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The antagonism by BW A868C of PGD_2 and BW245C activation of human platelet adenylate cyclase

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1 In glycerol-lysed human platelets, prostaglandin D_2 (PGD_2) and the hydantoin BW245C both activate adenylate cyclase in a biphasic manner. These activations are qualitatively different from those of carbacyclin, iloprost and prostaglandin E_2 (PGE_2) whose E/[A] curves can be adequately described by rectangular hyperbolae.

2 Prostaglandin E_1 (PGE_1) had E/[A] curves of slope significantly lower than that expected for a rectangular hyperbola.

3 The selective PGD_2 antagonist BW A868C shifts the first phase of the PGD_2 and BW245C E/[A] curves but has no effect on the second phase.

4 Applying a two-receptor model enables a pK_B to be derived for BW A868C of 9.11.

5 BW A868C has no effect on carbacyclin, iloprost, prostacyclin, PGE_1 and PGE_2 at a concentration 1,000 fold that of its K_B against PGD_2 and BW245C.

6 These results indicate that PGD_2 and BW245C are capable of activating adenylate cyclase in human platelets through the DP-receptor and by another mechanism as yet uncharacterized.

Introduction

Prostacyclin (PGI_2), stable analogues of prostacyclin such as carbacyclin and iloprost, prostaglandin E_1 (PGE_1) and prostaglandin D_2 (PGD_2) inhibit human platelet aggregation (Moncada *et al.*, 1976; Whittle *et al.*, 1978). Binding studies with radioligands suggest that whereas PGI_2, carbacyclin, iloprost and PGE_1 appear to interact at the same site, PGD_2 binds to a separate receptor (Siegl *et al.*, 1979). Similar binding studies have classified the hydantoin, prostaglandin-mimetic BW245C as acting on the PGD_2 receptor rather than the PGI_2 receptor (Town *et al.*, 1983; Hall & Strange, 1984).

The preceding paper (Giles *et al.*, 1989) describes a novel, competitive, high affinity, selective antagonist, BW A868C (3-benzyl-5-[6-carboxylhexyl]-1-[2-cyclohexyl-2-hydroxyethylamino]hydantoin), against PGD_2- and BW245C-inhibited platelet aggregation. As BW A868C was ineffective against PGI_2, PGE_1 and carbacyclin, these results support the classification of at least two separate prostaglandin receptors in human platelets.

Concomitant with the inhibition of platelet aggregation, all the above mentioned prostaglandin-mimetics elevate platelet intracellular cyclic AMP (Miller & Gorman, 1976; 1979; Tateson *et al.*, 1977) and have been shown to activate adenylate cyclase in human platelet membranes (Gorman *et al.*, 1977).

The activation of adenylate cyclase by PGI_2 and its stable analogues is quantitatively different from that of PGD_2. PGI_2 maximally stimulates human platelet cyclase to a much greater degree than PGD_2. Furthermore, we have extended the range of PGD_2 concentrations tested and observed a second phase of stimulation of adenylate cyclase by PGD_2, which is not apparent with carbacyclin, PGE_1, iloprost or prostacyclin.

This paper describes these observations and their partial characterization by use of the new selective PGD_2 antagonist BW A868C.

Methods

Platelet lysis

Human platelets were separated from peripheral blood and subsequently lysed by the method of Steer & Wood (1979). Human blood was collected by venepuncture into a bottle containing EDTA (Sigma, $1.2\,g\,l^{-1}$ of blood) as anticoagulant. Platelet-rich

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plasma (PRP) was obtained by centrifugation (1,000 g for 20 min at 4°C).

Platelets were isolated from the PRP by further centrifugation (4300 g for 10 min at 4°C) and resuspended in a buffer containing 1 mM EDTA, 150 mM NaCl, 10 mM Tris HCl, pH 7.6, 12 ml buffer being used per 34 ml of original blood. The platelets were recentrifuged under the same conditions and resuspended in 1–2 ml of the same buffer per 34 ml of original blood. This was layered onto a glycerol gradient in 150 mM NaCl (0%–40% v/v in 5% steps). The platelets were then centrifuged through the gradient (2000 g for 35 min at 4°C). The pellet was resuspended in a buffer containing 0.32 M sucrose, 5 mM Tris HCl, 1 mM EGTA and 1 mM 1,4-dithiothreitol (DTT, Sigma), pH 7.6; this buffer is hypotonic to the glycerol-loaded platelets and causes lysis. The lysed platelets were pelleted at 8700 g for 10 min at 4°C, washed 4 times with the same buffer and finally resuspended in that buffer at a protein concentration of 0.2 to 1.0 mg ml^{-1}.

Adenylate cyclase assay

Each experiment was done with a fresh platelet preparation. The standard reaction mixture contained 75 mM Tris/HCl (pH 7.6) (Sigma), 0.25 mM ATP (Boehringer), 1.25 mM $MgCl_2$, 1 mM cyclic AMP (Boehringer), 5 mM creatine phospate (Boehringer), 7 units creatine kinase (E.C. 2.7.3.2) (Boehringer), 0.5 mM 3-isobutyl-1-methyl xanthine (IBMX, Sigma), 10 μM GTP (Boehringer), 100 mM sucrose (B.D.H.), 0.3 mM EGTA (Sigma), 0.3 mM DTT (Sigma), enzyme protein (20 to 100 μg), [α-^{32}P]-ATP 1–2 μCi (NEN, 800 Ci mmol^{-1}), plus test substances in a final volume of 500 μl. All assays were performed in triplicate or quadruplicate. All additions were made with a randomised allocation of treatment to the assay tubes on ice. They were then transferred to a water bath and preincubated at 30°C. The time allowed for the tubes to equilibrate depended on the substance(s) being tested, but was usually 2 min. After preincubation [α-^{32}P]-ATP was added and the reaction carried out for 5 min before being stopped by the addition of 250 μl of 1% sodium dodecyl sulphate. After addition of 1.3 ml [^{3}H]-cyclic AMP (18–20,000 c.p.m., Amersham, 26 μCi mmol^{-1}) to each tube to monitor recovery, the labelled cyclic AMP was isolated by passage through Dowex AG-50 W (Bio-Rad) and alumina columns (Sigma, type WN-S) (Salomon *et al.*, 1974). The reaction was linear with platelet protein over the range used and for up to 2 h after the addition of [α-^{32}P]-ATP. The test substances had no effect on the recovery of cyclic AMP from the columns, nor on the linearity of the reaction and had reached a steady state in respect of cyclase activation within the perincubation period.

Compounds

BW245C 5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropyl hydration) and BW A868C were synthesized by Dr C. Caldwell and Dr M. Kelly respectively (Department of Medicinal Chemistry, Wellcome Research Laboratories). Iloprost was obtained from Amersham International, PGE_1 from Sigma and 5-E-carbacyclin from Upjohn Limited, U.S.A.

Carbacyclin, PGD_2, PGE_1, BW A868C and BW245C were all made up as stock solutions in 95% ethanol at 10 mg ml^{-1}.

These stocks were diluted, as appropriate, with adenylate cyclase buffer. All control buffers were adjusted to the same ethanol concentration.

Analysis of data

Where the data for a particular agonist were observed to produce monophasic E/[A] curves, simple logistics of the following general form were fitted:

$$\text{cyclic AMP produced} = \beta + \frac{\alpha}{1 + 10^{-n(\log [A] - \log [A_{50}])}} \quad (1)$$

where β is basal activity of adenylate cyclase, α the maximal response to agonist [A], $\log [A_{50}]$ is the location parameter and n the slope of the logistic.

The rectangular hyperbola (with n = 1) is a special case of the logistic and this model was preferred when the fit to the data was not significantly improved (as judged by an F-test) by the estimation of n.

Where the E/[A] curves of the agonist were clearly not monophasic then a more complex model was fitted. As BW A868C only seemed to move the first phase of the E/[A] curve the model assumed 2 receptors for the same agonist both of which activate adenylate cyclase but only the more sensitive one able to bind BW A868C. The model is essentially the sum of two logistic curves with separate location parameters and upper asymptotes, and common slopes. The location parameter of the first logistic is modified by BW A868C in line with simple competition. A further parameter, equivalent to the Schild-plot slope was included to allow for departures from simple competition.

The full model is:

$$\text{cyclic AMP produced} = \beta + \frac{\alpha'}{1 + 10^{-n(\log [A] - \log [A'_{50}] - \log(1 + 10r(pK)B + \log B))}} + \frac{\alpha''}{1 + 10^{-n(\log [A] - \log [A''_{50}])}} \quad (2)$$

where [B] is the concentration of antagonist (BW A868C), pK_B the negative logarithm of the antagonist dissociation constant, log $[A'_{50}]$ and log $[A''_{50}]$ the locations of the two phases of the E/[A] curve with upper asymptotes of α' and α'' respectively, and r is effectively the Schild-plot slope.

In practice the second logistic is incompletely defined as the second phase did not reach an asymptote over the range of agonist that it was practical to use. In consequence there was insufficient information to estimate slope, location and upper asymptote of the second logistic independently. Therefore the slope of the second logistic was set equal to the first.

Simple competitivity at the first site was assessed by checking for deviation of the Schild-plot slope (r) from unity that were consistent across data sets.

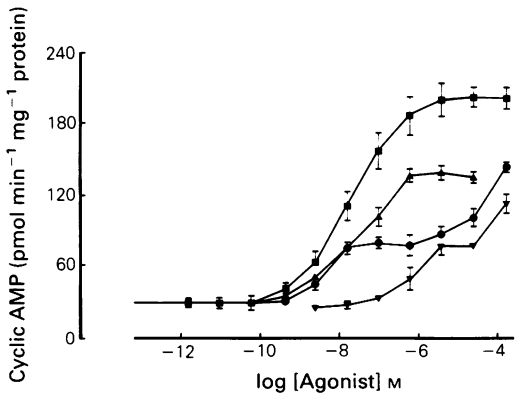

Figure 1 Representative E/[A] curves for the activation of adenylate cyclase in lysed human platelets from a single donor by carbacyclin (■), prostaglandin E_1 (PGE_1) (▲), BW245C (●) and PGD_2 (▼). The points are the means with s.e. (vertical bars) from triplicate determinations.

Results

Activation of platelet adenylate cyclase by prostaglandins and prostaglandin-mimetics

Prostaglandin D_2 (PGD_2) (Figures 1 and 2) and BW245C (Figures 1 and 3) both exhibit biphasic E/[A] curves for the activation of human platelet adenylate cyclase. This is a property not exhibited in the E/[A] curves of PGE_1 (Figure 1), carbacyclin (Figures 1 and 2), iloprost (Figure 2), prostacyclin and PGE_2 (not shown).

Fitting the E/[A] curves either by simple logistics, equation (1), (in the case of carbacyclin, PGE_2 and iloprost and prostacyclin) or by a more complex model (in the case of PGD_2 and BW245C), equation (2) as described in the methods, gave slopes not significantly different from that expected for rectangular hyperbolae. PGE_1 consistently was fitted better by a logistic of low slope (0.82 ± 0.03, mean ± s.e., $n = 7$ donors) than by a rectangular hyperbola. This is a finding which has been reported previously (Lombroso *et al.*, 1984). Table 1 gives maximal activations and concentrations giving half maximal stimulation (log $[A_{50}]$) for the agonists examined in platelets from a number of different donors. For PGD_2 and BW245C only the first phase of activation has been considered. PGD_2 and BW245C produced stimulations significantly lower than that of carbacyclin, iloprost, prostacyclin, PGE_2 and PGE_1, with iloprost and BW245C being the most potent (log $[A_{50}] = -8.3$ M) and PGE_2 being the least potent (log $[A_{50}] = -4.8$ M).

Table 1 The location parameters (log $[A_{50}]$s) and upper asymptote values (expressed as stimulation above basal activity) for the activation of human platelet adenylate cyclase by the prostaglandin-mimetics studied

Agonist	log $[A'_{50}]$ (M)	log $[A''_{50}]$ (M)	*Fold stimulation (relative to basal)*
PGE_2	-4.8 ± 0.04 (4)	N.A.	7.2 ± 0.40
PGE_1	-7.2 ± 0.06 (7)	N.A.	8.0 ± 0.80
Carbacyclin	-7.4 ± 0.10 (12)	N.A.	9.3 ± 1.00
Prostacyclin	-7.9 ± 0.10 (5)	N.A.	10.7 ± 0.40
Iloprost	-8.3 ± 0.09 (6)	N.A.	9.2 ± 0.90
PGD_2	-6.4 ± 0.10 (5)	-4.3 ± 0.07 (5)	2.9 ± 0.16^E
BW245C	-8.3 ± 0.08 (6)	-4.4 ± 0.05 (6)	2.7 ± 0.14^E

[E] Derived from first phase only.

log $[A_{50}]$s and maximal stimulations were obtained by fitting the E/[A] curves to the equations described in the methods. The estimates are the means for platelets from a number of different donors (in parentheses). Where there was no second phase of activation log $[A''_{50}]$ is not applicable (N.A.).

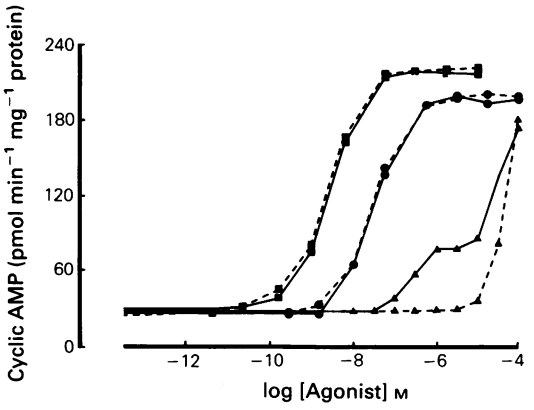

Figure 2 E/[A] curves for the activation of adenylate cyclase in lysed human platelets from a single donor by iloprost (■), carbacyclin (●) and prostaglandin D_2 (▲) in the absence (———) or presence (– – – –) of BW A868C (1.35 μM). The points are the means of triplicates, the s.e. bars have been omitted for clarity but never exceeded ±5% of the mean value.

Antagonism by BW A868C

BW A868C at 1.35 μM, a concentration more than 1,000 fold greater than its K_B in the platelet aggregation assay (Giles *et al.*, 1989), shifted the first phase of the PGD_2 E/[A] curve to the right but had no effect on the E/[A] curves for iloprost and carbacyclin (Figure 2). In similar experiments, but not shown, BW A868C at the same concentration shifted the E/[A] curve of BW245C but failed to shift E/[A] curves for prostacyclin, PGE_1 and PGE_2.

In the case of PGD_2, the two phases of activation were located too close together to allow a detailed analysis of BW A868C. Therefore, a pK_B for BW A868C was estimated using BW245C as the agonist because of the wider separation of the two phases (Figure 1).

Figure 3 shows a representative experiment in which BW A868C was used over the range of 5 nM to 1.22 μM. Only the first phase is affected, the right shift of the E/[A] curve approaching a limit at the highest concentrations of BW A868C. Fitting a two-receptor model where BW A868C competitively antagonized the higher affinity receptor for BW245C gave a pK_B of 9.11 ± 0.16 (s.e.) as estimated on 8 platelet preparations each from a different donor. Values for r other than unity gave better fits to two of the individual experiments but there was little sign of a consistent difference which was taken to indicate that the assumption of simple competitivity was reasonable. Deviations of n from unity were assessed in a similar way, and again no consistent differences were noted. The model was therefore fitted with n and r constrained to unity, and estimates of the pK_B and the remaining parameters for each experiment taken from this fit.

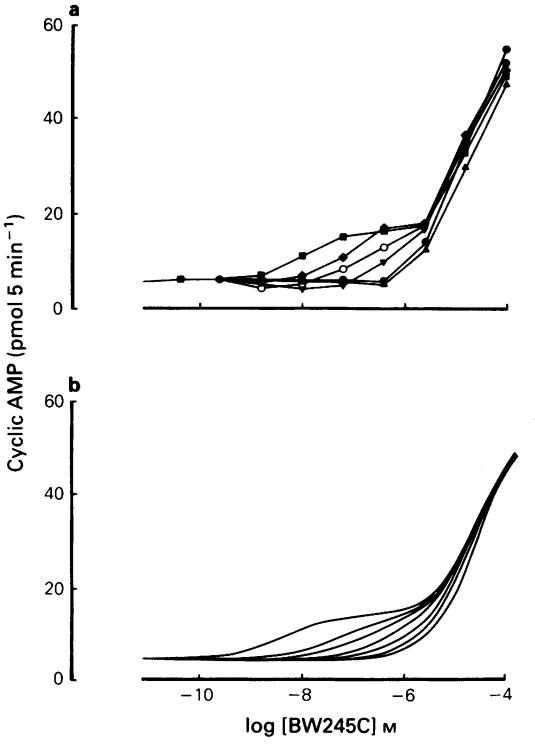

Figure 3 (a) E/[A] curves for the activation by BW245C of adenylate cyclase in lysed human platelets from a single donor in the presence of BW A868C at the following concentrations: 0 nM (■), 5 nM (◆), 15 nM (○), 45 nM (▼), 135 nM (●), 405 nM (▲) and 1215 nM (▲). The points are the means of triplicates. The s.e. bars have been omitted for clarity, but never exceeded ±5% of the mean value. (b) Fitted curves to the data shown in (a) using the two-receptor model described in Methods. The parameters estimated in this experiment were: $\beta = 4.9$, $\alpha' = 9.1$, $\alpha'' = 44.0$, $\log [A'_{50}] = -8.4$, $\log [A''_{50}] = -4.7$ and $pK_B = 9.4$.

Discussion

The finding in the preceding paper that BW A868C is a highly selective competitive DP-receptor antagonist offers a high affinity tool to help elucidate complex E/[A] curves of the type described in this paper for PGD_2 and BW245C.

The results show that BW A868C antagonizes only one of the two phases of activation of human platelet adenylate cyclase exhibited by both PGD_2 and BW245C. The degree of shift of this first phase translates into a pK_B value for BW A868C of 9.11, which is not significantly different from the 9.26

found in the whole platelet aggregation assay (Giles *et al.*, 1989). Like the aggregation assay, BW A868C did not give Schild-plot slope parameters of unity in two of the eight experiments. Of these two, one was significantly above and one significantly below unity. As there were no consistent deviations from unity and as the pooled value of r was 1.03 ± 0.05 (s.e.) it was taken that the criteria for BW A868C as a competitive antagonist was met in the cyclase assay. Neither did there appear to be an equilibrium problem as found in the aggregation assay. This was supported by experiments (not shown) where the progress of the adenylate cyclase activity was followed. These suggested that BW A868C at concentrations as low as that producing 20% inhibition were in a steady state with BW245C within the period of preincubation (2 min).

The second phase of activation by PGD_2 and BW245C is resistant to BW A868C and therefore appears not to be mediated through the same receptor and is not yet identified. It is possible that this phase is a reflection of some physical property of high concentrations of the prostaglandin-mimetic acting on adenylate cyclase itself or indirectly on the membrane environment of the enzyme. In estimating the pK_B for BW A868C we have ignored these possibilities and assumed that the second phase is receptor-mediated. This was done because a two-receptor model seemed a simple starting point with a pharmacological basis to which the data fitted tolerably well and because binding studies with [^{3}H]-iloprost and human platelets have shown that BW245C displaces this ligand with an IC_{50} of 78.3 μM (Hall & Strange, 1984). Assuming simple competition at the same site an estimated K_D of approximately 40 μM can be calculated. This value is very close to the ED_{50} computed for the second phase of adenylate cyclase activation by BW245C (Table 1), suggesting a specific receptor. Furthermore, using human platelet membranes we have found a similar close correspondence between the log [A_{50}] derived from adenylate cyclase assays and the log K_D derived from [^{3}H]-iloprost displacement studies for a large number of prostaglandin analogues.

In the rabbit jugular vein, BW245C produces a second relaxation phase thought to be through the EP_2-receptor (using the nomenclature of Kennedy *et al.*, 1982) rather than any other (Giles *et al.*, 1989). Displacement of [^{3}H]-iloprost by BW245C could be interpreted as the BW245C second phase in the platelet being mediated through the IP-receptor. We favour the IP-receptor as the second receptor in the human platelet because of agonist potencies in stimulating adenylate cyclase. Iloprost, PGI_2, carbacyclin and PGE_2 all show simple monophasic E/[A] curves with iloprost > PGI_2 > carbacyclin > PGE_2 which is the rank order expected for action on the IP-receptor (Dong *et al.*, 1986). The same authors show that for EP_2-receptors the rank order would be expected to be PGE_2 > carbacyclin > iloprost = PGI_2.

In conclusion this paper supports the classification of BW A868C as a highly selective, competitive antagonist at the DP-receptor with a pK_B of 9.11. This compound discriminates between the two mechanisms of activation of human platelet adenylate cyclase by PGD_2 and BW245C, one being characterized as through the DP-receptor and the other, as yet undefined, possibly through the IP-receptor.

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