl)-13,14-dihydro-13-aza-15$\alpha\beta$-ω-tetranor-TXA_2 ([^{125}I]-PTA-OH) binding to human platelets revealed a single affinity site (Narumiya *et al.*, 1986). On the other hand, Takahara *et al.* (1990) suggested that the TXA_2 receptor antagonist, GR32191, could discriminate two subtypes of the TXA_2 receptor in platelets. GR32191 binds reversibly to a site that mediates platelet shape change and increases $[Ca^{2+}]_i$, and irreversibly to a site linked to PLC activation and aggregation. Thus, TXA_2 receptor heterogeneity remains controversial from the pharmacological approach. Although the experiments on cloning and expression of cDNA for a human TXA_2 receptor derived from human placenta do not show the subtypes (Hirata *et al.*, 1991), Borg *et al.* (1994) have shown, using a specific antibody to the TXA_2 receptor, that a 52 kDa TXA_2 receptor exists in brain and aorta in addition to a 55 kDa TXA_2 receptor. Furthermore, TXA_2 receptor cDNA in human endothelial cells is reportedly different from that in human placenta, possibly reflecting alternative splicing of the cytoplasmic (carboxyl) tail (Raychowdhury *et al.*, 1994). They suggest that the human platelet may have one or more TXA_2 receptors that differ somewhat from either of those identified in placenta or endothelium, because human platelet RNA does not reveal a product when the RNA was reverse transcribed and amplified with oligonucleotide primers corresponding to the cytoplasmic tail nucleotide sequence, specific for the TXA_2 receptor identified in placenta or endothelium.

It is thought that an increase in $[Ca^{2+}]_i$ results in activation of calmodulin, which activates myosin light-chain kinase (MLCK) (Hathaway *et al.*, 1979). MLCK phosphorylates myosin light-chain (MLC) resulting in activation of actin-myosin interaction (Adelstein & Conti, 1975). In platelets, the 20 kDa MLC was phosphorylated during platelet activation induced by several agonists, such as thrombin (Itoh *et al.*, 1992). In addition, it has been reported that the release reaction and aggregation are associated with the Ca^{2+}-dependent phosphorylation of 20 kDa MLC (Nishikawa *et al.*, 1980). Therefore, it is generally accepted that Ca^{2+}-dependent activation of MLC is necessary for platelet activation including shape change and aggregation.

The present studies were designed to study TXA_2 receptor-mediated signal transduction with special reference to Ca^{2+} mobilization. The results obtained suggest that the shape change induced by TXA_2 receptor stimulation may not involve the $G_{q/11}$-phospholipase C-Ca^{2+} pathway, although the aggregation might involve the activation of the G_q family, PI hydrolysis and Ca^{2+} mobilization.

Methods

Platelet preparation

Whole blood was taken from the carotid artery of male rabbits weighing 2.5–3.5 kg under pentobarbitone anaesthesia (30 mg kg^{-1}, i.v.) with one seventh volume of ACD solution (65 mM citric acid, 85 mM trisodium citrate, 2% glucose) containing 5 μM indomethacin. Blood was centrifuged at 250 *g* for 10 min at room temperature and platelet-rich plasma (PRP) was obtained by centrifugation of the supernatant at 180 *g* for 5 min to remove contaminating erythrocytes and leukocytes. PRP was centrifuged at 650 *g* for 10 min and the pellet was resuspended in Tyrode-HEPES-albumin solution (composition, mM: NaCl 138, KCl 2.68, $MgCl_2.6H_2O$ 1.05, $NaHCO_3$ 4, HEPES 10, 0.1% glucose, 0.35% bovine serum albumin, indomethacin 5 μM, pH 6.35). Platelets were washed twice, and finally resuspended in Tyrode-HEPES-albumin solution (pH 7.35) at the optimum concentration.

Measurement of platelet shape change and aggregation

Platelet shape change and aggregation were monitored by light transmission in a 6-channel aggregometer (PAM-6C, Erma Co. Ltd., Tokyo). Washed platelets at $3-5\times 10^8$ ml^{-1} were preincubated in aggregometer cuvettes (total volume, 400 μl) with constant stirring at 37°C for 10 min. After addition of 1 mM $CaCl_2$ the platelet suspension was incubated for 5 min. Various inhibitors and antagonists were preincubated for 5 min before the addition of U46619. In shape change studies, 1 mM EGTA was added instead of 1 mM $CaCl_2$. Responses were normalized to the maximal response achieved in the control preparations.

Measurement of internal free calcium concentration

Changes in internal free calcium concentration ($[Ca^{2+}]_i$) were measured by monitoring the intensity of fura 2 fluorescence (Nakahata *et al.*, 1994). Washed platelets were incubated with 1 μM fura 2-AM at 37°C for 15 min. The platelets were washed twice and finally resuspended at about 1×10^8 ml^{-1}. Increases in $[Ca^{2+}]_i$ were measured in 1.5 ml of the platelet suspension in the quartz cell with constant stirring at 37°C using a fluorescence spectrophotometer (Hitachi, F-2000). Fura 2 fluorescence at 510 nm was monitored with excitation at 340 nm and 380 nm. $[Ca^{2+}]_i$ was calculated by using the K_d value of fura 2 to Ca^{2+} as 224 nM (Grynkiewicz *et al.*, 1985). For simultaneous recording of $[Ca^{2+}]_i$ and light transmission, another fluorescence spectrophotometer (Nihon Bunko, CAF-100) was used with constant stirring at 37°C. When CAF-100 was used, $[Ca^{2+}]_i$ was expressed as the ratio of 510 nm fluorescence intensities activated by 340 nm and 380 nm.

Measurement of total inositol phosphates

Washed platelets suspended in albumin-free Tyrode-HEPES solution (pH 7.35) were labelled with 25 μCi ml^{-1} [^{3}H]-*myo*-inositol at 37°C for 1 h. Platelets were washed with Tyrode-HEPES-albumin solution (pH 7.35), and resuspended at $3-6\times 10^8$ ml^{-1}. After platelet suspensions (360 μl) were preincubated for 10 min, they were incubated with drugs in the presence of 10 mM LiCl for 10 min in a final volume of 400 μl. Reaction was terminated by addition of 400 μl of ice-cold 10% trichloroacetic acid (TCA). The TCA extracts were washed three times with diethyl ether to remove TCA. Diethyl ether was removed by keeping the samples at 47°C for 30 min. Total [^{3}H]-inositol phosphates were separated by anion exchange column (AG 1X8, formate form, 100-200 mesh) as previously described (Nakahata *et al.*, 1992).

Preparation of platelet membranes

Platelets were lysed by sonication (five times for 15 s at intervals of 1 min) in ice-cold 5 mM EDTA-10 mM HEPES, pH 7.4. The homogenate was centrifuged at 40,000 *g* for 10 min at 4°C to obtain membranes. The membranes were washed twice with the above buffer and thereafter dissolved in a small volume (1–2 ml) of 0.32 M sucrose: 10 mM HEPES: 5 mM $MgCl_2$ (pH 7.4), and stored at −80°C until use.

Measurement of GTPase activity

Platelet GTPase activity was assessed by the method of Houslay *et al.* (1986) with minor modifications. Briefly, the reaction mixture contained (mM): Tris-HCl 50, (pH 7.4), $MgCl_2$ 5, dithiothreitol 1, NaCl 100, EGTA 0.5, ATP 1, phosphocreatine 12, [γ-^{32}P]-GTP 0.1 μM (30 Ci mmol^{-1}), creatine phosphokinase 50 units ml^{-1} and drugs tested. Platelet membranes were suspended in 10 mM Tris-HCl (pH 7.4) just before use. The reaction was initiated by the addition of 5 μg membranes and incubated for 5 min at 30°C. Thereafter 400 μl of ice-cold 5% Norit A in 20 mM phosphate buffer (pH 7.4) was added, vortexed and centrifuged at 15,000 r.p.m. for 2.5 min by a microfuge (Tomy Seiko, Co. Ltd., Tokyo). Aliquots (200 μl) of the supernatant were counted for radioactivity.

Binding assay

Saturation binding experiments were performed using [^{3}H]-SQ29548 and [^{3}H]-U46619. Platelets ($1-5\times10^8$) were incubated in Tyrode-HEPES-albumin solution (pH 7.35) with [^{3}H]-SQ29548 (0.3–150 nM) at 37°C or [^{3}H]-U46619 (0.5–200 nM) at 30°C for 5 min, respectively. After incubation, ice-cold 0.9% w/v NaCl (4 ml) was added to each tube and the reaction mixture was immediately filtered under reduced pressure through a Whatman GF/C glass fibre filter which was then washed three times with ice-cold 0.9% NaCl (4 ml). Nonspecific binding was defined by use of 10 μM S-145 for [^{3}H]-SQ29548 binding and 10 μM U46619 for [^{3}H]-U46619 binding. In competition binding experiments, platelets ($1-3\times10^8$) were incubated in the presence of 1 mM $CaCl_2$ or 1 mM EGTA for 5 min with [^{3}H]-SQ29548 (10 nM) at 37°C or with [^{3}H]-U46619 (10 nM) at 30°C. Nonspecific binding was defined as the amount of radioactivity bound in the presence of 100 μM U46619 or 1 μM ONO NT-126.

Measurement of adenosine 3', 5'-cyclic monophosphate (cyclic AMP)

Washed platelets were incubated in the aggregometer described above. The reaction was started by addition of drugs, and was terminated by addition of HCl to make a concentration of 0.1 N and by boiling for 2 min. After the sample was centrifuged at 1700 *g* for 10 min, cyclic AMP in the supernatant was determined by radioimmunoassay using a cyclic AMP assay kit (Yamasa Shoyu Co. Ltd.) as described by Honma *et al.* (1977).

Protein assay and data analysis

Protein was measured by dye-binding method using bovine serum albumin as a standard (Bradford, 1976). In binding experiments, the dissociation constant, K_d, and the maximum binding site, B_{max}, were obtained by Scatchard analysis. The inhibitory constant, K_i, was calculated from the IC_{50} of the inhibition curve using the Cheng & Prusoff equation (Cheng & Prusoff, 1973). The statistical difference of the values was determined by Student's *t* test. Each pA_2 was calculated as $-\log K_B$, having derived K_B from the dose-ratio (DR-1 = [B]/K_B).

Materials

Bovine serum albumin (BSA, fraction V, fatty acid-free) and 9, 11-dideoxy-9α, 11α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619) were obtained from Sigma (St. Louis, MO, U.S.A.). Fura 2/AM and RGDS (Arg-Gly-Asp-Ser) were purchased from Dojindo Laboratories (Kumamoto, Japan) and Peptide Institute, Inc. (Osaka, Japan), respectively. 1-(5-Indonaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-7) and (N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W-7) were obtained from Seikagaku Corporation (Tokyo, Japan). Prostaglandin E_1 and 5(z)-6-[(1R, 2R, 3R, 4S,)-3-(N-4-bromobenzensulphonylaminoethyl)bicyclo[2,2,1]heptane-2-yl]hex-5-enoic acid (ONO NT-126) were kindly given by Ono Pharmaceutical Co. Ltd. (Osaka, Japan). [1S-[1α, 2β(5Z), 3β, 4α]]7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2,2,1]hept-2-yl]-5-heptenic acid (SQ29548) was given by Squibb Japan Inc. (Tokyo, Japan). [1R[1α(Z), 2β, 3β, 5α]]-(+)-7-[5-[[(1, 1'-biphenyl)-4-yl]methoxy]-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptenoic acid hydrochloride (GR32191B) was given by Glaxo Groups Research Limited (Greenford, U.K.). 1-{β-3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1H-imidazole hydrochloride (SK&F96365) was given by SmithKline Beecham Pharmaceuticals (Welwyn, U.K.). 5Z-7-(3-endo-phenylsulphonylamino-(2,2,1)-bicyclohept-2-exo-yl)heptenoic acid (S-145) was given by Shionogi Pharmaceutical Co. Ltd (Osaka, Japan). [^{3}H]-SQ29548 (50.4 Ci mmol^{-1}), [^{3}H]-U46619 (20.7 Ci mmol^{-1}), [^{3}H]-*myo*-inositol (23.4 Ci mmol^{-1}), [γ-^{32}P]-GTP (30 Ci mmol^{-1}) and anti-G_q antibody QL were obtained from DuPont/ New England Nuclear (Boston, MA,

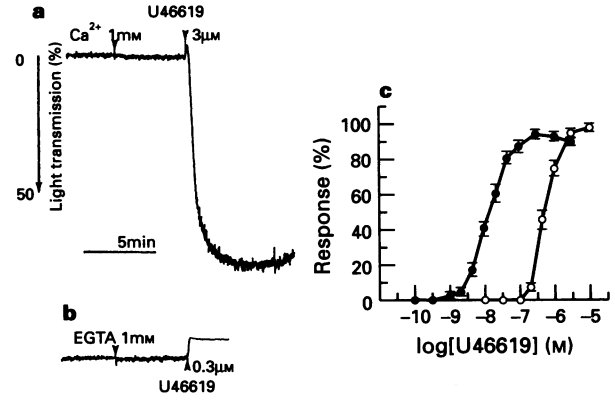

Figure 1 U46619-induced platelet shape change and aggregation measured in the presence or absence of external Ca^{2+} ions. (a) Platelets were preincubated with 1 mM $CaCl_2$ for 5 min at 37°C, and stimulated by U46619 to obtain aggregation. (b) Platelets were preincubated with 1 mM EGTA, and stimulated by U46619 to obtain shape change. (c) Concentration-response curves for platelet shape change (●) and aggregation (○). The measured responses were calculated as a percentage of the maximum response of 100%. Each point represents the mean ± s.e.mean (●; $n=14$; ○; $n=10$).

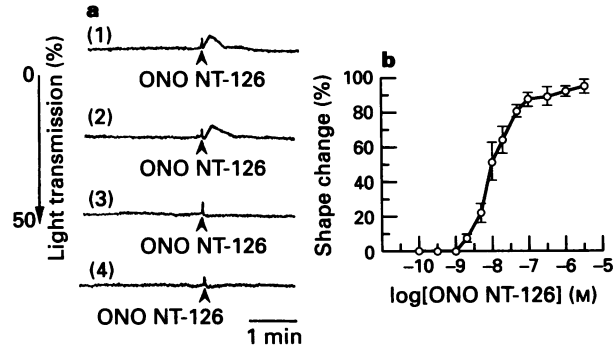

Figure 2 ONO NT-126-induced platelet shape change. (a) Platelets were preincubated with 1 mM $CaCl_2$ (1) or 1 mM EGTA (2) for 10 min at 37°C, and stimulated with 100 nM ONO NT-126. Platelets were preincubated with 1 μM SQ29548 (3) or 10 μM GR32191B (4) for 5 min in the presence of 1 mM EGTA, and stimulated with ONO NT-126. (b) Concentration-response curves of platelet shape change (○) for ONO NT-126 in the presence of 1 mM EGTA. The measured responses were calculated as percentages of the maximum response of 100%. Each point represents the mean ± s.e.mean ($n=6$).

U.S.A.). Cyclic AMP assay kit was obtained from Yamasa Shoyu Co. Ltd. (Choshi, Japan). Other chemicals or drugs were of reagent grade or of the highest quality available.

Results

U46619-induced shape change and aggregation

In the presence of external Ca^{2+}, U46619 caused platelet aggregation after transient shape change (Figure 1a). When external Ca^{2+} ions were chelated by 1 mM EGTA, U46619 caused a sustained shape change, but not aggregation (Figure 1b), indicating that U46619 could elicit shape change without utilizing external Ca^{2+}. U46619 elicited platelet shape change in lower concentrations than aggregation (Figure 1c). The pD_2 values were 6.24 ± 0.05 ($n = 10$) for aggregation and 7.87 ± 0.06 ($n = 14$) for shape change, respectively. The pD_2 values of these responses were significantly different ($P < 0.001$). Shape change occurred at a forty-times lower concentration of U46619 than aggregation.

In order to determine whether U46619-induced shape change and aggregation were mediated by TXA_2 receptor activation, we used three TXA_2 receptor antagonists i.e. ONO NT-126 (Nakahata *et al.*, 1990; 1992; Takano *et al.*, 1992), SQ29548 and GR32191B. Among these antagonists, ONO NT-126 by itself caused a transient shape change independent of external Ca^{2+}, without aggregation even if high concentrations were used and/or in the presence of external Ca^{2+} (Figure 2). ONO NT-126-induced shape change was concentration-dependent with the pD_2 of 7.98 ± 0.09 ($n = 6$) and inhibited by TXA_2 receptor antagonists, SQ29548 and GR32191B (Figure 2). The results suggest that ONO NT-126 has a partial agonist activity that can cause only shape change through TXA_2 receptor stimulation. ONO NT-126 (1 nM), SQ29548 (30 nM) and GR32191B (0.3 μM) caused parallel rightward shifts of the concentration-response curves for U46619-induced shape change (Figure 3a), suggesting that

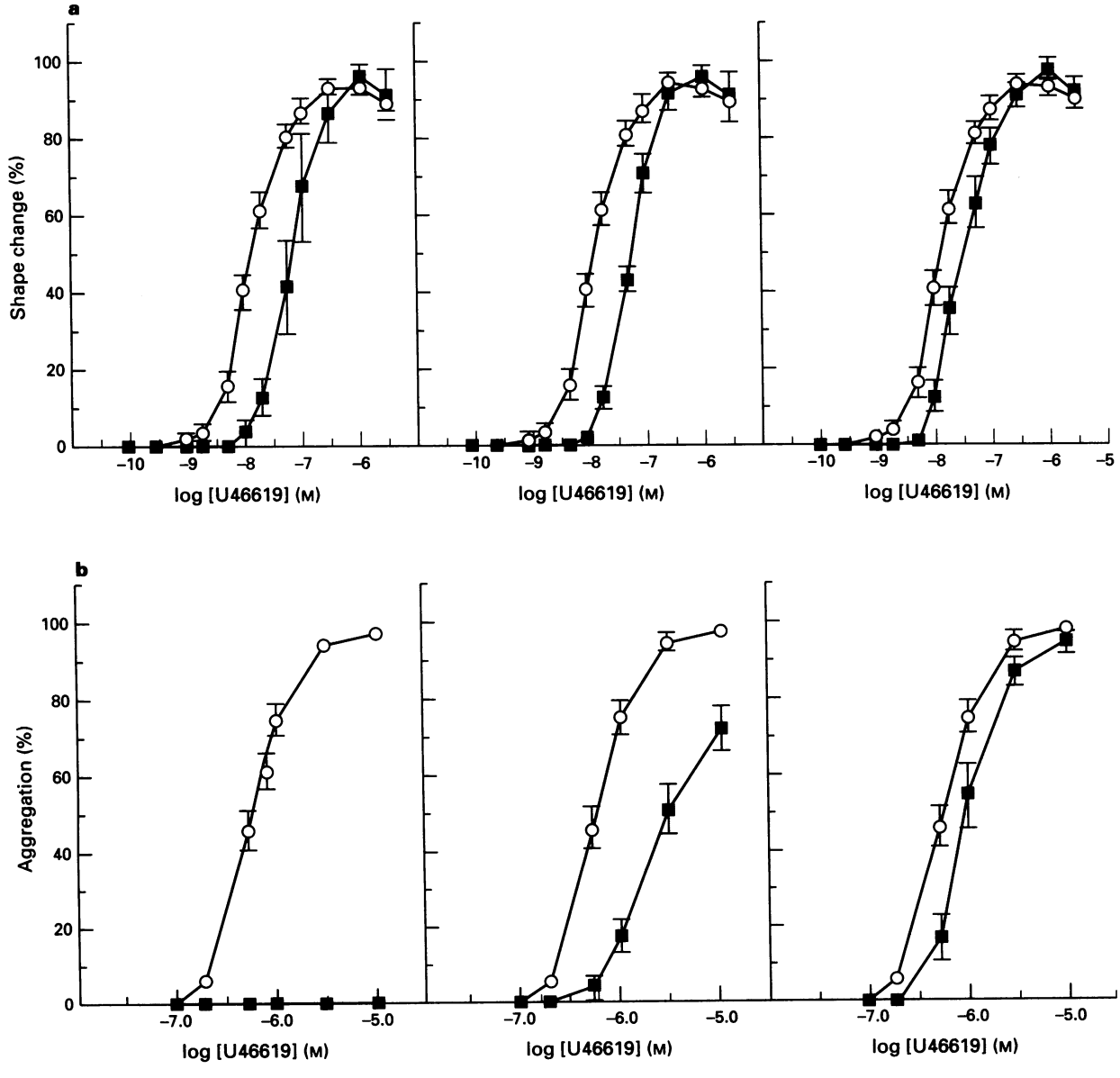

Figure 3 Effects of three TXA_2 antagonists on U46619-induced shape change (a) and aggregation (b). (a) Platelets were preincubated with 1 nM ONO NT-126 (left; ■, $n = 5$), 30 nM SQ29548 (middle; ■, $n = 4$) and 0.3 μM GR32191B (right; ■, $n = 4$) for 5 min at 37°C in the presence of 1 mM EGTA and stimulated with U46619; (○) U46619 alone. Each point represents the mean ± s.e.mean. (b) Aggregation was monitored in the presence of 1 mM $CaCl_2$ in a similar manner to (a). Symbols as in (a). Each point represents the mean ± s.e.mean of 6 determinations.

each antagonist inhibited shape change in a competitive manner and U46619-induced shape change was mediated via TXA_2 receptors. The pA_2 values for antagonism of U46619-induced shape change were 9.66 ± 0.24 ($n=5$) for ONO NT-126, 7.87 ± 0.03 ($n=4$) for SQ29548 and 6.50 ± 0.10 ($n=4$) for GR32191B, respectively. Next, we determined the effects of three antagonists on U46619-induced aggregation (Figure 3b). The pA_2 values in antagonizing U46619-induced aggregation were 8.30 ± 0.16 ($n=6$), 6.79 ± 0.08 ($n=6$) for SQ29548 and GR32191B, respectively. Each pA_2 value of SQ29548 or GR32191B in U46619-induced aggregation was not significantly different ($P > 0.05$) from that in the shape change, suggesting that SQ29548 and GR32191B could antagonize these responses with the same potencies. In contrast, ONO NT-126 at 1 nM non-competitively inhibited U46619-induced aggregation, although ONO NT-126 in the same concentration competitively inhibited the shape change.

Measurement of internal Ca^{2+} concentration and phosphoinositide hydrolysis

U46619 increased $[Ca^{2+}]_i$ in fura 2-loaded rabbit platelets (Figure 4). The increase in $[Ca^{2+}]_i$ in the presence of 1 mM Ca^{2+} was much larger than that in the presence of 1 mM EGTA, suggesting influx of Ca^{2+} from the external medium. U46619 increased $[Ca^{2+}]_i$ in a concentration-dependent manner with pD_2 values of 6.72 ± 0.10 ($n=5$) and 6.81 ± 0.07 ($n=5$) in the presence of 1 mM Ca^{2+} and 1 mM EGTA, respectively (Figure 4c). Moreover, these values were significantly different ($P < 0.001$) from the pD_2 of shape change, suggesting that shape change induced by U46619 might not be involved in Ca^{2+} mobilization.

U46619 concentration-dependently elicited PI hydrolysis in the presence or absence of external Ca^{2+} (Figure 5), quite similar to Ca^{2+} mobilization. U46619 accumulated total inositol phosphates much more in the presence of external Ca^{2+} than in the presence of 1 mM EGTA. The Ca^{2+} influx from external medium after TXA_2 receptor stimulation may contribute to further activation of PLC. The pD_2 values in the presence of 1 mM Ca^{2+} and 1 mM EGTA were 6.76 ± 0.01 ($n=3$) and 6.70 ± 0.07 ($n=3$), respectively and were not significantly different ($P > 0.1$) from each other. Furthermore, the value was similar to that of internal Ca^{2+} mobilization induced by U46619.

Effect of U46619 on GTPase activity

It has been proposed that the TXA_2 receptor couples to trimeric G protein, $G_{q/11}$, and $G_{q/11}$ acts as a transducer from the TXA_2 receptor to PLC (Shenker *et al.*, 1991). Then, GTPase activity in response to TXA_2 receptor stimulation was determined in platelet membrane preparations (Figure 6). U46619 activated GTPase in a concentration-dependent manner with the pD_2 value of 6.94 ± 0.12 ($n=6$). This value was similar to those for Ca^{2+} mobilization and PI hydrolysis, but different from that for shape change ($P < 0.001$). These results suggest that G protein-mediated PLC activation causes Ca^{2+} mobilization when stimulated by U46619 in rabbit platelets. U46619-induced activation of GTPase in platelet membranes was reduced by treatment of membranes with an anti-$G_{q/11}$ antibody QL for 90 min on ice (Figure 7). However, QL in high concentrations partially inhibited U46619-induced GTPase activation. Thus, we identified a G protein of G_q family as one of the G proteins coupled with TXA_2 receptors in rabbit platelets.

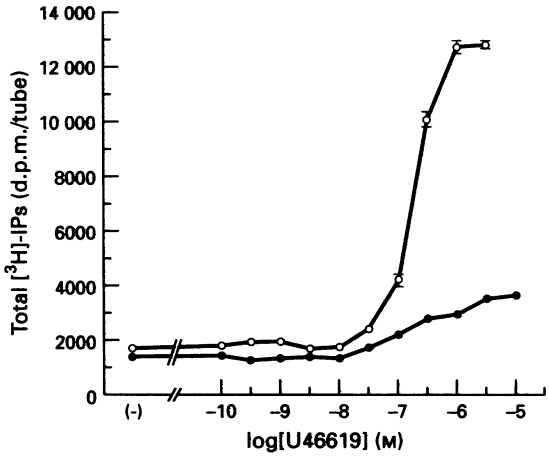

Figure 5 Effect of U46619 on PI hydrolysis in intact platelets. Platelets were labelled with 25 μCi ml^{-1} [^{3}H]-*myo*-inositol for 1 h at 37°C. Platelets were incubated with various concentrations of U46619 for 10 min in the presence of 1 mM $CaCl_2$ (○) or 1 mM EGTA (●). Each point represents the mean ± s.e.mean of 3 determinations.

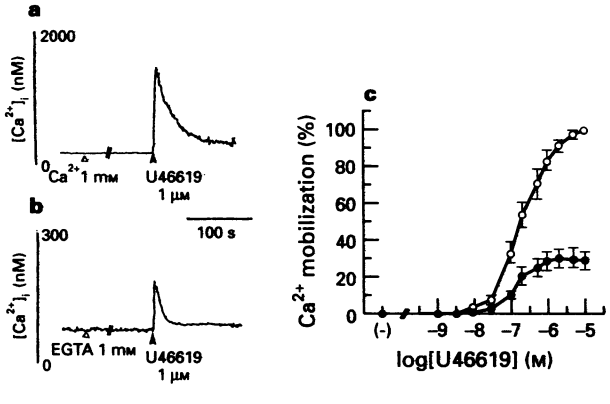

Figure 4 Effect of U46619 on $[Ca^{2+}]_i$. Platelets were incubated with 1 μM fura 2-AM for 15 min at 37°C. Platelets were stimulated with U46619 in the presence (a) or absence (b) of external Ca^{2+} ions. $[Ca^{2+}]_i$ was calculated as described in Methods. (c) Concentration-response curves for Ca^{2+} mobilization. U46619-induced increase in $[Ca^{2+}]_i$ in the presence of 1 mM EGTA (●) or 1 mM $CaCl_2$ (○) was calculated as the percentage of the maximum response in the presence of Ca^{2+}, taken as 100%. Each point represents the mean ± s.e.mean of 5 determinations.

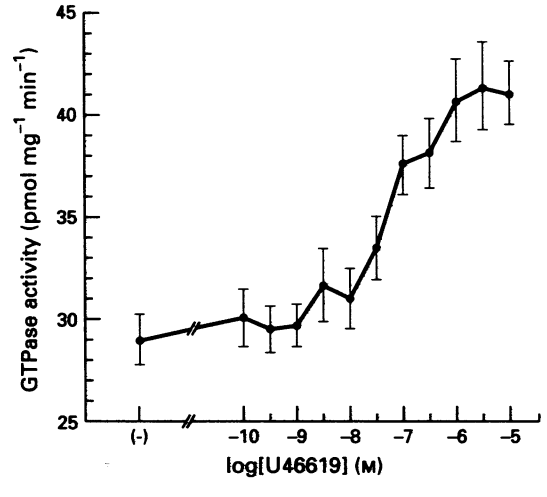

Figure 6 Effect of U46619 on GTPase activity in membrane preparations. Membranes (5μg) were stimulated with various concentrations of U46619 for 5 min at 30°C. Free [^{32}P]-Pi was counted as described in Methods. Each point represents the mean ± s.e.mean of 7 determinations.

Pharmacological characterization of U46619-induced shape change

It has been suggested that agonist-induced increase in $[Ca^{2+}]_i$ results in MLCK activation through calmodulin and that this event would correlate with platelet activation including shape change and aggregation. However, the pD_2 value of shape change was significantly different from Ca^{2+} mobilization in the absence of external Ca^{2+} (Figures 1c and 4c). Then, we examined the role of Ca^{2+} in U46619-induced shape change. W-7, a calmodulin antagonist, or ML-7, an MLCK inhibitor, had little effect on U46619 (0.3 μM)-induced shape change (Figure 8a). Moreover, we tried to monitor $[Ca^{2+}]_i$ and shape changes by a simultaneous recording (Figure 8b). U46619 at a concentration of 10 nM, which was close to the EC_{50} value for shape change, caused a sustained shape change, without affecting the $[Ca^{2+}]_i$ level.

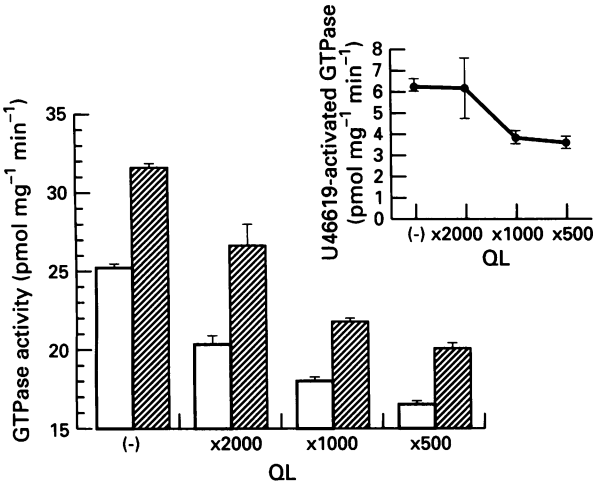

Figure 7 Effect of anti-$\alpha_{q/11}$ antibody QL on U46619-induced GTPase activation. Membranes were preincubated with various concentrations of QL for 90 min on ice. After preincubation for 5 min at 30°C, membranes (5 μg) were stimulated with (hatched column) or without (open column) 1 μM U46619 for 5 min. Free [^{32}P]-Pi was counted as described in Methods. Each column represents the mean ± s.e.mean of 3 determinations. Inset: U46619-induced activation of GTPase as calculated from the results.

Pharmacological characterization of U46619-induced aggregation

It is reported that fibrinogen binding to glycoprotein (GP) IIb/IIIa on platelet surface builds bridges between platelets (Phillips *et al.*, 1988). GP IIb and GP IIIa makes a complex at the ratio of 1:1 only when Ca^{2+} ions exist. Recently it has been suggested that GP IIb/IIIa acts as a Ca^{2+} channel (Fujimoto *et al.*, 1991). RGDS peptide (Arg-Gly-Asp-Ser) which blocks fibrinogen binding to GP IIb/IIIa was used to determine the role of GP IIb/IIIa on TXA_2 receptor-mediated aggregation (Figure 9). RGDS peptide inhibited both aggregation and Ca^{2+} mobilization induced by U46619 in a concentration-dependent manner, indicating the involvement of GP IIb/IIIa in TXA_2 receptor-mediated aggregation. Next we examined whether Ca^{2+} influx from the external medium was necessary for U46619-induced platelet aggregation. SK&F96365, a receptor-operated Ca^{2+} channel blocker (Merritt *et al.*, 1990), inhibited Ca^{2+} mobilization more effectively than aggregation (Figure 9). However, neither RGDS nor SK&F96365 affected the shape change induced by U46619 (data not shown). SK&F96365 at high concentrations increased $[Ca^{2+}]_i$ by itself, as a non-specific action of this drug (data not shown). These results suggest that the existence of Ca^{2+} ions in the external medium and/or Ca^{2+} influx from the external medium is important in causing aggregation in response to U46619.

Binding assay to intact rabbit platelets

To clarify TXA_2 receptor subtypes, a receptor binding assay in intact rabbit platelets was carried out by using [^{3}H]-SQ29548, a TXA_2 receptor antagonist, and [^{3}H]-U46619, a TXA_2 receptor agonist. Scatchard analysis of [^{3}H]-SQ29548 revealed a single binding site with a K_d of 14.88 nM and a B_{max} of 106.1 fmol/10^8 platelets (Figure 10a). The $-\log K_d$ for [^{3}H]-SQ29548 binding was similar to the pA_2 values obtained from shape change and aggregation. [^{3}H]-U46619 had also only one binding site with a K_d of 129.8 nM and a B_{max} of 170.4 fmol/10^8 platelets (Figure 10b). Next, we examined the dependency of external Ca^{2+} ions in the ligand binding to TXA_2 receptors, because U46619 in lower concentrations caused shape change in the presence of 1 mM EGTA. Unlabelled U46619 displaced [^{3}H]-SQ29548 or [^{3}H]-U46619 binding to intact rabbit platelets in a concentration-dependent manner in the presence or absence of external Ca^{2+} ions (Figure 11). However, there was no dependency on

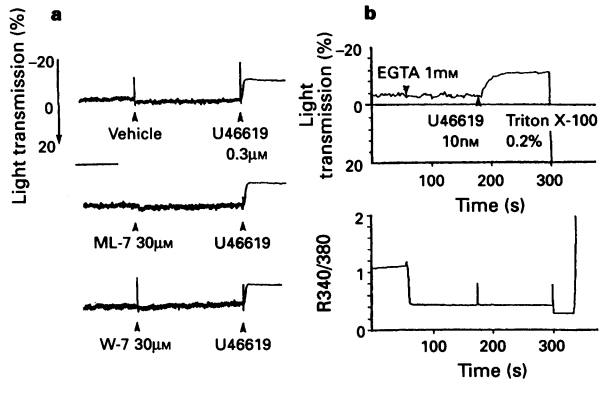

Figure 8 Characterization of U46619-induced shape change. (a) ML-7 or W-7 was preincubated with platelets in the presence of 1 mM EGTA for 5 min at 37°C before the stimulation with 0.3 μM U46619. Each trace represents a typical one from several experiments. (b) Fura 2-AM was loaded as described in Methods. Light transmission and fura 2 fluorescence at 340 nm and 380 nm were measured simultaneously.

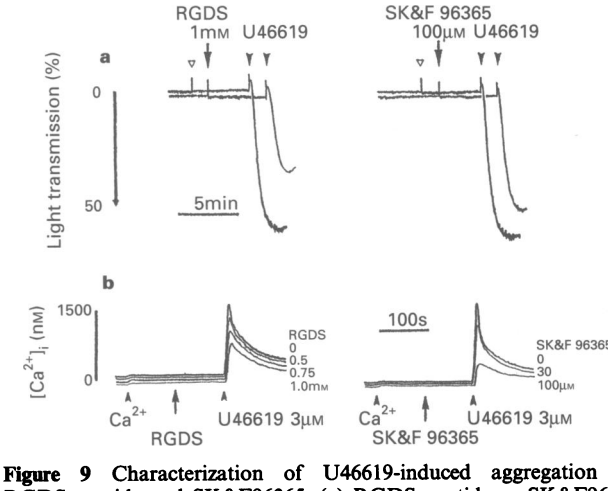

Figure 9 Characterization of U46619-induced aggregation by RGDS peptide and SK&F96365. (a) RGDS peptide or SK&F96365 was preincubated with platelets in the presence of 1 mM $CaCl_2$ before stimulation with 3 μM U46619. (b) Fura 2-loaded platelets were preincubated with RGDS or SK&F96365 in the presence of 1 mM $CaCl_2$ and stimulated with 3 μM U46619. $[Ca^{2+}]_i$ was calculated as described in Methods.

external Ca^{2+} ions in the binding of labelled-ligands to TXA_2 receptors. The results make it unlikely that Ca^{2+} ions change the affinity of the ligands for TXA_2 receptors. In inhibition of [^{3}H]-SQ29548 binding, the K_i values of U46619 were 179.5 nM and 107.4 nM and the Hill coefficients were 0.9951 and 1.144 in the presence and absence of external Ca^{2+}, respectively. For inhibition of [^{3}H]-U46619 binding, the K_i values of U46619 were 97.1 nM and 58.5 nM and the Hill coefficients were 0.889 and 0.838 in the presence and absence of external Ca^{2+}, respectively. Those K_i values were similar to the K_d value (129.8 nM) obtained from Scatchard analysis of [^{3}H]-U46619 binding. ONO NT-126 inhibited [^{3}H]-SQ29548 binding with K_i values of 2.81 nM and 2.16 nM in the presence and absence of external Ca^{2+}, respectively (Figure 12). The Hill coefficients were 0.890 and 0.969 in the presence and absence of external Ca^{2+}, respectively. For inhibition of [^{3}H]-U46619 binding, the K_i values of ONO NT-126 were 2.38 nM and 1.77 nM and the Hill coefficients were 1.375 and 0.808 in the presence and absence of external Ca^{2+}, respectively.

Effects of U46619 and ONO NT-126 on cyclic AMP levels

Cyclic AMP produced by activation of adenylate cyclase has been shown to be related to the desensitization process in TXA_2 receptor stimulation (Murray *et al.*, 1990). Cyclic AMP was determined to clarify its contribution to shape change or aggregation induced by TXA_2 receptor stimulation (Table 1). While prostaglandin E_1 (PGE_1) significantly increased cyclic AMP, neither ONO NT-126 nor U46619 affected cyclic AMP levels. Furthermore, when phosphodiesterase was inhibited by isobutyl methylxanthine (IBMX), ONO NT-126 or U46619 failed to increase cyclic AMP. Thus, cyclic AMP may not be involved in the action of ONO NT-126 or U46619.

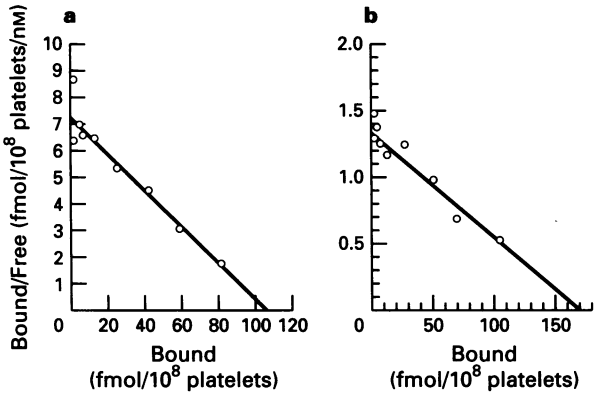

Figure 10 Scatchard analyses of [^{3}H]-SQ29548 (a) and [^{3}H]-U46619 (b) binding to intact platelets. Platelets were incubated with various concentrations of [^{3}H]-SQ29548 (a) or [^{3}H]-U46619 (b) for 5 min at 37°C or 30°C, respectively. Nonspecific binding was determined by 10 μM S-145 for [^{3}H]-SQ29548 and 10 μM U46619 for [^{3}H]-U46619. Each point represents the mean of three determinations.

Discussion

To clarify the mechanism of TXA_2-mediated shape change and aggregation, we examined TXA_2 receptor-stimulated signal transduction including receptor-binding, GTPase activation, formation of inositol phosphates and Ca^{2+} mobilization.

In investigations of the discrimination of TXA_2 receptor subtypes, Takahara *et al.* (1990) have shown previously that GR32191B inhibits non-competitively the aggregation induced by I-BOP, a TXA_2 agonist, while SQ29548 inhibits the aggregation competitively. In the present study, GR32191B as well as SQ29548 antagonized both shape change and aggregation competitively with similar potencies, indicating that the two antagonists recognize these responses in a similar manner. The K_d values of GR32191B are 316.2 nM ($pA_2 = 6.50$) for shape change and 162.2 nM ($pA_2 = 6.79$) for aggregation. The values are quite different from the K_d of 1.2–1.7 nM in human platelets (Takahara *et al.*, 1990; Armstrong *et al.*, 1993a), suggesting the presence of species differences for TXA_2 receptors. In rabbit aorta, the K_d of GR32191B is reported to be 61.7 nM ($pA_2 = 7.21$) and 70.8 nM ($pA_2 = 7.15$) (Lumley *et al.*, 1989; Yamamoto *et al.*, 1995). The K_d values of SQ29548 in the present study are 13.5 nM ($pA_2 = 7.87$) for shape change and 5.0 nM ($pA_2 = 8.30$) for aggregation, respectively. The values are similar to the K_d of 2.0 to 4.6 nM in human platelets (Takahara *et al.*, 1990; Armstrong *et al.*, 1993a), and to the K_d of 10.5 nM ($pA_2 = 7.98$) in rabbit aorta (Yamamoto *et al.*, 1995). Since the rate of dissociation of GR32191B is slow in human platelets (Armstrong *et al.*, 1993b), the difference in GR32191B action between the results of Takahara *et al.* (1990) and this study may relate to the preincubation time with the drug. On the other hand, ONO NT-126 inhibited non-competitively the U46619-induced aggregation, although it competitively inhibited the shape change. In addition, the EC_{50} of U46619 to elicit shape change was forty-times lower than that to elicit aggregation. From these pharmacological lines of evidence, it is thought that TXA_2 receptor subtypes exist in rabbit platelets, one of which

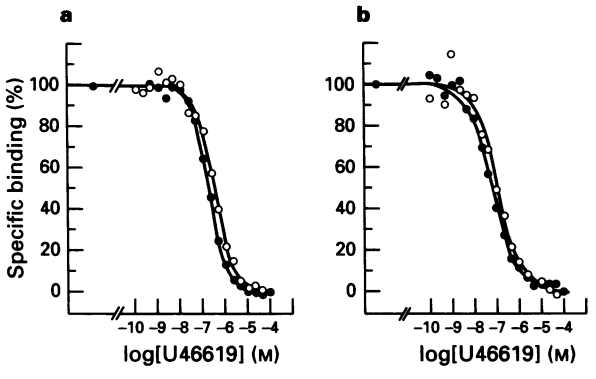

Figure 11 Inhibition of [^{3}H]-SQ29548 (a) and [^{3}H]-U46619 (b) binding by unlabelled U46619. Platelets were incubated with 10 nM [^{3}H]-SQ29548 (a) at 37°C or 10 nM [^{3}H]-U46619 (b) at 30°C in the presence of 1 mM $CaCl_2$ (○) or 1 mM EGTA (●). Nonspecific binding was determined in the presence of 100 μM U46619. Each point represents the mean of three determinations.

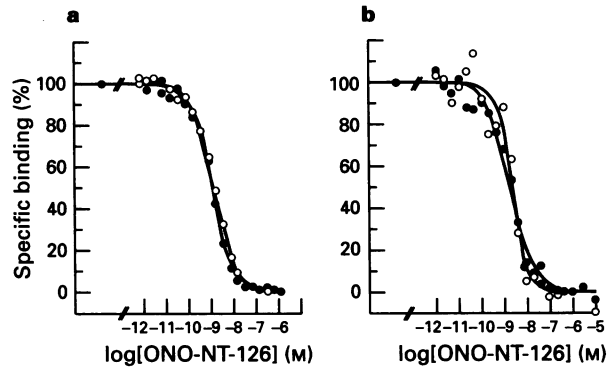

Figure 12 Inhibition of [^{3}H]-SQ29548 (a) or [^{3}H]-U46619 (b) binding by unlabelled ONO NT-126. Platelets were incubated with 10 nM [^{3}H]-SQ29548 (a) at 37°C or 10 nM [^{3}H]-U46619 (b) at 30°C in the presence of 1 mM $CaCl_2$ (○) or 1 mM EGTA (●). Nonspecific binding was determined in the presence of 1 μM ONO NT-126. Each point represents the mean of three determinations.

Table 1 Effects of ONO NT-126, U46619 and PGE_1 on cyclic AMP level in rabbit platelets

	Cyclic AMP (pmol/10^8 platelets)			
	−IBMX		*+IBMX (0.1 mM)*	
	+Ca^{2+} (1 mM)	*+EGTA* (1 mM)	*+Ca^{2+}* (1 mM)	*+EGTA* (1 mM)
No addition	1.45±0.054	1.52±0.092	3.28±0.325	2.83±0.050
ONO NT-126 (1 μM)	1.56±0.278	1.43±0.025	2.54±0.222	3.28±0.155
U46619 (1 μM)	1.39±0.041	1.36±0.055	3.25±0.250	3.11±0.171
PGE_1 (0.1 μM)	17.4±1.54*	13.7±0.769*	152.7±9.20*	110.0±9.16*

Rabbit washed platelets were incubated with the drug for 30 s in the presence or absence of isobutyl methylxanthine (IBMX, 0.1 mM). Cyclic AMP was determined by radioimmunoassay as described in Methods. Data represent mean±s.e.mean from three determinations. PGE_1 significantly increased cyclic AMP compared with control (no addition) in each condition (*$P<0.05$).

causes shape change and the other aggregation when stimulated by U46619. ONO NT-126 might inhibit the aggregation-linked receptor much more effectively than the shape change-linked receptor.

Scatchard analyses using [^{3}H]-SQ29548 and [^{3}H]-U46619 revealed 1-site of binding. Competitive inhibition of each radioligand binding by unlabelled U46619 was not altered in the presence or absence of external Ca^{2+}, suggesting that a ligand-receptor interaction is not influenced by the existence of external Ca^{2+}. Although the affinities of U46619 were somewhat increased by removal of external Ca^{2+}, there was no correspondence between the K_i values in the presence of 1 mM EGTA and the EC_{50} for shape change. ONO NT-126 could not discriminate between the receptor subtypes in the binding study, although the inhibition of aggregation by ONO NT-126 was much more effective than that of shape change. Therefore, the binding analyses with [^{3}H]-SQ29548 and [^{3}H]-U46619 did not allow discrimination between TXA_2 receptor subtypes in rabbit platelets. Previous biochemical studies demonstrated that differences in pH, by changing the histidine charge in TXA_2 receptors, allowed discrimination of TXA_2 binding sites in human platelets (Mayeux *et al.*, 1991). These authors showed that the affinity of I-BOP, a TXA_2 receptor agonist, for the receptor and the potency of I-BOP in inducing shape change were increased by lowering pH.

ONO NT-126 has interesting characteristics with respect to its effect on rabbit platelets, i.e. it causes shape change but not aggregation, it inhibits U46619-induced shape change in a competitive manner, and it inhibits U46619-induced aggregation in a non-competitive manner. ONO NT-126 as well as U46619 had no effect on adenylate cyclase: cyclic AMP system. Since ONO NT-126-induced shape change rapidly decreased and the inhibitory potency of ONO NT-126 was altered by incubation time, ONO NT-126 may desensitize a TXA_2 receptor. In rabbit aortic smooth muscles, ONO NT-126 inhibited TXA_2-induced contraction in a competitive manner (Yamamoto *et al.*, 1995). In human astrocytoma cells, [^{3}H]-SQ29548 binding was inhibited by ONO NT-126 with a K_i of 0.09 nM (Nakahata *et al.*, 1990). However, the Hill coefficient of ONO NT-126 for inhibition of binding is 0.706, suggesting that ONO NT-126 may not be a competitive antagonist of TXA_2 receptors in human astrocytoma cells. The fact that ONO NT-126 can discriminate between TXA_2 receptor-mediated shape change and aggregation in rabbit platelets, strongly suggests the existence of two TXA_2 receptors in rabbit platelets. However, ONO NT-126 fails to discriminate the binding site of [^{3}H]-U46619 in the present study. Therefore, an alternative interpretation of the results may be that there is a single TXA_2 receptor in rabbit platelets and the low occupancy of the receptor by an agonist induces shape change and the high occupancy induces aggregation. In this case, however, it is difficult to explain the different signal transduction for shape change and aggregation by TXA_2 receptor stimulation.

U46619 stimulated GTPase activity in a concentration-dependent manner. The EC_{50} value of U46619 in GTPase activation is similar to that for PI hydrolysis and Ca^{2+} mobilization, but quite different from that in shape change. Therefore, U46619-induced shape change may not involve the activation of G protein in plasma membranes, i.e. the shape change would be triggered by the other signalling besides the G protein-PLC-Ca^{2+} pathway. This hypothesis was supported by the results showing that neither ML-7 nor W-7 could inhibit U46619-induced shape change and that the shape change could be elicited without Ca^{2+} mobilization. Similar results have been reported by Simpson *et al.* (1986). This idea is consistent with the demonstration that neither IP_3 nor protein kinase C activation directly induces shape change (Estensen & White, 1974). However, the mechanism of TXA_2-induced shape change is so far unknown.

We recognized $G_{q/11}$ as one of the G proteins which communicates to TXA_2 receptors by anti $G_{q/11\alpha}$ antibody QL. However, the inhibition of U46619-elicited GTPase activity by QL was about 40% suggesting that other G proteins might also couple to the receptor. This result agrees with a previous report that stimulation of GTPase activity via TXA_2 receptors can only be partially (65–75%) inhibited by QL when the human platelet membrane is incubated for 60 min at 25°C (Shenker *et al.*, 1991). It has been reported that other G proteins of approximately 42 and 85 kDa distinct from the G_q family are also co-purified with TXA_2 receptors in human platelets (Knezevic *et al.*, 1993). G_i also couples functionally with human platelet TXA_2 receptors in a reconstituted system (Ushikubi *et al.*, 1994). Furthermore, G_{12} and G_{13}, which belong to the G_{12} family, have been reported to be coupled to TXA_2 receptors as well as thrombin receptors in human platelets (Offermanns *et al.*, 1994). These reports suggest that TXA_2 receptor-mediated signalling might not be restricted to $G_{q/11}$ which causes PLC activation and Ca^{2+} mobilization.

It has been suggested that external Ca^{2+} ions are indispensible for aggregation. Ca^{2+} ions are needed for construction of the GP IIb/IIIa complex (Fujimura & Phillips, 1983), and GP IIb/IIIa acts as the Ca^{2+} channel (Fujimoto *et al.*, 1991). The fact that RGDS and SK&F96365 prevent Ca^{2+} influx and aggregation, supports the idea that external Ca^{2+} are necessary for aggregation in mediating GP IIb/IIIa and Ca^{2+} influx. Ferrell & Martin (1989) suggest that GP IIb/IIIa activation causes tyrosine phosphorylation of many proteins. Furthermore, Gusovsky *et al.* (1993) have shown that Ca^{2+} influx by receptor stimulation causes tyrosine phosphorylation. The fact that the EC_{50} of U46619 in aggregation is somewhat higher than the EC_{50} for GTPase, PLC activation and Ca^{2+} mobilization suggests that other mechanisms besides PLC activation, such as tyrosine phosphorylation may be involved in eliciting aggregation.

Matsuoka *et al.* (1989) suggested that dibutyryl cyclic AMP antagonizes both shape change and aggregation, although 8-bromo cyclic GMP only inhibits aggregation stimulated by TXA_2 agonist. Recent reports have shown that TXA_2 receptor stimulation causes activation of several enzymes, such as tyrosine kinase in human (Oda *et al.*, 1992) and porcine platelets (Maeda *et al.*, 1993), and mitogen-activated protein kinase in

coronary artery smooth muscle cells (Morinelli *et al.*, 1994). Phosphoinositide 3-kinase is also activated by TXA_2 agonist in human platelets (Kucera & Rittenhouse, 1990). Therefore, further studies are needed to elucidate the mechanism of shape change and aggregation induced by TXA_2 receptor stimulation.

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