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PURIFICATION OF THE HUMAN BLOOD PLATELET THROMBOXANE A_2/PROSTAGLANDIN H_2 RECEPTOR PROTEIN

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Abstract—The human platelet thromboxane A_2/prostaglandin H_2 receptor has been purified 6100-fold to apparent homogeneity by a three-step chromatographic procedure with an overall yield of 6%. A 6-fold purification of the receptor was first achieved by chromatography of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)-solubilized membrane proteins from human platelets on a diethylaminoethyl (DEAE)-Sepharose column. The DEAE eluate fractions containing receptor activity were then applied to a newly developed affinity column using the cyclohexyl derivative of SQ30,741 (SQ31,491) as the immobilized ligand. Elution of the receptor from the affinity column with BM13.177 yielded a further purification of 1700-fold. An additional 4-fold receptor purification from the affinity column eluate was achieved by HPLC using GPC 500 and GPC 100 columns connected in tandem. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and silver staining of the HPLC eluate containing purified receptor revealed a single, distinct band with a molecular weight of 55,000. The receptor binding activity was detected with [^{3}H]SQ29,548 using a newly developed binding assay which involved immobilization of the receptor on polyethyleneimine-treated glass fiber filters. The binding of [^{3}H]SQ29,548 to the purified receptor was time dependent, saturable, reversible and highly specific. Unlabeled SQ29,548, BM13.505, and U46619 (but not thromboxane B_2 or 6-keto prostaglandin $F_{1\alpha}$) competed for [^{3}H]SQ29,548 binding to the purified receptor in a concentration-dependent manner. Scatchard analysis of [^{3}H]SQ29,548 binding to the purified receptor revealed the presence of a single class of high-affinity binding sites, with a K_d of 4 nM and a B_{max} of 17 nmol/mg protein.

The arachidonic acid metabolites prostaglandin H_2 (PGH_2‡) and thromboxane A_2 (TXA_2) are potent vasoconstrictors and platelet-aggregating agents [1, 2] which are thought to be involved in a variety of pathological conditions including thrombosis, atherosclerosis and angina [3, 4]. On this basis, considerable research effort has been directed toward elucidating the underlying mechanisms associated with TXA_2/PGH_2-induced cellular activation. Due to the relatively short biological half-life of TXA_2 and PGH_2, these studies have for the most part been limited to the use of stable TXA_2/PGH_2 mimetics, e.g. U46619, U44069 [5], I-BOP [6], and antagonists, e.g. 13-APA [7, 8], BM13.177 [9], I-PTA-OH [10], SQ29,548 [11], and S-145 [12]. Experiments employing tritiated derivatives of 13-APA [13] and U44069 [14] provided the first demonstration for the existence of discrete TXA_2/PGH_2 receptors associated with the human platelet membrane compartment. Similar results have been obtained utilizing other radiolabeled analogs, e.g. [^{125}I]PTA-OH [15], [^{3}H]U46619 [16], [^{3}H]SQ29,548 [17], [^{3}H]S-145 [18], and [^{125}I]BOP [6].

Although the precise mechanism by which TXA_2/PGH_2 receptor interaction leads to platelet activation is unclear, various studies have implicated elevations in intraplatelet calcium levels [19–21] as the signal transduction event. This intraplatelet calcium mobilization may directly derive from the platelet dense tubular system [22, 23], from activation of phosphoinositide metabolism [24, 25], or possibly from activation of calcium channels [26].

Separate studies have attempted to characterize the TXA_2/PGH_2 receptor itself. In whole platelets, photoaffinity labeling with azido [^{125}I]PTA-OH indicated receptor components in the range of 43, 39 and 27 kDa [27]. Membrane solubilization [28] followed by isoelectric focusing and photoaffinity labeling with azido [^{125}I]PTA-OH indicated receptor components comprised of 49 and 27 kDa proteins [29]. On the other hand, attempts to purify the platelet TXA_2/PGH_2 receptor have yielded different

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‡ Abbreviations: PGH_2, prostaglandin H_2; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio] - 1 - propanesulfonate; EGTA, ethyleneglycol-bis(β-amino-ethyl ether) *N,N'*-tetra-acetic acid; DEAE, diethylaminoethyl; 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; U46619, 15(*S*)-hydroxy-11,9-epoxymethano-prosta-5*Z*,13*E*- dienoic acid; BM 13.505, 4(2-(*p*-chloro-benzene sulfanylamino)-ethylbenzene acetic acid; BM 13.177, 4-[2-(benzenesulfonamido)-ethyl]-phenoxy-acetic acid; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; 6-keto $PGF_{1\alpha}$, 6-keto prostaglandin $F_{1\alpha}$; 13-APA, 13-azaprostanoic acid; S-145, 5*Z*-7-(3-endo-phenylsulfonylaminobicyclo[2.2.1]hept - 2 - exo - yl)hept-enoic acid; and PEI, polyethyleneimine.

molecular weight assignments. Specifically, subjecting solubilized platelet membranes to various procedures including affinity chromatography (using S-145) and size exclusion HPLC resulted in the purification of a 57 kDa protein to apparent homogeneity [30]. Using an oligonucleotide probe encoding a partial sequence of this protein, a human placental TXA_2 receptor was subsequently cloned and expressed in COS-7 cells [31]. The molecular weight value of 57,000 approximates a 60 kDa protein previously reported by us for the receptor in platelet membranes partially purified by size exclusion HPLC [32, 33].

Based on these disparate molecular weight assignments, a separate procedure was developed for purification of the platelet TXA_2/PGH_2 receptor protein. To this end, a new affinity column was prepared using the cyclohexyl derivative of SQ30,741 (SQ31,491), which has been shown to specifically antagonize TXA_2/PGH_2 platelet activation [34]. Utilization of this affinity column, in combination with diethylaminoethyl (DEAE) chromatography and size exclusion HPLC, resulted in the purification of a single protein of 55 kDa.

EXPERIMENTAL PROCEDURES

Materials

Out-dated platelet concentrates were obtained from Life Source Blood Services (Chicago), SQ29,548 and SQ intermediate (ethyl-[1*S*-[1α,2α(*Z*),3α,4α]] - 7 - [[3 - aminomethyl] - 7 - oxabicyclo[2.2.1]hept-2-yl]-5-heptenoate) for the synthesis of SQ31,491, ligand used for the affinity column, were provided by the Squibb Institute for Medical Research (NJ). BM13.177 and BM13.505 were provided by Dr. K. Stegmeier, Boehringer Mannheim GmbH (Mannheim, Germany). [^{3}H]SQ29,548 was purchased from DuPont (Boston, MA); U46619, CHAPS, and DEAE-Sepharose 6B were from the Sigma Chemical Co. (St. Louis, MO); TXB_2 and 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) was from the Upjohn Co. (Kalamazoo, MI); Affi-Gel 102, high molecular weight standards for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and dye reagents for protein assays were from Bio-Rad (Richmond, CA); HPLC GPC 100 column (macrosphere, 7 μm) was from Alltech (Deerfield, IL); HPLC GPC 500 (synchropak, 10 μm) from Synchrom, Inc. (Linden, IN); Asolectin from the American Lecithin Co. (Atlanta, GA); and Millex-HA filter unit (0.45 μm) from the Millipore Co. (Bedford, MA). GF/B filters were from Whatman (Hillsboro, OR).

Membrane preparation and solubilization

Typically, 12 units of out-dated human platelet concentrates (total volume: 600 mL) were pooled and incubated with 3 mM aspirin for 15 min to irreversibly block the platelet cyclo-oxygenase. The platelet concentrate was centrifuged at 160 *g* for 10 min to remove residual red blood cells, and prostacyclin (40 nM) was added to the concentrate to aid in resuspension of the platelets [35]. The platelets were pelleted by centrifugation at 1600 *g* for 20 min. The platelet pellet was suspended in 10% of the original volume in buffer A (25 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.4, 4°) plus prostacyclin (40 nM), and then centrifuged at 1600 *g* for 15 min. The resulting platelet pellet was then resuspended in 5% of the original volume using buffer A, sonicated (four 30-sec bursts with 30-sec intervals), and centrifuged at 1600 *g* for 5 min. The supernatant was transferred to a centrifuge tube, and the pellet was again suspended, sonicated, and centrifuged as described above. The supernatants were then combined and centrifuged at 100,000 *g* for 30 min (4°). The resulting platelet membrane pellet was solubilized by homogenization (10 strokes with a Teflon homogenizer) in 24 mL of buffer B (50 mM Tris–HCl, 10 mM CHAPS, 5 mM $MgCl_2$, pH 7.4, 4°). The solubilized mixture was centrifuged at 100,000 *g* for 30 min at 4°. This supernatant (containing approximately 2–3 mg/mL protein) was then used as the solubilized receptor preparation for further purification. The above procedure resulted in a 60–70% solubilization of the platelet membrane protein.

Receptor purification

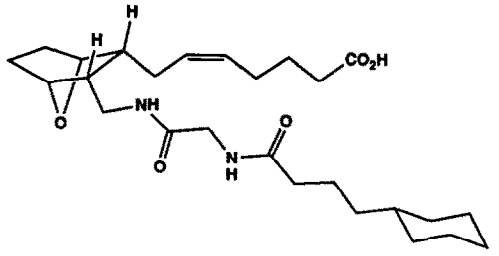
The following buffers were used for the various chromatography steps: buffer D: 20 mM Tris–HCl, 10 mM CHAPS, 20% glycerol, 500 mM KCl, 0.2 mM EGTA, 0.5 mg/mL Asolectin, pH 7.4; buffer E: buffer D containing 50 mM KCl; and buffer F: buffer D containing 100 mM KCl.

DEAE-Sepharose chromatography

Solubilized membranes (15 mL) were supplemented with KCl (50 mM final concentration) and Asolectin (0.5 mg/mL) and applied to a DEAE-Sepharose column (1.5 × 8 cm) equilibrated with buffer E. The column was washed with buffer E until all unbound proteins were eluted, and a linear gradient from 50 to 300 mM KCl (50 mL each) was then applied. The column eluate was monitored for UV absorbance at 280 nm, and fractions (4 mL) were collected for the measurement of specific binding activity, which is expressed as picomoles per milligram of protein. The fractions (15–21) having high receptor binding activity were combined and used for further purification by the affinity column.

Affinity chromatography

*Synthesis of affinity column ligand, [*1S-*[1*α*,2*α*(Z),3*α*,4*α*]] - 7 - [3 - [[[[(1 - oxocyclohexylpropyl)amino]acetyl]amino]methyl] - 7 - oxabicyclo-[2.2.1]hept-2-yl]-5-heptenoic acid* (**1**). The affinity column ligand **1** (SQ31,491, Fig. 1) was prepared according to the procedure of Nakane *et al.* [34] with only minor modification. Briefly, a stirred solution of *N*-(cyclohexylpropyl carbonyl)glycine (78 mg, 345 μmol) in *N,N*-dimethylformamide (DMF) (0.8 mL) and CH_2Cl_2 (10 mL) was cooled in a dry ice/acetone bath. To this solution was added 1-methylpiperidine (46 μL, 370 μmol) and isobutylchloroformate (50 μL, 370 μmol). This reaction mixture was stirred for 30 min, and ethyl-[1*S*-[1α,2α(*Z*),3α,4α]] - 7 - [[3 - aminomethyl] - 7 - oxabicyclo[2.2.1] hept-2-yl]-5-heptenoate hydrochloride (100 mg, 315 μmol) and 1-methylpiperidine (40 μL, 315 μmol) were added. The reaction mixture was



SQ 31,491

Fig. 1. Structure of affinity ligand.

allowed to warm to room temperature, stirred overnight, and then concentrated *in vacuo*. The residue was flash chromatographed (silica gel, eluted with 2% MeOH/$CHCl_3$) to yield the ethyl ester of **1** (124 mg, 80%) as an oil. The ester (252 μmol) was hydrolyzed in MeOH (10 mL) with NaOH solution (1.4 mL, 1 N) overnight. The reaction mixture was concentrated, and saturated NaCl solution was added and then acidified (pH 3, 1 N HCl) before extraction with $CHCl_3$ (30 mL × 3). The combined organic layers were washed (NaCl, 10 mL × 2), dried (Na_2SO_4), filtered, and concentrated. The compound was crystallized from $CHCl_3$ and hexane to give **1** as a white solid (105 mg, 72% overall yield): TLC, silica gel, 4% MeOH/$CHCl_3$, iodine detection, R_f = 0.15; m.p. 141–143° (reported, 141–143°, [34]); MS (DCI), (M + 1) 463; PMR ($CDCl_3$) δ 7.18 (m, 1H, NH), 6.94 (m, 1H, NH), 5.42 (m, 2H, —HC=CH—), 4.32 (s, 1H, =CH—O—CH—), 4.26 (s, 1H, —CH—O—CH), 4.0 (ABX, 2H, CO—CH_2—NH), 3.41–3.19 (m, 2H, —CH_2—NH—CO—), 2.35 (t, 2H, CH_2—COOH), 2.21 (t, 2H, CH_2—CO—NH), 2.20–0.78 (m, 27H).

Preparation of affinity gel. The carboxylate of the affinity column ligand **1** was covalently attached to Affi-Gel 102 via its amino group, thus providing a hydrophilic 6 atom spacer arm between the ligand and the gel. Affi-Gel 102 (1 mL, 15 μmol amine/mL gel) was washed thoroughly with 50 mL of 0.5 M NaCl, distilled water and dilute HCl (pH 4.6), using a sintered glass funnel, and then resuspended in 1 mL of dilute HCl (pH 4.6). Ligand **1** (18.5 mg, 40 μmol/mL) was dissolved in dioxane and aq. HCl of pH 4.6 (4 mL, 50%, v/v). To a stirred suspension of the washed Affi-Gel (1 mL, pH 4.6), a solution of ligand **1** was added, followed by the condensing agent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 77 mg, 400 μmol/mL). The mixture was gently shaken at room temperature for 20 hr. The gel was then washed with 50% dioxane, acetate buffer (pH 4.2)/0.5 M NaCl, $NaHCO_3$ buffer (pH 8.2)/0.5 M NaCl and 50 mM Tris–HCl (pH 7.4)/0.5 M NaCl. This affinity gel was stored in buffer (50 mM Tris–HCl, 0.5 M NaCl, 0.02% thimerosal, pH 7.4) at 4° until used. In an attempt to estimate the amount of ligand this procedure could link to the gel, another readily available thromboxane antagonist, 13-azaprostanoic acid [7] (40 μmol/mL) labeled with [^{3}H]-13-APA [36] (5×10^4 cpm) was subjected to the same chemistry. It was found that 2–3 μmol/mL of this latter derivative was covalently linked to the Affi-Gel, and it was thus assumed that similar amounts of the ligand **1** were incorporated into Affi-Gel in the aforementioned procedure.

The affinity matrix was packed into a column (0.5 mL, 0.8 × 1 cm) and pre-equilibrated with 20 bed volumes of buffer D. The solubilized membrane preparation (2 mL) was supplemented with 20% glycerol, 0.5 M KCl, 0.2 mM EGTA and 0.5 mg/mL Asolectin (final concentrations) and then incubated with 0.5 mL of pre-equilibrated affinity gel by rotating overnight at 4°. The unbound proteins were eluted in the initial three fractions as flow-through, and the column was washed with 9 mL of buffer D (1 mL/8 min). Receptor binding activity was then eluted sequentially with buffer D containing 10 and 50 mM BM13.177 (3 mL each) with a flow rate of 1 mL/8 min. After elution of the first 1-mL fraction, flow was stopped for 30 min and restarted to elute the subsequent 1-mL fractions. Since most of the receptor binding activity was eluted with 50 mM BM13.177, this concentration of BM13.177 was used in later experiments.

In separate experiments, DEAE column fractions which contained the partially purified receptor were incubated with 1 mL of the affinity gel, divided into two 0.5-mL columns, and eluted with BM13.177 as described above. All BM13.177-containing fractions were diluted 7-fold prior to measurement of binding activity.

Size exclusion HPLC using GPC 500 and GPC 100 columns

The GPC 500 (250 × 4.6 mm) and GPC 100 (250 × 4.6 mm) columns were connected in tandem and pre-equilibrated with buffer F. The affinity column fraction of the BM13.177 eluate was filtered through millex-HA (0.45 μm), and 0.5 mL was injected into the columns. The columns were eluted with buffer F at a flow rate of 0.2 mL/min. Fractions (0.2 mL) were collected and binding activity was measured. The receptor binding activity emerged as a single peak in fractions 23–30. The most highly purified receptor fractions (26–28) were combined and used for receptor characterization.

Binding assay

TXA_2 receptor activity in solubilized preparations was determined by a modified filtration binding assay procedure using [^{3}H]SQ29,548 as the radiolabeled ligand. The receptor was immobilized on GF/B filters [presoaked in 0.3% polyethyleneimine (PEI)] [37] by filtering 200–400 μL of the solubilized protein under vacuum. Samples which contained KCl were diluted (5-to 7-fold) with buffer C (50 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.4, 4°) before filtration. The filters were washed (2 × 5 mL) with buffer A (4°), and the vacuum was released. Buffer A (60 μL) was applied to the filter, followed by 20 μL of buffer A (total binding) or 20 μL of unlabeled 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 (2) with 5 mL of buffer A (4°), and the

radioactivity on the filter was measured by liquid scintillation spectrometry (Beckman LS 6800).

Saturation binding of the purified receptor was carried out with various concentrations (1–100 nM) of [^{3}H]SQ29,548 as described above, and nonspecific binding was determined with a 20 μM concentration of unlabeled SQ29,548. The saturation binding curve was transformed by the method of Scatchard [38]. The dissociation constant (K_d) and the number of binding sites (B_{max}) were obtained by the Equilibrium Binding Data Analysis (EBDA) program.

Polyacrylamide gel electrophoresis

SDS–PAGE was performed according to the method of Laemmli [39] using 7.5% polyacrylamide slab gels (20 × 15 × 0.15 cm). Typically, 60 μL of purified protein boiled in sample buffer with or without 25 mM dithiothreitol (DTT) was applied to the sample well. The apparent molecular weight of the purified receptor protein was determined using marker proteins of known molecular weight. The gels were stained with $AgNO_3$ according to the method of Morrissey [40].

Protein determination

Protein content of the solubilized membranes and the DEAE fractions was measured according to Bradford [41], with bovine serum albumin as the standard. Since, however, BM13.177 present in the affinity chromatography and HPLC fractions interferes with the Bradford assay, the protein content of these fractions was estimated by densitometric scanning (Ephortec TM, Joyce Loebl Densitometer) of the silver stained SDS–PAGE gels, with bovine serum albumin as the standard.

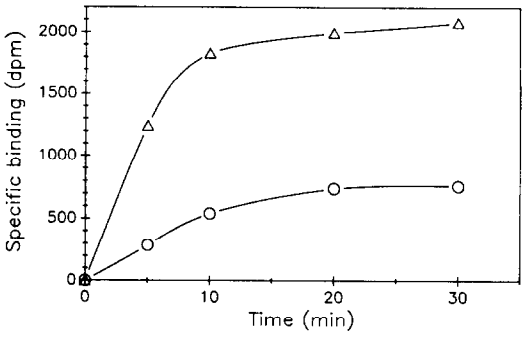


Fig. 2. Equilibrium binding of [^{3}H]SQ29,548 to solubilized platelet membranes by the conventional and modified filtration assay procedures. Conventional assay (O—O): solubilized platelet membranes (50 μg protein, 200 μL) were incubated (22°) with [^{3}H]SQ29,548 (2 nM) in the absence or presence of SQ29,548 (2 μM) for various time periods. The binding mixture was filtered through PEI-treated GF/B filters and washed with ice-cold buffer A (5 mL × 2). Modified assay (△—△): solubilized platelet membranes (50 μg protein, 200 μL) were filtered through PEI-treated GF/B filters and washed with ice-cold buffer A (5 mL × 2). Buffer A or SQ29,548 (2 μM) followed by [^{3}H]SQ29,548 (2 nM) was then applied to the filters and allowed to incubate for different time periods. The filters were subsequently washed with ice-cold buffer A (5 mL × 2). Data shown are specific binding values obtained by subtracting binding in the presence of 2 μM SQ29,548 from total binding data. For experimental details, see Experimental Procedures.

RESULTS

Binding assay

The modified filtration assay yielded highly reproducible receptor binding with less than a 10% standard deviation among triplicate samples. This sample variation was therefore equivalent to that observed in the conventional filtration technique, where incubation with the radiolabeled ligand is performed prior to filtration. In addition, a comparison between the two procedures demonstrated equivalent results in the following respects: (1) the degree of specific binding (approximately 90%); (2) the time to reach steady state (10–15 min) (Fig. 2); (3) the dissociation constants for [^{3}H]-SQ29,548 binding (K_d = 5–7 nM); and (4) competition for [^{3}H]SQ29,548 binding by unlabeled SQ29,548, U46619 and BM13.505.

On the other hand, the modified filtration assay offered certain advantages over the conventional technique. These include: (1) there was a roughly 3-fold increase in sensitivity, possibly due to increased receptor immobilization (Fig. 2); (2) the binding of [^{3}H]SQ29,548 could be measured in solubilized preparations containing agents which interfere with binding, e.g. BM13.177 (up to 7 mM) or *N*-acetylglucosamine (up to 0.5 M). The conventional filtration technique requires prior removal of BM13.177 by either dialysis or desalting column procedures, which in turn dilutes the receptor preparation; (3) the receptor could be readily concentrated by repetitive application of solubilized receptor to the filter; and (4) the immobilized receptor can be eluted from the filter with buffers containing high ionic strength [42]. However, this ionic interaction also limits the use of this procedure to receptor preparations which do not contain high salt concentrations, unless these preparations are diluted to less than 0.1 M prior to application to the filter.

Based on these considerations, the modified filtration assay was employed to measure [^{3}H]-SQ29,548 binding throughout the receptor purification procedures, and was especially useful in the measurement of binding activity in the affinity column and HPLC fractions.

DEAE chromatography

It can be seen (Fig. 3) that application of the solubilized membranes to a DEAE column resulted in binding activity appearing in certain fractions of the DEAE eluate. Specifically, the bulk of adsorbed proteins was gradually eluted with a linear salt gradient and reached a maximum elution at approximately 250 mM KCl. On the other hand, almost all of the binding activity was eluted at a lower ionic strength, i.e. between 70 and 180 mM KCl (fractions 14–24). Analysis of fractions 15–21 for protein concentration and binding activity revealed a 6-fold purification factor.

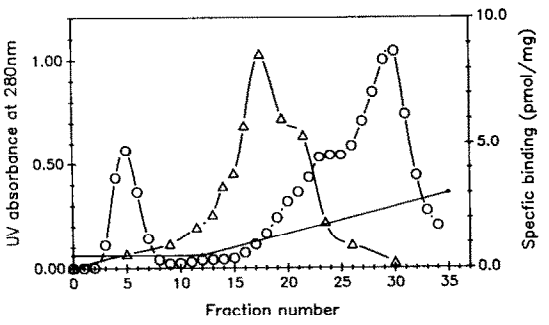


Fig. 3. DEAE-Sepharose chromatography of solubilized membranes. Solubilized membranes were applied to a DEAE-Sepharose column equilibrated with buffer E. The column was washed with buffer E, and eluted with a linear gradient of KCl from 50 to 300 mM and 4-mL fractions were collected. Key: (○—○) UV absorbance at 280 nm; (△—△) [^{3}H]SQ29,548 binding (pmol/mg protein); and (—) linear KCl gradient from 50 to 300 mM. For experimental details, see Experimental Procedures.

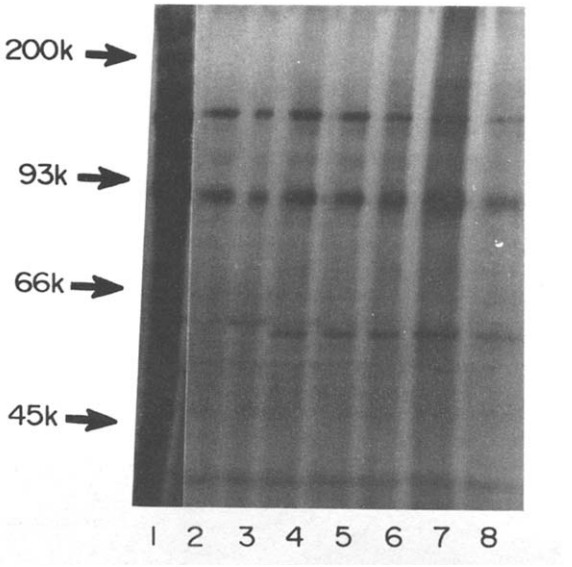


Fig. 4. Affinity column chromatography of solubilized membranes. SDS–polyacrylamide gel pattern (7.5% gel, nonreducing conditions) of each affinity column fraction. Lane 1: fractions 1–3 (flow-through); lane 2: fraction 12 (last fraction of wash); lane 3: fraction 13 (first fraction of 10 mM BM13.177); lane 4: fraction 14 (second fraction of 10 mM BM13.177); lane 5: fraction 15 (third fraction of 10 mM BM13.177); lane 6: fraction 16 (first fraction of 50 mM BM13.177); lane 7: fraction 17 (second fraction of 50 mM BM13.177); and lane 8: fraction 18 (third fraction of 50 mM BM13.177). Proteins were stained with silver reagent. For experimental details, see Experimental Procedures.

Affinity chromatography

To evaluate purification by affinity chromatography, it was first necessary to establish optimum binding conditions of the receptor to the affinity column. To this end, [^{3}H]SQ29,548 binding assays were performed with different buffers. Briefly, a comparison of buffers B and D at different salt concentrations (0–2 M KCl) demonstrated that the binding in buffer D was relatively insensitive to salt, with no decreases being seen up to 1 M KCl. On the other hand, increasing salt concentrations had a dramatic effect on the binding in buffer B, such that binding was inhibited by 70% at 1 M KCl. Since nonspecific ionic interaction of proteins to the affinity matrix is reduced at elevated salt concentrations, buffer D was selected for the affinity column chromatography.

The ability of the affinity gel to selectively absorb receptor protein was evaluated by incubating the solubilized membrane preparation with the affinity gel for 1–20 hr and assaying the supernatant for [^{3}H]-SQ29,548 binding. It was found that the supernatant binding activity rapidly decreased within 1 hr, reaching a minimum within 3 hr (data not shown). Continued incubation for an additional 17 hr did not result in a further decrease, with the maximum absorption of receptor being approximately 70%. There was also a selective absorption of the receptor, since total protein absorption was only 10% during this period of time.

After overnight incubation, the affinity gel was washed with 9 mL of buffer D and sequentially eluted with 10 and 50 mM BM13.177 in buffer D. Figure 4 illustrates the SDS–PAGE gel profile of the various affinity column eluate fractions. It was found that a distinct protein band at 55 kDa was specifically eluted in response to BM13.177, which provides evidence that the affinity gel selectively purified this protein. Furthermore, gel lanes containing the 55 kDa protein (4–8) corresponded to the fractions containing the [^{3}H]SQ29,548 binding activity, with the highest specific binding observed in lane 7 (50 dpm in lane 2 vs 1200 dpm in lane 7).

In addition to the 55 kDa protein, the BM13.177 eluate also contained major bands at 140 and 90 kDa. Reduction with DTT resulted in the appearance of four bands with molecular weights of 120,000, 110,000, 90,000 and 55,000 (results not shown). These findings suggest that the 140 kDa protein was probably glycoprotein (GP)IIb, whereas a component of the 90 kDa protein was GPIIIa [43]. Furthermore, reduction with DTT had no significant effect on the migration of the 55 kDa band on SDS–PAGE. Due to the contamination of the BM13.177 eluate fractions with these and other proteins, DEAE column chromatography was employed to partially purify the receptor prior to affinity chromatography.

DEAE plus affinity chromatography

Figure 5 illustrates the specific binding profile in different affinity column fractions. It can be seen that some binding activity was associated with fractions 1–16, which represented receptor not bound by the affinity matrix. During the 15 mL wash with buffer D (fractions 17–31) there was no significant elution of binding activity. On the other hand, 50 mM BM13.177 elution resulted in substantial binding activity appearing in fractions 32–35, with peak binding in fraction 33.

Figure 6 illustrates the gel profile obtained by the

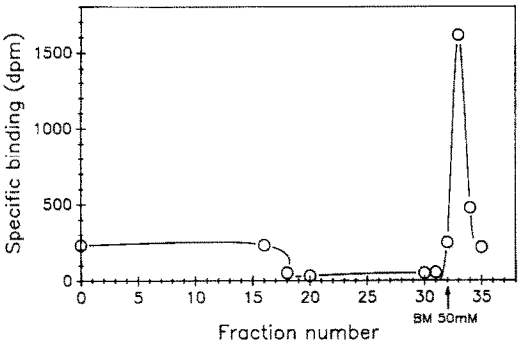


Fig. 5. DEAE-Sepharose chromatography followed by affinity chromatography. [^{3}H]SQ29,548 binding profile of affinity column fractions: flow-through (fractions 1–16); wash (fractions 17–31); and 50 mM BM13.177 elution (fraction 32–35). For experimental details, see Experimental Procedures.

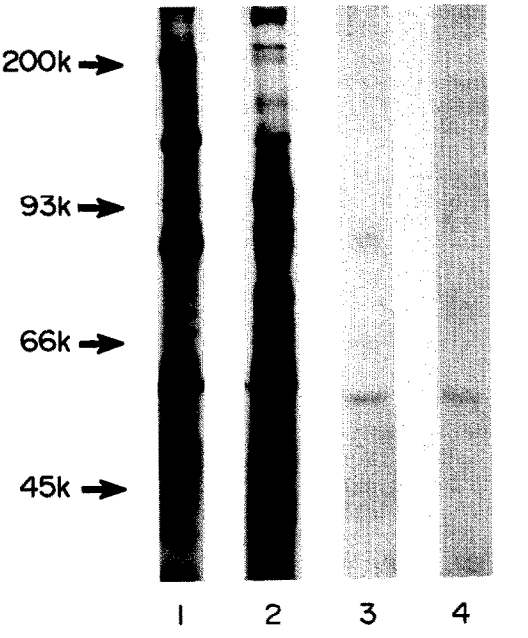


Fig. 6. SDS–polyacrylamide gels of different purification steps (7.5% gel, nonreducing conditions). Lane 1: solubilized membranes; lane 2: eluate from DEAE chromatography alone (fractions 15–21 of Fig. 3); lane 3: BM13.177 (50 mM) eluate (fraction 33 of Fig. 5) from affinity chromatography of DEAE fractions 15–21; lane 4: HPLC eluate (fractions 26–28 of Fig. 7) of the sample containing lane 3. For experimental details, see Experimental Procedures.

combination of DEAE and affinity chromatography. It can be seen that the BM13.177 eluate contained only two major bands corresponding to 90 and 55 kDa (lane 3), with a binding activity 1700-fold higher than that of solubilized membranes. Furthermore, reduction with DTT did not change the R_f value of the 90 kDa protein, indicating that this protein was not GPIIIa. Therefore, prepurification with DEAE chromatography (lane 2) selectively removed GPIIb–IIIa, which represented a major contaminant of the affinity column eluate. On the other hand, an unidentified 90 kDa protein appeared to co-purify with the 55 kDa protein. Interestingly, this 90 kDa protein has the same mobility on SDS–PAGE as GPIIIb or GPIV.

DEAE plus affinity chromatography plus HPLC

As a final approach to separate the 90 kDa protein from the 55 kDa protein, size exclusion HPLC was employed. In these experiments, GPC 500 and GPC 100 columns connected in tandem were used to further purify the affinity column eluate from samples that had undergone DEAE chromatography. It can be seen (Fig. 7, lower panel) that fractions 26–30 contain a single, distinct band at 55 kDa. Furthermore, the binding activity (Fig. 7, upper panel) co-eluted with these fractions, indicating that the 55 kDa band represented the purified receptor. On the other hand, no binding activity was detected in fractions 18–21 which contained the 90 kDa protein.

Table 1 summarizes the various procedures used to purify the platelet TXA_2/PGH_2 receptor. Clearly, the greatest degree of purification was achieved with affinity chromatography, which yielded approximately 300-fold purification with a 23% recovery. Both the DEAE and HPLC procedures added an additional 6-fold and 4-fold purification, respectively. Recovery for DEAE ion exchange chromatography was 54 and 50% for HPLC. Taken together, the overall purification scheme (to achieve a homogeneous receptor fraction) resulted in a 6100-fold purification factor with a 6% recovery, starting from a solubilized membrane preparation.

Receptor characterization

Figure 8 depicts a time course of [^{3}H]SQ29,548 binding to the purified receptor. It was found that binding reached a steady state within approximately 10 min, which is consistent with that observed in solubilized membranes (Fig. 2). The binding of [^{3}H]-SQ29,548 to the purified receptor was also found to be saturable, reaching saturation at a 50 nM concentration of the ligand. Transformation of this saturation binding curve by Scatchard analysis yielded a straight line (Hill coefficient of 1.076), a K_d of 4 nM and a B_{max} of 17 nmol/mg protein (Fig. 9). These findings suggest that the purified receptor protein exhibits a single class of binding sites and has a K_d comparable to that found in whole platelets [16, 17], platelet membranes [17] and solubilized platelet membranes (unpublished results). Furthermore, since 1 mg of the 55 kDa protein represented 18 nmol, it appears that 95% of the purified receptor is capable of binding [^{3}H]SQ29,548 with a 1:1 stoichiometry.

Regarding specificity of [^{3}H]SQ29,548 binding by the purified receptor, Fig. 10 demonstrates the identical specificity and potency profile previously observed for whole platelets [17], platelet membranes [17], and solubilized membranes (data not shown). Thus, binding was inhibited in a concentration-dependent manner by agents known to interact at

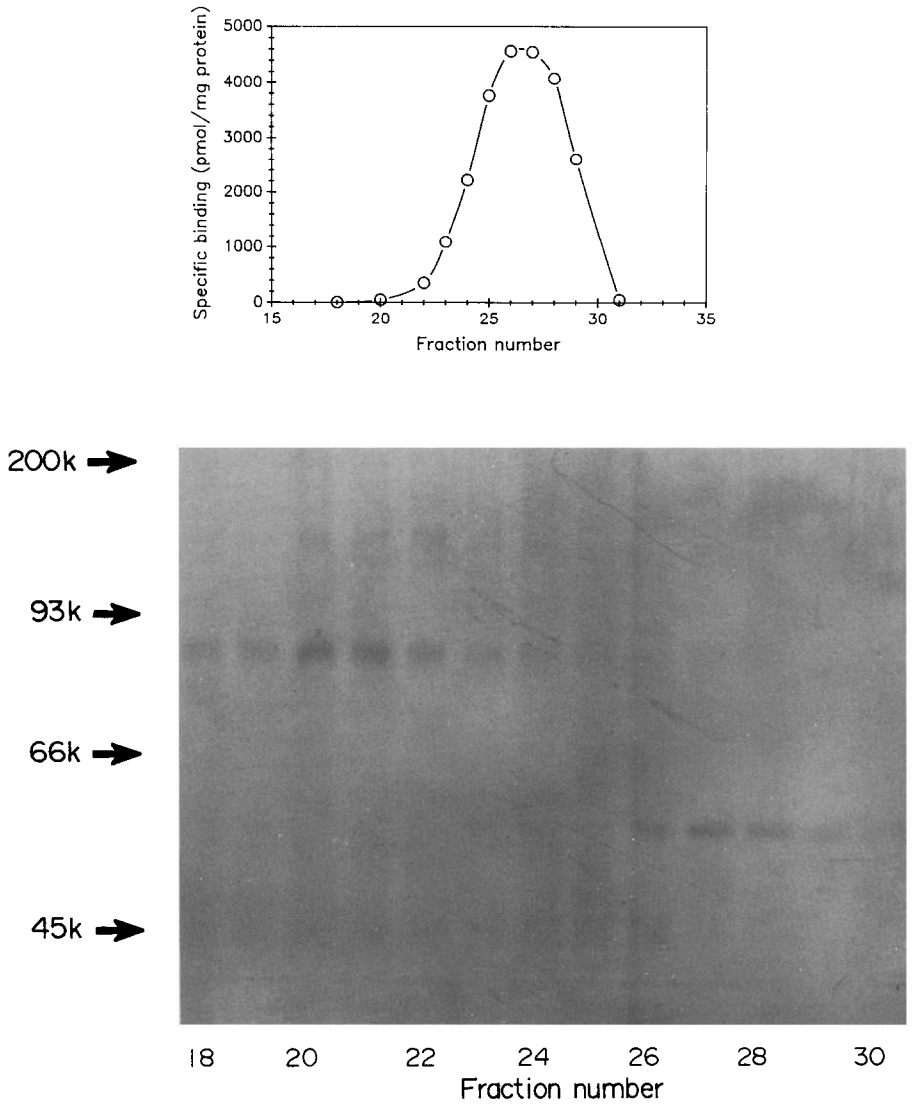


Fig. 7. Size exclusion HPLC of eluates from affinity chromatography preceded by DEAE chromatography. Upper panel: [^{3}H]SQ29,548 binding profiles of HPLC eluate fractions. Lower panel: SDS–PAGE analysis of HPLC eluate fractions. Results are typical of more than five separate experiments. For additional details, see Experimental Procedures.

Table 1. Summary of the purification of the TXA_2/PGH_2 receptor from human platelets

Fraction	Protein (μg)	Specifically bound: Total (pmol)	Specifically bound: pmol/mg protein	Yield (%)	Purification (fold)
Solubilized membranes	32,000	22.34	0.70	100	1
DEAE	3000	12.08	4.03	54	6
Affinity	2.3	2.77	1203	12	1700
HPLC	0.3	1.28	4282	6	6100

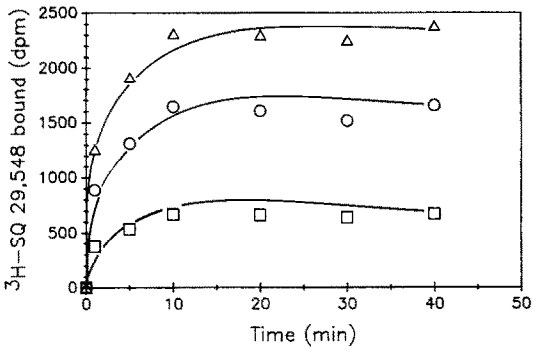


Fig. 8. Time course of [^{3}H]SQ29,548 binding to the purified receptor. Purified receptor (3 ng) was incubated with [^{3}H]-SQ29,548 (4 nM) on the assay filters for various time periods. At 1, 5, 10, 20, 30 and 40 min filters were washed with ice-cold buffer A (5 mL 2). Specific binding (○—○) of the radioligand to the receptor was determined as total binding (△—△) minus nonspecific binding (□—□), described in Experimental Procedures.

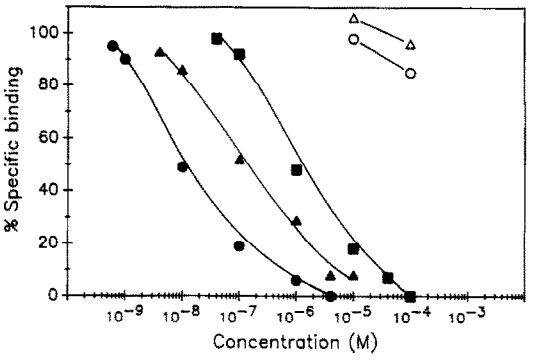


Fig. 10. Competition of [^{3}H]SQ29,548 binding to the purified receptor by various ligands. Purified receptor (5 ng) was incubated with [^{3}H]SQ29,548 (4 nM) and different concentrations of various ligands. Binding was performed as described in Experimental Procedures. Key: (●—●) SQ29,548; (▲—▲) BM13.505; (■—■) U46619; (○—○) TXB_2 and (△—△) 6-keto $PGF_{1\alpha}$.

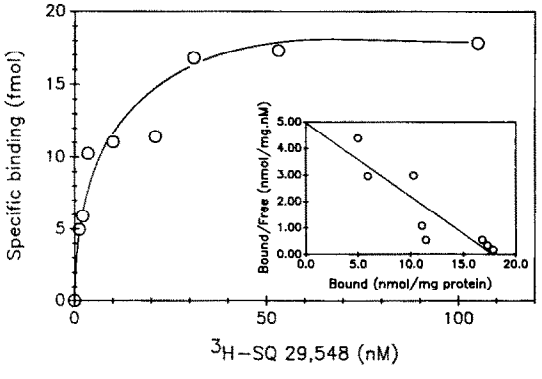


Fig. 9. Saturation binding of [^{3}H]SQ29,548 to the purified receptor. The purified receptor (1 ng) was incubated with 1–100 nM [^{3}H]SQ29,548 for 20 min. Nonspecific binding was determined using 20 μM unlabeled SQ29,548. Insert: Transformation of the saturation binding data by Scatchard analysis. For additional details, see Experimental Procedures.

the TXA_2/PGH_2 receptor level, i.e. SQ29,548, BM13.505 and U46619, but was not inhibited by agents that do not alter receptor activity, i.e. TXB_2 and 6-keto $PGF_{1\alpha}$. Using the IC_{50} values from these inhibition curves, the K_i values for the various ligands can be approximated using the Cheng–Prusoff relationship [44]. It was found that this approximation yielded K_i values of 3, 50 and 220 nM for SQ29,548, BM13.505 and U46619, respectively. These values are roughly equivalent to the K_d values previously reported for these agents [16, 17].

DISCUSSION

The present results describe the purification of the human platelet TXA_2/PGH_2 receptor to apparent homogeneity. This purification was achieved by employing three separate chromatographic procedures. Initially, a new affinity column matrix was developed using SQ31,491 coupled to Affi-Gel 102. When incubated with solubilized platelet membranes, this affinity matrix adsorbed approximately 70% of the TXA_2/PGH_2 receptor activity, indicating that it substantially bound solubilized receptor. Elution of the bound receptor from the affinity gel was accomplished with the TXA_2/PGH_2 antagonist BM13.177. Analysis of the eluted fractions by SDS–PAGE demonstrated that the affinity column specifically concentrated a 55 kDa protein, and that the highest degree of [^{3}H]SQ29,548 binding activity (300-fold greater than solubilized membranes) co-eluted with this protein. On the other hand, the 55 kDa-enriched fractions also contained other proteins, most notably proteins in the range of 90 and 140 kDa, which could account for the observed binding activity. However, reduction with DDT demonstrated that the 140 kDa protein was GPIIb, and the 90 kDa protein band was partially GPIIIa [43]. The presence of GPIIb–IIIa in the affinity column eluate was presumably due to its high concentration in platelet membranes.

Based on these findings, attempts were made to separate GPIIb–IIIa using DEAE ion exchange chromatography and evaluate the binding activity in the eluted fractions. It was found that [^{3}H]SQ29,548 binding protein(s) eluted prior to GPIIb–IIIa, indicating that this glycoprotein complex was not responsible for the observed binding activity. Furthermore, DEAE chromatography increased the specific activity of binding by 6-fold relative to solubilized membranes, presumably due to the removal of GPIIb–IIIa. The DEAE eluate containing the bulk of binding activity was then subjected to affinity chromatography. SDS–PAGE of the affinity column eluate demonstrated that [^{3}H]SQ29,548 binding was localized in fractions enriched in 90 and 55 kDa proteins. These findings indicated that the

most likely candidate for the TXA_2/PGH_2 receptor was the 90 or 55 kDa protein.

The next series of experiments was undertaken to separate these proteins and localize binding activity in the eluted fractions. This separation was achieved by size exclusion HPLC. These experiments involved a three-step chromatographic procedure, i.e. DEAE chromatography, followed by affinity chromatography, followed by HPLC. Analysis of the HPLC eluate by SDS–PAGE demonstrated highly efficient separation of the 90 and 55 kDa proteins. Furthermore, although some [^{3}H]SQ29,548 binding activity was associated with fractions containing both the 90 and 55 kDa proteins, the binding activity was concentrated in the homogeneous 55 kDa fractions.

These findings provide evidence that the TXA_2/PGH_2 receptor activity is localized in a 55 kDa protein, which is in agreement with our previous estimate [32, 33], and recently published reports in which a form of the receptor was purified to homogeneity [30] and subsequently cloned and expressed [31]. On the other hand, this molecular weight assignment is not in agreement with estimates derived from photoaffinity labeling [27] and isoelectric focusing coupled with photoaffinity labeling [29]. In these studies a 43 kDa, a 39 kDa or a 27 kDa protein was suggested to be the TXA_2/PGH_2 receptor. The purified protein isolated in the present experiments exhibited a pharmacological binding profile indistinguishable from that observed for the TXA_2/PGH_2 receptor in other studies using whole platelets [16, 17], platelet membranes [13, 45] and solubilized membranes ([28], present study). Thus, [^{3}H]SQ29,548 bound to the purified receptor in a specific, time dependent, and saturable manner. Scatchard analysis revealed a K_d of 4 nM, which is almost identical to that previously reported. The present results also demonstrate that the purified receptor possesses a B_{max} of 17 nmol/mg protein and a single binding site for [^{3}H]SQ29,548. These values are in close agreement with those reported for [^{3}H]-S-145 binding in a purified preparation [30], and further support the notion that the TXA_2/PGH_2 receptor is represented by a 55 kDa protein.

On the other hand, the finding that this 55 kDa protein co-purifies (by affinity chromatography) with a 90 kDa protein suggests that the native form of the TXA_2/PGH_2 receptor, when bound to the ligand on the affinity matrix, may exist as a complex with another protein(s). If this were the case, the 90 kDa protein would be expected to co-elute with the receptor during affinity chromatography. Alternatively, it is also possible that the 90 kDa protein represents a separate form of the TXA_2/PGH_2 receptor which has a low affinity for binding. Affinity chromatography, due to a high ligand concentration, may be capable of concentrating this protein, even though its activity would not be easily detected in the binding assay. Clearly the potential significance of the 90 kDa protein warrants further investigation.

In summary, the present results demonstrate a three-step procedure for purification of the human platelet TXA_2/PGH_2 receptor protein to apparent homogeneity. Compared to a previously published method for receptor purification [30], the techniques reported here: (1) were relatively rapid and did not require recycling of the affinity column product for several days to isolate receptor protein; (2) did not require removal of the eluting ligand prior to assay of binding activity; and (3) yielded a distinct and homogeneous SDS–PAGE band at 55 kDa. Receptor protein is currently being isolated for antibody production, reconstitution studies in phospholipid vesicles, further comparison with the placental TXA_2 receptor, and investigation of the potential role of the 90 kDa protein in human platelet TXA_2/PGH_2 receptor activity.

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