Octimibate, a potent non-prostanoid inhibitor of platelet aggregation, acts via the prostacyclin receptor

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1 Octimibate, 8-[(1,4,5-triphenyl-1H-imidazol-2-yl)oxy]octanoic acid, is reported to have antithrombotic properties. This is in addition to its antihyperlipidaemic effects which are due to inhibition of acyl-CoA:cholesterol acyltransferase (ACAT). The aim of this study was to investigate the mechanism of the antithrombotic effect of octimibate, and to determine whether the effects of octimibate are mediated through prostacyclin receptors.

2 In suspensions of washed (plasma-free) human platelets, octimibate is a potent inhibitor of aggregation; its IC_{50} is approx. 10 nM for inhibition of aggregation stimulated by several different agonists, including U46619 and ADP. The inhibitory effects of octimibate on aggregation are not competitive with the stimulatory agonist; the maximal response is suppressed but there is no obvious shift in potency of the agonist. In platelet-rich plasma, octimibate inhibits agonist-stimulated aggregation with an IC_{50} of approx. 200 nM.

3 Octimibate also inhibits agonist-stimulated rises in the cytosolic free calcium concentration, $[Ca^{2+}]_i$, in platelets. Both Ca^{2+} influx and release from intracellular stores are inhibited. The effects of octimibate on aggregation and $[Ca^{2+}]_i$ are typical of agents that act via elevation of adenosine 3':5'-cyclic monophosphate (cyclic AMP). Similar effects are seen with forskolin, prostacyclin (PGI_2) and iloprost (a stable PGI_2 mimetic).

4 Octimibate increases cyclic AMP concentrations in platelets and increases the cyclic AMP-dependent protein kinase activity ratio. Octimibate stimulates adenylyl cyclase activity in human platelet membranes, with an EC_{50} of 200 nM. The maximal achievable activation of adenylyl cyclase by octimibate is 60% of that obtainable with iloprost. Octimibate has no effect on the cyclic GMP-inhibited phosphodiesterase (phosphodiesterase-III), which is the major cyclic AMP-degrading enzyme in human platelets.

5 Octimibate inhibits, apparently competitively, the binding of [3H]-iloprost (a stable PGI_2 mimetic) to platelet membranes; the estimated K_i is 150 nM.

6 The platelets of different species show considerable differences in the apparent potency of their inhibition of aggregation by octimibate; platelets from cynomolgus monkeys are 3 fold more sensitive than those from humans, while rat, cat and cow platelets are 50, 100, and 250 fold less sensitive than human platelets. The sensitivity of these different species to iloprost, however, varies over a range of only 10 fold with no obvious difference between primates and non-primates.

7 Octimibate appears to be a potent agonist (aggregation), or partial agonist (adenylyl cyclase), at prostacyclin receptors and is the first non-prostanoid agent of this type to be identified. The species differences in relative potency of octimibate and iloprost may reflect the existence of receptor subtypes.

Introduction

Octimibate, 8-[(1,4,5-triphenyl-1H-imidazol-2-yl)oxy]octanoic acid, has been described as having antihyperlipidaemic, anti-atherosclerotic and antithrombotic properties (European Patent Specification, EP-B-130526). Octimibate (10–100 mg kg⁻¹, daily) has been shown to reduce both plasma cholesterol and atherosclerotic lesions in fat-fed rabbits and minipigs (Lautenschlager *et al.*, 1986; Rucker *et al.*, 1988), and it causes a shift from esterified to free cholesterol in cholesterol-loaded macrophages (Schmitz *et al.*, 1985). These

antihyperlipidaemic and antiatherosclerotic effects of octimibate have been ascribed to its inhibition of the cholesterol esterifying enzyme, acyl-CoA:cholesterol acyltransferase (ACAT). However, the antithrombotic activity of octimibate cannot be explained by its inhibition of ACAT. *In vitro*, aggregation of human platelets induced by adenosine diphosphate (ADP), arachidonic acid, collagen, platelet-activating factor and thrombin was inhibited by octimibate with IC_{50} values in the range of 1–9 μ M. *In vivo*, ADP-induced thrombus formation in the microcirculation of the hamster cheek pouch was reduced after octimibate doses of 10–100 mg kg⁻¹ (Lautenschlager *et al.*, 1986). Octimibate thus appears to be a functional antagonist of platelet aggregation in that it inhibits aggregation stimulated by a variety of agonists, rather than acting as a specific receptor antagonist. However, the way in which octimibate inhibits platelet aggregation has not been described to date.

Here, we have explored the mechanism of action of octimibate, and provide evidence that it acts (at least in part) via the prostacyclin (PGI_2) receptor to inhibit platelet aggregation.

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Figure 1 The structures of octimibate (8-[(1,4,5-triphenyl-1H-imidazol-2-yl)oxy]octanoic acid), iloprost and prostacyclin (PGI₂).

The effects of octimibate on human platelets have been compared to PGI₂ and iloprost (a stable PGI₂ mimetic); the structures of these three compounds are shown in Figure 1. We have investigated the effects of octimibate on platelet aggregation, cytosolic free calcium concentration ($[Ca^{2+}]_i$), adenosine 3': 5'-cyclic monophosphate (cyclic AMP) concentrations and cyclic AMP-dependent protein kinase activity, adenylyl cyclase activity, and binding of [³H]-iloprost to platelet membranes. Since the potency of iloprost is reported to vary in different species (Sturzebecher & Losert, 1987; Armstrong *et al.*, 1989), the effects of octimibate and iloprost have been compared on inhibition of aggregation of platelets from several different species. Many prostanoid agonists at the PGI₂ receptor have been described (Oliva & Nicosia, 1987). However, octimibate is the first non-prostanoid agonist to be described for the PGI₂ receptor on any cell-type or tissue, including platelets.

Methods

Preparation of human platelets

Blood was freshly collected from healthy volunteers, who gave informed consent, and platelets were prepared as previously

described (Hallam *et al.*, 1984). For experiments with platelet-rich plasma (PRP), blood was collected into 1/10th volume citrate dextrose (7 mM citric acid, 139 mM trisodium citrate, 93 mM glucose), and was centrifuged for 5 min at 500 *g*. The upper layer of PRP was removed, and was diluted 1/2 with buffer containing (mM): NaCl 145, KCl 5, MgCl₂ 1, HEPES 10 (pH 7.4 at 37°C) and glucose 10. The final platelet density was approx. 1.5×10^8 cells ml⁻¹. The resulting 50% PRP was incubated with aspirin (100 µM) for 15 min at 37°C before use in the aggregation experiments.

For experiments with washed platelets, blood was collected into 1/7th volume acid citrate dextrose (citric acid 65 mM, trisodium citrate 85 mM, glucose 110 mM), and was centrifuged for 5 min at 500 *g*. The upper layer of PRP was removed, and aspirin (100 µM) was added. The PRP was then centrifuged for 15 min at 200 *g*, and the resultant platelet pellet was resuspended (at approx. 1.5×10^8 cell ml⁻¹) in medium containing (mM): NaCl 145, KCl 5, MgCl₂ 1, CaCl₂ 0.2, HEPES 10 (pH 7.4 at 37°C), glucose 10 and apyrase 10 µg ml⁻¹. For experiments to measure $[Ca^{2+}]_i$, the PRP was incubated with quin2 AM (20 µM) for 30 min at 37°C before centrifugation.

Essentially similar methods were used for preparation of platelets from different animal species.

Measurement of aggregation

Aggregation was measured at 37°C in a 4 channel aggregometer (PAP-4 from Biodata Corp.). For the experiments with washed platelets, CaCl₂ (1 mM) and fibrinogen (1 mg ml⁻¹) were added to aliquots of cells just before use. Aliquots (0.7 ml) of PRP or washed platelets were continuously stirred, and the test compound was added 2 min before the aggregatory stimulus. The final concentration of dimethylsulphoxide (DMSO) was 0.1%. The extent of aggregation was assessed 4 min after addition of the stimulus, and the effects of the compounds were measured as % inhibition of the control response in the absence of inhibitor (vehicle present). Aggregation was stimulated by U46619 (1 µM) or ADP (20 µM), except where dose-response curves for U46619 and ADP are shown.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured as previously described (Rink *et al.*, 1982; Hallam *et al.*, 1984). The PRP was incubated with quin2 AM, as described above, and the washed quin2-loaded platelets were used for these experiments. Quin2 fluorescence was measured at 340 nm excitation, 500 nm emission in a Perkin-Elmer LS-5 fluorimeter at 37°C. $[Ca^{2+}]_i$ was calculated from the quin2 fluorescence as previously described (Tsien *et al.*, 1982). CaCl₂ (1 mM) or EGTA (1 mM) were added to cells (0.7 ml) as required. The test compound was added 2 min before the stimulatory agonist (ADP 20 µM). The final concentration of DMSO was 0.1%. The effects of the compounds were measured as % inhibition of the initial peak in fluorescence evoked by the stimulus.

Measurement of the cyclic AMP-dependent protein kinase activity ratio and of platelet cyclic AMP content

Suspensions of washed human platelets (prepared as above) were incubated with the test compounds for 2 min at 37°C. The incubations were terminated with 9 volumes of homogenizing buffer containing (mM): KH₂PO₄ 10, EDTA 10, isobutyl methylxanthine 0.5, Triton X-100 (0.2% w/v), pH 6.8 (Seiler *et al.*, 1987). The resulting homogenates were used directly in the cyclic AMP-dependent protein kinase activity ratio assay (Murray *et al.*, 1990a). Phosphotransferase activity was measured using malantide (a synthetic dodecapeptide) as substrate in the absence and presence of exogenous cyclic AMP. Activity in the absence of exogenous cyclic AMP is a measure of the amount of active enzyme in the platelets following incubation with the test compound, and activity in the presence of exogenous cyclic AMP is a measure of the total

amount of enzyme present in the platelets. The cyclic AMP-dependent protein kinase activity ratio therefore measures the amount of cyclic AMP-dependent protein kinase that is active in the platelets after the 2 min incubation with the test compound as a fraction of the total activity.

For measurement of platelet cyclic AMP content, parallel aliquots of washed platelets were incubated with the test compounds for 2 min at 37°C. These incubations were terminated by addition of 0.1 volumes of HClO₄ (3 M). After 30 min at 4°C, these extracts were centrifuged for 1 min at 12,000 *g*. Perchloric acid was removed from aliquots (0.2 ml) of the supernatant by addition of 0.25 ml of a 1:1 mixture of 1,1,2,2-dichlorodifluoroethane and tri-*n*-octylamine, followed by thorough mixing then centrifugation (Sharps & McCarl, 1982). The upper aqueous layer was taken for measurement of cyclic AMP. Cyclic AMP was assayed with the kit detailed in the Materials section, and platelet cyclic AMP content is expressed as pmol/10⁸ platelets.

Preparation of human platelet membranes

Platelet-rich plasma concentrates were obtained from the Blood Transfusion Service, Cambridge, and membranes were prepared by a modification of the method of Edwards *et al.* (1987). The PRP was centrifuged for 15 min at 800 *g*, and the supernatant was discarded. The rest of the procedure was carried out at 4°C. The platelet pellets were resuspended in buffer containing 0.25 mM EDTA and 5 mM Tris-Cl, homogenized with a Dounce homogenizer (10 strokes with a loose pestle), and then centrifuged at 26,000 *g* for 20 min. The pellet was washed twice by resuspension in buffer containing 0.25 mM EDTA and 50 mM Tris-Cl, followed by homogenization, then centrifugation at 26,000 *g* for 10 min. The final membrane pellet was homogenized in buffer containing 0.25 mM EDTA and 50 mM Tris-Cl, and stored at -70°C. This membrane suspension contained approximately 12 mg protein ml⁻¹. All Tris buffers were adjusted to pH 7.4 at 20°C. Protein determinations were according to the method of Bradford (1976).

Measurement of platelet membrane adenylyl cyclase activity

Membranes were prepared as described above, and adenylyl cyclase activity was measured by a modification of the method of Salomon *et al.* (1974). The incubation (total volume of 50 µl) contained ATP 100 µM, [α -³²P]-ATP 1–2 µCi, phosphocreatine 20 mM, creatine kinase 40 U ml⁻¹, myokinase 40 U ml⁻¹, GTP 10 µM, cyclic AMP 1 mM, EGTA 100 µM, MgCl₂ 5 mM, EDTA 10 µM, Tris-Cl 50 mM, pH 7.4 at 20°C, approximately 30 µg platelet membrane protein, 0.4% dimethylsulphoxide (DMSO) and the test compounds. The incubations, for 10 min at 37°C, were initiated by addition of platelet membranes and terminated by addition of 100 µl of a solution containing cyclic AMP 40 mM, [³H]-cyclic AMP (approx. 10,000 c.p.m.), ATP 10 mM and 1% SDS, followed by addition of 1 ml of water. [α -³²P]-ATP and [³²P]-cyclic AMP were separated by a two step chromatography method, Dowex, followed by alumina (Salomon *et al.*, 1974). [³H]-cyclic AMP was used to monitor recovery of cyclic AMP from the columns, and cyclic AMP formation was expressed as pmol cyclic AMP min⁻¹ mg⁻¹ protein.

Inhibition of [³H]-iloprost binding to platelet membranes

Membranes were prepared as described above, and inhibition of [³H]-iloprost binding was measured as described by Hall & Strange (1984). The incubation (total volume of 270 µl) contained Tris-Cl 50 mM (pH 7.4 at 20°C), MgCl₂ 4 mM, EDTA 40 µM, [³H]-iloprost 10 nM, membrane protein 0.4–0.8 mg, 1.85% DMSO, and various concentrations of unlabelled iloprost or octimibate. For determination of non-specific binding, 20 µM unlabelled iloprost was included. (Specific binding was

85–90% of total binding). The tubes (triplicates for each condition) were set up on ice, and then incubated for 30 min at 37°C. The incubations were terminated by rapid filtration on Whatman GF/B filters using a Brandel harvester. The filters were washed three times with Tris-Cl 50 mM, MgCl₂ 5 mM, pH 7.4 at 20°C, and then counted for radioactivity.

From two preliminary experiments measuring binding of [³H]-iloprost as a function of [³H]-iloprost concentration, *K_D* values of 16.7 nM and 22.6 nM were obtained. A mean value of 19.6 nM was then used in the Cheng and Prusoff equation (Cheng & Prusoff, 1973) to estimate the *K_i* values for iloprost and octimibate from the IC₅₀ values obtained in binding inhibition experiments. Data were fitted to a one site model using Grafit (Erithacus Software Ltd., Staines, Middx).

Analysis of data

Concentration-inhibition curves were fitted to the logistic equation by computer using the programme ALLFIT (DeLean *et al.*, 1978). This analysis yields values for the IC₅₀ and the Hill coefficient.

Materials

Octimibate was synthesized, according to the method described by Lautenschlager *et al.* (1986), in the Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, Welwyn, and was dissolved in DMSO. Iloprost was obtained from Schering AG, Postfach 650311, D-1000 Berlin 65, Germany, and was dissolved in ethanol (0.5 mg 10 µl⁻¹) then diluted to 0.5 mg ml⁻¹ in a solution containing 0.9% NaCl and 10 mM Tris-Cl, pH 8.3. PGI₂ was from Sigma, and was dissolved at 1 mg ml⁻¹ in a solution containing 90% ethanol and 10 mM NaOH, then diluted in a solution containing 0.9% NaCl and 10 mM Tris, pH 8.5. U46619 (dideoxy-11 α , 9 α -epoxymethano-prostaglandin F_{2a}) was from The Upjohn Company, Kalamazoo, MI, U.S.A. ADP, GTP and EDTA were from Boehringer. Quin2 acetoxymethyl ester (quin2 AM) was from Calbiochem, EGTA was from Fluka, and the Dowex cation exchange resin (AG50W-X4, H⁺ form) was from Bio-Rad. [α -³²P]-ATP (10–50 Ci mmol⁻¹), [³H]-cyclic AMP (30 Ci mmol⁻¹), the cyclic AMP assay kit (RPA 508) and [³H]-iloprost (TRK 839) were from Amersham International plc. All other materials were from Sigma or BDH.

Results

Effect of octimibate on platelet aggregation

Figure 2 shows the effect of octimibate, iloprost, PGI₂ and forskolin on aggregation of washed human platelets stimulated with the thromboxane mimetic, U46619 (1 µM). Figure 2a shows typical aggregation traces for platelets stimulated with U46619, following a 2 min preincubation with various concentrations of octimibate. This clearly shows that octimibate is a potent inhibitor of U46619-stimulated aggregation, with maximal inhibition at 25 nM octimibate. From the dose-response curve shown in Figure 2b, the IC₅₀ for inhibition of U46619-stimulated platelet aggregation by octimibate is 10.5 ± 0.4 nM (*n* = 9), and the Hill coefficient of the inhibition curve is 3.5 ± 0.4. Figure 2b also shows inhibition of aggregation by PGI₂ (IC₅₀ = 0.36 ± 0.01 nM, Hill coefficient = 3.4 ± 0.3, *n* = 1), iloprost (IC₅₀ = 0.89 ± 0.03 nM, Hill coefficient = 3.5 ± 0.5, *n* = 8) and forskolin (IC₅₀ = 106 ± 10 nM, Hill coefficient = 2.7 ± 0.6, *n* = 1).

Octimibate inhibited platelet aggregation stimulated by a variety of agonists in addition to U46619. ADP-stimulated aggregation was potently inhibited by octimibate with an IC₅₀ of 8.4 ± 0.1 nM, *n* = 4 (see Table 1 and Figure 3). Octimibate also inhibited aggregation stimulated by vasopressin (0.5 µM), 5-hydroxytryptamine (2.5 µM with 10 µM adrenaline) or thrombin (0.1 U ml⁻¹) with IC₅₀ values of 2 nM, 18 nM and 50 nM

Figure 2 The effects of octimibate, iloprost, PGI₂ and forskolin on aggregation of washed human platelets stimulated with U46619. (a) Typical aggregation traces showing U46619 (1 μM)-stimulated aggregation of washed platelets in the presence of varying concentrations of octimibate (0–25 nM) added 2 min before U46619. (b) Dose-response curves for inhibition of aggregation by PGI₂ (▲), iloprost (■), octimibate (●) and forskolin (Δ). The compounds were added 2 min before U46619 (1 μM). Percentage inhibition is calculated as inhibition of the maximal extent of aggregation measured 4 min after addition of U46619. Data are means of 8 experiments with iloprost, 9 experiments with octimibate, and are single experiments with PGI₂ and forskolin; vertical bars show s.e.mean; where not shown, the error bars are within the symbol size.

respectively. Since octimibate inhibited aggregation stimulated by several different agonists, it is unlikely to be acting as a receptor antagonist. Such functional inhibition would therefore be expected to be 'non-competitive' with the agonist. Figure 3 demonstrates clearly that the inhibitory effect of octimibate on ADP- and U46619-evoked aggregation of washed platelets was not competitive. The dose-response curves for ADP (Figure 3a) and U46619 (Figure 3b) showed a decrease in maximum effect with increasing concentrations of octimibate, while there was no detectable shift in EC₅₀ for the agonist. The inhibitory effect of iloprost showed similar characteristics to that of octimibate (results not shown).

Octimibate also inhibited aggregation of platelets in plasma, although its potency was considerably reduced com-

Figure 3 Effect of octimibate on aggregation of washed human platelets stimulated by various concentrations of ADP (a) or U46619 (b). Washed platelets were incubated for 2 min with the concentrations of octimibate indicated, and then challenged with varying concentrations of ADP or U46619. (Where a range of octimibate concentrations are shown, this indicates that different concentrations were needed on different platelet preparations to obtain the same % inhibition.) Aggregation is shown as the extent of aggregation (arbitrary units) 4 min after addition of the stimulus. Data are means of 3 experiments, s.e.mean shown by vertical bars; where not shown, the error bars are within the symbol size.

pared to that with washed platelets. The IC₅₀ for inhibition of U46619-stimulated aggregation of platelet-rich plasma by octimibate was 207 ± 4 nM ($n = 10$), compared to 10.5 ± 0.4 nM ($n = 9$) for inhibition of U46619-stimulated

Table 1 Effects of octimibate and iloprost on human platelets

	Octimibate	Iloprost	Ratio octimibate/iloprost
K_i (nM) for binding	153 ± 28 (7)	31 ± 4 (14)	4.9
Stimulation of adenylyl cyclase			
EC ₅₀ (nM)	204 ± 8 (7)	6.8 ± 0.7 (15)	30
Relative maximum effect	0.57 ± 0.02 (7)	1.0	
Inhibition of rise in $[Ca^{2+}]_i$			
IC ₅₀ (nM) for washed platelets stimulated by ADP	9.8 ± 0.4 (3)	1.7 ± 0.1 (3)	5.8
Inhibition of aggregation			
IC ₅₀ (nM) for washed platelets stimulated by U46619	10.5 ± 0.4 (9)	0.9 ± 0.03 (8)	11.7
stimulated by ADP	8.4 ± 0.1 (4)	0.7 ± 0.05 (4)	12.0
Inhibition of aggregation			
IC ₅₀ (nM) for platelet-rich plasma stimulated by U46619	207 ± 4 (10)	1.6 ± 0.09 (5)	129
stimulated by ADP	226 ± 4 (3)	1.4 ± 0.03 (3)	161

Figure 4 The effects of octimibate, iloprost, PGI₂ and forskolin on ADP-stimulated increases in [Ca²⁺]_i in quin2-loaded washed human platelets. (a) Typical quin2 fluorescence traces (calibrated to [Ca²⁺]_i) showing ADP (20 μM)-stimulated increases in [Ca²⁺]_i in washed platelets in the presence of various concentrations of octimibate (0–25 nM) added 2 min before ADP. Experiments were carried out in the presence of extracellular Ca²⁺ (1 mM CaCl₂ added) or in the absence of extracellular Ca²⁺ (1 mM EGTA added). (b) Dose-response curves for inhibition of the ADP-stimulated increase in quin2 fluorescence by octimibate in the presence of extracellular Ca²⁺ (total rise in [Ca²⁺]_i; ○) or in the absence of extracellular Ca²⁺ (Ca²⁺ release from intracellular stores; ●). Experimental details are as described in (a). Percentage inhibition is calculated as inhibition of the maximum peak in fluorescence measured after addition of ADP. Data are means ± ranges of duplicate determinations in one experiment. (Where not shown, the error bars are within the symbol size.) (c) Dose-response curves for inhibition of the ADP-stimulated increase in quin2 fluorescence in the presence of extracellular Ca²⁺ by PGI₂ (▲), iloprost (■), octimibate (●) and forskolin (△). The compounds were added 2 min before ADP (20 μM). Percentage inhibition is calculated as inhibition of the maximum peak in fluorescence measured after addition of ADP. Data are means of 5 experiments with octimibate, 3 experiments with iloprost and forskolin, and a single experiment with PGI₂; vertical bars show s.e.mean; where not shown, the error bars are within the symbol size.

aggregation of washed platelets. Similar results were obtained for ADP-stimulated platelets, IC₅₀ values of 226 ± 4 nM ($n = 3$) and 8.4 ± 0.1 nM ($n = 4$) in the presence and absence of plasma respectively. The presence of plasma had little effect on the inhibitory activity of iloprost; the IC₅₀ values were 1.6 ± 0.09 nM ($n = 5$) in plasma, compared to 0.9 ± 0.03 nM ($n = 8$) on washed platelets stimulated with U46619. These results are summarised in Table 1.

The inhibitory effects of both octimibate and iloprost were reversible. Platelet-rich plasma was incubated with concentrations of compound that caused 100% inhibition of aggregation, then centrifuged, and the platelets were resuspended in buffer (as described in the method section for preparation of washed platelets). When these platelets were then challenged with an aggregatory stimulus (U46619), there was no inhibition of aggregation.

Effect of octimibate on agonist-stimulated $[Ca^{2+}]_i$ rises in platelets

Figure 4 shows the effect of octimibate, iloprost, PGI_2 and forskolin on $[Ca^{2+}]_i$ responses of washed human platelets stimulated with ADP ($20 \mu M$). Figure 4a shows typical fluorescence traces, calibrated to $[Ca^{2+}]_i$, for quin2-loaded platelets stimulated with ADP in the presence or absence of extracellular Ca^{2+} (1 mM $CaCl_2$ or 1 mM EGTA added respectively). The platelets were preincubated for 2 min with the concentrations of octimibate shown. The figure clearly shows that octimibate is a potent inhibitor of ADP-stimulated rises in $[Ca^{2+}]_i$, both in the presence or absence of extracellular Ca^{2+} . In the presence of extracellular Ca^{2+} , the ADP-stimulated rise in $[Ca^{2+}]_i$ is due to a combination of Ca^{2+} release from intracellular stores and influx of extracellular Ca^{2+} (Rink & Sage, 1990). Under these experimental conditions, where the cells are heavily loaded with a $[Ca^{2+}]_i$ indicator dye (quin2) to buffer changes in $[Ca^{2+}]_i$ (Merritt *et al.*, 1990), 25–30% of the rise in fluorescence is due to Ca^{2+} release from intracellular stores and the rest is due to influx of extracellular Ca^{2+} (compare control traces plus and minus extracellular Ca^{2+} in Figure 4a). The inhibitory effects of octimibate in the presence of extracellular Ca^{2+} are therefore predominantly due to inhibition of Ca^{2+} influx. When the platelets are incubated in the absence of extracellular Ca^{2+} (1 mM EGTA added), the ADP-stimulated rise in $[Ca^{2+}]_i$ is due to release from intracellular stores. This is also shown in Figure 4a, which shows inhibition of this response by octimibate. Both Ca^{2+} release from intracellular stores and influx of extracellular Ca^{2+} show similar sensitivities to inhibition by octimibate, as shown in Figures 4a and 4b. Figure 4b shows dose-response curves from a single experiment for inhibition of total $[Ca^{2+}]_i$ rises and Ca^{2+} release from intracellular stores by octimibate. The IC_{50} values for octimibate were 9.0 ± 0.6 nM (Hill coefficient 1.3 ± 0.1) and 11.6 ± 0.8 nM (Hill coefficient 1.2 ± 0.1) respectively.

Figure 4c shows dose-response curves for inhibition of the total ADP-stimulated increase in $[Ca^{2+}]_i$ by octimibate ($IC_{50} = 9.8 \pm 0.4$ nM, Hill coefficient 1.8 ± 0.1 , $n = 5$), PGI_2 ($IC_{50} = 0.16 \pm 0.01$ nM, Hill coefficient 1.5 ± 0.2 , $n = 1$), iloprost ($IC_{50} = 1.7 \pm 0.11$ nM, Hill coefficient 2.4 ± 0.5 , $n = 3$), and forskolin ($IC_{50} = 370 \pm 26$ nM, Hill coefficient 2.2 ± 0.4 , $n = 3$). For each of these compounds, similar concentrations were required to inhibit both Ca^{2+} release from intracellular stores and influx of extracellular Ca^{2+} : $67 \pm 4\%$ ($n = 3$) and $81 \pm 3\%$ ($n = 3$) inhibition respectively by 25 nM octimibate; $62 \pm 6\%$ ($n = 3$) and $65 \pm 2\%$ ($n = 3$) inhibition respectively by 2.1 nM iloprost; 74% ($n = 1$) and 70% ($n = 1$) inhibition respectively by 0.27 nM PGI_2 ; $61 \pm 3\%$ ($n = 3$) and $71 \pm 7\%$ ($n = 3$) inhibition respectively by 500 nM forskolin.

Effect of octimibate and iloprost on cyclic AMP metabolism in platelets

Figure 5 shows the effect of octimibate and iloprost on the cyclic AMP content (Figure 5a) and the cyclic AMP-dependent protein kinase activity ratio (Figure 5b) of human platelets following parallel 2 min incubations at $37^\circ C$. It is clear that both iloprost and (to a lesser extent) octimibate have stimulated increases in cyclic AMP content and activation of cyclic AMP-dependent protein kinase (an increase in the activity ratio). It should be noted that, at concentrations of octimibate and iloprost that are required to inhibit platelet aggregation (Figure 2 and Table 1), the stimulated increases in cyclic AMP content and cyclic AMP-dependent protein kinase activity are very small. This stimulated increase in platelet cyclic AMP content could be due to either stimulation of adenylyl cyclase or inhibition of cyclic nucleotide phosphodiesterase.

Figure 6 shows that both octimibate and iloprost activate adenylyl cyclase in human platelet membranes. The EC_{50} for activation of adenylyl cyclase by octimibate is 204 ± 8 nM

Figure 5 The effects of octimibate and iloprost on cyclic AMP levels (a) and the cyclic AMP-dependent protein kinase activity ratio (b) in washed human platelets. Platelet suspensions were incubated for 2 min in parallel incubations with various concentrations of octimibate (●) or iloprost (■), and following termination of the incubations, cyclic AMP levels (a) or cyclic AMP-dependent protein kinase activity ratios (cA-PrK) (b) were measured. Data are means of 1–5 (mostly 4) experiments; vertical bars show s.e.mean; where not shown, the error bars are within the symbol size.

($n = 7$), Hill coefficient is 1.4 ± 0.1 . The EC_{50} for activation by iloprost is 6.8 ± 0.7 nM ($n = 15$), Hill coefficient is 1.2 ± 0.1 . The maximum effect of octimibate relative to that of iloprost is $57 \pm 2\%$ ($n = 7$). The maximum effect of PGI_2 was similar to that of iloprost (results not shown). High concentrations of octimibate ($> 10 \mu M$) appear to inhibit adenylyl cyclase activ-

Figure 6 The effects of octimibate and iloprost on adenylyl cyclase activity in human platelet membranes. Adenylyl cyclase activity is shown as pmol cyclic AMP formed min⁻¹ mg⁻¹ protein during a 10 min incubation at $37^\circ C$. Dose-response curves are shown as means of 7 experiments for octimibate (●) and 15 experiments for iloprost (■); vertical bars show s.e.mean, where not shown, the error bars are within the symbol size.

Figure 7 Inhibition of [^3H]-iloprost binding to platelet membranes by iloprost or octimibate. [^3H]-iloprost binding is shown as % specific binding, after subtraction of nonspecific binding, during a 30 min incubation at 37°C . Results are shown as means of triplicates from a single representative experiment with iloprost (■) and octimibate (●). The curves were obtained by fitting the data to a single site model. Vertical bars show s.e. mean; where not shown, the error bars are within the symbol size.

ity. This is most probably due to a nonspecific effect, since concentrations of octimibate $> 10\ \mu\text{M}$ cause red blood cell haemolysis.

An alternative or additional means for elevation of cellular cyclic AMP could be inhibition of phosphodiesterase. The major cyclic AMP-metabolising phosphodiesterase in platelets is the cyclic GMP-inhibited phosphodiesterase, phosphodiesterase III, (Murray *et al.*, 1990b). Octimibate, at concentrations up to $1\ \mu\text{M}$, had no effect on the activity of this enzyme.

Inhibition of [^3H]-iloprost binding by octimibate

Figure 7 shows curves for inhibition of specific binding of [^3H]-iloprost to human platelet membranes by unlabelled iloprost or octimibate; the results are from a single typical experiment carried out in triplicate. For both ligands, the data appear to fit well to a single site analysis. Octimibate inhibited completely the specific binding of [^3H]-iloprost. The IC_{50} values for iloprost and octimibate were $46.5 \pm 5.2\ \text{nM}$ ($n = 14$) and $231 \pm 42\ \text{nM}$ ($n = 7$) respectively. These IC_{50} values correspond to K_i values of $30.9 \pm 3.5\ \text{nM}$ ($n = 14$) for iloprost and $153 \pm 28\ \text{nM}$ ($n = 7$) for octimibate. Maximal binding of [^3H]-iloprost was $0.96 \pm 0.22\ \text{pmol mg}^{-1}$ protein ($n = 5$). Both this value and the K_d of $19.6 \pm 3.0\ \text{nM}$ ($n = 2$, with range) for [^3H]-iloprost are consistent with values reported by others (e.g. Hall & Strange, 1984).

Effects of octimibate on aggregation of platelets from different species

Table 1 summarises all the results obtained with octimibate and iloprost on human platelets, and Table 2 shows the effects of octimibate and iloprost on aggregation of washed platelets from several different species. From Table 2, it is evident that octimibate is a potent inhibitor of aggregation of human and cynomolgus monkey platelets, but is 20–200 fold less potent

on all other species tested (similar results were obtained using platelet-rich plasma). The potency of iloprost varied by no more than a factor of 10 between species, with no obvious differences between primates and non-primates. Table 2 also shows the ratio of potency of octimibate/iloprost for each species. It is evident that octimibate is, relatively, at least 20 fold more potent on primates than non-primates, when considering the species variation of both octimibate and iloprost.

Discussion

In addition to its antihyperlipidaemic and antiatherosclerotic actions, octimibate has been reported to have antithrombotic activity with inhibition of platelet aggregation at concentrations of $1\text{--}9\ \mu\text{M}$ (Lautenschlager *et al.*, 1986). Here, we have shown that octimibate is considerably more potent for inhibition of human platelet aggregation than was previously reported. We have shown that octimibate inhibits platelet aggregation with an IC_{50} of about $10\ \text{nM}$ for washed platelets and about $200\ \text{nM}$ for platelets in plasma. The reduction in potency of octimibate in plasma is presumably due to binding to plasma proteins. As the inhibitory effect was not competitive with the stimulatory agonist (U46619 or ADP), octimibate was not acting as a receptor antagonist.

Many agonists that stimulate platelet aggregation also evoke rises in $[\text{Ca}^{2+}]_i$ (Rink *et al.*, 1982; Hallam *et al.*, 1984; Rink & Sage, 1990), and inhibition of agonist-stimulated rises in $[\text{Ca}^{2+}]_i$ can result in inhibition of agonist-stimulated aggregation (Sage & Rink, 1985; Merritt *et al.*, 1989). We have shown that octimibate inhibits agonist-stimulated $[\text{Ca}^{2+}]_i$ rises in platelets with an IC_{50} value of about $10\ \text{nM}$, a similar potency to that for inhibition of aggregation. Agonist-stimulated rises in $[\text{Ca}^{2+}]_i$ are due to both release of Ca^{2+} from intracellular stores and influx of extracellular Ca^{2+} (Rink & Sage, 1990). Octimibate inhibited both Ca^{2+} release from stores and influx over the same dose-range, thus it cannot be acting as a plasma membrane Ca^{2+} channel blocker since a Ca^{2+} channel blocker would be expected to inhibit selectively influx of Ca^{2+} (Merritt *et al.*, 1989). Agents, such as forskolin, PGI_2 and PGD_2 , that elevate cyclic AMP are known to inhibit agonist-stimulated $[\text{Ca}^{2+}]_i$ rises in platelets (Feinstein *et al.*, 1983; Rink & Sanchez, 1984). Forskolin has been found to inhibit both Ca^{2+} release from internal stores and influx with similar potency, and also to inhibit aggregation (Sage & Rink, 1985). Here, we have confirmed that both forskolin and PGI_2 (and iloprost, a stable PGI_2 mimetic), inhibit agonist-stimulated platelet aggregation and rises in $[\text{Ca}^{2+}]_i$ due to both store release and influx, with similar effects to those of octimibate. Experiments were therefore carried out to see if octimibate evoked increases in cyclic AMP in platelets.

We have shown that octimibate, as well as iloprost, does evoke increases in platelet cyclic AMP content and increases the platelet cyclic AMP-dependent protein kinase activity ratio (i.e. the proportion of the enzyme that is active following incubation of platelets with the test compound). At concentrations of octimibate or iloprost that inhibit platelet aggregation, the stimulated increases in cyclic AMP or cyclic AMP-dependent protein kinase activity ratio are almost undetectable. Such a result was not unexpected, since a similar

Table 2 Inhibition of washed platelet aggregation from different species

Species	Stimulus	IC_{50} (nM)		Ratio (octimibate/iloprost)
		Octimibate	Iloprost	
Human	U46619	10.5	0.9	12
Cynomolgus monkey	U46619	3.8	1.0	4
Guinea-pig	U46619	204	0.7	296
Dog	PAF	310	1.9	163
Rat	ADP	520	1.6	325
Cat	U46619	1100	4.0	275
Cow	PAF	2700	8.5	318

relation between elevation of cyclic AMP and inhibition of platelet aggregation has already been demonstrated for the phosphodiesterase inhibitor, siguazodan, whereby very small increases in cyclic AMP are associated with inhibition of platelet aggregation (Murray *et al.*, 1990b). The octimibate-stimulated rise in cyclic AMP could be due to either inhibition of phosphodiesterase or stimulation of adenylyl cyclase. The main phosphodiesterase isozyme found in platelets is the cyclic GMP-inhibited phosphodiesterase (Murray *et al.*, 1990b), and we have shown that the activity of this enzyme is not affected by octimibate, but octimibate does stimulate adenylyl cyclase activity in platelet membranes.

Platelets are known to have several receptors, including PGI₂, prostaglandin D₂ (PGD₂) and adenosine (A₂), that are coupled to stimulation of adenylyl cyclase (Miller & Gorman, 1979; Huttemann *et al.*, 1984; Ashby, 1989; Keen *et al.*, 1989a) and inhibit aggregation (Moncada *et al.*, 1976; Rink & Sage, 1990). Iloprost is a stable PGI₂ mimetic (Gryglewski & Stock, 1987), and inhibition of [³H]-iloprost binding can be used to assay the interaction of ligands with the PGI₂ receptor (Hall & Strange, 1984). We have shown that octimibate inhibits completely the binding of [³H]-iloprost to platelet membranes, with an estimated K_i of 150 nM. This result suggests that octimibate binds to the same site as iloprost, the PGI₂ receptor, although additional binding sites for octimibate to other receptor-types cannot be excluded. The K_i value for inhibition of binding of [³H]-iloprost by octimibate (150 nM) is similar to the EC₅₀ value for activation of adenylyl cyclase (200 nM). These results suggest the major (or only) site of action of octimibate in stimulating adenylyl cyclase in platelets is at the PGI₂ receptor. The K_i value for inhibition of binding of [³H]-iloprost by iloprost (30 nM) is greater than the EC₅₀ value for activation of adenylyl cyclase (7 nM), which suggests that there could be spare receptors for activation of adenylyl cyclase by iloprost.

Octimibate also inhibited [³H]-iloprost binding and activated adenylyl cyclase in membranes of NCB-20 cells (Kelly, Keen & MacDermot, unpublished observations), a cell line reported to have receptors for PGI₂ (Blair *et al.*, 1980; Blair & MacDermot, 1981). Desensitization of PGI₂ receptors is reported to be homologous in NCB-20 cells (Kelly *et al.*, 1990), and iloprost-pretreatment of these cells reduced considerably their subsequent response to octimibate as well as iloprost (Kelly, Keen & MacDermot, unpublished observations). This result provides further support for the hypothesis that the effects of octimibate are mediated (at least in part) through the same receptor as that for iloprost (i.e. the PGI₂ receptor).

Interaction of octimibate with the PGI₂ receptor could explain some of the antiatherosclerotic effects of octimibate. Octimibate has been reported to increase cholesterol efflux from cholesterol-loaded macrophages by causing a shift from esterified to free cholesterol (Schmitz *et al.*, 1985; Lautenschlager *et al.*, 1986). These effects of octimibate have been assumed to be due to inhibition of cholesterol esterification by ACAT. However, PGI₂ is known to decrease the lipid content of

cholesterol-loaded macrophages (Willis *et al.*, 1986) and vascular smooth muscle cells where it elevates cyclic AMP and activates neutral cholesterol ester hydrolase through cyclic AMP-dependent phosphorylation (Hajjar, 1986). Some of the antiatherosclerotic effects of octimibate could therefore be due to PGI₂ receptor-mediated activation of neutral cholesterol ester hydrolase in addition to the previously reported inhibition of ACAT.

Although octimibate appears similar to iloprost in its spectrum of activities, some differences are also apparent. The maximum activation of adenylyl cyclase obtained with octimibate is considerably less than that obtained with iloprost or PGI₂; the maximum activation seen with octimibate was 60% of that seen with iloprost. This result might suggest that octimibate is acting as a partial agonist at the PGI₂ receptor. An alternative or additional explanation is that there is a nonspecific inhibitory effect superimposed. The decrease in adenylyl cyclase activity at very high concentrations of octimibate appears to be due to nonspecific effects, since high concentrations of octimibate cause red blood cell haemolysis. From these results it is therefore not possible to determine whether octimibate is a full or a partial agonist at the PGI₂ receptor, and further experiments were not possible due to the problems of insolubility and inhibition of adenylyl cyclase activity at high concentrations of octimibate.

Octimibate showed considerably more species variation than either iloprost (Sturzebecher *et al.*, 1987; Armstrong *et al.*, 1989), PGI₂ (Whittle & Moncada, 1984; Oliva & Nicosia, 1987) or other PGI₂ mimetics including carbacyclin (Oliva & Nicosia, 1987). Octimibate was highly selective for inhibition of primate platelet aggregation compared to other species. This differential species sensitivity of octimibate might indicate the presence of different receptor subtypes in primates compared to other species. Obviously such results highlight the need for caution when interpreting results obtained on tissue from other species and from animal models.

To date, all known PGI₂ mimetics are prostanoid-type molecules (Oliva & Nicosia, 1987). Octimibate is structurally very different, and is a novel and potent inhibitor of platelet aggregation. The species variation of octimibate points to differences between the biological effects of octimibate and iloprost. It was therefore of interest to see whether octimibate could distinguish between platelet and vascular PGI₂ receptors, since such a compound could have significant therapeutic potential (Keen *et al.*, 1989b). The results of these studies are reported in the following paper (Merritt *et al.*, 1991).

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