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SUBCELLULAR LOCALIZATION OF A THROMBOXANE A_2/PROSTAGLANDIN H_2 RECEPTOR ANTAGONIST BINDING SITE IN HUMAN PLATELETS*

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Abstract—The subcellular localization of a binding site for the competitive thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) antagonist, 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), was determined. Subcellular fractions of platelets were prepared by glycerol lysis or nitrogen cavitation, and were characterized by the use of enzymatic markers specific for plasma membranes, endoplasmic reticulum (dense tubular system), mitochondria, granules, and cytosolic constituents. The K_d and density of binding sites in the subcellular fractions were determined by Scatchard analysis of equilibrium binding data. The K_d and B_{max} for [^{125}I]-PTA-OH determined in the lysates were 49 ± 11 nM and 4.1 ± 1.7 pmol/mg protein respectively (N = 6). The K_d values were not significantly different in any of the fractions assayed. The binding sites were coenriched (4.5 ± 0.66 fold) with the enzymatic markers for plasma membranes (3.7 ± 0.5 fold) and dense tubular system (2.4 ± 0.4 fold). The binding sites were not coenriched with markers for cytoplasmic constituents, mitochondria, or granules. The ability of the TXA_2/PGH_2 mimetic U46619 to compete with [^{125}I]-PTA-OH for the binding site was also determined for the various subcellular fractions. The IC_{50} for U46619 was 5.4 ± 1.2 μM in the lysate, and was not significantly different in the subcellular fractions. These data suggest that the binding site is the TXA_2/PGH_2 receptor described previously. These data are consistent with the notion that the putative TXA_2/PGH_2 receptor is localized in the plasma membranes and/or the dense tubular system.

Thromboxane A_2 (TXA_2) and prostaglandin H_2 (PGH_2) activate human platelets through one or more receptors within the platelet [1]. Both TXA_2 and PGH_2, and their stable analogs, produce a number of morphological and biochemical changes in platelets during activation [2]. These changes include an increase in intracellular free calcium, arising from both intracellular and extracellular sources [3], increased poly-phosphoinositide turnover [4], protein phosphorylation [5], shape change [6], release of granules into the extracellular medium [7], and aggregation [1]. This multiplicity of events induced by TXA_2/PGH_2 raises the possibility that their putative receptors may be localized to one or more subcellular sites.

The radioiodinated TXA_2/PGH_2 antagonist, 9,11-dimethylmethano-11,12-methano-16-(3-iodo-4-hydroxyphenyl)-13,14-dihydro-13-aza-15α, β-ω-tetranor TXA_2 [^{125}I]PTA-OH [8], has been used recently to describe the binding properties of putative TXA_2/PGH_2 receptor in intact human platelets [9], and in crude and solubilized particulate fractions prepared from human platelets [10, 11]. However, the subcellular localization of the putative receptor remains to be determined. Localization of the human platelet TXA_2/PGH_2 receptor in one or more subcellular fractions may lead to a better understanding of the role of the TXA_2/PGH_2 receptor in platelet function. This report describes the subcellular localization of the putative TXA_2/PGH_2 receptor in human platelets.

METHODS

I-PTA-OH and [^{125}I]-PTA-OH were synthesized as described previously [8, 10] and purified by either TLC [10] or HPLC. HPLC purification was performed on a Whatman Partisil-5-ODS-3 reverse phase column with a mobile phase of methanol–0.2 M ammonium acetate (65:35) at a flow rate of 1 ml/min. Under these conditions, the elution order of the reactants and products is unreacted ^{125}I (3.8 min), PTA-OH (7.2 min), [^{125}I]-PTA-OH (10.1 min), and di-iodo-PTA-OH ([^{125}I]$_2$-PTA-OH)

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(15.3 min). Under the reaction conditions used, however, no detectable $[^{125}I]_2$-PTA-OH was formed. Fractions were collected at 1-min intervals, and the second radioactive peak (corresponding to [^{125}I]-PTA-OH) was diluted with 10–20 ml of methanol and stored at −20° for up to 2 months. [^{125}I]-PTA-OH is chemically stable under these storage conditions. Na[^{125}I] and 5-hydroxy[side chain-2-^{14}C]tryptamine creatinine sulfate were obtained from the Amersham Corp. (Chicago, IL). Omnifluor was purchased from New England Nuclear, and Solubilizer for Aqueous Samples from Research Products International (Mt. Prospect, IL). Scintillation grade toluene and ammonium acetate (HPLC grade) were obtained from Fisher (Norcross, GA). U46619 (9α,11α-methanoepoxy $PGF_{2\alpha}$) was purchased from Upjohn Diagnostic (Kalamazoo, MI). Sucrose was obtained from Beckman (Palo Alto, CA). Methanol was obtained from American Scientific Products (Muskegon, MI). All other reagents and suppliers were of the highest purity available from Fisher or Sigma (St. Louis, MO).

Glycerol lysis preparation. Human platelets were collected by standard thrombocytopheresis procedures, using acid citrate dextrose (2.45 g dextrose, 2.2 g sodium citrate, 730 mg citric acid per 100 ml solution) as the anticoagulant, from normal volunteers who had not received medication for 2 weeks prior to the procedure. Written informed consent was obtained from all volunteers. This protocol was approved by the Medical University of South Carolina Institutional Review Board. Typically, approximately 5000 ml of whole blood was processed yielding about 400 ml of platelet concentrate. The contamination of the concentrate by red and white blood cells was less than 1%. EDTA and indomethacin (5 mM and 10 μM final concentration respectively) were added to the concentrate to prevent platelet activation, and the platelets were pelleted by centrifugation at 3000 *g* for 10 min at 4°.

Lysates were prepared by the glycerol lysis procedure of Barber and Jamieson [12]. The platelets were resuspended in 100 ml isotonic saline containing 25 mM Tris–HCl, pH 7.4, at 4° by gentle rubbing with a rubber policeman, followed by pipetting back and fourth several times to break up visible clumps. The resuspended platelets were carefully layered over two 100-ml 0–40% glycerol gradients prepared in isotonic saline buffer, containing 1 mM EDTA. The platelets were slowly pelleted by centrifugation at 1200 *g* for 30 min at 4° in a swinging bucket rotor, followed by centrifugation at 3000 *g* for 10 min in a fixed angle rotor to produce a final pellet. The supernatant glycerol solution was discarded, and the pellet was resuspended in 60 ml of lysis buffer containing 0.25 M sucrose, 25 mM Tris–HCl, 1 mM EDTA, pH 7.4, at 4°. Swirling alone, as described by Barber and Jamieson [12], did not adequately resuspend the pellet, and many large clumps remained, so the pellet was resuspended with six passes in a tight-fitting glass dounce homogenizer.

The lysates were layered over a 27% (w/w, δ = 1.115 at 5°) sucrose cushion (4 × 25 ml) containing 25 mM Tris–HCl, pH 7.4, at 4° and 1 mM EDTA in an Ultraclear (Beckman) centrifuge tube (filling volume 38.5 ml), and centrifuged at 65,000 *g* for 180 min at 4°. Four protein-containing zones were collected. The top zone, designated zone 1, was the soluble fraction of the platelets. The first particulate fraction, designated zone 2, was collected at the interface between zone 1 and the sucrose layer. The sucrose layer, designated zone 3, also contained a significant amount of protein which could be pelleted by centrifugation at 106,000 *g* for 1 hr. The pellet at the bottom of the sucrose layer was designated as zone 4. Zones 2 and 3 were diluted 1:1 with the Tris–sucrose–EDTA buffer, and pellets were prepared from these fractions by centrifugation at 106,000 *g* for 60 min at 4° in a Beckman Type 30 rotor. These pellets and the pellet from zone 4 were resuspended in Tris–sucrose without EDTA to a protein concentration of 1–10 mg/ml, divided into 500-μl aliquots, frozen in liquid nitrogen, and stored at −70° until assayed.

Nitrogen cavitation preparation. Platelets were collected by thrombocytopheresis and processed by a modification of the method of Kaplin *et al.* [13]. To each 10 γ of platelet-rich plasma was added 1 ml of citrate solution (38 mM citric acid and 75 mM sodium citrate). The platelets were pelleted by centrifugation for 15 min at 2000 *g* at 4°. All subsequent steps were performed on ice. The pellet was resuspended in 100 ml of Tris–citrate buffer (65 mM Tris, 100 mM KCl, 12 mM citrate, pH 6.4, at 4°) and washed once by centrifugation as described above. The final pellet was resuspended in 100 ml Tris–citrate buffer containing 0.4 mM phenylmethylsulfonylfluoride, aprotinin (1 trypsin inhibitory unit per ml), and 2 mM benzamidine HCl. The platelets were lysed three times by explosive decompression using a Parr cell disruption bomb (Parr Instrument Co., Moline, IL) equilibrated with nitrogen at 1000 psi for 12–15 min at 4°. The homogenate was collected and centrifuged at 1000 *g* for 10 min at 4°. The supernatant was removed and saved, and the pellet was resuspended in 50 ml lysis buffer and relysed. The second lysate was collected and centrifuged as described above, the supernatant was pooled with the first supernatant, and the pellet was resuspended in 50 ml homogenizing buffer and relysed. The third lysate and the two supernatants were pooled and designated the lysate. The lysate was centrifuged at 1000 *g* for 20 min at 4°. The supernatant was carefully decanted off the pellet and carried through the rest of the procedure. The pellet was resuspended in 25 ml of lysis buffer, saved, and designated F1. The 1000 *g* supernatant was centrifuged at 12,000 *g* for 20 min at 4°. The supernatant was carefully decanted off this pellet and saved. The pellet was resuspended in 25 ml of lysis buffer, saved, and designated F2. The supernatant was centrifuged at 106,000 *g* for 60 min at 4°. The supernatant was designated F4, and the pellet resuspended in 6 ml lysis buffer, saved, and designated F3.

F2 was layered (in 7-ml aliquots) over three sucrose cushions composed of 30 ml of 1.6 M (δ = 1.21 at 5°) sucrose, 25 mM Tris–HCl and 5 mM EDTA, pH 7.4, at 4°, and centrifuged at 112,700 *g* for 50 min at 4°. This centrifugation yielded three organelle-containing zones: a narrow band at the interface of the homogenizing buffer and the 1.6 M

sucrose layer (G1), a diffuse zone throughout the 1.6 M sucrose layer (G2), and a pellet (G3). The interface was collected by careful aspiration. The sucrose layer was collected by careful decantation and diluted 1:1 with 25 mM Tris–HCl, pH 7.4. The interface and diluted sucrose layers were centrifuged at 106,000 *g* for 60 min at 4°. The resulting pellets, and the pellet from the gradient centrifugation was resuspended in a small volume of homogenizing medium at a protein concentration of 1–10 mg/ml. All fractions were divided into 1-ml aliquots frozen in liquid nitrogen and were stored at −70° until assay.

Enzyme assays. β-D-Glucuronidase activity was used as a marker for lysosomal granules, and was measured by the determination of the rate of release of phenolphthalein from phenolphthalein glucuronide using a modification of the method of Stahl and Fishman [14], with the addition of 0.15% Triton X-100.

Glucose-6-phosphatase was used as a marker for endoplasmic reticulum (dense tubular system) and was measured by the determination of the rate of release of inorganic phosphate from glucose-6-phosphate using the method of Aronson and Touster [15]. Inorganic phosphate was determined in the supernatants using a modification of the method of Fiske and SubbaRow as described by Aronson and Touster [15]. In two experiments, NADPH-cytochrome *c* reductase activity was used as an additional marker for endoplasmic recticulum (dense tubular system) and was measured following the method of Guengerich [16].

Succinate dehydrogenase activity was used as a marker for mitochondria and was measured by the determination of the rate of reduction of oxidized cytochrome *C* by a modification of the method of Tisdale [17]. Monoamine oxidase activity, an outer mitochondrial membrane enzyme [18], was also measured as an alternative marker for mitochondria, and to ensure that the membrane-enriched fractions were not contaminated with mitochondrial membranes. Monoamine oxidase activity was determined by a modification of the method of Robinson *et al.* [19], by following the oxidation of [^{14}C]-benzylamine. Assays were conducted at 37° in 12 × 75 mm silanized glass tubes containing: 0.2 M phosphate buffer, pH 7.2, ~100,000 cpm [^{14}C]-benzylamine (1 mM) and 10–300 μg of protein from each fraction in a total volume of 1 ml. Blank tubes containing buffer, [^{14}C]-benzylamine but no protein were also carried through the procedure. The reaction was allowed to proceed for 1 hr and was stopped by the addition of 0.1 ml of 60% perchloric acid. The entire mixture was added to a glass vial containing counting solution without solubilizer and shaken well. Under these conditions, the counting assay solution mixture formed two phases. The [^{14}C]-benzylamine remained in the aqueous phase, and the [^{14}C]-benzaldehyde extracted into the organic phase. Typically blanks were only 1% or less of the total counts. Enzyme activity was determined from the specific activity of the [^{14}C]-benzaldehyde formed. Under these assay conditions, enzyme activity was linear with time to at least 90 min, and up to 400 μg protein.

Phosphodiesterase activity was used as a marker for plasma membranes and was measured by the determination of the rate of release of *p*-nitrophenol from bis-*p*-nitrophenylphosphate, using a modification of the method of Taylor and Crawford [20], with the addition of 0.15% Triton X-100. The reaction was stopped with glycine–sodium dodecyl sulfate buffer (pH 11.7) as described for β-glucuronidase [15].

Lactate dehydrogenase activity was used as a marker for the soluble, cytosolic fraction, and was determined at room temperature by the spectrophotometric method of Wroblewski and LaDue [21] using a Sigma kit.

β-Thromboglobulin levels were used as a marker for α granules and were determined using a radioimmunoassay kit from Amersham. The samples were subjected to five freeze–thaw cycles to ensure complete release of β-thromboglobulin, and serial dilutions of each fraction were prepared in distilled water for the assay. Samples were assayed at three different dilutions. Immunoreactivity in the fractions was parallel with the standard curve.

Serotonin (5-HT) content was used as a marker for dense granules. [^{14}C]-5-HT creatinine sulfate was added to the platelet-rich plasma (PRP) to a final concentration of 0.5 μM. The PRP was then incubated for 15–20 min at 37° in a shaking water bath. [^{14}C]-5-HT was determined by liquid scintillation counting of 0.1 to 1 ml aliquots of the various fractions. The uptake of [^{14}C]-5-HT was typically 80–90% of the amount added. Greater than 95% of the [^{14}C]-5-HT that was taken up was released by thrombin (0.1 to 0.5 units/ml). The identity of the ^{14}C associated with the various fractions as [^{14}C]-5-HT was confirmed by thin-layer chromotography on silica gel G using ethyl acetate–isopropanol–ammonium hydroxide (45:35:20) to develop the plates. Of the radioactivity in the fractions examined, 91% co-migrated with authentic 5-HT.

Protein was determined by a modification of the method of Lowry *et al.* [22].

Preparation of fractions for electron microscopy. Small pellets of the various platelet fractions were submerged in half-strength Karnovsky's fixative [23] (pH 7.4) for 24–48 hr, triply washed in 0.1 M sodium-cacodylate buffer (pH 7.4) containing 7.5% sucrose, post-fixed for 1 hr in the same buffer with 2% OsO_4, briefly rinsed in distilled H_2O, and stained *en bloc* in 0.5% uranyl acetate buffered with 0.1 M sodium-hydrogen-maleate (pH 5.0). Following another brief rinse in distilled water, the pellets were dehydrated routinely in graded alcohols and propylene oxide. Each pellet was then fragmented with a sharpened orange stick, and the resulting pieces were embedded in epoxy resins. Sections of several randomly selected blocks of each pellet stained with lead citrate and saturated uranyl acetate were examined in a Hitachi HS8-2 electron microscope and characterized without prior knowledge of their presumed origin or composition.

Radioligand-binding assays. Binding of [^{125}I]-PTA-OH to the various fractions was used as a marker for the TXA_2/PGH_2 receptor. Binding assays were conducted using a modification of a previously described method [10]. Assays were performed in silanized glass tubes (12 × 75 mm) at 30° for 30 min. Incubations (200 μl) contained 25–50 μg

protein, 25 mM Tris–HCl buffer, pH 7.4, ~100,000 cpm of [^{125}I]-PTA-OH (0.2 nM) and various concentrations of either I-PTA-OH or U46619. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (2 mM) was included in the assay mixture. The reaction was terminated by dilution with 4 ml of ice-cold 25 mM Tris–HCl (pH 7.4) containing 2 mM CHAPS and filtered under reduced pressure through Whatman GF/C glass fiber filters that had been pretreated with 0.3% polyethylenimine for at least 1 hr. The filters were washed with three additional 4-ml aliquots of the ice-cold buffer. CHAPS has been used previously to successfully solubilize the receptor [11], and was found to decrease the non-displaceable binding.

Data analysis. Data from equilibrium binding studies of [^{125}I]-PTA-OH were analyzed by a two-site model using a Simplex computer program [24, 25] as previously described for binding to a crude membrane preparation [10]. The data in Table 1 were analyzed by a Mann–Whitney U test. Values were considered to be significantly different at the $P < 0.05$ level. Studies of the competition by U46619 for the [^{125}I]-PTA-OH binding sites were analyzed by constructing log-logit plots of the percent inhibition of displaceable binding of [^{125}I]-PTA-OH versus concentration. The IC_{50} was defined as the concentration of U46619 giving 50% inhibition of displaceable [^{125}I]-PTA-OH binding.

RESULTS

Glycerol lysis preparation. Figure 1 shows the distribution of the marker enzymes in each of the various fractions. The data are presented as relative enrichment (ratio of the specific activity) compared to the lysate. Thus, enrichment of a particular enzyme in a fraction would be indicated by a value greater than one. Recovery of protein applied to the gradient in the various fractions was as follows: zone 1, 67 ± 2.5%; zone 2, 1.1 ± 0.2%; zone 3, 4.3 ± 0.8%; zone 4, 23 ± 1.6% for a total recovery of 95 ± 2.3%. Zone 1 was enriched only in lactate dehydrogenase activity and was disenriched in all other markers. Zone 2 was enriched in the plasma membrane marker, phosphodiesterase, and in the dense tubular system marker, glucose-6-phosphatase, and was disenriched in the mitochondrial, lysosomal and cytosolic markers. The electron micrograph of zone 2 (Fig. 2A) shows a homogenous preparation of small unilamellar vesicles with no granules or mitochondria present. Zone 3 was also enriched in both membrane markers and disenriched in mitochondrial and cytosolic markers. There was, however, a slight (1.5-fold) enrichment in the lysosomal marker, β-glucuronidase. The major constituent of this fraction was unilamellar vesicles that were somewhat larger than those in zone 2, with no mitochondria present, but there was slight contamination by granular constituents (Fig. 2B). Zone 4 was enriched primarily in the mitochondrial marker, succinate dehydrogenase, but was also enriched to a lesser extent in both membrane markers. There was no enrichment of the lysosomal marker in this zone, and the cytosolic marker was disenriched. Electron micrographs of this fraction (not shown) revealed that it contained mitochondria and granules, but it was also contaminated by large membrane vesicles and insoluble cytoplasmic constituents.

In addition to the markers discussed above, monoamine oxidase and NADPH–cytochrome *c* reductase

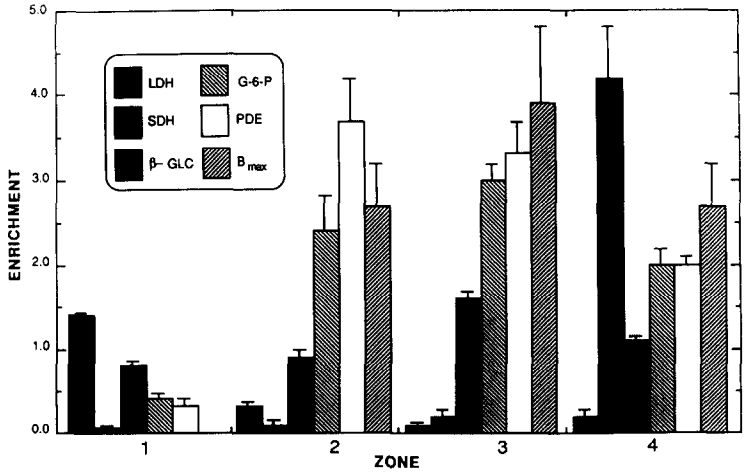

Fig. 1. Distribution of marker enzymes and B_{max} for [^{125}I]-PTA-OH for subcellular fractions prepared by glycerol lysis. Data are presented as the mean ± SEM from six to seven preparations prepared as described in Methods. The determination of enrichment is described in Results. Abbreviations are as follows: LDH, lactate dehydrogenase; SDH, succinate dehydrogenase; β-GLC, β-glucuronidase; G-6-P, glucose-6-phosphatase; and PDE, phosphodiesterase. Zones are as described in Results. Enzyme activities in the lysate were as follows: phosphodiesterase, 0.028 ± 0.007 nmol/μg protein/hr; glucose-6-phosphatase, 0.408 ± 0.047 nmol/μg protein/hr; β-glucuronidase, 0.060 ± 0.012 nmol/μg protein/hr; succinate dehydrogenase, 0.741 ± 0.150 nmol/μg protein/hr; and lactate dehydrogenase, 1.02 ± 0.24 units. These values are in the same range as those previously obtained for platelet lysates [26].

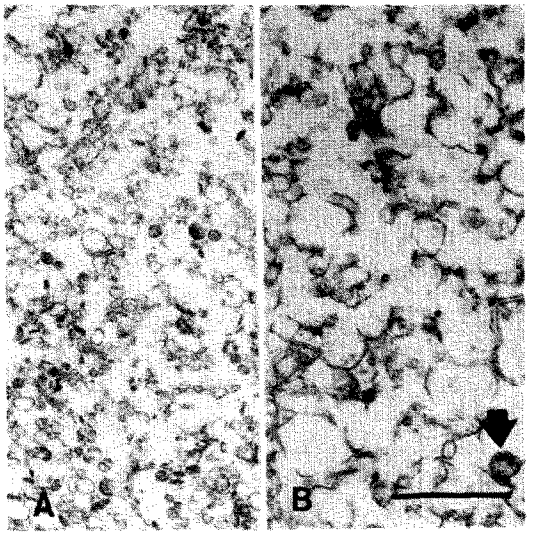

Fig. 2. Electron micrographs of zones 2 and 3 from the glycerol lysis preparation. (A) Zone 2 is comprised of a uniform population of unilamellar membrane vesicles approximately to 0.2 μm in diameter. (B) Zone 3 contains predominately uniform membrane vesicles approximately 0.6 μm in diameter as well as occasional dense cored vesicles (granules, arrow). Bar = 1 μm.

activity were measured in two preparations. The distribution of monoamine oxidase activity, a constituent of the outer mitochondrial membrane, followed a pattern similar to that found for succinate dehydrogenase activity. Zones 1, 2, and 3 were disenriched in monoamine oxidase, whereas zone 4 was enriched 2.9-fold in monoamine oxidase (N = 2). These data are consistent with a lack of contamination of zones 2 and 3 by mitochondrial membranes. Likewise, NADPH–cytochrome *c* reductase followed a pattern similar to that found for glucose-6-phosphatase, strengthening the evidence that zone 2 contains significant amounts of dense tubular system (data not shown), in contrast to the report of Barber and Jamieson that this fraction is derived primarily from plasma membrane [12]. In one preparation, the distribution of β-thromboglobulin in the fractions was determined. No β-thromboglobulin was detectable in zones 1 or 2, but there was some detectable activity in zone 3 and considerable enrichment (5-fold) in zone 4.

The binding of the TXA_2/PGH_2 receptor antagonist [^{125}I]-PTA-OH was used as a marker for the TXA_2/PGH_2 receptor. There was no specific binding of [^{125}I]-PTA-OH in zone 1, the soluble fraction. Specific binding was observed in the other three zones, and it was characterized further with equilibrium binding studies to determine binding site density and its affinity for the ligand in each of the zones. There was no significant change in the K_d for [^{125}I]-PTA-OH in any of the fractions compared to the lysate (Table 1). There was a significant ($P < 0.05$) increase in the B_{max} in all of the zones when compared to the lysate by a Mann–Whitney U test. There was no significant difference in the B_{max} values compared between zones.

Table 1. Comparison of K_d and B_{max} values from the glycerol lysis preparations

	K_d (nM)	B_{max} (pmol/mg protein)
Lysate	49 ± 11	4.1 ± 1.7
Zone 2	59 ± 17	8.6 ± 2.8*
Zone 3	99 ± 31	18 ± 7.5*
Zone 4	94 ± 30	13 ± 3.8*

Data are expressed as mean ± SEM for six preparations. Experiments were performed as described in Methods.

* Significantly different from lysate ($P < 0.05$) as determined by the Mann–Whitney U test. There was no significant difference between zones as determined by the Mann–Whitney U test.

The ability of the TXA_2/PGH_2 mimetic U46619 [7] to compete with [^{125}I]-PTA-OH for its binding site in the lysate and in each of the zones was used as a measure of the specificity of the binding. Figure 3 shows the competition curves for U46619 in each of the zones assayed. U46619 inhibited [^{125}I]-PTA-OH binding in the lysate with an IC_{50} of 5.4 ± 1.2 μM (N = 5), similar to that found previously for crude and solubilized membrane preparations [9, 10]. The IC_{50} values for zones 3 and 4 were 5.9 ± 1.4 μM (N = 5) and 2.4 ± 0.6 μM (N = 5) respectively. There was no significant difference ($P > 0.05$) between the IC_{50} values for the lysate and zone 3 or 4. The IC_{50} for U46619 in zone 2 was determined in only one preparation due to low yields of protein and was found to be 2.5 μM.

Nitrogen cavitation preparation. Differential centrifugation of the lysate produced by nitrogen cavitation yielded four fractions F1, the 1000 *g* pellet, contained 15% of the lysate protein. F2, the 12,000 *g* pellet, previously described as being enriched in mitochondria and granules [13], contained 16% of the total recovered protein. F3, the 106,000 *g* pellet, contained 7.4% of the protein; and F4, the 106,000 *g* supernatant, considered to be the soluble or cytosolic fraction contained 60% of the protein. The total recovery of protein was 98%. Subfractionation of F2 over a sucrose cushion yielded three protein-containing zones: G1, the interface at the top of the 1.6 M sucrose; G2, the 1.6 M sucrose layer, and G3, the pellet at the bottom of the 1.6 M sucrose layer.

Figure 4 shows the enrichment of the markers for some of the fractions. F1 was slightly enriched in all of the particulate markers assayed, and was disenriched in the cytosolic marker, lactate dehydrogenase (data not shown). F2 was enriched primarily in the granule markers β-thromboglobulin (4.5-fold) and to a lesser extent 5-HT (2.4-fold), succinate dehydrogenase (2.3-fold) and phosphodiesterase (1.7-fold). The B_{max} was enriched 2.0-fold in this fraction. F3 was enriched only in phosphodiesterase (2.7-fold) and disenriched in all other markers. The greatest enrichment of binding (3.4-fold) was found in this fraction. Fraction F4 was enriched only in lactate dehydrogenase and was disenriched in all other markers (data not shown). Subfraction G1 was enriched in all organelle markers, with the greatest enrichment occurring for the mitochondrial marker

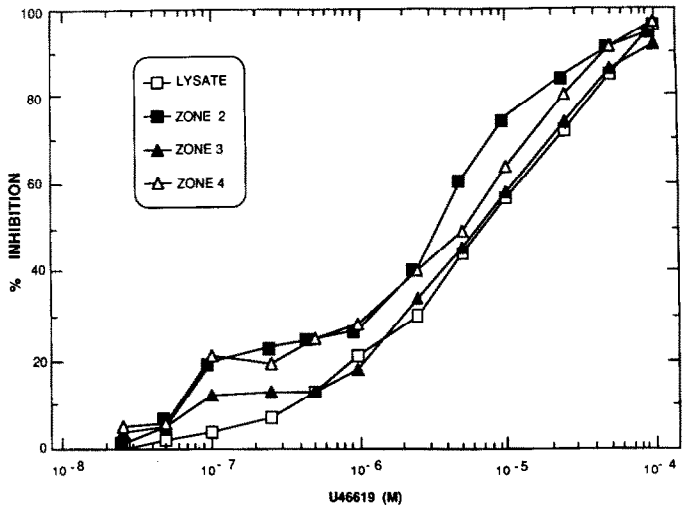

Fig. 3. Competition of [^{125}I]-PTA-OH with the TXA_2/PGH_2 mimetic U46619. Data are presented as the mean of triplicate determinations for five preparations except for zone 2 which is the mean of triplicate determinations for one preparation as described under Methods.

(4.8-fold), but the B_{max} was enriched only 1.6-fold. Subfraction G2 showed only slight enrichment in organelle markers (data not shown). Subfraction G3 was enriched primarily in the two granule markers, with slight contamination by mitochondria and membranes. Enrichment of binding in this fraction was only 1.9-fold. The electron micrographs of F2 and G3 shown in Fig. 5 confirm the results of the enzyme assays. Figure 5A shows the electron micrograph of F2, which appears to be composed primarily of mitochondria and granules with some contaminating membranes. Figure 5B shows the electron micrograph of G3, which appears to consist almost entirely of granules, with slight contamination by mitochondria and membranes.

DISCUSSION

While there is indirect evidence that the platelet TXA_2/PGH_2 receptor may be located on either the plasma membrane or the dense tubular system (*vide infra*), such studies do not rule out localization of the receptor on other platelet organelles and, given the multiplicity of effects of TXA_2/PGH_2 mimetics on platelets, the receptor may reside on more than one organelle. For example, U46619 has been shown to enhance the release of $^{45}Ca^{2+}$ from bovine pulmonary artery mitochondria [27]. Thus, it is important to examine all organelles for possible localization of the receptor.

The present report describes the localization of

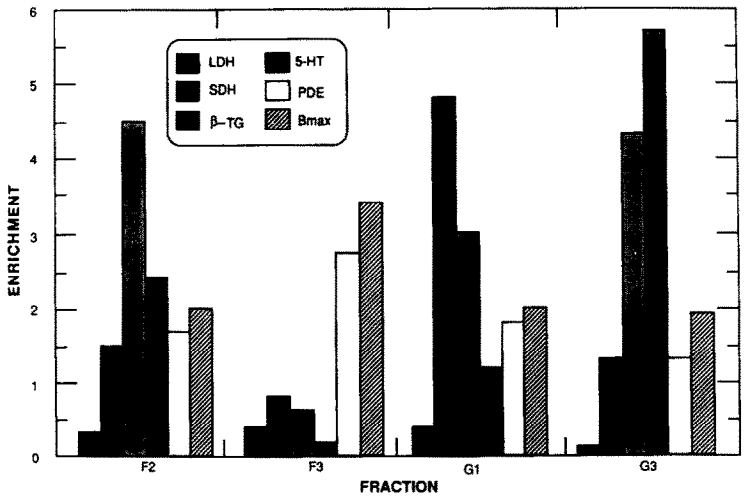

Fig. 4. Distribution of marker enzymes and B_{max} for [^{125}I]-PTA-OH for the subcellular fractions prepared by nitrogen cavitation. Data are presented as the mean of two to three determinations for one preparation prepared as described in Methods. Abbreviations are as described in the legend of Fig. 1 with the following addition: β-TG, β-thromboglobulin; and 5-HT, [^{14}C]-5-hydroxytryptamine.

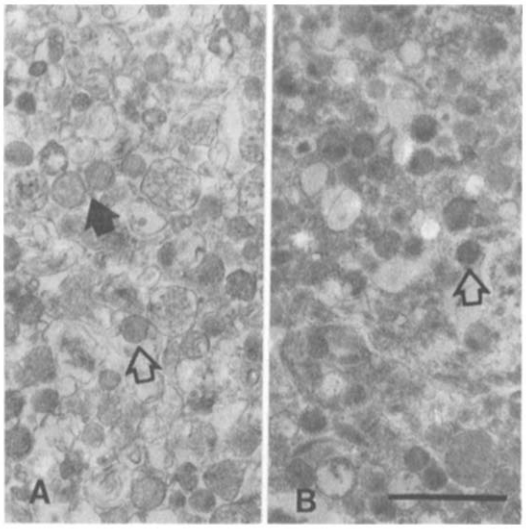

Fig. 5. Electron micrographs of fractions F2 and G3 from the nitrogen cavitation preparation. (A) Fraction F2 is comprised mostly of mitochondria (open arrow) and granules (closed arrow). (B) Fraction G3 is comprised mostly of granules (open arrow). Bar = 1 μm.

the binding site for [^{125}I]-PTA-OH in subcellular fractions of human platelets prepared using two different cell disruption techniques. The binding site appears to be located on either (or both) the plasma membrane or dense tubular system, and does not appear to be located on the mitochondria, or any of the three types of granules, nor does it appear to be a constituent of the cytoplasm. Support for this notion is derived from the fact that the greatest enrichment of binding occurred only in those fractions that were enriched in the activities of phosphodiesterase (plasma membranes) and glucose-6-phosphatase and NADPH-cytochrome *c* reductase (dense tubular system). That the binding site is not located within the soluble, cytosolic fraction is indicated by a lack of enrichment of binding in those fractions enriched in lactate dehydrogenase, a marker for the cytoplasm. The binding site does not appear to be located on the mitochondria since its enrichment did not follow the enrichment of the mitochondrial markers (succinate dehydrogenase and monoamine oxidase). Likewise, the binding site does not appear to be located on any of the granules since the enrichment of binding did not follow the granule markers (5-HT and β-thromboglobulin). Thus, it appears that the binding site is located on either the dense tubular system or on the plasma membranes, or both. While a high voltage free flow electrophoresis procedure has been described for resolving plasma membranes and dense tubular system [28], the equipment required for this technique is not readily available. Additionally, there is a requirement for pretreatment of the platelets with neuraminidase in order to achieve good separation, a treatment which might alter the characteristics of the PGH_2/TXA_2 receptor.

Evidence that the binding site represents the putative TXA_2/PGH_2 receptor is supported in part by the observation that U46619, a TXA_2/PGH_2 mimetic, competed with [^{125}I]-PTA-OH for binding to the various fractions. The IC_{50} for U46619 in the fractions was not significantly different from the IC_{50} for U46619 obtained in the lysate, which in turn was similar to the IC_{50} determined previously for U46619 in crude and solubilized membrane preparations [9, 11]. Thus, the binding of [^{125}I]-PTA-OH in these preparations would appear to be specific for the putative TXA_2/PGH_2 receptor, as has been determined previously [9–11]. Clearly, additional confirmatory data that the binding site is the receptor would be valuable; however, the amounts of protein obtained in these studies precluded more extensive studies.

There is indirect evidence in the literature for localization of the TXA_2/PGH_2 receptor within a membrane fraction of the platelet. It was first suggested that TXA_2 and PGH_2 might elicit their effects by acting at the dense tubular systems [29, 30], since the dense tubular system is the site of synthesis of PGH_2 and TXA_2 [31–33] and is the major storage site of releasable calcium within the platelet [29]. Gerrard *et al.* [33] reported that arachidonic acid and the prostaglandin endoperoxides are capable of promoting release of calcium from a membrane preparation enriched in the dense tubular system. Rybicki *et al.* [34], using a similar preparation, found that the arachidonic acid-stimulated release could be blocked by the TXA_2/PGH_2 antagonist 13-azaprostanoic acid, consistent with the localization of the receptor in the dense tubular system. These studies were extended by Mayo *et al.* [35] who found that U46619 could promote calcium release in a preparation enriched in the dense tubular system. In studies on intact platelets, U46619 has been reported to cause a release of intraplatelet membrane-bound calcium, as determined using chlortetracycline fluorescence [36]. Additionally, it has been reported that added arachidonic acid can release intraplatelet calcium, and this effect is blocked by the TXA_2/PGH_2 receptor antagonist 13-azaprostanoic acid [37, 38]. Taken together, these data are consistent with the localization of the receptor in the dense tubular system.

In contrast to these reports, Carey *et al.* [39] have reported that neither PGH_2, TXA_2 nor U46619 is capable of stimulating calcium release from a highly purified preparation of dense tubular system membranes. The discrepancy between these and the earlier studies may be due to contamination of the preparations in the earlier studies by plasma membranes, or to loss of functional receptors during preparation of the dense tubular system membranes. Also, it has been reported that the increase in intraplatelet-free calcium by arachidonic acid or U46619, as measured by quin2 fluorescence, is due mainly to an influx of extracellular calcium [3]. These studies suggest that the receptor may reside on the plasma membrane. Additionally, a monoclonal antibody to β_2-microglobulin blocks the aggregation and secretion responses to TXA_2 and U46619 in a manner similar to 13-azaprostanoic acid [40]. The antibody did not block the responses to thrombin or ADP. These data are consistent with competitive antagonism of the TXA_2/PGH_2 receptor and also suggest that the receptor may reside on the plasma membrane.

Localization of the TXA_2/PGH_2 receptor on the plasma membrane would be consistent with the localization of many other pro-aggregatory receptors, which are located on the platelet plasma membrane. These include glycoprotein V, a receptor for thrombin [41], the GPIIb/III complex for fibrinogen [42], GPIb for von Willebrand Factor [43], the ADP receptor [44], and the α_2-adrenergic receptor [45].

In summary, it appears that the binding site for the TXA_2/PGH_2 antagonist [^{125}I]-PTA-OH, and by inference the TXA_2/PGH_2 receptor, is located either within the dense tubular system or the plasma membrane, or both. Absolute localization awaits the development of additional techniques for the study of the TXA_2/PGH_2 receptor, such as histocytochemical localization using antibodies directed at the receptor, photoaffinity radiolabeling and autoradiography or improved fractionation techniques for separation of plasma membranes from the dense tubular system.

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