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Short communication

A study of prostacyclin mimetics distinguishes neuronal from neutrophil IP receptors

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Abstract

The prostacyclin mimetics BMY 45778 (3-[4-(4,5-diphenyl-2-oxazolyl)-5-oxazolyl]phenoxy]acetic acid), BMY 42393 (2-[3-[2-(4,5-diphenyl-2-oxazolyl)ethyl]phenoxy]acetic acid) and EP 185 (*rac* 5-endo-(6′-carboxyhex-2′*Z*-enyl)-6-exo-(*p*-methoxyphenyl-phenyl-methylazino)-bicyclo[2.2.2]oct-2-ene) inhibited rat neutrophil aggregation stimulated by *N*-formyl-methionyl-leucyl-phenylalanine (IC_{50} = 20, 462, and 1195 nM respectively). In contrast only BMY 45778 (1–10 μM) produced any significant inhibition (10–20%) of the spontaneous activity of rat colon. BMY 45778 (10 μM) also attenuated the inhibitory effect of the prostacyclin analogue cicaprost on rat colon, whereas BMY 42393 and EP 185 did not. BMY 45778 appears to be a low affinity partial agonist at prostacyclin receptors on rat colon and its low potency in rat colon compared with rat neutrophils suggests the presence of a different prostacyclin receptor located on enteric neurones.

Keywords: Prostacyclin receptor; Cicaprost; Non-prostanoid prostacyclin mimetic; Neutrophil, rat; Colon, rat

1. Introduction

Prostacyclin is best known as an endogenous inhibitor of platelet function and a potent vasodilator, and therefore it is not surprising that prostanoid IP receptors have been characterised most extensively in blood platelets and vascular smooth muscle (Coleman et al., 1994). Another potentially important action of prostacyclin is suppression of white cell activation, including human monocytes/macrophages (Wise et al., 1991) and rat neutrophils (Wise and Jones, 1994). However, not all prostacyclin (IP receptor mediated) effects are inhibitory. For example the prostacyclin analogue cicaprost, which is a highly specific IP receptor agonist, stimulates enteric neurones inducing release of acetylcholine and substance P in the guinea-pig ileum (Jones and Lawrence, 1993) and release of inhibitory NANC (non-adrenergic, non-cholinergic) transmitters in the rat colon (Qian and Jones, 1995); these actions of cicaprost are abolished by tetrodotoxin.

In addition to prostacyclin analogues, a number of compounds that show little structural resemblance to prostacyclin have been shown to be agonists at both platelet and vascular IP receptors. The first agents to be described (e.g. EP 157, *rac* 5-endo-(6′-carboxyhex-2′*Z*-enyl)-6-exo-diphenylmethoxyiminomethyl-bicyclo-[2.2.2.]oct-2-ene) were derivatives of prostaglandin H_2 (Armstrong et al., 1986). EP 157 competed for [^{3}H]iloprost binding to platelets and inhibited platelet aggregation in a variety of species. EP 157 produced relatively small elevations of cyclic AMP in rat and pig platelets and inhibited adenylyl cyclase activity in response to iloprost in a manner typical of a partial agonist acting at IP receptors. Later agents (e.g. octimibate) lacked a typical prostanoid ring system and have been termed 'non-prostanoid prostacyclin mimetics' (Meanwell et al., 1994).

In the present study we have chosen to examine the effects of three more recently identified agents which activate IP receptors in human platelets. EP 185 (*rac* 5-endo-(6′-carboxyhex-2′*Z*-enyl)-6-exo-(*p*-methoxy-phenyl-phenyl-methylazino)-bicyclo[2.2.2]oct-2-ene), which is related to EP 157, inhibits ADP-induced human platelet aggregation (Jones et al., 1993) and competes for [^{3}H]iloprost binding to human platelet membranes with an approximate IC_{50} value of 40 nM (unpublished observations). BMY 42393 (2-[3-[2-(4,5-di-

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phenyl-2-oxazolyl)ethyl]phenoxy]acetic acid) and BMY 45778 (3-[4-(4,5-diphenyl-2-oxazolyl)-5-oxazolyl]phenoxy]acetic acid) are related to octimibate (Meanwell et al., 1994). BMY 42393 is a broad-spectrum inhibitor of platelet aggregation and shows all the properties expected of a mechanism of action involving activation of IP receptors (Seiler et al., 1994). For example, BMY 42393 competes for [^{3}H]iloprost and [^{3}H]prostaglandin E_1 binding to platelet membranes, but has little effect on [^{3}H]prostaglandin E_2, [^{3}H]prostaglandin D_2 or [^{3}H]SQ 29548 (thromboxane receptor antagonist) binding. BMY 42393 also stimulates platelet cyclic AMP production, activates cyclic AMP-dependent protein kinase, and prevents thrombin-induced increases in intracellular calcium. BMY 45778 is more potent than BMY 42393 and has a similar range of effects on human platelets (Meanwell et al., 1994). In particular, 24 h incubation of platelets with BMY 45778 causes desensitization of the cyclic AMP response to iloprost, a phenomenon generally associated with activation of IP receptors. In this paper we report our finding that these prostacyclin mimetics show quite different profiles as inhibitors of rat neutrophil aggregation and as stimulators of rat enteric neurones.

2. Materials and methods

2.1. Materials

The following chemicals were used: fMLP (*N*-formyl-methionyl-leucyl-phenylalanine) and nicotine hydrogen tartrate (Sigma Chemical Co., USA); NIH thioglycollate broth (Difco, USA). EP 185 was supplied by Dr N.H. Wilson of the Department of Pharmacology, University of Edinburgh. The gifts of BMY 42393 and BMY 45778 (Bristol-Myers Squibb, USA) and cicaprost (Schering, Germany) are gratefully acknowledged.

2.2. Neutrophil aggregation

Peritoneal neutrophils, elicited by intraperitoneal injection of 3 ml of NIH thioglycollate broth (0.26 g/ml), were isolated from male Sprague-Dawley rats weighing 190–230 g (Wise and Jones, 1994). Neutrophils were finally resuspended at 5×10^6 cells/ml in Hepes-buffered saline (HBS: NaCl, 145 mM; KCl, 5 mM; $MgCl_2$, 1 mM; Hepes acid, 10 mM; glucose, 10 mM; pH 7.55). The resulting cell suspension contained approximately 90% neutrophils with a cell viability of > 96% as judged by the trypan blue exclusion test.

Neutrophil aggregation was measured with a Chrono-log platelet aggregometer set at 37° C and 800 rpm as described in Wise and Jones (1994). The cell suspension (500 μl) was incubated in siliconized cuvettes for 1 min with the addition of $CaCl_2$ (1 mM) and indomethacin (3 μM). Test drugs (5 μl) were then added for 2 min followed by an EC_{50} concentration of fMLP (16 nM).

2.3. Rat isolated colon preparation

Male Sprague-Dawley rats, weighing 250–300 g, were fasted overnight and killed by stunning and exsanguination. A 2 cm length of the proximal ascending colon was removed and suspended in a 10 ml organ bath containing Krebs solution at 37° C (Krebs: NaCl, 118 mM; KCl, 4.7 mM; $CaCl_2$, 2.5 mM; $MgSO_4$, 1.0 mM; KH_2PO_4, 1.18 mM; $NaHCO_3$, 25 mM; glucose, 10 mM; indomethacin, 1 μM; gassed with 95% O_2/5% CO_2). Contractions of the longitudinal muscle (resting tension about 0.3 g) were measured with Grass FT03 isometric transducers connected to a MacLab data acquisition system (ADInstruments, Australia).

Preparations were allowed about 60 min to equilibrate, with frequent washing. Several submaximal doses of cicaprost were tested on each preparation to ensure a stable level of sensitivity, then agonist doses were added for 2 min and the response calculated as a percentage of the control resting spontaneous activity. To examine the effect of the prostacyclin mimetics on the response to inhibitory agonists, the test compounds were added 10 min before cicaprost (10 nM) or nicotine (3 μM). To examine the effect of BMY 45778 in more detail, cumulative concentration-response curves to cicaprost were obtained following 10 min incubation with either Krebs solution or BMY 45778 (10 μM).

2.4. Data analysis

IC_{50} values (concentration of test compound producing 50% of own maximal inhibitory response) were calculated using the four-parameter logistic curve fitting program ALLFIT (De Lean et al., 1978). To determine agonist ranking for inhibition of neutrophil aggregation, IC_{30} values (concentration of test compound producing 30% of maximum inhibition) were calculated. Results are expressed as mean ± S.E.M. Statistical analysis was performed using an unpaired Student's *t*-test.

3. Results

3.1. Inhibition of rat neutrophil aggregation

Cicaprost produced a concentration-dependent inhibition of fMLP-stimulated neutrophil aggregation to a maximum of 52% inhibition, with an IC_{50} value of 2 nM (Fig. 1). Both BMY 42393 and BMY 45778 produced log concentration-response curves parallel to that of cicaprost, again showing a maximal effect of

approximately 60% inhibition and IC_{50} values of 462 nM and 20 nM respectively (Fig. 1).

At concentrations up to 1 μM, EP 185 inhibited fMLP-stimulated neutrophil aggregation with a potency similar to that of BMY 42393 (Fig. 1). However, at concentrations greater than 1 μM, EP 185 produced almost complete inhibition of neutrophil activity which could not be ascribed to any solvent effect (matching concentrations of ethanol had no effect on aggregation) or to loss of cell viability (assessed by the trypan blue exclusion test on cell samples taken immediately after the aggregation test). The IC_{50} value of EP 185 was 1195 nM.

3.2. Inhibition of rat colon contractility

In the first set of experiments using discrete dosing, cicaprost inhibited the spontaneous activity of the rat colon with an IC_{50} value of 6 nM. Neither EP 185 nor BMY 42393 had any effect on colonic activity at 0.1–10 μM, whereas BMY 45778 produced a small degree of inhibition which was significant at 1 μM ($P < 0.05$; Fig. 2). The response ($80 \pm 3\%$ inhibition, $n = 4$) to a single dose of cicaprost (10 nM) was unaffected by either 10 μM EP 185 ($80 \pm 6\%$, $n = 4$) or 10 μM BMY 42393 ($84 \pm 6\%$, $n = 4$). However, the combination of cicaprost (10 nM) and BMY 45778 (10 μM) produced only $49 \pm 5\%$ inhibition ($n = 4$, $P < 0.01$). In contrast the inhibitory action of nicotine (3 μM) was not inhibited by BMY 45778 (10 μM): control $87 \pm 3\%$ ($n = 4$); combination $91 \pm 3\%$ inhibition ($n = 4$).

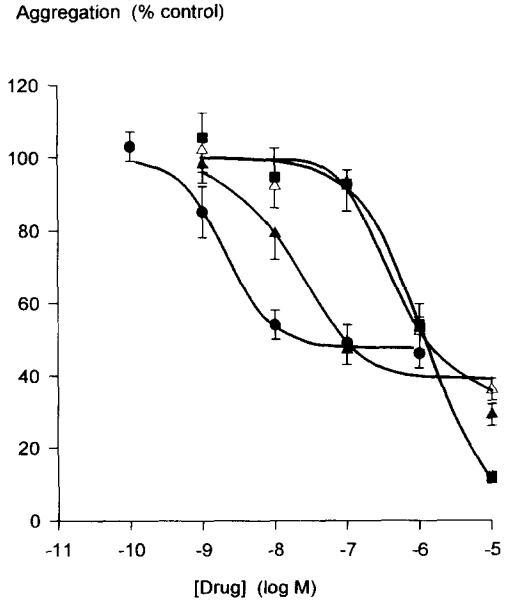

Fig. 1. The effects of prostacyclin mimetics on fMLP-induced neutrophil aggregation. Rat neutrophils were incubated for 2 min with test compound prior to the addition of an EC_{50} concentration of fMLP (16 nM). Data shown are means of at least 4 separate determinations; vertical error bars represent S.E.M. Cicaprost (●), BMY 42393 (△), BMY 45778 (▲), and EP 185 (■).

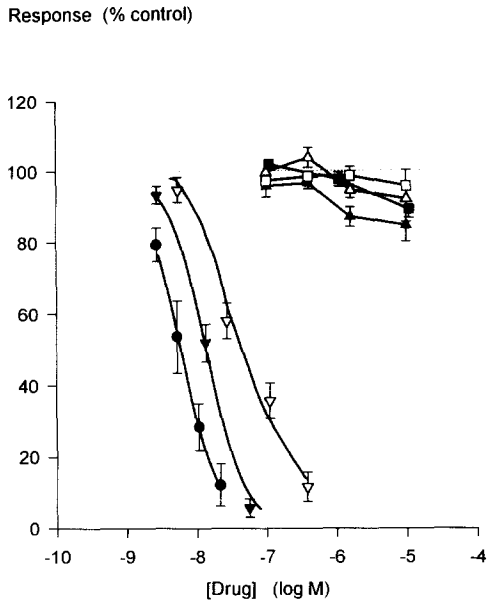

Fig. 2. The effects of prostacyclin mimetics on the spontaneous contractile activity of rat colon. Data shown are means of at least 4 separate determinations; vertical error bars represent S.E.M. Agonist drugs were tested for 2 min. Discrete dosing schedule: cicaprost (●), BMY 42393 (△), BMY 45778 (▲), EP 185 (■), and solvent control (0.14–14 mM DMSO) for BMY 42393 and BMY 45778 (□). Cumulative dosing schedule: cicaprost (▼), cicaprost in the presence of 10 μM BMY 45778 (▽).

In a second set of experiments using cumulative dosing, the cicaprost IC_{50} value was 14 ± 2 nM. In these experiments, BMY 45778 (10 μM) produced little inhibition (< 5%, $n = 4$) and shifted the log concentration-response to cicaprost to the right; dose ratio = 3.8 at the 50% response level (Fig. 2).

4. Discussion

In the present study the potency of cicaprost in inhibiting fMLP-stimulated rat neutrophil aggregation and in inhibiting the spontaneous activity of rat colon is very similar to that reported for inhibition of aggregation of rat washed platelets (Armstrong et al., 1989) and is strongly suggestive of the involvement of IP receptors in mediating these inhibitory responses.

As reported previously, rat neutrophil activity can be inhibited by agents which elevate cyclic AMP such as the IP receptor agonists cicaprost and iloprost, as well as EP_2 receptor agonists such as prostaglandin E_2, and in all cases, maximal inhibition never exceeded 60% of the control response to fMLP (Wise and Jones, 1994). Therefore the extra inhibitory effect of EP 185, and to a lesser extent BMY 42393 and BMY 45778, observed in the present study suggests that EP 185 may

have some inhibitory activity unrelated to the production of cyclic AMP.

The order of potency of the IP receptor agonists for the inhibition of rat neutrophil aggregation in the present study is cicaprost > BMY 45778 > BMY 42393 = EP 185, with equi-effective molar ratios relative to cicaprost of 1.0, 8, 139 and 192 respectively. For the inhibition of human platelet aggregation measured in platelet-rich plasma, the order of potency is cicaprost > BMY 45778 > EP 185 > BMY 42393; equi-effective molar ratios are 1.0, 34, 125–375 and 1500 respectively (data for cicaprost and EP 185 from Jones et al., 1993; BMY 42393 from Seiler et al., 1994 and BMY 45778 from Meanwell et al., 1994). However such a comparison may be misleading since the prostacyclin mimetics exhibit a high degree of protein binding relative to the hydrophilic prostacyclin analogues (Jones et al., 1993; Meanwell et al., 1994) and, in contrast with the human platelet experiments, the rat neutrophil studies are performed in protein-free solutions. Therefore in an attempt to compensate for the protein binding properties of BMY 45778, BMY 42393 and EP 185, we have recalculated the equi-effective molar ratios relative to BMY 45778, i.e. 0.12, 1.0, 18 and 24 in rat neutrophils, and 0.03, 1.0, 44 and 3.7–11 in human platelets, for cicaprost, BMY 45778, BMY 42393 and EP 185 respectively. Thus the absolute potency of IP agonists appears to be merely 4-fold less in rat neutrophils compared with human platelets, but their order of potency in the different species appears similar.

These observations contrast with the evidence for the heterogeneity of platelet IP receptors that has been presented previously in an extensive study of the effects of iloprost, cicaprost, 6a-carba-prostacyclin and EP 157 on platelets from a variety of species (Armstrong et al., 1989). IP receptors on human, pig and horse platelets were found to be similar in nature but distinct from those of rabbit and rat platelets. Octimibate and related compounds were also able to demonstrate species specificity, being 10- to 50-fold less potent in inhibiting rat and rabbit platelets compared with human platelets (Seiler et al., 1990, 1994; Merritt et al., 1991a; Meanwell et al., 1994). Together these observations suggest that whilst rat neutrophils and human platelets appear to express similar IP receptors, different IP receptors are expressed on rat platelets. Coleman et al. (1994) have suggested that there is no convincing evidence for subclassification of IP receptors and that any differences probably result from species variants of the IP receptor, rather than from true subtypes. A more extensive study of these IP agonists on rat platelets (both in platelet-rich plasma and washed preparations) is clearly required to clarify these observations.

Of the three prostacyclin mimetics studied, only BMY 45778 had any inhibitory effect on rat colon motility. The maximum effect was only about 15% of the cicaprost maximum, and in preparations with a lower sensitivity to cicaprost, BMY 45778 had little effect. BMY 45778 also attenuated the inhibitory effect of cicaprost, but not that of nicotine; the latter also stimulates enteric neurones in the colon to release inhibitory NANC transmitters (Qian and Jones, 1995). These results suggest that BMY 45778 acts specifically, but with low efficacy, on IP receptors in the rat colon. Partial agonist activity has been reported previously for octimibate on human coronary and mesenteric arteries (Merritt et al., 1991b) and the human monocytic cell line Mono Mac 6 (Wise et al., 1991), and for EP 157 in pig platelets (Armstrong et al., 1986) with antagonism of the full agonist iloprost. It has been suggested that both across-species and within-species differences of IP agonists can be accounted for in terms of a single IP receptor with different coupling efficiencies in different species and tissues. In this respect, greater coupling efficiency has been reported for a cloned murine IP receptor present in a CHO cell line compared with the native receptor in P-815 mastocytoma cells in response to iloprost (Namba et al., 1994).

However, with respect to the BMY 45778 data, the argument for a single rat IP receptor with different coupling efficiencies is more difficult to sustain. BMY 45778 is the most potent member of the octimibate series reported so far, with an IC_{50} of 27 nM in human platelet-rich plasma. The inhibitory potency of BMY 45778 on rat neutrophils is also high, with measurable effects present at 10 nM. In terms of binding affinity, BMY 45778 competes with [^{3}H]iloprost binding to human platelet membranes with an IC_{50} of only 5 nM (Meanwell et al., 1993). This contrasts with the low affinity of BMY 45778 for IP receptors in the rat colon; applying the Schild equation for competitive antagonism to the second set of results gives an apparent K_d of 4 μM. A similar argument applies to EP 185 and BMY 42393, which compete for [^{3}H]iloprost binding to human platelet membranes with IC_{50} values of 40 nM (unpublished observations) and 171 nM (Seiler et al., 1994) respectively, but at 10 μM have absolutely no inhibitory effect against cicaprost on the rat colon.

In conclusion, the different profiles of activity of the three prostacyclin mimetics relative to cicaprost on the rat colon compared to the rat neutrophil can be reasonably explained by the existence of IP receptor subtypes, with the prostacyclin mimetics showing very low binding affinities for the rat colon subtype. Full confirmation of these observations will require further evidence that these prostacyclin mimetics indeed act at IP receptors in the rat neutrophil. Comparison with literature data on these and other prostacyclin mimetics suggests that IP receptors are similar on rat neutrophils and human platelets, but differ from IP receptors on rat colon (enteric neurones) and rat platelets.

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