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ISOELECTRIC AND MASS CHARACTERIZATION OF HUMAN PLATELET THROMBOXANE A_2/PROSTAGLANDIN H_2 RECEPTORS

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To further characterize the human thromboxane A_2 (TXA_2)/prostaglandin H_2 (PGH_2) receptor, preparative isoelectric focusing (IEF) was performed on solubilized platelet membranes. TXA_2/PGH_2 receptors, assayed by specific binding of the TXA_2/PGH_2 antagonist [^{125}I]PTA-OH, were electrofocused at pH 5.6. Scatchard analysis of IEF fraction pH 5.6 revealed a 180-fold concentration of TXA_2/PGH_2 receptors (B_{max}=3650±228 pM/mg focused, 19±4 pM/mg unfocused) with no change in binding affinity (Kd=47±7 nM focused, 36±14 nM unfocused). SDS-polyacrylamide gel electrophoresis of photoaffinity-labelled electrofocused receptors revealed concentration of specifically labelled proteins having molecular masses of 49,000 and 27,000 Daltons. These results suggest that the human platelet TXA_2/PGH_2 receptor has a pI of 5.6, molecular mass of 49,000 Daltons, and may exist as a dimer. Preparative IEF should prove useful in the eventual purification of this receptor.

Thromboxane A_2 (TXA_2) stimulates platelet aggregation through interaction with specific membrane receptors (1,2). Abnormalities of TXA_2 production and of the platelet TXA_2/PGH_2 receptor have been noted in some cardiovascular disorders (3,4). The platelet TXA_2/PGH_2 receptor has been extensively characterized using equilibrium binding studies in intact platelets (5), platelet membranes (6), and solubilized platelet membranes (7) with the radiolabelled TXA_2/PGH_2 antagonist [^{125}I]PTA-OH. Recently, Mais et. al. were successful in photoaffinity labelling the TXA_2/PGH_2 receptor in intact platelets (8) permitting estimates of its pI and molecular weight. However, meaningful analysis of the results in that report is difficult due to apparent specific labelling of multiple proteins. The present study characterized the platelet TXA_2/PGH_2 receptor using a recently developed technique of preparative isoelectric focusing, allowing partial purification and recovery of active receptor, and photoaffinity labelling.

Abbreviations: I-PTA-OH, 9, 11-dimethylmethano-11,12-methano-16-(3-iodo-4-hydroxyphenyl)-13,14-dihydro-13-aza-15$a\beta$-ω-tetranor-TXA_2; I-PTA-azido, 9,11-dimethylmethano-11,12-methano-16-(3-iodo-4-azidophenoxy)-13,14-dihydro-13-aza-15$a\beta$-ω-tetranor-TXA_2; SQ29,548, [[1S]1α,2β(5Z),3β,4α]-7-[3-[[2-[(phenylamino)carbonyl]-hydrazino]methyl]-7-oxabicyclo[2.2.1]-heptenoate.

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MATERIALS AND METHODS

Chemicals [^{125}I]PTA-OH (9) and [^{125}I]PTA-azido (10) were synthesized from their precursers (gifts from Dale E. Mais and Perry V. Halushka, Charleston, S.C.) as described elsewhere (5,10). SQ29,548 (11) was a gift from Martin Ogletree, Squibb Institute for Medical Research, Princeton, N.J. Sephadex G-75 (Ultradex), ampholines and all other electrophoresis supplies were from LKB. All other chemicals were of reagent grade from Sigma or Fisher.

Preparative Isoelectric Focusing Expired platelet concentrates were purchased from a local blood bank. Membranes prepared by freeze-thawing and dounce homogenization of washed platelets were diluted to a protein concentration of 25 mg/ml in 25 mM Hepes (pH 7.4) containing 10 μM indomethacin and 75 μg/ml phenylmethylsulfonyl fluoride (Hepes buffer) and stored at -70°C until use. Membranes were solubilized by addition of 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) to a final concentration of 40 mM followed by centrifugation at 200,000xg for 60 minutes (4°C). The supernatant fraction (solubilized membranes) was either used immediately for preparative IEF or was diluted 1:10 in 25 mM Hepes (pH 7.4) for binding studies.

Preparative IEF was performed on a horizontal bed of Sephadex G-75 in 2% ampholines and 2 mM CHAPS. Three or five ml of solubilized membranes were applied to the gel and electrofocused at 10°C for 18 hours at 8 W constant power. Following a filter paper print of the gel for Coomassie blue staining, the gel was grid fractionated, the pH of each fraction was determined in situ at 10°C, and focused proteins were eluted from individual gel fractions by addition of 1 ml of Hepes buffer and centrifugation through small columns. Each fraction was assayed for protein concentration using the method of Bradford (12) with gamma globulin as the standard. [^{125}I]PTA-OH binding was assayed as a marker for TXA_2/PGH_2 receptors.

Radioligand Binding Studies Binding of [^{125}I]PTA-OH was assayed using a modification of a previously described procedure (7). The buffer was 25 mM Hepes, 1 mM CHAPS (pH 7.4). Reactants were incubated for 30 minutes at 30°C. The reaction was terminated by vacuum filtration over Whatman GF/A glass fiber filters pre-soaked in 1% polyethyleneimine. Non-specific binding was determined by addition of 5 μM unlabelled I-PTA-OH or 10 μM SQ29,548. Equilibrium binding data were analyzed using LIGAND (13) to determine binding capacity (B_{max}) and dissociation constants (K_d).

Photoaffinity labelling of washed platelets with [^{125}I]PTA-azido was as described (10). Photoaffinity labelling of electrofocused receptor was accomplished by addition of 10^7 cpm of [^{125}I]PTA-azido to 1 ml of IEF fraction with or without 10 μM unlabelled I-PTA-OH or SQ29,548 to determine specific labelling. The reactants were allowed to equilibrate in the dark for 30 minutes prior to UV photolysis for three minutes. As photoincorporation of [^{125}I]PTA-azido was very low at these concentrations of electrofocused membranes (approximately 1 mg/ml), unbound tracer was separated from bound tracer by elution over 1x10 cm Sephadex G-50 columns which retained approximately half of the added tracer. Proteins were resolved according to molecular mass on 10% discontinuous SDS-polyacrylamide gels by the method of Laemmli (14). After silver staining, autoradiography was performed to visualize photolabelled proteins.

RESULTS

Isoelectric Focusing After preparative IEF specific [^{125}I]PTA-OH binding (displaced by unlabelled I-PTA-OH and SQ29,548) focused in a single peak of activity at pH 5.6 (n=7, figure 1). In two experiments a smaller peak of activity was also seen at pH 6.0 but, unlike the pH 5.6 fraction, [^{125}I]PTA-OH binding to this fraction was displaceable only by unlabelled I-PTA-OH and not by SQ29,548. These results were consistent whether or not the pH of the electrofocused fractions was individually titrated to 7.4 prior to the binding assay. In addition, [^{125}I]PTA-OH binding to solubilized membranes was found to be stable over a pH range of 4.8 to 8.0 (table 1). Therefore, subsequent experiments were performed on IEF fractions without titrating the pH.

Scatchard analysis (figure 2) of [^{125}I]PTA-OH binding to solubilized membranes prior to IEF revealed a single binding site with a K_d of 36±14 nM and a binding capacity of 19±4 pM/mg

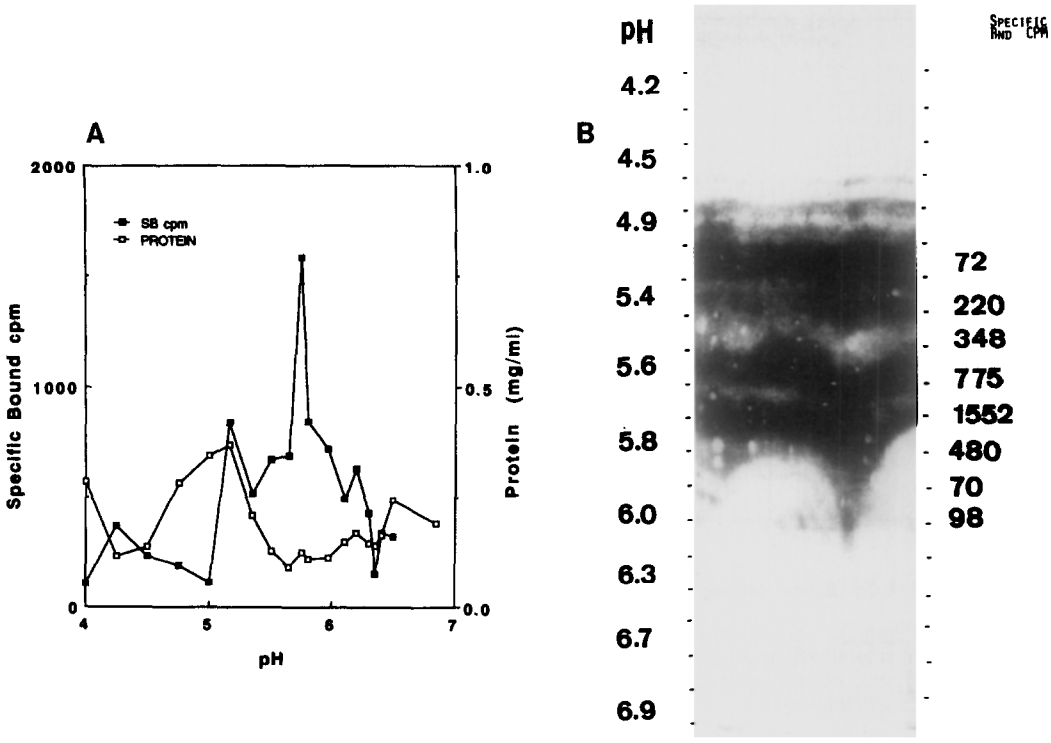

Figure 1. Preparative IEF of solubilized human platelet TXA2/PGH2 receptors. A) Graphic demonstration of [^{125}I]PTA-OH specific binding and protein concentration related to pH profile after IEF B) Coomassie blue stain of filter paper print of horizontal bed IEF. Fraction pH is shown on the left border, [^{125}I]PTA-OH specific binding (cpm/100 µl) on the right border.

(n=3). After IEF, Scatchard analysis of the pH 5.6 fraction revealed a *K*d of 47±7 nM and a binding capacity of 3650±228 pM/mg (n=3). Thus, IEF concentrated the TXA2/PGH2 receptor approximately 180-fold.

Table 1. Effect of pH on [^{125}I]PTA-OH binding

pH	Vehicle	5 µM I-PTA-OH
4.0	1134±23*	930±15
4.8	2579±56	967±18
5.3	2566±42	998±17
6.0	2561±39	789±12
6.5	2362±37	877±16
7.1	2522±43	842±18
7.5	2788±62	800±12
8.0	2450±28	813±7

Results (cpm) are the means ±SEM of triplicate determinations from a single solubilized membrane preparation (2 mg/ml). Total binding was determined in the presence of vehicle and non-specific binding determined by addition of 5 µM unlabelled I-PTA-OH. * denotes significant ($p<0.05$) difference by one way ANOVA.

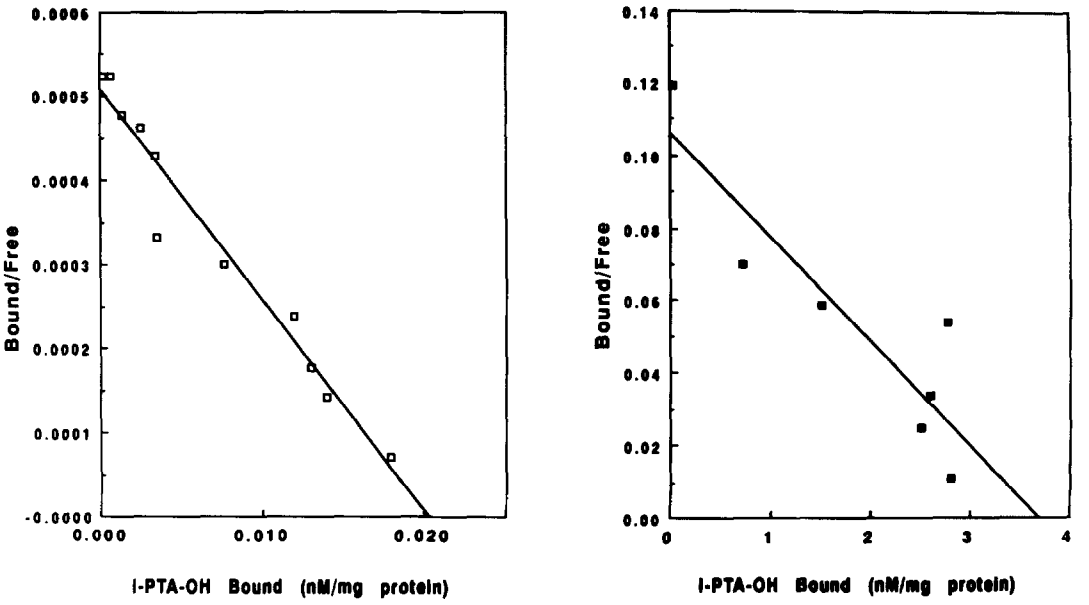

Figure 2. Scatchard plots (LIGAND program) of [^{125}I]PTA-OH specific binding to solubilized membranes (left figure, open squares) and pH 5.6 IEF fraction (right figure, closed squares). Data are presented as the means of three separate experiments each.

Photoaffinity Labelling In washed platelets [^{125}I]PTA-azido specifically labelled three proteins of molecular masses 49 kD, 41 kD, and 27 kD (figure 3a). These results were not dependent on whether I-PTA-OH or SQ29,548 were used as specific competitors.

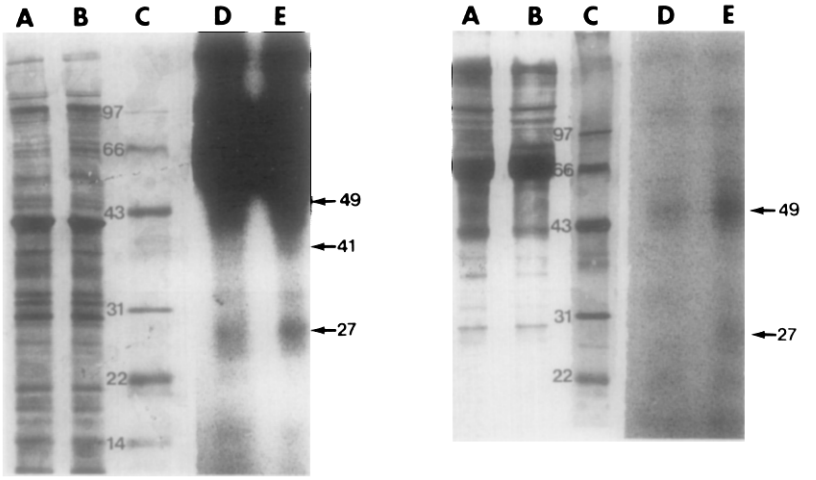

Figure 3. Representative SDS-polyacrylamide gel electrophoresis and corresponding autoradiograph of [^{125}I]PTA-azido photoaffinity-labelled intact platelets (left) and pH 5.6 IEF fraction (right). Specific photoaffinity labelling was assessed by addition of 10 μM SQ29,548. *Lane A*, silver stain plus SQ29,548; *Lane B*, silver stain plus vehicle; *Lane C* molecular weight standards; *Lane D*, autoradiogram plus SQ29,548; *Lane E*, autoradiogram plus vehicle. Arrows indicate specifically labelled proteins and molecular masses.

The pH 5.6 IEF fraction was photolabelled. Although silver staining demonstrated that numerous protein bands were present after IEF, autoradiography showed concentration of two [^{125}I]PTA-azido specifically labelled proteins of molecular mass 49 kD and 27 kD (figure 3b). Many of the non-specifically photolabelled proteins as well as the specifically labelled 41 kD protein were excluded after IEF.

DISCUSSION

The present study demonstrates that the human platelet TXA2/PGH2 receptor can be concentrated and recovered in active form after preparative isoelectric focusing. Two biophysical properties of the receptor, the pI and the molecular mass, are described.

The human platelet TXA2/PGH2 receptor has been extensively characterized in equilibrium binding studies using several different radiolabelled TXA2/PGH2 antagonists and agonists (5,15,16). Competition of [^{125}I]PTA-OH binding with stable TXA2/PGH2 analogs (5) and with authentic TXA2 and PGH2 (17) indicate that its binding site on platelets represents the TXA2/PGH2 receptor. Preliminary steps toward isolation and purification of this receptor have been taken with the solubilization of the receptor in active form (7) and the recent development of a specific radioactive photoaffinity ligand (8,10). However, methods of significantly concentrating the receptor in active form have not been previously reported.

In the present study, after preparative IEF, [^{125}I]PTA-OH binding activity was concentrated at pH 5.6. Mais et al (8) also have reported a pI for the platelet TXA2/PGH2 receptor of 5.6 as well as 4.9, 5.1, and 5.3 after analytical IEF of photoaffinity-labelled receptor performed under denaturing conditions (8M urea). While it is possible that the use of urea by Mais allowed for resolution of several different receptor isoforms by protein unfolding (18), in light of the present findings it is likely that some of the "specifically" photoaffinity-labelled proteins do not represent the TXA2/PGH2 receptor.

Preparative IEF concentrated, at pH 5.6, two specifically photoaffinity-labelled proteins having molecular masses of 49 and 27 kD. Burch et al (7) reported a hydrodynamic molecular weight of the CHAPS solubilized platelet TXA2/PGH2 receptor of 140 kD. This estimate may be high because of inclusion of associated peptides, such as GTP-binding proteins (19), in the TXA2/PGH2 receptor-CHAPS complex. Mais et al (8) reported specific photoaffinity labelling ([^{125}I]PTA-azido) in washed platelets of three proteins having molecular masses of 43, 39, and 27 kD. In the present study [^{125}I]PTA-azido photolabelling of intact platelets resulted in three specifically labelled proteins of 49, 41, and 27 kD mass. It is likely that the 41 and 39 kD proteins and the two 27 kD proteins in the two studies are identical. However, under the conditions of this study, the largest specifically labelled protein clearly did not co-migrate with the 43 kD molecular mass standard. This difference in the two studies cannot be explained.

After preparative IEF the 49 kD and 27 kD proteins were concentrated and the 41 kD protein was excluded. The loss of the 41 kD protein suggests that it represented false or "pseudo-specific" labelling of the protein. Co-concentration of the 49 and 27 kD affinity-labelled proteins at pH 5.6 suggests that the human platelet TXA2/PGH2 receptor may be a hetero-dimer or that, as previously postulated (20), different subtypes of platelet TXA2/PGH2 receptors may exist.

In conclusion, the human platelet TXA_2/PGH_2 receptor can be concentrated 180-fold and recovered in active form after preparative IEF. The receptor has a pI of 5.6 and a molecular mass of approximately 49 kD.

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