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Labeling of Human Platelet Plasma Membrane Thromboxane A_2/Prostaglandin H_2 Receptors Using SQB, a Novel Biotinylated Receptor Probe

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ABSTRACT. This study reports the synthesis, biological evaluation, and application of a new biotinylated derivative 1-[[1*S*-[1α,2α(*Z*),3α,4α]]-7-[3-[[[[(1-oxocyclohexylpropyl)amino]acetyl]amino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoyl]-2-[hexahydro-2′-oxo-1*H*-thieno[3′,4′*d*]imidazole-4′-pentanoyl]hydrazine (SQB) of the thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) receptor antagonist [1*S*-[1α,2α(*Z*),3α,4α]]-7-[3-[[[[(1-oxocyclohexylpropyl)amino]acetyl]amino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (SQ31,491). SQB was synthesized by reacting SQ31,491 with biotin hydrazide, and the product was purified by flash chromatography. It was found that SQB specifically inhibited platelet aggregation in response to U46619 with an IC_{50} of 275 nM. On the other hand, SQB did not inhibit adenosine diphosphate or A23187-induced aggregation. Competition binding studies revealed that SQB produced a concentration-dependent inhibition of [^{3}H]-[1*S*-[1α,2β(5*Z*),3β,4α]]-7-[3-[[2[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid ([^{3}H]SQ29,548) specific binding in 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)-solubilized platelet membranes, with a K_i of 220 nM. The shape of the SQB inhibition binding curve was indistinguishable from that produced by the TXA_2/PGH_2 receptor antagonist BM13.177. Finally, incubation of gel-filtered platelets or platelet-rich plasma with SQB and fluorescein isothiocyanate (FITC)-avidin demonstrated fluorescent labeling of platelet plasma membrane TXA_2/PGH_2 receptors. Furthermore, this SQB-FITC fluorescent labeling was reduced significantly by co-incubation of the platelets with the TXA_2/PGH_2 antagonist SQ29,548. Based on the ability of SQB-FITC-avidin to label intact platelets, it can be concluded: (1) that a pool of platelet TXA_2/PGH_2 receptors resides in the plasma membrane; and (2) that the binding domains for these receptors are oriented at or near the external membrane surface. Collectively, these data demonstrate that SQB is a highly specific probe for TXA_2/PGH_2 receptors, which should be of significant value for receptor localization studies in platelets and other tissues. BIOCHEM PHARMACOL 52;5:763–770, 1996.

KEY WORDS. SQB; TXA_2/PGH_2 receptor; receptor binding domains; biotinylated receptor probe

The arachidonic acid metabolites TXA_2† and PGH_2 [1, 2] are known to be intimately involved in the process of hemostasis as well as the development of thromboembolic disorders [3–5]. Thus, interaction of TXA_2/PGH_2 with a membrane-associated receptor [6–13] leads to the full range of platelet responses including shape change, aggregation, and granular secretion. Although the precise mechanisms associated with this activation response are presently unknown, previous studies have demonstrated that occupation of platelet TXA_2/PGH_2 receptors leads to the mobilization of intraplatelet calcium stores [14–18]. In this regard, evidence has been provided that the TXA_2/PGH_2 receptor protein functions in close association with at least one G-protein, i.e. G_q [19, 20], resulting in activation of phospholipase C (PLC) [21–23]. In this model, TXA_2/PGH_2-mediated PLC activation leads to the generation of inositol triphosphate (IP_3), which in turn mobilizes calcium from internal storage pools, i.e. the platelet dense tubular system (DTS) [24]. On the other hand, separate studies have suggested that subclasses of TXA_2/PGH_2 receptors may exist in platelets [25–27], and that these subclasses may be involved in separate functional responses, i.e. shape change and aggregation. Thus, in addition to being coupled to PLC, evidence has been provided that activation of the TXA_2/PGH_2 receptor is directly involved in calcium release from

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† *Abbreviations:* TXA_2, thromboxane A_2; PGH_2, prostaglandin H_2; SQ29,548, [1*S*-[1α,2β(5*Z*),3β,4α]]-7-[3-[[2[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; SQB, 1-[[1*S*-[1α,2α(*Z*),3α,4α]]-7-[3-[[[[(1-oxocyclohexylpropyl)amino]-acetyl]amino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoyl]-2-[hexahydro-2′-oxo-1*H*-thieno[3′,4-*d*]imidazole-4′-pentanoyl]hydrazine; U46619, 15(*S*)-hydroxy-11,9-epoxymethano-prosta-5*Z*,13*E*-dienoic acid; BM 13.177, 4-[2-(benzenesulfonamido)-ethyl]phenoxyacetic acid; FITC, fluorescein isothiocyanate; MDS, molecular dynamics simulation; and SQ31,491, [1*S*-[1α,2α(*Z*),3α,4α]]-7-[3-[[[[(1-oxocyclohexylpropyl) amino]acetyl]amino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid.

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the DTS [28, 29] independent of IP_3 formation [30, 31]. This direct action of TXA_2/PGH_2 is also supported by the finding that the DTS contains both cyclo-oxygenase and thromboxane synthase activity [32], and is therefore capable of synthesizing both PGH_2 and TXA_2.

Consequently, the evidence to date suggests that TXA_2/PGH_2 receptors function to elevate the levels of intraplatelet calcium, and that this is the primary mechanism by which platelet activation occurs through the TXA_2/PGH_2 signal transduction pathway. On the other hand, definitive documentation supporting the location of TXA_2/PGH_2 receptors (or their subpopulations) on platelet plasma membranes and/or internal membrane structures remains to be established.

Based on these considerations, experiments were undertaken to develop a novel probe for TXA_2/PGH_2 receptor localization. Specifically, the TXA_2/PGH_2 receptor antagonist SQ31,491 [33] was coupled at the carboxyl terminal with biotin to form the new biotinylated derivative SQB. This compound was demonstrated to be a highly selective inhibitor of TXA_2/PGH_2-mediated platelet aggregation and [^{3}H]SQ29,548 binding. Furthermore, fluorescence measurements of platelets preincubated with SQB-FITC-avidin demonstrated specific ligand association with platelet plasma membranes. These findings suggest that SQB will prove to be a valuable probe for localizing TXA_2/PGH_2 receptors on platelet membrane structures.

MATERIALS AND METHODS

Materials

Ethyl-[1S-[1α,2α(Z),3α,4α]]-7-[[3-aminomethyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoate hydrochloride and SQ29,548 were provided by the Squibb Institute for Medical Research, Princeton, NJ. BM13.177 was a generous gift from Boehringer Mannheim, Mannheim, Germany. SQ31,491 was synthesized as previously described [33, 34]. Biotin hydrazide, ADP, A23187, U46619, BSA (once recrystallized, essentially globulin free), goat anti-biotin IgG, biotinylated rabbit anti-goat IgG, rhodamine-avidin, and poly-L-lysine were purchased from the Sigma Chemical Co., St. Louis, MO. [^{3}H]SQ29,548 was obtained from DuPont (Boston, MA), and GF/B filters were from Whatman (Hillsboro, OR). Sepharose 2B was purchased from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). Human platelet-rich plasma was obtained from Life Source Blood Services (Chicago, IL). FITC conjugated avidin was purchased from Vector Laboratories (Burlingame, CA). Paraformaldehyde was obtained from Polysciences (Warrington, PA) and Sepharose CL-2B was obtained from Pharmacia, Inc. (Piscataway, NJ). All other chemicals were reagent grade.

Synthesis of SQB

The SQB analog was synthesized as summarized in Fig. 1. Dimethylformamide (DMF) was distilled from potassium hydride and dichloromethane (CH_2Cl_2) from phosphorus pentoxide. Both solvents were stored over activated 4Å molecular sieves under nitrogen. To a stirred solution of SQ31,491 (5 mg, 10.8 μmol) in CH_2Cl_2 (0.4 mL), 1-methylpiperidine (12.96 μmol, 1.43 μL) was added under nitrogen. The mixture was then cooled in an acetone-dry ice bath (−78°), and isobutyl chloroformate (12.96 μmol, 1.68 μL) was added. Forty-five minutes later, the reaction was warmed to −25° and stirred for an additional 35 min. A pre-warmed (80°) solution of biotin hydrazide (11 mg, 42.6 μmol) in DMF (1.5 mL) was added under nitrogen. This reaction mixture was stirred (−25° for 30 min) and then allowed to warm to room temperature. The residue was diluted with CH_2Cl_2, washed twice with saturated NaCl, dried with $MgSO_4$, filtered, and concentrated. The crude product was subjected to flash chromatography [$CHCl_3$/MeOH, 90/10; R_f ($CHCl_3$/MeOH, 80/20) = 0.6], and the purity of the biotinylated derivative was established by reverse-phase HPLC. Chemical ionization mass spectroscopy of the purified derivative yielded *m/e* 703(M + 1).

Measurement of Platelet Aggregation

Platelet-rich plasma was isolated from CPD (citrate-phosphate-dextrose)-anticoagulated human blood as described [35], and platelet aggregation in response to U46619 (0.6 μM), ADP (2 μM), or the divalent cation ionophore A23187 (4 μM) was measured by the turbidimetric method [36] using a model 400 Lumi-aggregometer (Chronolog Corp., Havertown, PA). In aggregation experiments utilizing ADP and A23187 as the agonists, the platelet-rich plasma was pretreated with indomethacin (20 μM) to inhibit endogenous TXA_2/PGH_2 production. The effect of SQB or SQ29,548 on platelet aggregation was determined by incubating various concentrations of the inhibitors with platelet-rich plasma for 5 min prior to the addition of agonists.

[^{3}H]SQ29,548 Receptor Binding Assay

Evaluation of [^{3}H]SQ29,548 binding to solubilized platelet TXA_2/PGH_2 receptors was performed by glass fiber filtration as previously described [34]. Specifically, the solubilized receptors were immobilized on GF/B filters (presoaked in 0.3% polyethyleneimine) by filtering 200–400 μL of the solubilized protein under vacuum. The filters were washed (2 × 5 mL) with buffer (25 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4, 4°), and the vacuum was released. Buffer (60 μL) was applied to the filter, followed by either 20 μL of buffer (total binding), 20 μL of competing substances (competition binding), or 20 μL of cold SQ29,548 (2 μM, nonspecific binding). After a 5-min incubation period, 20 μL of [^{3}H]SQ29,548 (2 nM) was applied to the filter and allowed to incubate for an additional 20 min at room temperature. The filters were then washed (two times) with 5 mL of buffer (4°), and the radioactivity on the filter was measured by liquid scintillation spectrometry (Beckman LS 6800). Under these conditions, [^{3}H]SQ29,548 specific binding was determined to be 85%.

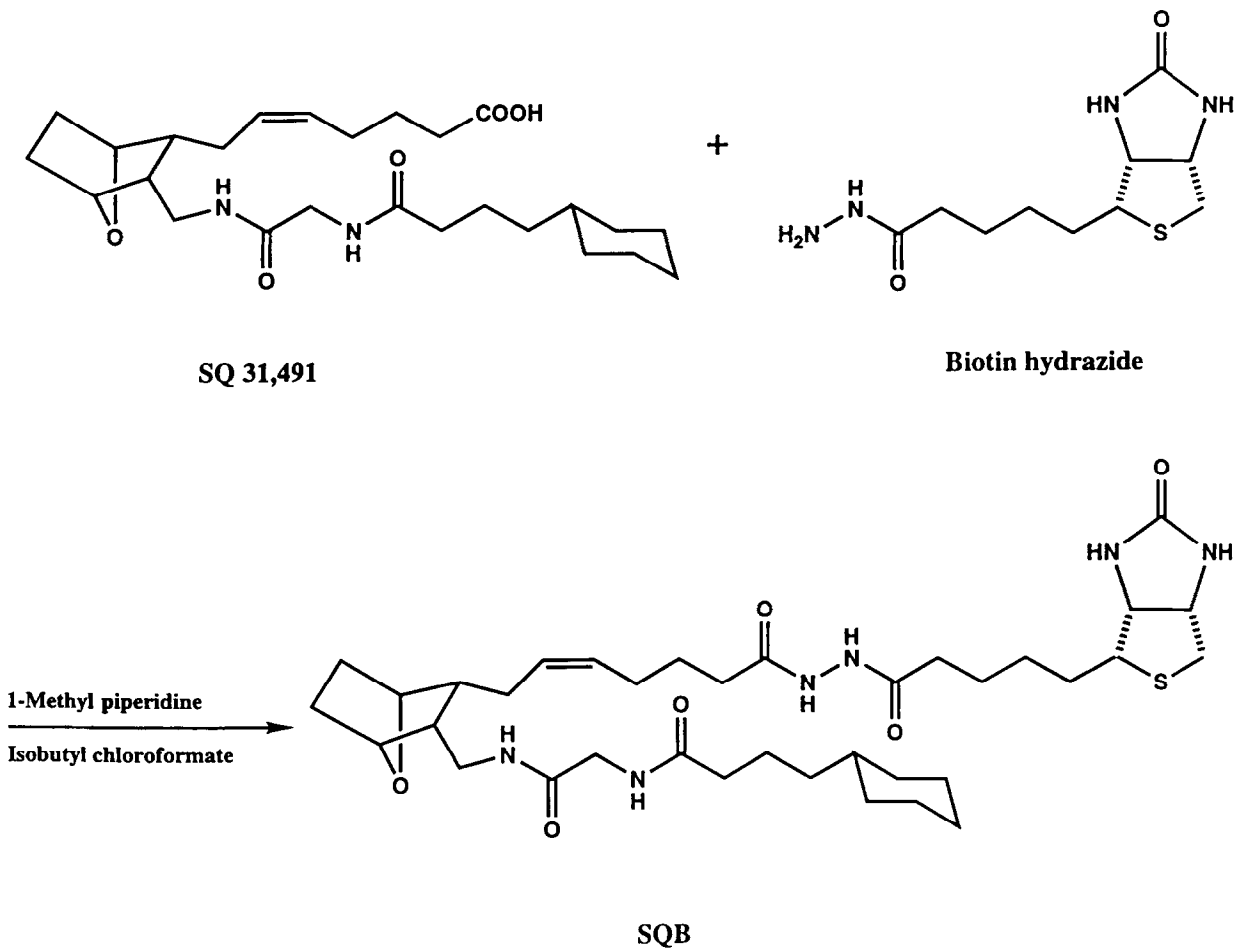

FIG. 1. Synthetic scheme for SQB.

Measurement of Platelet-Associated SQB–FITC Fluorescence

Platelet SQB fluorescence was determined using a photon-counting microspectrofluorometer [14]. Briefly, platelets were isolated from acid-citrate dextrose-anticoagulated human blood by differential centrifugation followed by gel filtration on Sepharose 2B and resuspension in modified Tyrode's buffer as described [37]. Resting platelets (1.6×10^9/mL) were incubated with 2 μM SQB (plus SQ29,548 vehicle) for 15 min at 22°. In competition experiments, platelets were co-incubated with 2 μM SQB and 25 μM SQ29,548 for 15 min at 22°. Following staining with 5 μL FITC-avidin (75 nM) for an additional 15 min at 22°, the platelets were sedimented through silicone oil, and cell-associated fluorescence was determined by microspectrofluorometry as previously described [14], using a 4× objective at excitation and emission wavelengths of 487 and 531 nm, respectively.

In separate experiments, the association of SQB with platelets in their native plasma was evaluated. This was done to avoid the possibility that gel filtration of platelets may, in some way, alter the distribution or orientation of the membrane-associated TXA_2/PGH_2 receptor protein. In these studies, platelet-rich plasma was incubated with 1 μM SQB (plus SQ29,548 vehicle) for 15 min at 22°. In separate competition experiments, the platelet-rich plasma was incubated with 1 μM SQB plus 10 μM SQ29,548 for 15 min at 22°. Following staining with 20 μL FITC-avidin (300 nM) for an additional 10 min at 22°, 1-mL samples of the platelet-rich plasma were filtered rapidly through 7 mm GF/B filters (presoaked with 3% BSA for 12 hr to decrease nonspecific protein binding). The platelets remaining on the filters were then washed rapidly with 2 mL of Modified Tyrode's buffer, and the filter manifold was positioned under the microspectrofluorometer objective (4×) for FITC fluorescence determinations.

Immunofluorescence Microscopy

Immunofluorescent staining of gel-filtered platelets was performed as previously described [38]. Briefly, resting platelets were fixed with 1% paraformaldehyde on ice for 1 hr. Unreacted aldehyde was blocked with NH_4Cl-Tris-buffered saline, pH 7.4, and the cells were permitted to settle on polylysine-coated glass coverslips. The intact cells were then rinsed with Tris-buffered saline containing 0.1%

BSA and incubated for 20 min with 25 μM SQB. Following rinsing, cells were stained sequentially with goat anti-biotin IgG, biotinylated rabbit anti-goat IgG followed by rhodamine-avidin, and were mounted on a droplet of FITC-Guard (Testog Inc., Chicago, IL). Platelets were viewed with a Jenval phase/fluorescence microscope equipped with an HBO 50-W mercury lamp, and an IVF1 epifluorescence condenser with BP 485 and 546 excitation filters, BP 520-560 and LP590 barrier filters and were photographed with Tri-X panchromatic film.

MDS

MDS with annealing was used to establish the maximum upper chain length corresponding to a minimum energy conformation. The MM2 force field with extended parametrization was used, and the extended conformation shown in Fig. 8 is 2.1 kcal/mol above the global energy minimum realized from the MDS trajectory. The statistical average length of the upper side chain will therefore be less than the maximum length shown.

RESULTS

Figure 2 illustrates the effects of SQB on human platelet aggregation. It can be seen that concentrations of SQB in the range of 275–500 nM produced concentration-dependent inhibition of U46619 (0.6 μM)-induced aggregation, with 50% inhibition (IC_{50}) occurring at approximately 275 nM SQB. On the other hand, SQB had no effect on aggregation induced by ADP (2 μM) or A23187 (4 μM) even at concentrations as high as 5 μM (Fig. 3). For comparative purposes, aggregation experiments were also performed using the well-characterized TXA_2/PGH_2 receptor antagonist SQ29,548 [12] as the inhibitor. It was found that SQ29,548 produced 50% inhibition of U46619-induced aggregation at approximately 5 nM, and did not interfere with ADP- or A23187-induced aggregation even at 50 nM. These results therefore establish that the pharmacological profile of SQB for inhibition of platelet aggregation is identical to that of SQ29,548. Thus, the coupling of biotin to SQ31,491 did not alter the specificity profile of the molecule for inhibition of TXA_2/PGH_2-mediated platelet aggregation.

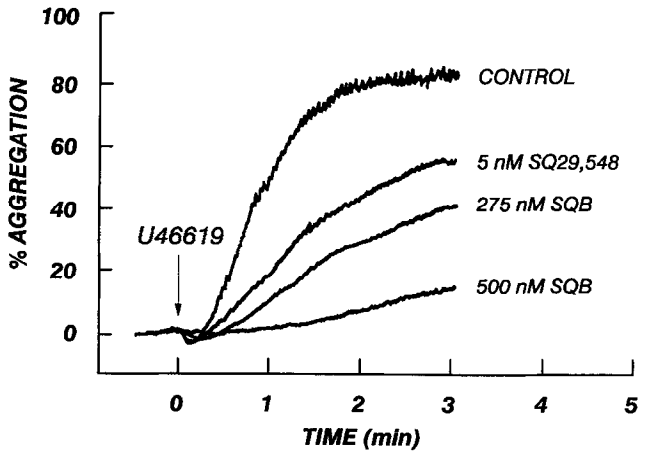

FIG. 2. Effect of SQB on U46619-induced human platelet aggregation. Platelet-rich plasma was incubated with 275 nM SQB, 500 nM SQB, 5 nM SQ29,548, or vehicle for 5 min prior to addition of U46619 (0.6 μM). These aggregation curves are representative of multiple traces obtained from three separate blood donors.

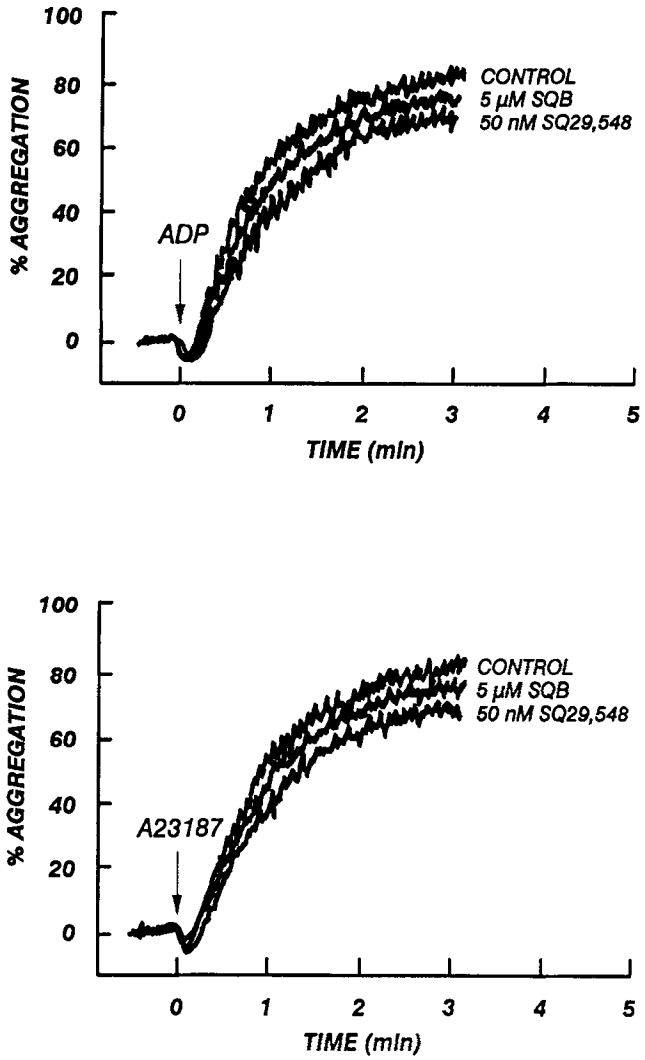

FIG. 3. Effect of SQB on ADP- or A23187-induced human platelet aggregation. Platelet-rich plasma was incubated with 5 μM SQB, 50 nM SQ29,548, or vehicle prior to addition of ADP (2 μM) (top panel) or A23187 (4 μM) (bottom panel). These aggregation curves are representative of multiple traces obtained from three separate blood donors.

Radiolabeled binding experiments were next undertaken to determine whether SQB produced its inhibitory effects through direct antagonism at the level of the platelet TXA_2/PGH_2 receptor. In these studies, platelet TXA_2/PGH_2 receptors were solubilized, and the ability of SQB to compete for [^{3}H]SQ29,548 (2 nM) binding was determined. It can be seen (Fig. 4) that the SQB inhibition

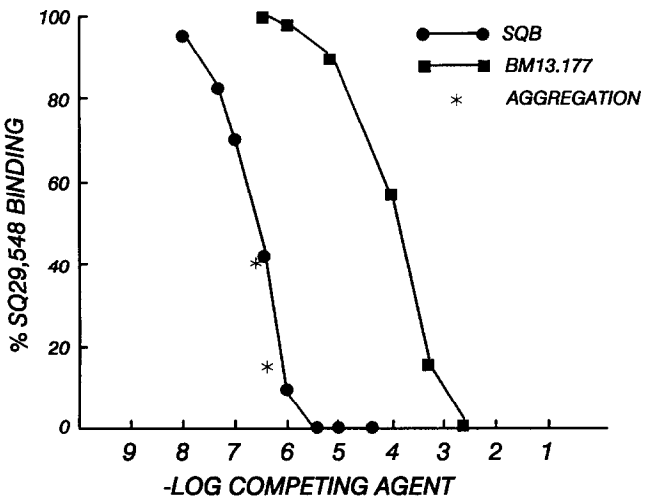

FIG. 4. Competition binding curves of SQB and BM13.177 for solubilized platelet TXA_2/PGH_2 receptors. The effects of various SQB and BM13.177 concentrations on [^{3}H]SQ29,548 binding were determined in 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)-solubilized platelet membranes. See Materials and Methods for details. The asterisks refer to the percent of U46619-aggregation observed at the corresponding SQB concentrations (275 and 500 nM). Each point represents the mean of triplicate values obtained from three separate blood donors.

binding curve is a characteristic sigmoidal shape indicative of direct competition for the TXA_2/PGH_2 receptor site. For comparative purposes, parallel binding studies were also performed using a structurally unrelated TXA_2/PGH_2 receptor antagonist, BM13.177 [9]. It can be seen (Fig. 4) that even though BM13.177 was found to be approximately 2.5 log units less potent than SQB in competing for [^{3}H]SQ29,548 binding, the shape of its binding inhibition curve was indistinguishable from that associated with SQB. Furthermore, transformation of the SQB inhibition binding curve by the Cheng–Prusoff relationship [11] yielded a K_i for SQB of 220 nM, which is in good agreement with the IC_{50} = 275 nM value for inhibition of U46619-induced platelet aggregation.

In subsequent experiments, SQB was evaluated for its ability to fluorometrically label TXA_2/PGH_2 receptors in intact platelets. Specifically, gel-filtered platelets were pre-incubated with SQB (2 μM) for 15 min, followed by incubation with FITC-avidin. The platelets were sedimented by centrifugation, and the supernatant was aspirated. Platelet-associated SQB–FITC fluorescence was then determined by the procedures described in Materials and Methods. As can be seen in Fig. 5, microspectrofluorometric examination of the SQB-treated platelets revealed a dramatic increase (650%) in platelet fluorescence relative to FITC-avidin-treated control platelets, i.e. an increase from 2550 cps to 16,410 cps. This finding provides direct visual evidence for the interaction of SQB with human platelet membranes. Furthermore, the specificity of SQB in directly labeling TXA_2/PGH_2 receptors was demonstrated by the finding that co-incubation with the competing TXA_2/PGH_2 antagonist, SQ29,548 (25 μM), resulted in a 76% reduction in the SQB–FITC associated fluorescence intensity.

The ability of SQB to specifically label TXA_2/PGH_2 receptors on intact platelets was confirmed by rapid filtration experiments using platelet-rich plasma. Thus, Fig. 6 illustrates that incubation of platelet-rich plasma with 1 μM SQB also resulted in a substantial increase (500%) in platelet-associated SQB–FITC fluorescence, i.e. from 12,530 cps to 63, 270 cps. As was observed with gel-filtered platelets, competition with the TXA_2/PGH_2 receptor antagonist SQ29,548 (10 μM) resulted in a significant reduction (61%) in the SQB–FITC platelet fluorescence levels.

Finally, the ability of SQB to label platelet TXA_2/PGH_2 receptors was visualized by immunofluorescence microscopy. It was found (Fig. 7) that incubation of gel-filtered platelets with SQB resulted in a fluorescent rim staining pattern consistent with plasma membrane receptor localization.

The above experiments investigating platelet aggregation, radioligand binding, and fluorescent labeling therefore establish that SQB specifically binds to the human platelet TXA_2/PGH_2 receptor protein.

DISCUSSION

The present study reports the synthesis, characterization, and application of a new biotinylated probe for platelet TXA_2/PGH_2 receptors. In this regard, SQB was found to be a highly specific inhibitor of TXA_2/PGH_2-mediated platelet aggregation. Thus, SQB blocked aggregation induced by the TXA_2/PGH_2 mimetic U46619 with an IC_{50} of 275 nM, but had no significant effect on aggregation induced by either ADP or the divalent cation ionophore A23187. This finding establishes that the inhibitory properties of SQB are specific to the TXA_2/PGH_2-signal transduction pathway. In separate radioligand binding experiments using solubilized TXA_2/PGH_2 receptors, it was found that SQB effectively competed for [^{3}H]SQ29,548 binding to the receptor protein, and the shape of the binding inhibition curve was comparable to that produced by the known TXA_2/PGH_2 receptor antagonist BM13.177. Furthermore, application of the Cheng–Prusoff relationship to this SQB inhibition binding curve yielded a K_i of 220 nM. The close agreement between the IC_{50} for inhibition of aggregation (275 nM) and the K_i for inhibition of [^{3}H]SQ29,548 binding (220 nM) provides evidence that SQB specifically interacts with the TXA_2/PGH_2 receptor-ligand binding domain(s), and that this receptor interaction is the mechanism by which SQB inhibits TXA_2/PGH_2-mediated platelet aggregation.

Based on these findings, experiments were conducted to determine whether SQB was capable of fluorometrically labeling TXA_2/PGH_2 receptors in intact gel-filtered platelets or platelets in their native plasma. It was found that incubation of platelets with SQB did indeed result in significant fluorescent labeling in both gel-filtered platelets

and platelet-rich plasma. The specificity of this labeling for TXA_2/PGH_2 receptors was demonstrated, in turn, by the finding that competition with the well-characterized TXA_2/PGH_2 antagonist SQ29,548 substantially reduced the fluorescence labeling intensity.

The implications inherent in these fluorescent labeling experiments are 2-fold. First, because of a molecular size of 68 kDa, avidin does not have access to internal platelet membrane components. This notion of plasma membrane labeling was confirmed by our findings (Fig. 7) that SQB-treated platelets revealed a fluorescence rim-staining pattern. Consequently, the ability of the SQB–avidin complex to label intact platelets constitutes the first direct evidence for the existence of TXA_2/PGH_2 receptors on platelet plasma membranes. Second, these studies provide evidence that the ligand binding domain(s) for platelet plasma membrane TXA_2/PGH_2 receptors cannot reside in the cytoplasmic regions of the receptor protein. This conclusion is based on MDSs performed on SQB. Specifically, MDS revealed that the maximum molecular distance from the avidin binding region on the biotin functional group to the bicyclic head group of SQB is 20.5 Å (Fig. 8). However, since this distance of 20.5 Å represents the extended conformation of the molecule, the statistical average length of the upper side chain will actually be less than that shown. Furthermore, it is also necessary to consider that the biotin functional group must project a certain distance above the plasma membrane to permit binding to avidin (Fig. 9). Thus, the maximum allowable depth of insertion of the SQB molecule into the plasma membrane would be considerably less than 20.5 Å. On the other hand, the width of platelet plasma membranes is typically 70–90 Å [39]. Consequently, the molecular span of SQB would not permit interaction with a cytoplasmic oriented ligand binding domain(s) on this receptor protein. Based on these considerations, and the demonstrated ability of avidin to complex

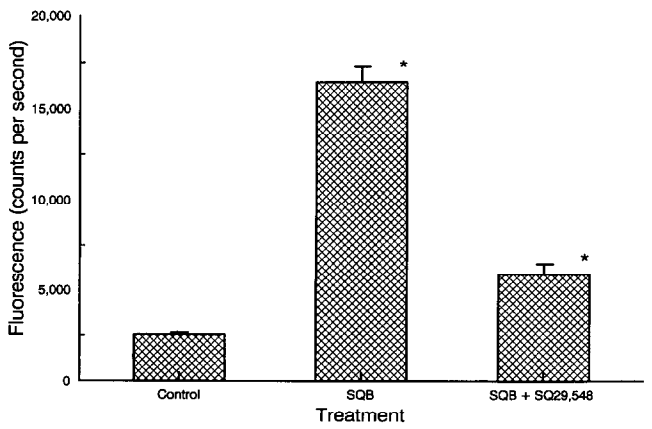

FIG. 5. SQB labeling of TXA_2/PGH_2 receptors in intact platelets. Gel-filtered platelets were incubated with SQB and FITC-avidin in the absence or presence of the TXA_2/PGH_2 receptor antagonist SQ29,548. See Materials and Methods for details. Each bar value represents the fluorescence mean ± SEM of triplicate values obtained from three separate blood donors. Key: (*) $P < 0.001$.

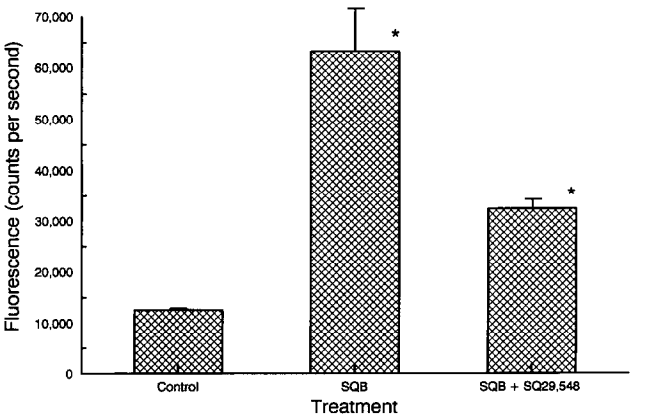

FIG. 6. SQB labeling of TXA_2/PGH_2 receptors in platelet-rich plasma. Platelet-rich plasma was incubated with 1 μM SQB and FITC-avidin in the absence or presence of the TXA_2/PGH_2 receptor antagonist SQ29,548. See Materials and Methods for details. Each bar value represents the mean ± SEM of quadruplicate values obtained from three separate blood donors. Key: (*) $P < 0.02$.

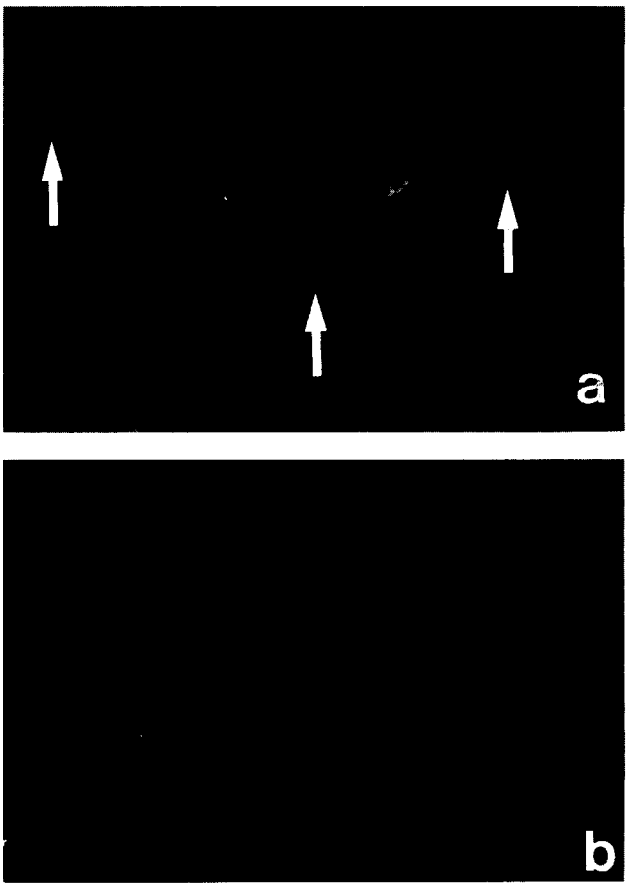

FIG. 7. Fluorescence micrographs of platelets labeled with SQB. Panel a: gel-filtered platelets were incubated with SQB, and stained sequentially with goat anti-biotin IgG, biotinylated rabbit anti-goat IgG, followed by rhodamine-avidin. Panel b: gel-filtered platelets not pretreated with SQB were stained sequentially with goat anti-biotin IgG, biotinylated rabbit anti-goat IgG, followed by rhodamine-avidin. Micrographs are representative of the results obtained from three separate experiments; magnification = 1500×.

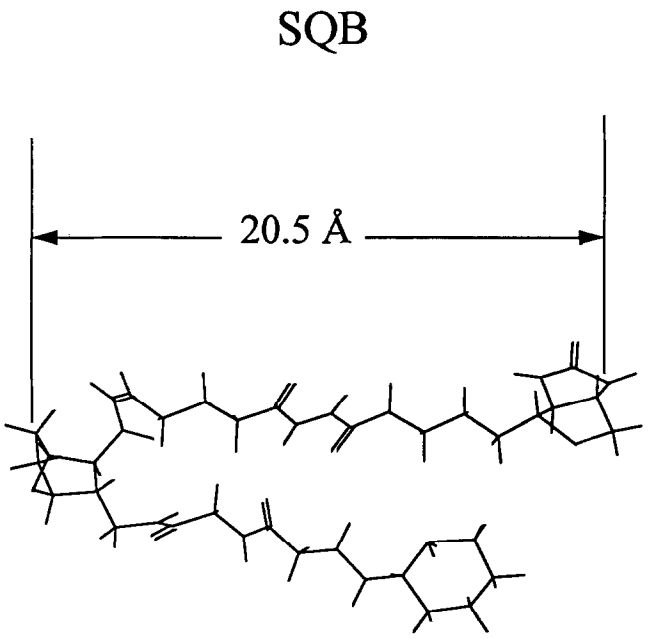

FIG. 8. Estimation of maximum SQB upper chain length using molecular dynamics simulation (see Materials and Methods for details).

with receptor-associated SQB, one must therefore conclude that the binding domain(s) for the TXA_2/PGH_2 receptor protein resides at or near the external aspect of the platelet plasma membrane, i.e. at a depth of less than 20.5 Å.

While these studies establish the presence of TXA_2/PGH_2 receptors on platelet plasma membranes, they do not preclude the existence of different TXA_2/PGH_2 receptor populations in association with separate, internal platelet membrane structures. As previously mentioned, the platelet DTS not only contains the bulk of internally releasable calcium, but it also possesses both cyclo-oxygenase and thromboxane synthase activity. Thus, this single internal structure has the synthetic capability to generate TXA_2 and to serve as its target for receptor-mediated calcium release.

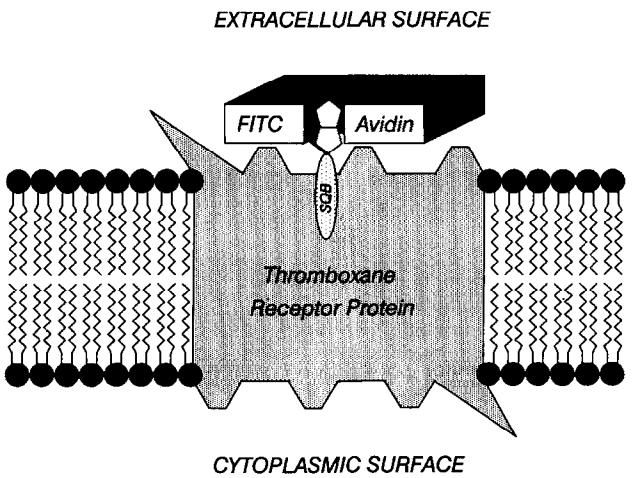

FIG. 9. Schematic representation of SQB–FITC-avidin association with platelet plasma membrane TXA_2/PGH_2 receptors.

Clearly, additional studies will be required to address this interesting possibility.

In summary, SQB has been shown to bind to human platelet TXA_2/PGH_2 receptors in a highly specific and competitive fashion. Furthermore, the present use of this probe has established that at least one pool of platelet TXA_2/PGH_2 receptors resides in the plasma membrane compartment, and that the ligand binding domain(s) for this receptor pool is presumably oriented near the extracellular membrane surface. Collectively, these findings indicate that SQB should provide a novel approach for evaluating the cellular localization/orientation of TXA_2/PGH_2 receptors, or for tracking possible receptor redistribution as a consequence of cellular activation.

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