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PHOTOAFFINITY LABELLING OF A THROMBOXANE A_2/PROSTAGLANDIN H_2 ANTAGONIST BINDING SITE IN HUMAN PLATELETS

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The diazonium salt of 9,11-dimethylmethano-11,12-methano-16-(4-aminophenoxy)13,14-dihydro-13-aza-15αβ-ω-tetranor TXA_2 (PTA-POA) was synthesized and used as a photoaffinity ligand for the putative human platelet TXA_2/PGH_2 receptor. Incubation of human platelet membranes with the diazonium salt of PTA-POA followed by photolysis at 290 nm(hν) resulted in a 40% decrease in the specific binding of [^{125}I]PTA-OH as measured in the radioligand binding assay. Co-incubation with a TXA_2/PGH_2 agonist followed by photolysis resulted in no decrease in specific binding. Incubation of the diazonium salt of PTA-POA with solubilized platelet membranes without photolysis followed by Scatchard analysis resulted in no change in the K_d for [^{125}I]PTA-OH (38 nM) and the preparation which was incubated with the diazonium salt (42 nM). However, the B_{max} for [^{125}I]PTA-OH binding was reduced from 2.4 pmole/mg protein for control to 1.4 pmole/mg protein. These studies show that the diazonium salt of PTA-POA may be a useful photoaffinity ligand for human platelet TXA_2/PGH_2 receptors.

Human platelets aggregate in response to thromboxane A_2 (TXA_2) and prostaglandin H_2 (PGH_2) through stimulation of specific receptors (1). Recently, a specific binding site for the TXA_2/PGH_2 antagonist I-PTA-OH (2) has been described in washed human platelets (3) and human platelet membranes (4). In addition, this binding site has been solubilized from human platelets in active form using the detergent CHAPS (5) and its hydrodynamic properties characterized (6).

Abbreviations. PTA-POA, 9,11-dimethylmethano-11,12,methano-16-(4-aminophenoxy)13,14-dihydro-13-aza-15αβ-ω-tetranor-TXA_2; I-PTA-OH, 9,11-dimethylmethano-11,12-methano-16-(3-iodo-4-hydroxyphenyl)-13,14-dihydro-13-aza-15αβ-ω-tetranor-TXA_2; SQ26655, [1S-(α,2β(5Z), 3α(1E, 3S), 4α)]-7-[3-(3-hydroxy-1-octenyl)-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; CHAPS, 3-[(cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; DZ, diazonium salt; TXA_2, thromboxane A_2; PGH_2, prostaglandin H_2.

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Photoaffinity ligands have been frequently used to characterize a variety of receptors. However, to date there have been no photoaffinity ligands synthesized in the eicosanoid field. To further the characterization and purification of the TXA_2/PGH_2 receptor we report herein the synthesis and evaluation of a photoaffinity ligand.

MATERIALS AND METHODS

The synthesis of PTA-POA (IV) is shown in Figure 1. The 13-azapinane intermediate (I) was a gift of ONO Pharmaceutical Co., Osaka, Japan.

(p-Nitrophenoxy)-2,3 epoxypropane (II). Into 50 ml of dry acetone was added 3.0 g (22 mmoles) of 4-nitrophenol, 44 mmoles of anhydrous K_2CO_3 and 3.5 g (44 mmoles) of epichlorohydrin. This solution was refluxed for 18 hrs followed by addition of 50 ml of water. The mixture was extracted with 2 x 50 ml of ethyl ether and the ether extracts washed with 2 x 25 ml portions of 1N NaOH. The ether extracts were dried over $MgSO_4$ followed by removal of the ether under vacuum. The residue was distilled under reduced pressure to give the desired epoxide in 85% yield (121-123° at 0.5 mm). Mass spectral data was consistent with the assigned structure; $[M]^+ = 195$, $[M-43]^+ = 162$.

9,11-Dimethylmethano-11,12-methano-16-(4-nitrophenoxy) 13,14-dihydro-13-aza-15αβ-ω-tetranor TXA_2 (III). Into 3 ml of dry methanol was added 50 mg (180 μmoles) of amine I and 38 mg (197 μmoles) of epoxide II. This solution was refluxed under an atmosphere of argon for twenty hours followed by removal of the methanol under vacuum. The methyl ester was hydrolysed by stirring the residue in 3 ml of a 1:1 mixture of THF and 0.2 N LiOH overnight at room temperature. The product was purified on a silica column using $CHCl_3$ and MeOH(9:1) as the mobile phase. A yellow glass was obtained (68% yield, 56 mg). The methyl ester of III showed $[M]^+ = 474$, $[M - 152]^+ = 322$, $[M-182]^+ = 292$.

9,11-Dimethylmethano-11,12-methano-16-(4-aminophenoxy)13,14-dihydro-13-aza-15αβ-ω-tetranor TXA_2 (PTA-POA)(IV). The nitro compound was reduced using ammonia-ferrous sulfate method described previously (7) in a 60% yield after purification on a silica column. The methyl ester of IV showed $[M]^+ = 444$, $[M-152]^+ = 292$.

PTA-POA was dissolved in 500 μl of 3 N acetic acid to give a concentration of 500 μM and cooled to 0°C. To this solution was added 10 μl of 135 mM $NaNO_2$ dissolved in water and the reaction allowed to sit on ice for ten minutes. The diazonium salt was then diluted into the membrane or solubilized membrane preparation to a final concentration of 500 nM and the preparation incubated for one hour at 0°C followed by photolysis at 0°C for 30 minutes in a 3 ml quartz cuvette in a spectrophotometer (American Instrument Co., Silver Spring, MD.) set at a wavelength of 290 nm.

Platelet membranes (4) and solubilized membranes (5) were prepared as previously described. Following photolysis, the membranes were centrifuged and resuspended in 10 ml of buffer a total of three times to remove unreacted ligand. The solubilized preparation was passed through a Sephadex G-25 column to remove the unreacted ligand. [^{125}I]PTA-OH binding assays were carried out as described previously (3-6). [^{125}I]PTA-OH was prepared and stored as previously described (3). All other reagents were of the highest purity available from Sigma (St. Louis, MO, USA). SQ 26655 was a gift of Dr. D.N. Harris of Squibb Institute for Medical Research (Princeton, NJ).

RESULTS

The chemical structure of PTA-POA is shown in Figure 1 while the structures of the diazonium salt (DZ) and the presumed photolysis product are shown in Figure 2. The highly reactive arylcation is generated from the diazonium salt following exposure to ultraviolet radiation. In order to establish if this reactive species may incorporate into the TXA_2/PGH_2 receptor, human platelet membranes were prepared and incubated with the diazonium salt (500 nM) for 60 min at 0°C. Following the 30 min photolysis reaction and washing of the membranes, binding assays using the radioligand [^{125}I]PTA-OH were performed to determine the number of specifically displaceable bound counts. Table 1 shows the combined results from three experiments and are shown as a percent of control. The control membranes, received only vehicle. Neither irradiation of the membranes nor incubation of the membranes with the diazonium salt alone resulted in any significant change in the number of displaceable counts. In contrast, membranes incubated with the diazonium salt and irradiated had 43 ± 8% of the displaceable bound counts compared to control. To insure that the loss in displaceable bound counts was specific, the membranes were also incubated in the presence of the TXA_2/PGH_2 agonist SQ26655 (50 μM)(8). As indicated in Table 1, SQ26655 prevented the diazonium salt from being covalently linked

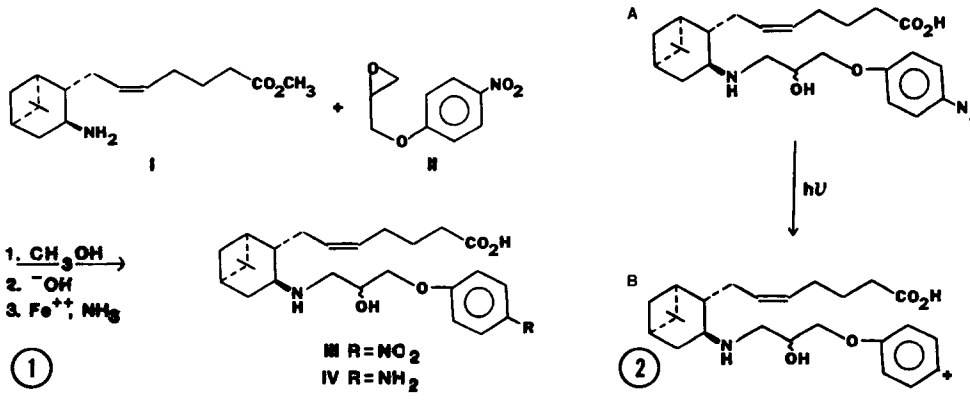

Figure 1. Synthetic pathway to photoaffinity ligand PTA-POA.

Figure 2. Structure of diazonium salt of PTA-POA(A) and photolysis product(B).

Table 1. Effect of the diazonium salt of PTA-POA on Binding of [^{125}I]PTA-OH to Human Platelet Membranes

Preparation	Displaceable Counts % of Control
DZ	102 ± 2
DZ + hν	43 ± 8
hν	87 ± 7
DZ + hν + SQ 26655	88 ± 4

Mean ± S.E.M., n = 3.
Platelet membranes were prepared as previously described (4). The control preparation was incubated with the diazonium salt (DZ) vehicle only and carried through the washings done for the other membrane preparations. The final concentration of SQ 26655 was 50 μM. Binding studies with [^{125}I]PTA-OH were conducted as previously described (3). The displaceable counts for the controls in each of the experiments were 936, 1673 and 495. The protein concentration per assay for each experiment ranged from 75 μg/tube to 130 μg/tube and were determined by the method of Lowry (13).

to the receptor after photolysis since the displaceable bound counts were essentially that of the control.

In a similar manner, two experiments were performed on solubilized platelet membranes. Membranes solubilized in CHAPS were incubated with the diazonium salt of PTA-POA (500 nM) followed by photolysis. In control experiments, the solubilized preparation was incubated with the diazonium salt but not photolyzed. Each of these preparations were passed through a

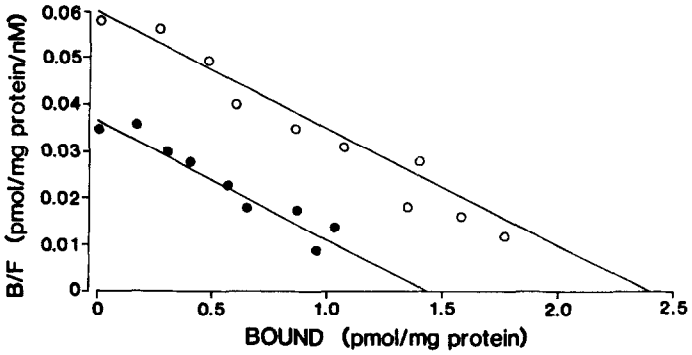

Figure 3. Representative Scatchard analysis of equilibrium binding data for [^{125}I]PTA-OH in solubilized human platelet membranes. Solubilized platelet membranes were incubated with 500 nM of diazotized PTA-POA for 60 min at 0°C followed by irradiation with light at 290 nm(●). The solubilized preparation was passed through a Sephadex G-25 column to remove excess ligand followed by equilibrium binding with [^{125}I]PTA-OH. The control was incubated with the diazonium salt of PTA-POA but was not irradiated(○).

Sephadex G-25 column to remove unreacted ligand followed by equilibrium binding with [^{125}I]PTA-OH. The results of one of the experiments are shown as a Scatchard plot in Figure 3. The K_d values for the two preparations were unchanged, being 38 and 42 nM for the control and photolyzed preparation, respectively. However, the B_{max} value was reduced 40%, from 2.4 pmole/mg protein for control to 1.4 pmole/mg protein for the photolyzed preparation. In an additional experiment, the K_d values were 32 nM for both the control and irradiated preparation and the B_{max} was 1.56 pmoles/mg protein for the control and 0.80 pmole/mg protein for the irradiated sample. Photolysis alone in the absence of the diazonium salt had no effect on the K_d or B_{max} values (data not shown).

DISCUSSION

Photoaffinity labelling is a well documented method used for the purification and characterization of biological receptors. It is also a useful pharmacological tool for the study of spare receptors. Herein we report the synthesis of the first photoaffinity ligand for use in the eicosanoid field and present evidence of its photo-incorporation into the putative human platelet TXA_2/PGH_2 receptor.

The design of PTA-POA is based on findings that the ω-side chain of prostaglandin and thromboxane derivatives can be modified without subsequent loss in biological activity (9-10). While the use of arylazido photoaffinity probes is the most widely described, photolysis of aryldiazonium salts are known to generate the corresponding arylcation which is hyper-reactive and not subject to rearrangement (11). Recently, aryldiazonium salts have been successfully utilized as photoaffinity probes of the nicotinic acetylcholine receptor (12).

Structurally, PTA-POA is closely related to other 13-azapinane derivatives developed in our laboratory which possess biological activity (2,9). Only when platelet membranes or solubilized membranes were photolyzed in the presence of the diazonium salt of PTA-POA was there a decrease in specific

binding. This suggests that the generation of a reactive species is necessary for the irreversible incorporation of the ligand into the putative TXA_2/PGH_2 receptor. In addition, the prevention of the reduction in displaceable counts induced by the photolyzed diazonium salt in the presence of SQ26655 supports the specificity of the incorporation. These results suggest that the diazonium salt of PTA-POA covalently incorporates into the human platelet TXA_2/PGH_2 receptor and provides a useful tool for the further characterization of this receptor. Futhermore, these studies suggest that additional photoaffinity ligands can be made for other eicosanoid receptors using this similar chemical approach, i.e. ω side chain modifications.

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