Characteristics of the binding of [³H]-GR32191 to the thromboxane (TP-) receptor of human platelets

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1 The interaction of the specific thromboxane (TP-) receptor blocking drug, [³H]-GR32191 with human intact platelets and platelet membranes has been investigated *in vitro*.

2 On intact platelets, association of specific [³H]-GR32191 binding at 37°C was biphasic, with an initial rapid component and a slower secondary phase. Dissociation experiments indicated displacement from two sites with *t*₁ values of 8.1 and 65.6 minutes. *K*_d values derived from the kinetic rate constants for the rapid onset/offset and slow onset/offset phases were 0.4 and 0.5 nM respectively.

3 Competition binding of [³H]-GR32191 and GR32191 on intact platelets gave an IC₅₀ of 2.3 nM. Scatchard analysis indicated a single class of binding site with a *K*_d of 2.2 nM. Further analysis of the data yielded a Hill slope of –1.0 again indicating an interaction at a single binding site. Saturation binding experiments gave a similar estimate of the *K*_d value for [³H]-GR32191 to that obtained from competition binding experiments. A possible explanation for the biphasic interaction of the GR32191 in intact platelets may lie in restriction of its access to and egress from a population of TP-receptors.

4 In platelet membranes at 37°C, specific [³H]-GR32191 binding was complete within 5 min with a calculated association rate constant of $3.2 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$. Dissociation of [³H]-GR32191 was relatively slow, with measurable specific binding persisting for >40 min. Analysis of these data yielded a *t*₁ of 17.7 min and a dissociation rate constant of 0.04 min^{–1} and indicated dissociation from a single site. The *t*₁ for dissociation appeared to be related to the contact time of platelet membranes with [³H]-GR32191. Derivation of a *K*_d from the kinetic rate constants gave a value of 0.13 nM.

5 Competition binding of [³H]-GR32191 and GR32191 to platelet membranes gave an IC₅₀ value of 3.5 nM. Scatchard analysis of these data indicated a single binding site with a *K*_d of 2.1 nM. Saturation binding experiments with [³H]-GR32191 yielded similar IC₅₀ and *K*_d values to those from competition experiments.

6 In further competition binding experiments, the TP-receptor agonists U-46619, STA₂, EP171 and 9,11-azo PGH₂ and antagonists SQ29,548, BM 13.177 and EP092 all competed with specific [³H]-GR32191 binding on intact platelets and, where determined, on platelet membranes. All compounds fully displaced specific [³H]-GR32191 binding. However, where tested, the IC₅₀ values for a particular compound were always greater when [³H]-GR32191 was the radioligand than when [³H]-SQ29,548 was used. At the concentrations used in these studies (2 and 5 nM respectively), platelets appeared to bind approximately twice as much [³H]-GR32191 as [³H]-SQ29,548.

7 In conclusion, the interaction of [³H]-GR32191 with human intact platelets was complex but the data were consistent with an action at a single class of binding site; from competition experiments this appears to be the functional TP-receptor. The interaction of the drug with this binding site is, however, characterized by a slow dissociation. This characteristic was confirmed in studies with platelet membranes and does not therefore appear to be an artefact of diffusion. Estimates of the *K*_d of the drug differed depending on the method of determination. Because of the slow dissociation of [³H]-GR32191, those relying upon equilibrium of the radioligand with competing agent may be unreliable. The rate of dissociation also appeared to be related to the contact time of drug with receptor. An explanation for this phenomenon may lie in the ability of GR32191 to induce a change in the conformational state or location of the human platelet TP-receptor.

Keywords: Human platelet; thromboxane A₂ receptor blocking drug; radioligand binding; thromboxane A₂ receptor

Introduction

Thromboxane (Tx) A₂ is a potent platelet aggregatory and vasoconstrictor agent which has been implicated in the pathophysiology of occlusive vascular disease (FitzGerald *et al.*, 1987). To exert its effects, TxA₂ appears to act at a specific receptor which has been termed a TP-receptor (Coleman *et al.*, 1984). GR32191 is a potent and specific TP-receptor blocking drug which prevents human platelet aggregation and contraction of vascular smooth muscle induced by TxA₂

and its mimetics (Lumley *et al.*, 1989; Hornby *et al.*, 1989). The interaction of GR32191 with the human platelet TP-receptor has been supported by its ability to compete for binding with the TP-receptor radioligand [¹²⁵I]-PTAOH, the *K*_i for inhibition of binding being in close agreement with the *K*_d value obtained from functional studies (Lawrence *et al.*, 1988). However, in these functional studies, GR32191 appeared to antagonize U-46619-induced platelet aggregation in a non-competitive manner, with slopes of the Schild plot being significantly greater than unity (Lumley *et al.*, 1989). In addition, the attainment of the peak aggregatory response to U-46619 was slowed in the presence of GR32191. At concentrations approximately 10–30 times greater than the estimated pA₂ value, an unsurmountable antagonism of U-46619-in-

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duced platelet aggregation was seen. This profile of GR32191 appeared unique to platelets since the antagonism of U-46619-induced contraction of vascular smooth muscle was competitive and surmountable (Lumley *et al.*, 1989).

The aim of the present study was therefore to investigate the binding of GR32191 to the human platelet. To achieve this, [³H]-GR32191 was prepared and binding investigated both in intact platelets and in a platelet membrane preparation. For comparison with results observed with [³H]-GR32191, binding studies were also performed with [³H]-SQ29,548 (Hedberg *et al.*, 1988). This TP-receptor blocking drug potentially antagonizes U-46619-induced human platelet aggregation (Lumley *et al.*, 1989). However, unlike GR32191, the antagonism produced by SQ29,548 appears to be competitive, with slopes of the Schild regression not significantly different from unity (Lumley, unpublished). In addition no marked slowing of the aggregatory response to U-46619 is observed in the presence of SQ29,548. In binding studies [³H]-SQ29,548 associates and dissociates rapidly from human platelets and appears to represent a relatively uncomplicated radioligand for studying the platelet TP-receptor (Hedberg *et al.*, 1988).

Methods

Preparation of human platelet and platelet membrane suspensions

Human blood (180 ml) was withdrawn from the ante-cubital vein and mixed with 36 ml acid-citrate-dextrose solution (see Drugs used) before centrifugation at 250 g for 20 min to give platelet-rich plasma (PRP). Following addition of prostacyclin (30 nM) the PRP was further centrifuged at 450 g for 20 min to give a platelet pellet. For intact platelet studies the pellet was resuspended in 15 ml of assay buffer. For membrane studies, platelet pellets were frozen at -20°C, thawed, resuspended in assay buffer and the suspension homogenized on ice in a Ystral homogenizer.

Radioligand assay

Each assay tube contained 360 µl buffer, [³H]-GR32191 (final concentration of 2 nM added in 10 µl), 50 µl buffer or test compound and 80 µl of intact platelets or platelet membrane suspension.

Association experiments To determine the association rate of [³H]-GR32191, platelets or platelet membranes were incubated with 2 nM of the radioligand at 37°C for various periods of time. Incubation was terminated by the addition of 3 ml of ice-cold assay buffer and rapid filtration under suction through Whatman GF/B glass fibre filters using a Brandell cell harvester. Filters were rinsed three times with 3 ml of ice-cold buffer, placed in counting vials containing 3 ml of scintillant and counted in a scintillation counter.

Dissociation experiments Association of 2 nM [³H]-GR32191 with whole platelets or platelet membranes was achieved by incubation at 37°C for 30 min before dissociation was initiated by the addition of 5 µM unlabelled GR32191. Samples were incubated at 37°C for various lengths of time after the addition of unlabelled GR32191 before the reaction was stopped as above. In some experiments the dissociation of [³H]-SQ29,548 (5 nM) was determined in the same way.

Competition experiments Drugs tested for competition with [³H]-GR32191 binding were added either to platelet membranes or intact platelets in concentrations ranging from 0.1 nM to 10 µM simultaneously with 2 nM [³H]-GR32191 and incubated for 30 min at 37°C before termination of the reaction as above. When aggregatory agonists were studied, 4 nM cicaprost (Sturzebecher *et al.*, 1986) was included to prevent

platelet aggregation. Inhibition experiments using [³H]-SQ29,548 were carried out as for GR32191, except that the concentration of radioligand used was 5 nM.

Saturation experiments Increasing concentrations of [³H]-GR32191 (2–50 nM) were incubated for 30 min at 37°C with either intact platelets or membranes before terminating the reaction.

Non-specific binding (NSB) In all experiments described above, NSB was determined at either each or selected time points by co-incubating 5 µM GR32191 with samples containing 2 nM [³H]-GR32191. In some experiments on intact platelets, EP092 (5 µM) was used instead of GR32191.

Analysis of data

All analyses were performed only on data (counts min⁻¹, c.p.m.) expressed as specific binding utilising published methods/programmes (see Barlow, 1983).

Association/dissociation Association of [³H]-GR32191 with both intact platelets and platelet membranes was analysed by linear regression of log₁₀ bound [³H]-GR32191 at equilibrium (B_{eq} ; c.p.m.) divided by B_{eq} minus the amount bound at any given time (B_t) against time, using a computerised least-squares fit to a straight line ('Linefit'). K_{obs} was determined from the slope of the regression $\times 2.303$ and the association rate constant (k_{+1}) determined as

$$k_{+1} = \frac{K_{obs} - k_{-1}}{[L]}$$

where k_{-1} and $[L]$ are the dissociation rate constant and the radioligand concentration respectively.

The dissociation rate constant (k_{-1}) was determined using the data from each individual dissociation curve by a least-squares fit of bound [³H]-GR32191 (B) to time ('Expofit') according to the equation $B = B_0 e^{-k_{-1}t}$ where B_0 is the c.p.m. prior to addition of excess GR32191 ('time zero') and $k = k_{-1}$. The time to half dissociation ($t_{1/2}$) was calculated from the k_{-1} value as:

$$t_{1/2} = \frac{0.693}{k_{-1}}$$

With some data (see Figure 3), points lay consistently above or below the fitted line suggesting that a two phase dissociation process may be involved. In these instances data were fitted to $B = B_0[e^{-k_{-1}t} + e^{-k_{-2}t}]$ using the programme 'Doublexp' which yielded two rate constants.

Competition studies IC₅₀ values were determined from competition studies by analysing specifically bound [³H]-GR32191 (c.p.m.) versus concentration of competing agent using 'Polyfit' (Barlow, 1991).

Saturation binding K_d values were determined from saturation binding data using a direct fit to a hyperbola using 'Hypmic'.

Scatchard analysis K_d was determined from Scatchard analysis which was performed on data from both competition and saturation experiments. A plot of bound (fmol/assay tube) divided by free (nM) [³H]-GR32191 versus bound [³H]-GR32191 (fmol/assay tube) was analysed by linear regression ('Linefit'). The negative reciprocal of the slope of the line was equivalent to K_d . Competition data were also analysed using a Hill plot of the data according to the equation, $\log B/B_{max} - B$ vs $\log [L]$ ('Inhibition') with a B_{max} estimated from the Scatchard plot of the same data.

For all graphical presentations of data, the linear regression programme 'Linefit' was used to obtain the line of best fit.

Drugs used

The assay buffer (pH 7.4) had the following composition (mM): NaCl 100, glucose 5, Tris HCl 50 and contained indomethacin (1 μM). Acid citrate-dextrose solution was prepared by dissolving disodium hydrogen citrate (2 g) and glucose (3 g) in 120 ml of distilled water. All reagents were Analar grade and obtained from BDH. Prostacyclin (PGI_2) sodium salt (Schering A.G.) was prepared as a 50 $\mu\text{g ml}^{-1}$ stock solution in 50 mM Tris-HCl, pH 9.0; dilutions were prepared in saline. Cicaprost (ZK96480, Schering) was dissolved in saline to give a stock solution of 50 $\mu\text{g ml}^{-1}$. Indomethacin (Sigma) was prepared as a 10 mM solution in absolute ethanol; dilutions were prepared in saline. $[^3\text{H}]$ -GR32191 hydrochloride salt (specific activity 48.3 Ci mmol^{-1} ; >96% pure) was tritiated at Amersham and incorporated two tritium atoms in the alkyl carboxylic acid chain of the GR32191 molecule (Figure 1) and was supplied as a 50 μM solution in ethanol. Dilutions were prepared in the assay buffer to give a final concentration of ethanol of 0.04% v/v in each assay tube. GR32191 (1 α -(6'-carboxyhex-3'-enyl)-2 β -(N-piperidino)-3 α -hydroxy-5 α -(4''-biphenylmethoxy)-cyclopentane; hydrochloride salt, Glaxo Group Research) was prepared as an 2.0 mg ml^{-1} solution in saline. U-46619 (11 α , 9 α -epoxymethano PGH_2 , Upjohn Diagnostics) was prepared as its sodium salt by dissolving in approximately 1% w/v NaHCO_3 in saline. Dilutions of these drugs were prepared using the assay buffer. $[^3\text{H}]$ -SQ29,548 (2 α -(6'-carboxyhex-2'-enyl)-3 α -(1'-(N-(phenylcarbamoyl)-hydrazino) methyl)-7-oxa-bicyclo [2,2,1] heptane, specific activity 30–60 Ci mmol^{-1}) was purchased from NEN. EP171 ((rac) 9 α ,11 α -epoxy-10 α -homo-16-p-fluorophenoxy-w-tetranor-15S-hydroxy-prosta-SZ, 13E-dienoic acid) and EP092 ((rac) 9 α ,11 α -ethano-1-methyl-13 (N-phenyl thio-carbamoyl) hydrazono-w-heptanor-prosta-SZ-enoic acid) were synthesized in the Department of Pharmacology, Edinburgh University whilst the following were obtained as indicated; 9,11-azo PGH_2 (Upjohn), STA $_2$ (11 α -carba-9,11-thia TXA $_2$, Ono), BM 13.177 (4-[2-[[[4-chlorophenyl] sulphonyl]amino] ethyl] benzeneacetic acid, Glaxo), SQ29,548 Squibb. Stock solutions of the prostanoids (5–10 mg ml^{-1}) were prepared in ethanol and stored at -20°C . Aqueous solutions were obtained by adding the calculated molar equivalent of NaOH, evaporating to dryness and dissolving the residue in warm saline. Further dilutions were prepared in assay buffer. PGI_2 , cicaprost, U-46619 and GR32191 stock solutions were stored at -20°C between experiments. All drugs were stored on ice during an experiment.

Figure 1 The chemical structure of $[^3\text{H}]$ -GR32191.

Results

Intact platelets

The rate of association of $[^3\text{H}]$ -GR32191 to intact platelets at 37°C was slow and complex. Although 80% of binding was

Figure 2 The onset of $[^3\text{H}]$ -GR32191 (2 nM) binding to human intact platelets at 37°C . (a) Individual preliminary experiments showing total binding assessed over 90 min indicate an initial rapid (approximately 80% of maximum) phase of binding over 10 min followed by a more prolonged phase. In (b) and (c), the fast onset (0–10 min) and slower onset (10–60 min) specific binding phases are shown from separate series of experiments ($n = 4$ and 13 respectively). These data were analysed separately to obtain k_{+1} values using mean B_{eq} values, calculated from individual experiments, of 10 min for the slow component and 53 min for the fast component. Specific binding ($\bullet$) was determined at total binding ($\square$) minus non specific binding (NSB) (Δ), the latter being determined in the presence of 5 μM unlabelled GR32191. In (b), NSB was determined at 10 min whereas in (c) it was assessed routinely at virtually all time points to allow for an accurate assessment of the slow binding component. At time points where NSB was not determined, specific binding was calculated from an average value of NSB from adjacent time points. In (c), specific binding at 10 min was significantly less than binding at 30, 40, 50 and 60 min ($P < 0.05$, Student's t test).

Table 1 Association and dissociation rate constants and affinity constants for the interaction of [³H]-GR32191 with human platelets and platelet membranes *in vitro*

Measured variables	Platelet membranes	n	Fast component	Intact platelets	
				n	Slow component
Association rate constant (k_{+1}) ($\times 10^8 \text{ M}^{-1} \text{ min}^{-1}$)	3.2 ± 0.8	(4)	1.9 ± 0.3^a	(4)	0.19 ± 0.0004^b
k_{obs}	0.69 ± 0.16	(4)	0.46 ± 0.05^a	(4)	0.05 ± 0.01^b
Dissociation rate constant (k_{-1}) (min^{-1})	0.044 ± 0.004	(8)	0.093 ± 0.015	(4)	0.011 ± 0.001
t_1 (min)	17.7 ± 2.8	(8)	8.1 ± 1.5	(4)	65.6 ± 8.8
K_d (nM) c	2.1 ± 0.2	(8)		2.2 ± 0.1^c	(4)
d	1.8 ± 0.1	(4)		2.8 ± 0.03^e	(6)

^aFor calculation of k_{obs} , B_{eq} value taken at 10 min.

^bFor calculation of k_{obs} , B_{eq} value taken between 30–60 min (mean, 53 ± 3 min).

K_d was derived from ^c competition experiments between 2 nM [³H]-GR32191 and increasing concentration of GR32191 and ^d saturation binding experiments using [³H]-GR32191. ^e K_d measured on intact platelets for both fast and slow sites together.

complete within 10 min, equilibration of a slower component required 30–60 min. Figure 2a shows data from two preliminary experiments. Because of this complex profile, the fast component (0–10 min) and slow component (10–60 min) were analysed separately, with parallel comprehensive analysis of NSB in the case of the latter (Figures 2b and c). Values for k_{obs} and k_{+1} for the two components are shown in Table 1.

Initial experiments to examine the dissociation of [³H]-GR32191 from intact platelets, indicated displacement from two sites. The best fit of the data from an initial series of experiments is shown in Figure 3a. Experiments were repeated (Figure 3b; $n = 4$), the log plot of these data being curvilinear (Figure 3c). Each individual dissociation curve was analysed separately by 'Doublexp' to derive t_1 and k_{-1} values for the fast and slow offset components separately (Table 1).

In some experiments in intact platelets the TP-receptor blocking drug, EP092 (5 μM ; K_d 10 nM; Jones *et al.*, 1984), was used to determine the NSB of [³H]-GR32191 for comparison with the value obtained with GR32191 itself (5 μM). In these experiments no significant difference was observed in the two determinations of NSB, with mean ($n = 4$) values of 876 ± 42 and 756 ± 85 c.p.m. being obtained with EP092 and GR32191 respectively.

Scatchard analysis of [³H]-GR32191 binding in intact platelets was determined by two methods. Firstly, intact platelets were incubated with 2 nM [³H]-GR32191 at 37°C for 30 min simultaneously with increasing concentrations of unlabelled GR32191. The mean competition curve is shown in Figure 4a and the mean IC_{50} for unlabelled GR32191 from individual experiments was 2.3 ± 0.1 nM ($n = 4$). Scatchard analysis of each experiment gave a straight line (mean $r = 0.90 \pm 0.05$) suggesting a single class of binding site with a K_d of 2.2 ± 0.1 nM (Figure 4b). Because of the suggestion of two binding sites from the dissociation experiments in intact platelets, data was also analysed by 'Inhibition'. Analysis of the data gave a Hill slope of -1.0 ± 0.08 also suggesting the existence of one class of binding site. In the second method, intact platelets were incubated at 37°C for 30 min with increasing concentrations of [³H]-GR32191. The individual saturation curves (data not shown) gave a mean K_d of 2.3 ± 0.2 nM ($n = 6$). Transformation of these data into Scatchard plots gave straight lines (mean $r = 0.91 \pm 0.02$), again indicating one class of binding site with a mean K_d of 2.8 ± 0.3 nM. The K_d values obtained by division of the measured kinetic rate constants (k_{-1}/k_{+1}) obtained with intact platelets for the fast onset/offset and slow onset/offset components were 0.5 nM and 0.4 nM respectively. This was some 5 fold lower than the K_d values obtained from both Scatchard analysis and functional experiments (1.7 ± 0.2 nM) of GR32191 antagonism of U-46619-induced human platelet aggregation (Lumley *et al.*, 1989).

Platelet membranes

Figure 5a shows the association binding of 2 nM [³H]-GR32191 to platelet membranes. At 37°C, binding was virtually complete within 5 min and plateaued at this level for up to 20 min. The plot of $\log B_{\text{eq}}/B_{\text{eq}} - B_i$ versus time (Figure 5b) for individual experiments gave a mean ($\pm$ s.e. mean, $n = 4$) k_{obs} of 0.69 ± 0.16 and k_{+1} of $3.2 \pm 0.8 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$. After incubation of 2 nM [³H]-GR32191 with platelet membranes for 30 min at 37°C, addition of 5 μM unlabelled GR32191 reversed specific binding, but this dissociation was very slow (Figure 5c). Even 40 min after the addition of unlabelled GR32191, binding of [³H]-GR32191 had not returned to the level of non-specific binding. Figure 5d shows the mean plot of the log bound ligand against time which yields a straight line consistent with dissociation from one site. Each individual dissociation curve was analysed separately and gave a mean ($n = 8$) t_1 value of 17.7 ± 2.8 min and dissociation rate constant k_{-1} value of $0.044 \pm 0.004 \text{ min}^{-1}$. To investigate the possibility that the rate of dissociation of GR32191 from the platelet TP-receptor was related to the contact time, dissociation experiments were carried out using platelet membranes pre-incubated with 2 nM [³H]-GR32191 for 5–10 and 15–20 min. The mean ($n = 5$ –6) t_1 values obtained at these times were 8.5 ± 0.2 and 12.3 ± 1.7 min, respectively.

Scatchard analysis of binding of [³H]-GR32191 to platelet membranes was determined, as for intact platelets, by two different methods. The mean IC_{50} for competition between unlabelled GR32191 and 2 nM [³H]-GR32191 was 3.5 ± 0.2 nM ($n = 8$) (Figure 6a). When these data were transformed into Scatchard plots (Figure 6b), straight lines (mean $r = 0.93 \pm 0.01$) were obtained indicating one class of binding site with a mean K_d of 2.1 ± 0.2 nM. Increasing concentrations of [³H]-GR32191 gave saturation binding curves (data not shown) with a mean K_d value of 1.6 ± 0.2 nM ($n = 4$). Again the Scatchard plots were straight lines (mean $r = 0.93 \pm 0.02$) indicating one class of binding site with a K_d of 1.8 ± 0.1 nM. These values again correlate well with the K_d value from functional studies. However, the K_d value obtained by division of the measured mean kinetic rate constants (k_{-1}/k_{+1}) was 0.14 nM and is therefore lower than the K_d value obtained from either Scatchard analysis or functional studies.

Competition experiments

The IC_{50} values for competition between either 2 nM [³H]-GR32191 or 5 nM [³H]-SQ29,548 and a range of TP-receptor agonists and antagonists for binding to intact human platelets and, where tested, to platelet membranes is shown in Table 2. SQ-29,548 is a TP-receptor antagonist with a reported K_d of 5 nM (Hedberg *et al.*, 1988). The drug dis-

Figure 3 The dissociation of specific [³H]-GR32191 binding from human intact platelets at 37°C. Platelets were pre-incubated with 2 nM [³H]-GR32191 for 30 min at which time dissociation was instigated by the addition of 5 μ M unlabelled GR32191 (time 0). In (a), dissociation data from a series (n = 3) of experiments has been fitted either to a single exponential (broken line) or to the equation $B = B_0[e^{-k_1t} + e^{-k_2t}]$ (solid line). Note that the scatter of data about the solid line is uniform compared with that around the broken line (and has a reduced standard deviation). The half times for the single exponential (broken arrow) and for the two phase dissociation (solid arrows) are indicated on the abscissa scale. In the experiments depicted in (b), each point represents the mean $\pm$ s.e.mean of 4 determinations. In all experiments in (a) and (b), determinations of control binding and nonspecific binding (NSB) were performed at the beginning ('0' min) and end ('40' min) of each experiment and showed that these values did not significantly change over this period. Mean (n = 7) control values of 5262 ± 37 and 5461 ± 158 c.p.m. and NSB values of 970 ± 53 and 826 ± 58 c.p.m. were obtained at the two times respectively. In (c), the log plot of the dissociation data is curvilinear and t_1 and k_{-1} values for a fast and slow component were determined from individual experiments by computer analysis (see text and Table 1).

Figure 4 (a) Competition binding between 2 nM [³H]-GR32191 and unlabelled GR32191 in intact platelets. Each point represents the mean $\pm$ s.e.mean of 4 determinations. (b) Scatchard plot of the data in (a).

sociates from the TP receptor of human platelets very rapidly. For example, on intact platelets at 37°C it had a measured mean (n = 3) t_1 of 1.7 ± 0.14 min (this study). It was a consistent finding in these paired inhibition experiments that, at 2 and 5 nM respectively, the specific binding of [³H]-GR32191 to intact platelets was approximately twice that of [³H]-SQ29,548 (8065 ± 365 versus 3205 ± 296 c.p.m., n = 16). Scatchard analysis of a paired saturation binding experiment, performed in quadruplicate on a single preparation of platelet membranes, gave a K_d of 1.7 nM and an x intercept of 97.3 fmol for [³H]-GR32191 and values of 4.6 nM and 56.9 fmol for [³H]-SQ29,548. A part of these differences in binding of the two radioligands appeared to be accounted for by a difference in their specific activities (see Drugs used). The mean (n = 4, total counts added for each radioligand, 43991 ± 10760 c.p.m. for 2 nM [³H]-GR32191 and 78116 ± 5956 c.p.m. for 5 nM [³H]-SQ29,548, supported this view. Competition curves for U46619, EP171, STA₂ and SQ29,548 are shown in Figure 7. For all TP-receptor agonists and antagonists tested, the radioligand binding curves obtained with [³H]-GR32191 for the TP agonists lie to the right of those found using [³H]-SQ29,548 (Table 2 and Figure 7). However, all compounds tested were able to displace fully all specific [³H]-GR32191 and [³H]-SQ29,548 binding.

Discussion

The antagonism of TP-receptor-mediated human platelet aggregation by GR32191 is characterized by a slowing of the aggregatory response to agonist, suppression of its maximum effect and Schild regression slopes of greater than unity

Figure 5 Onset (a and b) and dissociation (c and d) of specific $[^3\text{H}]\text{-GR32191}$ (2 nM) binding to human platelet membranes at 37°C . (a) Specific binding ($\bullet$) was determined as total binding ($\square$) minus nonspecific binding (NSB) ($\triangle$) the latter being determined in the presence of $5\ \mu\text{M}$ unlabelled GR32191. NBS was not determined at each time point (see legend to Figure 2). Each point represents the mean $\pm$ s.e. mean of 4 determinations. (b) Log transformation of the specific binding data from experiments shown in (a). K_{obs} and association rate constant (k_{+1}) values quoted in text were derived from similar plots from individual experiments. In (c), membranes were pre-incubated with 2 nM $[^3\text{H}]\text{-GR32191}$ for 30 min at 37°C and then dissociation instigated by the addition of $5\ \mu\text{M}$ unlabelled GR32191 (time 0). Specific binding data (i.e. total binding minus NSB) ($\bullet$) are shown, each point representing the mean $\pm$ s.e. mean of 8 determinations. Determinations of control binding and NSB were performed at the beginning (0 min) and end (40 min) of each experiment and showed that these values did not change significantly over this period. Mean ($n=8$) control values of 2298 ± 220 and 2371 ± 250 c.p.m. and NSB values of 643 ± 95 and 673 ± 79 c.p.m. were obtained at the two times respectively. (d) Log transformation of dissociation data from the individual experiments from (c). The slope of the plot gives the dissociation rate constant k_{-1} .

(Lumley *et al.*, 1989). In addition, the drug has a duration of action upon platelets in man, which far exceeds its detection in plasma (Thomas & Lumley, 1990). The present study has examined *in vitro*, the binding characteristics of GR32191 to the human platelet TP-receptor in an attempt to explain some of these phenomena. For this purpose $[^3\text{H}]\text{-GR32191}$ was synthesized, the resultant radioligand possessing high specific activity. The specific $[^3\text{H}]\text{-GR32191}$ binding studied appeared to occur with the platelet TP-receptor since it was fully prevented by a range of TP-receptor agonists and antagonists. In the present study, the interaction of $[^3\text{H}]\text{-GR32191}$ with both intact platelets and platelet membranes was investigated.

In intact platelets a complex picture emerged, with both association and dissociation of $[^3\text{H}]\text{-GR32191}$ occurring in two distinct phases. With association, although 80% of binding was complete within 10 min at 37°C , equilibration of a slower component required 30–60 min. Similarly, following a 30 min incubation, dissociation $t_{1/2}$ values of approximately 8 and 66 min were observed for the two phases. These data could be taken as evidence for $[^3\text{H}]\text{-GR32191}$ interacting at two sites. However, calculation of K_d values from the kinetic rate constants for the fast onset/offset and slow onset/offset phases yielded very similar values. In support of this, Scatchard analysis of competition and saturation binding data

with $[^3\text{H}]\text{-GR32191}$ in intact platelets appeared to indicate binding of the drug at a single site. Takahara and colleagues (1990) have also studied the binding of $[^3\text{H}]\text{-GR32191}$ to human intact platelets. Association of the drug appeared to occur at a single site, whereas dissociation was biphasic and best described by a two site model. The slow dissociation component seen with $[^3\text{H}]\text{-GR32191}$ was more pronounced than that seen in the present study, possibly a reflection of the higher concentration of drug used (10 nM versus 2 nM in the two studies respectively).

One possible explanation for the association/dissociation profile observed in intact platelets with $[^3\text{H}]\text{-GR32191}$ in both the present study and that of Takahara and associates is that, in addition to binding to TP-receptors on the platelet surface, the drug also undergoes binding to an identical but 'internalised' TP-receptor. Being a zwitterion, GR32191 is highly charged at neutral pH and this would be expected to slow its passage, in both directions, across biological membranes so explaining the slow onset and offset. Studies with $[^3\text{H}]\text{-SQ29,548}$ also indicated the possible existence of such an internalised pool of receptors (Hedberg *et al.*, 1988). An alternative explanation is that a proportion of the TP-receptor population, with which the drug interacts, may become internalised. The latter hypothesis is supported by data *in vivo* in man where GR32191 treatment appears to

Figure 6 (a) Competition binding between 2 nM [³H]-GR32191 and unlabelled GR32191 in platelet membranes. In (a) each point represents the mean ± s.e. mean of 8 determinations. (b) Scatchard plot of the data in (a).

result in a reduction of platelet TP-receptor density (see below) (Takahara *et al.*, 1990; Armstrong *et al.*, 1990). We are not aware of a similar biphasic association/dissociation profile being reported with other antagonists, although it is well established in studies on β -adrenoceptors that internalisation of the receptor occurs upon exposure to agonist which then results in a reduced access, and therefore binding, with certain ligands (Harden, 1983).

In the simpler membrane preparation, where potential artefacts arising from internalisation of receptors and diffusion of drugs are minimized, the onset of [³H]-GR32191 binding was relatively rapid, reaching equilibrium at 37°C within 5 min. In contrast, the rate of dissociation following a 30 min incubation was slow at 37°C, with measurable specific binding persisting for greater than 40 min with a t_1 value of 17.7 min. This dissociation t_1 was directly related to the contact time of the drug, being 8.5 min with incubations of 5–10 min and 12.3 min with incubations of 15–20 min. Analysis of both association and dissociation data for [³H]-GR32191 indicated interaction of the drug with a single site. From competition binding and saturation binding experiments with membranes, mean K_d values of approximately 2 nM were obtained. Scatchard analysis of these data also indicated interaction at a single binding site. In addition, the K_d values obtained agree closely with the corresponding value (1.7 nM) determined from functional studies on human platelets (Lumley *et al.*, 1989).

A feature of the present study on both membranes and intact platelets was that the K_d value determined from the kinetic rate constants, k_{+1} and k_{-1} , was lower than that determined from Scatchard analysis of both competition and saturation data and from functional experiments. Clearly, K_d values determined by all of these methods should theoret-

Table 2 IC_{50} values for competition between [³H]-GR32191 or [³H]-SQ29,548 and a range of TP-receptor agonists and antagonists for binding to human intact platelets

	IC_{50} (nM)	
	[³ H]-GR32191	[³ H]-SQ29,548
<i>TP-agonists</i>		
U-46619	947 ± 134	220 ± 21
	1220 ± 150 ^a	
STA ₂	205 ± 39	87 ± 19
	420 ± 80 ^a	
EP171	23.3 ± 2.0	5.4 ± 0.8
9,11-azo PGH ₂	314.9 ± 15.6	NT
<i>TP antagonists</i>		
GR32191	2.3 ± 0.12	1.2 ± 0.12
SQ29,548	17.5 ± 2.1	5.7 ± 0.2
BM13.177	13,400 ± 1,200	NT
EP092	226.5 ± 22.5	NT

Values are the mean (± s.e. mean) from 4 experiments. For each drug, individual IC_{50} values for the two radioligands were obtained from paired experiments using blood from the same individual. NT = not tested.

^aValue, obtained on human platelet membranes.

cally be the same. However, the slow dissociation of the drug from platelets would suggest that, in some situations (see below) true equilibrium of a competing agent with the platelet TP-receptor may not occur, the so-called 'hemi-equilibrium' state (see Rang, 1966). Thus, the K_d value determined from experiments assumed to be at equilibrium, such as competition and functional studies may be unreliable, whereas those derived from kinetic experiments may be the more accurate.

The present study points towards interaction of specifically bound [³H]-GR32191 with a single site on human platelets. As mentioned, from competition experiments with a range of TP-receptor agonists and antagonists, specific [³H]-GR32191 binding appeared to occur with the platelet TP-receptor. In paired experiments the ability of the same range of compounds to compete with [³H]-SQ29,548 for binding was also studied. In general the IC_{50} values obtained with this range of compounds using [³H]-SQ29,548, agree with those reported by others for it and other TP-receptor radioligands such as [³H]-U-46619 and [¹²⁵I]-PTA-OH with values (range) for U-46619 of 9–125 nM, STA₂ 27–220 nM, EP171 2.9 nM and SQ29,548 8–25 nM (Mais *et al.*, 1985; Kattelman *et al.*, 1986; Narumiya *et al.*, 1986; Liel *et al.*, 1987; Hedberg *et al.*, 1988; Lawrence *et al.*, 1988; Jones *et al.*, 1989). In contrast, IC_{50} values obtained with [³H]-GR32191 for any given ligand were always greater than those obtained with [³H]-SQ29,548 despite the fact that all compounds were able to displace fully both radioligands. In addition, at the radioligand concentrations used in these studies, platelets appeared to bind approximately twice as much [³H]-GR32191 as they did [³H]-SQ29,548. Nevertheless, SQ29,548 was able to displace fully all the [³H]-GR32191 specific binding.

The radioligands were used at concentrations (2 and 5 nM respectively), close to their K_d values determined from functional studies or Scatchard analysis of competition data. In addition both compounds possessed similar specific activities. However, as indicated above, the 'true' K_d value for GR32191 may be closer to the value of 0.5 nM derived kinetically. Thus in terms of receptor occupancy, 2 nM [³H]-GR32191 would produce a disproportionately greater amount of binding than 5 nM [³H]-SQ29,548 and would therefore require correspondingly greater concentrations of competing ligand to displace it. The K_i values for U-46619, STA₂, EP171 and GR32191, determined from the experiments using [³H]-SQ29,548, were 117, 46.3, 2.9 and 0.64 nM respectively. Normally in the Cheng-Prusoff equation, the K_d of the radioligand is known whereas the K_i of the competing ligand is unknown. However, by utilising the above [³H]-SQ29,548-derived K_i values in the Cheng-Prusoff equation, applied to

Figure 7 Competition between 2 nM [^3H]-GR32191 (■) or 5 nM [^3H]-SQ29,548 (●) and (a) U46619, (b) STA₂ (c) EP171, and (d) SQ29,548 for binding to intact platelets. For each compound, inhibition experiments were performed using both radioligands in the same preparation of platelets to allow for direct comparison of results. Results are mean $\pm$ s.e. mean of 4 determinations.

the corresponding data for each compound using [^3H]-GR32191 as the radioligand, the K_d of [^3H]-GR32191 can be calculated as the unknown. From this exercise, K_d values for [^3H]-GR32191 of 0.3–0.8 (mean 0.5) nM are derived which are lower than those derived from Scatchard analysis or from functional studies and closer to the values derived from the kinetic constants.

The slow dissociation of [^3H]-GR32191 seen in the present study could explain the *in vitro* profile seen with the drug on platelets (slowing of aggregation, suppression of agonist maxima, Schild regression slopes greater than unity) (Lumley *et al.*, 1989; Hornby *et al.*, 1989). In the presence of a slowly dissociating antagonist, an agonist can only readily equilibrate with unoccupied receptors, the so-called 'hemi-equilibrium' state (see Rang, 1966). Equilibration of the agonist with antagonist-occupied receptors will be slower and be dictated by the dissociation rate of the complex. If the agonist requires only low occupancy to achieve a given response, then the response measured may not show any deviation from that predicted by a competitive model. However, when the agonist occupancy required for the given response is high, a slowing of the response will ensue, even in the presence of relatively small amounts of antagonist. With higher concentrations of antagonist, an agonist response may not occur at all, either because the biological response is not observed for long enough or, deactivation or desensitization processes in the cell nullify the slowly rising activation. In the case of platelet aggregation, it has been calculated that U-46619 requires approximately 40% receptor occupancy to achieve a full effect (Armstrong *et al.*, 1985). With this level of occupancy required by the agonist and in the presence of a slowly dissociating antagonist such as GR32191, a hemi-equilibrium state would be anticipated with consequential slowing of the response and suppression of the maxima.

Interestingly, in platelets, a maximum shape change res-

ponse to U-46619 requires only 5% receptor occupancy (Armstrong *et al.*, 1985) indicating that this response is better coupled than is aggregation. In the case of the shape change, parallel rightward displacements of the U-46619 concentration-effect curve are observed in the presence of concentrations of GR32191 which suppress the aggregation responses to the same agonist (Lumley, unpublished). In this context, it is interesting to note that an apparent 'resistance' of U-46619-induced shape change to suppression by GR32191 compared with aggregation has been cited as evidence for these responses being mediated by different TP-receptors (Takahara *et al.*, 1990). In the latter study, platelets were exposed to GR32191 and then washed to remove the reversibly bound drug, U-46619-induced aggregation, phosphoinositide hydrolysis and 5-hydroxytryptamine secretion were inhibited, yet shape change and elevation of cytosolic Ca^{2+} induced by the agonist were not. However, in these studies, only a single, high concentration of U-46619 was used (1 μM). This would produce a supramaximal effect upon shape change but a sub- or just-maximal effect upon aggregation. It is quite feasible, therefore, that following exposure to GR32191 complete inhibition of aggregation could be observed whereas shape change, whilst being significantly antagonized, would appear to be unaffected because of the supra-maximal stimulus used. Construction of full concentration-effect curves for shape change and aggregation might resolve this question. However, it would seem premature at this stage to invoke the existence of two TP-receptor populations in human platelets.

The same *in vitro* functional profile on platelets, to that seen with GR32191, has been observed with other structurally-related compounds such as AH23848 (Brittain *et al.*, 1985). The slow dissociation of [^3H]-GR32191 from human platelet TP-receptors would not appear to be simply a function of its high potency since [^3H]-SQ29,548, which is of

similar potency ($K_d = 5$ nM), dissociates rapidly from human platelet membranes with a t_1 of 1.7 min at 37°C (this study). Furthermore, whilst SQ29,548 potently antagonizes TP-receptor agonist-induced aggregation of human platelets, no slowing of the aggregatory response, depression of the agonist maxima nor high slopes of the Schild regression is observed (Takahara *et al.*, 1990; Lumley, unpublished data). It is also interesting to note that, in contrast to platelets, on vascular smooth muscle GR32191 behaves as a fully surmountable and competitive TP-receptor blocking drug (Lumley *et al.*, 1989). This, like the shape change response of platelets described above, could reflect a better coupling of TP-receptor stimulation in vascular smooth muscle and therefore a less pronounced hemi-equilibrium phenomenon in this tissue.

It is also likely that slow dissociation of GR32191 from the platelet TP-receptor also, in part, accounts for its long duration *in vivo* in man following termination of oral dosing (Thomas & Lumley, 1990). However, such a mechanism alone seems unlikely to explain a duration in excess of 4 days seen in such studies (Thomas & Lumley, 1990; Takahara *et al.*, 1990). It is possible that the nature of the GR32191 binding changes with time, the binding being stronger the longer the drug is in contact with the TP-receptor. As mentioned above, the dissociation t_1 of [³H]-GR32191 from platelet membranes appeared to be directly related to the initial contact time of the drug. In a further recent study (Armstrong *et al.*, 1990), we have presented evidence that

prolonged incubation of human platelets with GR32191 *in vitro* or 'exposure' to the drug *in vivo* leads to a reduction in the density of platelet TP-receptors. Takahara and colleagues (1990) have also demonstrated a similar phenomenon, with suppression of platelet aggregation in patients receiving GR 32191 being more closely correlated with a reduction in TP-receptor density than with plasma drug levels. The mechanism of this reduction in receptor number is, as yet, unknown.

In summary, the present study indicates that GR32191 interacts with a single site on human platelets which has the characteristics of the TP-receptor. In platelet membranes the interaction of the drug with the receptor is characterized by a slow dissociation which is directly related to contact time. In intact platelets the association and dissociation profile of GR32191 are more protracted, possibly due to interaction of the drug with 'internalised' receptors. Because of this complex profile, estimation of a true K_d value for the compound is difficult. The characteristics of the interaction of GR32191 with the human platelet TP-receptor probably explain its profile of action *in vitro* and may go some way to explaining its long duration following administration to man.

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