Br. J. Pharmac. (1987), **91**, 223–227

Binding of thromboxane A_2/prostaglandin H_2 agonists to human platelets

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1 The competition of [^{125}I]-9, 11 dimethylmethano-11, 12 methano-16-(3-iodo-4- hydroxyphenyl)-13, 14-dihydro-13-aza 15$\alpha\beta$-ω-tetranor-thromboxane A_2 ([^{125}I]-PTA-OH), a thromboxane A_2/prostaglandin H_2 receptor antagonist, with a series of thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) mimetics for binding to the putative TXA_2/PGH_2 receptor in washed human platelets was studied.

2 The rank order potency for the series of mimetics to compete with [^{125}I]-PTA-OH for binding was compared with their rank order potency for induction of platelet aggregation. The rank order potency for the mimetics to compete with [^{125}I]-PTA-OH for binding was ONO-11113>SQ-26655> U44069> U46619 = 9, 11-azo PGH_2> MB28767. This rank order potency was highly correlated with their rank order potency for inducing platelet aggregation ($r = 0.992$).

3 Changes in the intra or extracellular concentrations of Na^+ did not have a significant effect on the competition between U46619 and [^{125}I]-PTA-OH for binding to the putative receptor.

4 In summary, it appears that these TXA_2/PGH_2 mimetics activate human platelets through the putative TXA_2/PGH_2 receptor.

Introduction

Thromboxane A_2 (TXA_2) and prostaglandin H_2 (PGH_2) are labile proaggregatory agents derived from arachidonic acid (Hamberg *et al.*, 1975). Because of their extreme lability, they are unsuitable for quantitative pharmacological studies or radioligand binding assays that characterize their putative receptors. To overcome these problems, stable analogues of thromboxane A_2 (TXA_2) and prostaglandin H_2 (PGH_2) have been synthesized and their pharmacological effects on platelet function characterized (Bundy, 1975; Corey *et al.*, 1975; Coleman *et al.*, 1981). Although these stable analogues all aggregate platelets presumably via activation of the TXA_2/PGH_2 receptor, there have not been any studies simultaneously characterizing their rank order of potency in radioligand competition assays and aggregation assays under comparable conditions.

[^{125}I]-9, 11 dimethylmethano-11, 12 methano-16-(3-iodo-4-hydroxyphenyl)-13, 14-dihydro-13-aza 15$\alpha\beta$-ω-tetranor thromboxane A_2 ([^{125}I]-PTA-OH) is a competitive TXA_2/PGH_2 receptor antagonist that binds to the putative human platelet TXA_2/PGH_2 receptor (Mais *et al.*, 1985a; Saussy *et al.*, 1985) and is a useful tool with which to explore the interaction of stable TXA_2/PGH_2 mimetics with their putative receptor(s). This paper describes the rank order of potency of a series of TXA_2/PGH_2 agonists in competing with [^{125}I]-PTA-OH for binding to the putative TXA_2/PGH_2 receptor and inducing platelet aggregation.

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Methods

Platelet aggregation studies

Blood was drawn via venipuncture from normal human volunteers into syringes containing 5 mM EDTA and 10 μM indomethacin (final concentrations). The donors had not taken any medications for at least 10 days, and informed consent was obtained from all volunteers. This study was approved by the Institutional Review Board for Human Research. The

blood was centrifuged at 175 g for 20 min at room temperature, and the platelet rich plasma (PRP) was pipetted into plastic tubes. The PRP was centrifuged at 800 g for 20 min at room temperature, and the platelet pellet was resuspended in buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM glucose, and 10 μM indomethacin, pH 7.4 at 37°C) to a final concentration of 2.5×10^8 platelets ml^{-1}. In some experiments, NaCl was replaced with an equimolar concentration of N-methyl-D-glucamine. $CaCl_2$ was added to a final concentration of 250 μM. Platelet aggregation was monitored in silanized glass cuvettes in a Chronolog model 300 aggregometer (Havertown, PA) by previously published methods (Born, 1962). Following a 1 min preincubation (37°C), the washed platelets (475 μl) were aggregated with varying concentrations of agonists and the response observed for 2 min. Dose-response curves were constructed and the EC_{50} values were obtained. The EC_{50} value is defined as the concentration required to produce 50% of the maximum aggregation occurring 1 min after addition of the agonists.

Radioligand binding assays

Incubations (200 μl) were carried out at 37°C for 30 min in 12×75 mm silanized glass tubes which contained 5×10^7 washed platelets. The assay buffer consisted of 50 mM Tris-HCl (pH 7.4 at 37°C), either 100 mM NaCl or N-methyl-D-glucamine (NMDG) (as a sodium substitute) (Limbird *et al.*, 1982) 5 mM glucose and 10 μM indomethacin. The assay mixture also contained ~0.1 nM [^{125}I]-PTA-OH (~50,000 c.p.m.) and various concentrations of the competing agonists (10^{-9} to 10^{-4} M). The reaction was terminated by the addition of 4 ml of ice-cold 50 mM Tris-HCl and 100 mM NaCl (omitted in sodium substitution experiments), pH at 4°C followed by rapid filtration through Whatman GF/C glass fibre filters (Whatman, inc.; Clifton, N.J., U.S.A.). The filters were washed three more times with 4 ml of ice-cold buffer and the entire filtration process was complete within 10 s. Nondisplaceable binding was defined as that remaining in the presence of 750 nM I-PTA-OH and was between 50 to 60% of the total binding. Analysis of the data yielded an IC_{50} value defined as the concentration of competing ligand required to produce a 50% displacement of specifically bound [^{125}I]-PTA-OH from its binding site. The K_d for [^{125}I]-PTA-OH is approximately 20 nM (Mais *et al.*, 1985b; Narumiya *et al.*, 1986).

Sodium content of platelets

The PRP was separated into two tubes and centrifuged at 800 g for 20 min at room temperature. Platelets were resuspended in either Na^+-free buffer (50 mM Tris, 100 mM NMDG, 5 mM dextrose and 10 μM indomethacin at a final pH 7.4) or in Tris-NaCl buffer (50 mM Tris, 100 mM NaCl, 5 mM dextrose and 10 μM indomethacin, pH 7.4) to a concentration of 5×10^8 platelets ml^{-1}. Incubations (3.0 ml) containing 7.5×10^8 platelets (total number) were at 37°C for 30 min. The incubation mixture, chosen to simulate the conditions of the binding assay, consisted of either Tris-NMDG buffer, Tris-NaCl buffer or Tris-NaCl buffer containing monensin (30 μM). The incubation was terminated by rapidly chilling the tubes in an ice-bath. Subsequent steps were carried out at 4°C with the exception of the centrifugations done at room temperature. To assess the extracellular volume remaining with the platelets, [^{14}C]-inulin (200,000 c.p.m.) was added to the incubation mixture and the radioactivity in the final pellet was measured. We verified that [^{14}C]-inulin did not bind to the platelets. One ml of incubation mixture containing [^{14}C]-inulin was layered over 0.2 ml ice-cold 0.25 M sucrose in a plastic conical tube (Beckman Instruments, Inc.) and centrifuged for 1 min at 10,000 g in a Beckman Microfuge (Feinstein *et al.*, 1977). The supernatant was aspirated, the pellet rinsed with 1 ml ice cold 0.25 M sucrose and centrifuged again under the same conditions. The supernatant was aspirated, residual fluid wiped from the tube with a cotton swab and the pellet resuspended in 10 mM CHAPS-Tris buffer to lyse the platelets. Na^+ was quantitated by use of a flame photometer. The intraplatelet Na^+ concentrations were: 18 ± 7, 54 ± 11 and 101 ± 6 mEq l^{-1} ($n = 3$/group) for NMDG, vehicle and monensin (30 μM)-treated platelets, respectively. Both NMDG and monensin-treated platelet Na^+ concentrations were significantly different from NaCl controls ($P < 0.01$, ANOVA).

Materials

ONO-11113 (9, 11-epithio-11, 12-methano-TXA_2) was a gift from ONO Pharmaceutical Company (Osaka, Japan). U46619 (15S-hydroxy-11α, 9α-(epoxymethano) prosta-5Z, 13E-dienoic acid) and U44069 (15S-hydroxy-11α, 9α-(methanoepoxy) prosta-5Z, 13E-dienoic acid) were purchased from the Upjohn Company (Kalamazoo, MI, U.S.A.). 9, 11-azo-PGH_2 (9, 11 azo prostaglandin H_2) and SQ26655 ([1S-(1α, 2β (5Z), 3α (1E, 3S)), 4α]-7-[3-(3-hydroxy-1-octenyl)-7-oxabicyclo [2.2.1] hept-2-yl]-5 heptenoic acid) were gifts from Dr Martin Ogletree, Squibb Institute for Medical Research. MB28767 ((±)11-deoxy-16-phenoxy-17, 18, 19, 20 tetranor prostaglandin E_1) was a gift from Dr Michael Caton, May & Baker Ltd. I-PTA-OH was synthesized as previously described (Mais *et al.*, 1984). [^{125}I]-PTA-OH was synthesized as previously described by the chloramine T method (Mais *et al.*, 1985b). The reaction mixture was subjected to h.p.l.c. on a Whatman ODS-3 reverse phase-

Table 1 EC_{50} values for platelet aggregation and IC_{50} values for competition with [^{125}I]-PTA-OH in washed platelets by agonists

Agonist	*EC_{50}* (nM)		*IC_{50}* (nM)	
ONO-11113	15 ± 2	(10)	32 ± 7	(7)
SQ26655	16 ± 2	(14)	56 ± 7	(4)
U44069	44 ± 2	(7)	76 ± 10	(4)
9, 11-azo PGH_2	59 ± 5	(11)	165 ± 57	(6)
U46619	61 ± 6	(12)	140 ± 13	(9)
MB28767	1303 ± 160	(6)	5860 ± 1999	(5)

Data are expressed as mean ± s.e.mean. The number in parentheses represents the number of experiments. EC_{50} values; ONO-11113 = SQ26655 > U44069 > 9, 11 azo PGH_2 = U46619 > MB28767. IC_{50} values; ONO-11113 ≥ SQ26655 > U44069 > U46619 = 9,11-azo PGH_2 > MB28767. > signifies a value significantly different ($P < 0.05$) from the preceding value. ≥ = $P = 0.05$.

column and eluted with MeOH and 0.1 M NH_4Ac (65:35). [^{125}I]-PTA-OH was prepared fresh daily from the stock solution in the following manner. An aliquot was evaporated to dryness under a stream of nitrogen and redissolved in an appropriate volume of assay buffer. CHAPS, 3-[(cholamidopropyl)-dimethylammionio]-1-propanesulphonate and all other reagents were of highest purity available from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Statistics

Radioligand binding data were subjected to a log-logit transformation analysis and the IC_{50} value and slope were determined. For binding data that had a slope significantly different from −1, an iterative simplex computer programme for nonlinear regression analysis (Nelda & Mead, 1965; Lam, 1970) fitting the data to either a one or two-site model was used. A Mann Whitney U test was used to determine whether a significant difference existed in the potencies of the agonists in the binding and aggregation assays.

Results

Aggregation studies

Washed platelets were aggregated with the agonists and their EC_{50} values obtained (Table 1). The relative potencies were as follows: ONO-11113 = SQ-26655 > U44069 > 9, 11-azoPGH_2 = U46619 > MB28767 (> signifies differences at $P < 0.05$).

Radioligand binding studies

The competition of the agonists with [^{125}I]-PTA-OH for its binding site in washed platelets was determined (Table 1). The slopes of the lines in the log-logit transformation analysis were not significantly different from −1 for all the agonists except MB28767 which had a value of −0.74 ± 0.04 ($n = 5$). MB28767 also failed to displace completely [^{125}I]-PTA-OH from its binding site. These competition studies were further analysed as both one- and two-site models by the Simplex programme. The fit for a two site model was not significantly ($P > 0.05$) better than that obtained for a one site model. The relative potencies for the agonists are ranked as follows: ONO-11113 ≥ SQ26655 > U44069 > U46619 = 9, 11-azo-PGH_2 > MB287767. A highly significant ($P < 0.01$) correlation was found between the potency of the six agonists in the binding studies compared to their potency in the aggregation studies ($r = 0.992$). However, the IC_{50} values were consistently higher than the EC_{50} values.

Effects of Na^+ on agonist binding

Sodium has previously been found to influence the binding of some agonists to their putative receptors (Tsai & Lefkowitz, 1978). To determine whether sodium could alter agonist binding, several series of experiments were performed. Washed platelets were incubated in buffer containing 100 mM NaCl plus the cationophore monensin (30 μM) to increase intracellular Na^+ (Motulsky & Insel, 1983) or the NaCl was replaced with 100 mM NMDG. The IC_{50} for U46619 in the absence of NaCl was significantly lower than that in the presence of NaCl, 166 ± 25 nM compared to 217 ± 30 nM, respectively ($P < 0.05$ using a paired Student's t test, $n = 5$). In contrast to the above small effect of removing Na^+ from the buffer incubation with 100 mM NaCl plus monensin to increase intracellular Na^+ did not change the IC_{50} values for U46619, 256 ± 9 nM and 265 ± 9 nM, respectively.

To determine whether the change in the IC_{50} value for U46619 in the binding assay represented a significant effect on platelet function, the effects of NMDG on U46619-induced platlet aggregation were determined. The EC_{50} for U46619 was 104 ± 18 nM ($n = 7$) in the presence of NaCl and was 113 ± 13 nM ($n = 7$) in the absence of NaCl. However, in several of the experiments the maxium aggregation response obtained was less in the platelets that were incubated in the NMDG buffer than in the platelets incubated in the NaCl buffer.

Discussion

This study demonstrates that a group of structurally dissimilar TXA_2/PGH_2 agonists compete with [^{125}I]-PTA-OH for a binding site on washed human platelets with a rank order of potency similar to their rank order of potency in inducing aggregation. These observa-

tions confirm and extend previous studies with several of these agonists in radioligand binding assays. However, in those studies there was no direct comparison made to their aggregatory potencies under similar assay conditions (Hung *et al.*, 1983; Armstrong *et al.*, 1983; Narumiya *et al.*, 1986). In the binding study, the agonists had a slope not significantly different from −1 in the log-logit plots except for MB28767. Thus, with the exception of MB28767, the agonists appear to be interacting with a single class of binding sites, presumably the putative TXA_2/PGH_2 receptor. Since the fit for a two site model for MB28767 was not significantly better than a one site model, it is not possible that MB28767 is interacting with two differing sites. Collectively these data support the concept that [^{125}I]-PTA-OH is binding to the putative TXA_2/PGH_2 receptor and that these agonists are interacting with that receptor.

While the rank order potencies for the agonists in the binding assay and aggregation studies agreed with each other, the EC_{50} values for the aggregation studies were consistently lower than the IC_{50} or the calculated K_d values, which are virtually the same as the IC_{50} values (Cheng & Prusoff, 1973), found in the binding assay. The reasons for the differences in the absolute potencies of the agonists in the two assays is unknown. Several explanations are tenable. If spare receptors exist for TXA_2/PGH_2 in platelets then one might expect to see a higher IC_{50} compared to the EC_{50}. Indeed, we have some preliminary data which supports this contention (unpublished observations). Alternatively, the IC_{50} values might correlate better with some other platelet function, such as secretion, myosin light chain phosphorylation or exposure of fibrinogen receptors (Gerrard & Carroll, 1981; Morinelli *et al.*, 1983; Seiss *et al.*, 1983).

It has been previously found that sodium may decrease receptor affinity for some platelet agonists, particularly those that are inhibitory for adenylate cyclase (Tsai & Lefkowitz, 1978). For example, the affinity of agonists for the platelet α_2-adrenoceptor is decreased by sodium (Connolly & Limbird, 1983a). It has been postulated that occupation of the platelet TXA_2/PGH_2 receptor, may result in the inhibition of adenylate cyclase (Claesson & Malmsten, 1977). Thus, it might have been expected that Na^+ would decrease the affinity of U46619 for its receptor. While there was a statistically significant increase in the IC_{50} value for the U46619 in the presence of NaCl, it was probably not of biological significance. This is borne out in part by the fact that the EC_{50} values for U46619-induced aggregation were the same in the presence of NaCl, confirming a previous observation (Connolly & Limbird, 1983b). Monensin also failed to alter the IC_{50} value for U46619 in the binding assay, while it clearly increased intraplatelet Na^+ concentrations. Thus it would appear that Na^+ has no modulating effect on the binding of TXA_2/PGH_2 agonists to their receptor. More recent evidence supports the notion that the TXA_2/PGH_2 receptor is linked to phospholipase C and phosphatidylinositol bisphosphate turnover (Seiss *et al.*, 1985). Thus, one might not expect to see an effect of Na^+ on the binding affinity of TXA_2/PGH_2 agonists.

In summary, the results described here support the notion that the group of TXA_2/PGH_2 mimetics used in these studies and [^{125}I]-PTA-OH bind to the putative TXA_2/PGH_2 receptor. [^{125}I]-PTA-OH should be a useful tool to help further characterize the putative TXA_2/PGH_2 receptor.

The technical assistance of Ms M. Garvin, Ms A. Boehm and Mr David deHoll is gratefully acknowledged. This research was supported in part by HL36838, HL29566 and HL07260. P.V.H. is a Burroughs Wellcome Scholar in Clinical Pharmacology.

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(Received November 11, 1986.
Revised February 2, 1987.
Accepted February 3, 1987).