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A photoaffinity label for the thromboxane A_2/prostaglandin H_2 receptor in human blood platelets

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A photoactive iodoarylazide derivative (I-APA-PhN_3) of the competitive thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) antagonist 13-azaprostanoic acid is evaluated. Upon photoactivation, the compound was found to inhibit specifically and irreversibly human platelet aggregation induced by the TXA_2/PGH_2 mimetic U46619. In receptor-binding studies using [^{3}H]U46619, I-APA-PhN_3 exhibited an IC_{50} of 300 nM for inhibition of U46619 binding. Photoactivation of I-APA-PhN_3 resulted in an irreversible 58% reduction in specific binding of U46619. This compound and its corresponding radio-iodinated form will prove to be useful tools for the isolation and purification of the TXA_2/PGH_2-binding protein in human platelets.

Platelet; Thromboxane receptor; Photoaffinity label; U46619

1. INTRODUCTION

Through their actions as potent vasoconstrictors and platelet-aggregating agents, thromboxane A_2 (TXA_2) and prostaglandin H_2 (PGH_2) are likely contributors to the pathogenesis of thrombosis, atherosclerosis and angina. This has resulted in considerable interest in the nature of the TXA_2/PGH_2 receptor. A number of specific antagonists [1–5] and radioligands [6–10] have been employed to study the TXA_2/PGH_2-binding site, and one of the major goals of research in this area is the complete purification and characterization of this protein.

In this connection, the development of photoaffinity labelling techniques using radioactive probes has aided greatly in the identification and purification of hormone and drug receptors [11,12]. Thus, we have synthesized 17,18,19,20-tetranor-16-(3′-iodo-4′-azidophenyl)-13-azaprostanoic acid (I-APA-PhN_3, fig.1), a derivative of the competitive TXA_2/PGH_2 antagonist *trans*-13-azaprostanoic acid (13-APA). I-APA-PhN_3 incorporates a photoactive iodophenylazide moiety and is easily radio-iodinated [13–15]. We report here the

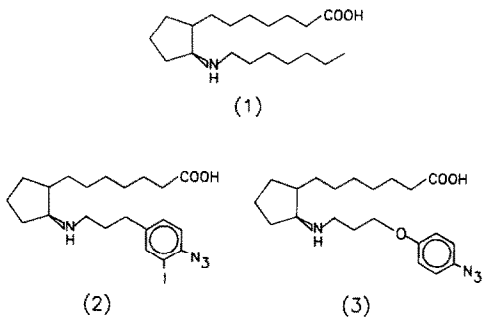

Fig.1. Structures of 13-APA (1), I-APA-PhN_3 (2) and APA-O-PhN_3 (3).

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biological characterization of this new photoaffinity label for the TXA_2/PGH_2 receptor.

2. MATERIALS AND METHODS

2.1. *Aggregation studies*

The synthesis of I-APA-PhN_3 has been described [13,14]. Platelet-rich plasma (PRP) was prepared from blood collected into 0.38% citrate-phosphate-dextrose-adenine from healthy, drug-free donors. All aggregations were performed by the turbidometric method [16], and contained 20 μM indomethacin. Drugs or vehicle (95% ethanol) were added 1 min prior to the aggregating agent. In photoactivation studies, PRP containing 20 μM I-APA-PhN_3 was subjected to 30 min of UV irradiation from a 100 W mercury lamp. The platelets were sedimented by centrifugation, resuspended in buffer A (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.5 mM Na_2HPO_4, 5 mM glucose, 10 mM Hepes, pH 7.4), and aggregated as described above.

2.2. *Receptor studies*

[^{3}H]U46619 (22.4 Ci/mmol, gift of Dr E. Do, New England Nuclear, Boston, MA) was employed as a radiolabelled TXA_2/PGH_2 mimetic [9,17].

2.2.1. Binding inhibition studies

Platelets were treated with 1 mM aspirin, removed from plasma and resuspended in buffer B (138 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 5.5 mM glucose, 25 mM Tris-HCl, pH 7.4) to a cell count of $3–5 \times 10^8$/ml. For the binding inhibition study, platelets were incubated in darkness for 5 min with 1 nM [^{3}H]U46619 in the presence of vehicle (total binding) or with increasing concentrations of I-APA-PhN_3. Nonspecific binding was assessed in a separate incubation in the presence of 1 μM unlabelled U46619. Specific binding is defined as the difference between total and nonspecific binding, and was 70–75% of total binding. Aliquots (1 ml) of the binding incubation mixture were filtered rapidly under vacuum through Whatman GF/C filters and washed 3 times with 5 ml ice-cold buffer B. [^{3}H]U46619 activity on the filters was determined in a Beckman LS6800 liquid scintillation spectrometer.

2.2.2. Photoactivation studies

Cells were incubated in darkness for 5 min with 20 μM I-APA-PhN_3 or vehicle, after which samples were divided into two equal portions. One portion from the I-APA-PhN_3 and control groups was subjected to photolysis for 2 min, while the other was incubated in darkness for an additional 2 min. Cells were then washed and resuspended three times in buffer B containing 3.8% (w/w) bovine serum albumin (Sigma, St. Louis, MO) to remove free I-APA-PhN_3, and resuspended in buffer B at a concentration of $3–5 \times 10^8$/ml. The binding assay was conducted as described for the inhibition studies.

3. RESULTS

Aggregation induced by 3 μM U46619 (the minimum dose necessary to produce maximal aggregation in PRP) was completely inhibited by 10 μM I-APA-PhN_3, as shown in fig.2C. In contrast, 25 μM 13-APA was required to achieve the same degree of inhibition at the same agonist dose (fig.2B). Furthermore, it was found that I-APA-

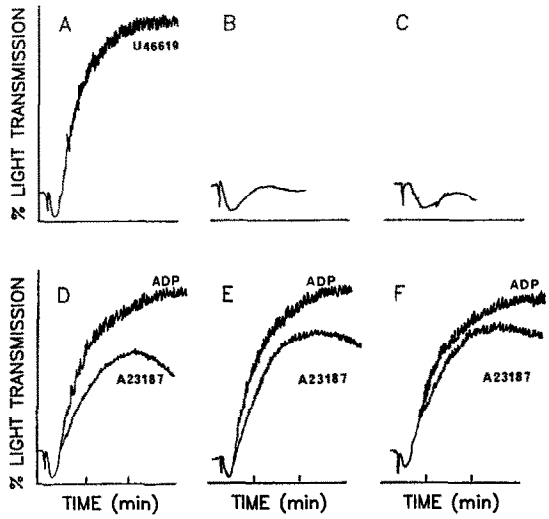

Fig.2. Effects of 13-APA and I-APA-PhN_3 on platelet aggregation. PRP was incubated with 20 μM indomethacin (all traces) and ethanol vehicle (A,D), 25 μM 13-APA (B,E) or 10 μM I-APA-PhN_3 (C,F) for 1 min prior to agonist addition. Agonist doses were 3 μM U46619 (A–C), 4 μM A23187 and 5 μM ADP (D–F, as shown).

PhN_3, like 13-APA, was pharmacologically specific since it had no effect on either ADP or A23187 induced aggregation (fig.2E,F). Fig.3 shows that after incubation with I-APA-PhN_3 under photoactivating conditions and removal of free drug, U46619 aggregation remained greater than 90% inhibited (fig.3C). On the other hand, resuspension completely reversed inhibition of U46619 aggregation by the competitive antagonist, 13-APA (fig.3B). As before, A23187- and ADP-induced aggregation was unaffected by either I-APA-PhN_3 or 13-APA (fig.3E,F).

To demonstrate further that the pharmacological effects of I-APA-PhN_3 on platelet aggregation resulted from action at the TXA_2/PGH_2 receptor, binding studies using [^{3}H]U46619 were performed. I-APA-PhN_3 was found to inhibit the specific binding of [^{3}H]U46619 in a dose-dependent manner, with the concentration for 50% inhibition being approx. 300 nM (fig.4).

Irreversible inhibition of U46619 binding by photoactivation of I-APA-PhN_3 is illustrated in fig.5. Platelets were irradiated or dark incubated

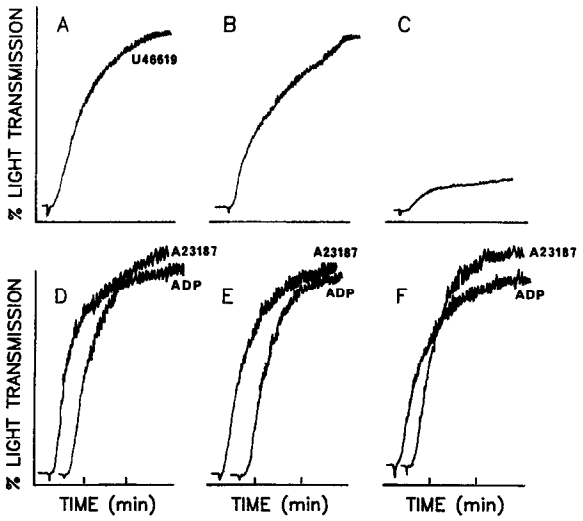

Fig.3. Irreversibility of aggregation inhibition after photolysis. PRP was incubated with vehicle (A,C), 50 μM 13-APA (B,E) or 20 μM I-APA-PhN_3 (C,F) for 30 min in the presence of UV light. Platelets were sedimented by centrifugation and resuspended in buffer A. Following addition of 20 μM indomethacin, platelets were aggregated to 0.2 μM U46619 (A–C), 2 μM A23187 and 5 μM ADP (D–F, as shown).

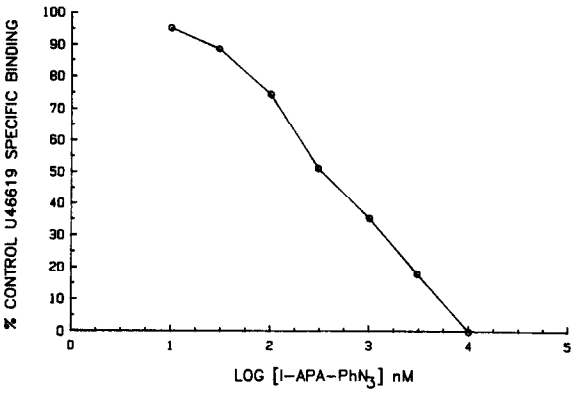

Fig.4. Inhibition of specific binding of [^{3}H]U46619 by I-APA-PhN_3 in aspirin-treated human platelets resuspended in buffer B. Each point is the average of 2 experiments performed in duplicate.

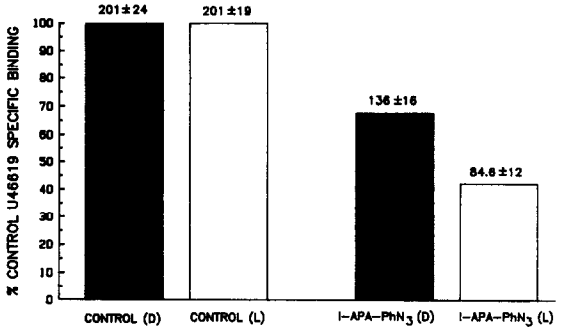

Fig.5. Photoactivation and irreversible inhibition of [^{3}H]U46619 binding by I-APA-PhN_3. Aspirin-treated platelets resuspended in buffer B were incubated 5 min in darkness with 20 μM I-APA-PhN_3 or its vehicle (controls). Samples were divided and either photolysed for 2 min [control (L) and I-APA-PhN_3 (L)] or left in darkness an additional 2 min [control (D) and I-APA-PhN_3 (D)], followed by washing 3 times with buffer B containing 3.8% (w/w) bovine serum albumin. After resuspension in buffer B (without albumin) at 3–5 $\times$ 10^8 platelets/ml, [^{3}H]U46619 binding was assessed. Values above bars are specifically bound dpm/10^8 platelets (mean $\pm$ SE, n = 7).

with 20 μM I-APA-PhN_3 or its vehicle (control), washed three times to remove drug and resuspended. It was found that exposure to UV light alone had no effect on the binding of [^{3}H]U46619. However, photolysis in the presence of I-APA-PhN_3 [I-APA-PhN_3 (L) in fig.5] resulted in an irreversible 58% inhibition of specific U46619

binding compared to control cells. In the absence of photolysis, incubation of cells with I-APA-PhN_3 [I-APA-PhN_3 (D) in fig.5] resulted in a smaller loss (32%) of U46619 specific binding. This small amount of receptor apparently inactivated in the absence of photolysis may be due to inadequate removal of the drug by the washing procedure or a dark reaction in which the compound is nonphotochemically incorporated into the receptor.

4. DISCUSSION

In recent years the use of radioactive photoaffinity labelling techniques has been of significant value in the isolation, purification and characterization of receptor proteins from a variety of cells. Thus, the incorporation of a radiolabel into a photolabile molecule provides a unique means of marking receptors throughout the various stages of protein purification. To this end, we have synthesized two potential photoaffinity probes for the TXA_2/PGH_2 receptor: I-APA-PhN_3 and a phenoxyarylazide 13-APA derivative (APA-O-PhN_3, fig.1) [13,14].

In APA-O-PhN_3 the photoactive arylazide group is attached to the molecule via an ether linkage. Presumably, because of the lability of this phenoxide bond, the molecule could not be iodinated. In addition, photoactivation of the molecule resulted in irreversible but non-specific inhibition of human platelet aggregation (not shown). Based on these characteristics APA-O-PhN_3 was not considered to be a useful photoaffinity label for the receptor. Subsequent to synthesis of our compounds, another phenoxy-linked molecule has also been reported as a photoaffinity probe for the TXA_2/PGH_2 receptor [18]. However, this aryldiazonium salt, like our phenoxy compound, was not iodinated.

In contrast to APA-O-PhN_3, the methylene series was readily iodinated to give I-APA-PhN_3 and the corresponding radiolabelled derivative, ^{125}I-APA-PhN_3, having a specific activity of 600 Ci/mmol [13,14]. These compounds, one of which is radiolabelled, are the first reported photoaffinity probes for the TXA_2/PGH_2 receptor. Furthermore, the results reported here demonstrate the following: (i) an iodophenylazide group can be incorporated into the structure of 13-APA without loss of biological potency and specificity; (ii) I-APA-PhN_3 can irreversibly inhibit platelet aggregation induced by U46619; (iii) I-APA-PhN_3 can irreversibly inhibit binding of [^{3}H]U46619 to its binding site, presumably the TXA_2/PGH_2 receptor; and (iv) photoactivation of the molecule results in a significant increase in the irreversible receptor blockade. Taken together, these data indicate that I-APA-PhN_3 and its corresponding radio-iodinated derivative will be valuable tools for molecular characterization, isolation and purification of the putative TXA_2/PGH_2 receptor.

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