Phencyclidine binds to blood platelets with high affinity and specifically inhibits their activation by adrenaline

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The ion channel probe phencyclidine [1-(1-phenylcyclohexyl)piperidine; PCP] selectively inhibited aggregation, secretion and ultrastructural changes in platelets induced by adrenaline, but did not affect activation induced by other common platelet agonists such as α -thrombin, ADP, collagen or ionophore A23187. [3 H]PCP bound to platelets with high affinity (K_d , 134 ± 33 nM; 3600 ± 1020 sites/platelet), as did the thienyl analogue [3 H]TCP {1-[1-(2-thienyl)cyclohexyl]piperidine}. PCP binding to platelets was increased 3–4-fold in *N*-methylglucamine buffer in the absence of Na⁺ ions. Binding was unaffected by haloperidol and was only weakly inhibited (EC_{50} 10–20 μ M), without significant stereoselectivity by the two sets of stereoselective ligands, dexoxadrol/levoxadrol and (+)MK801/(–)MK801. Binding of PCP was not competed for by adrenaline or yohimbine. Only the high-affinity binding of [3 H]PCP to platelets was blocked by prior treatment of the platelets with the covalent affinity probe Metaphit, and these platelets no longer aggregated in response to adrenaline although they responded normally to α -thrombin, ADP and collagen. These results suggest that platelets contain high-affinity receptors for PCP that can modulate adrenaline-induced platelet activation.

INTRODUCTION

Phencyclidine [1-(1-phenylcyclohexyl)piperidine, PCP] is a drug that was first utilized as a fast-acting anaesthetic, although its legal use in humans was abandoned because of accompanying hallucinatory effects and maniacal excitement. These psychomimetic effects of PCP are thought to be exerted through its binding to high-affinity PCP receptors in the brain which are associated with the *N*-methyl-D-aspartate (NMDA) receptor complex for the excitatory amino acids glutamate and aspartate (Foster & Fagg, 1987). The receptor complex contains ion channels controlling Na⁺/K⁺ exchange and the translocation of Ca²⁺ from the exterior to the interior of the cell (Albuquerque *et al.*, 1981; Blaustein & Ickowicz, 1983; Bartschat & Blaustein, 1986) which leads to activation of the arachidonate cascade (Dumuis *et al.*, 1988).

Ion movements also play an important role in the activation of platelets and can lead to changes in cytoplasmic pH and membrane potential, as well as in the translocation of Ca²⁺ both intracellularly and across the plasma membrane, possibly through the action of receptor-operated ion channels (Sweatt *et al.*, 1988; Salzman & Ware, 1988; Zschauer *et al.*, 1988; Garcia-Sancho *et al.*, 1989). Since platelets have long been studied as accessible models of neurotransmitter function (Pletscher, 1988), we have now examined their interaction with PCP. We have found that PCP binds to a high-affinity receptor in platelets and specifically inhibits platelet activation by adrenaline.

MATERIALS AND METHODS

Reagents

PCP and 1-[1-(2-thienyl)cyclohexyl]piperidine (TCP) were obtained from the Research Technology Branch of the National Institute on Drug Abuse, Rockville, MD, U.S.A. [3 H]PCP (47.6 Ci/mmol) and [3 H]TCP (55 Ci/mmol) were from

NEN–Dupont, Boston, MA, U.S.A. (+)MK801 and (–)MK801 were kindly provided by Dr. Patricia Contreras, G.D. Searle and Co. Dexoxadrol and levoxadrol (Jacobson *et al.*, 1987), and Metaphit (Rafferty *et al.*, 1985) were prepared as previously described. Adrenaline, *N*-methylglucamine and ADP were purchased from Sigma (St. Louis, MO, U.S.A.), and arachidonic acid was from Nuchek Prep (Elysian, MN, U.S.A.). Cyclic AMP radioimmunoassay kits were purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.).

Human blood, anticoagulated with citrate/phosphate/dextrose/adenine (CPD-A1), was obtained from volunteer donors through American Red Cross Blood Services and was processed to platelet-rich plasma (PRP) within 3 h of collection. Platelets were isolated from PRP by gel filtration (Lages *et al.*, 1975) or washing (Tandon *et al.*, 1989).

Platelet activation

Aggregation was carried out using a suspension (0.4 ml) of gel-filtered platelets [(1–2) $\times 10^8$ platelet/ml] incubated for 10 min at 37 °C with Ca²⁺ (1 mM) and fibrinogen (1 mg/ml) prior to the addition of adrenaline. The agent to be tested was usually added at this time, although preincubations for as little as 1 min were equally effective. From the technical viewpoint, it should be noted that individual donors differ greatly in their sensitivity to adrenaline and that the response of gel-filtered platelets decreases rapidly on standing. It is important that control samples and samples containing inhibitors be utilized at the same time after gel filtration, for example by the use of a multi-channel aggregometer.

To measure secretion, PRP was initially incubated for 30–45 min at room temperature with 35 nM-[3 H]5-HT (5'-hydroxy[1,2- 3 H(n)]tryptamine creatinine sulphate; 30.4 Ci/mmol; NEN–Dupont) prior to gel filtration (Tandon *et al.*, 1989). Platelet suspensions (300 μ l of 1.5×10^8 /ml) were incubated for 15 min at 37 °C in an aggregation cuvette with

Abbreviations used: PCP, phencyclidine [1-(1-phenylcyclohexyl)-piperidine]; TCP, 1-[1-(2-thienyl)cyclohexyl]piperidine; PRP, platelet-rich plasma; 5-HT, 5-hydroxytryptamine.

various concentrations of PCP prior to the addition of adrenaline to a final volume of 400 μ l. Imipramine was not utilized at this stage as it was found to antagonize the inhibitory effects of PCP, and PCP itself inhibits the re-uptake of 5-HT (Arora & Meltzer, 1980). The progress of aggregation was monitored for 5 min prior to the addition of 80 μ l of stop buffer containing imipramine (Harmon & Jamieson, 1986). Samples were centrifuged (15 000 g, 0.5 min) and supernatant radioactivity was determined.

Ultrastructure

Samples of PRP with or without PCP (final concn. 200 μ M) were stirred on a platelet aggregometer with a final concentration of 5.0 μ M-adrenaline. At 3 min after addition of adrenaline the samples were fixed by combining with an equal volume of 0.1 % glutaraldehyde in White's saline solution [a 10 % solution of a 1:1 mixture of (a) 2.4 mM-NaCl, 0.1 mM-KCl, 46 mM-MgSO₄, 64 mM-Ca(NO₃)₂·H₂O and (b) 0.13 mM-NaHCO₃, 8.4 mM-NaH₂PO₄ and 0.1 g of Phenol Red/litre, pH 7.4. After 15 min the samples were centrifuged to pellets (1400 g, 5 min) and the supernatant fixative was removed and replaced with 3 % glutaraldehyde in the same buffer. The samples resuspended in the second aldehyde fixative were maintained at 4 °C for 30 min, then sedimented to pellets as above. Supernatant solution was removed and replaced with 1 % osmic acid in distilled water containing 1.5 % potassium ferrocyanide for 1 h at 4 °C. All samples were dehydrated in a graded series of alcohol, and embedded in Epon 812. Thin sections cut from the plastic blocks on an ultramicrotome were examined after enhancement of contrast with uranyl acetate and lead citrate in a Philips 301 electron microscope.

Binding assays

Binding assays were carried out in triplicate using 4×10^7 washed platelets in a total volume of 0.1 ml of Tyrode's/Hepes buffer, pH 8.0, for 10 min at 4 °C; the concentration of ³H-labelled ligand (³H]PCP) or (³H]TCP) was 35 nM unless otherwise specified. Studies designed to assess the involvement of Na⁺ in PCP binding were carried out in Tyrode's/Hepes buffer wherein Na⁺ had been replaced by an equivalent amount of *N*-methylglucamine at pH 8.0.

The assay mixture was centrifuged (15 000 g, 5 min) in a Microfuge (Beckman Instruments, Palo Alto, CA, U.S.A.). The supernatant solution containing free ligand was carefully and completely removed by aspiration, and the end of the tube was cut off and the platelet pellet (bound ligand) was dissolved in 0.5 ml of Protosol (NEN-Dupont) for 2 h. Following this, the solution was neutralized with 70 μ l of acetic acid, mixed with 5 ml of OCS or ACS II (Amersham-Searle, Arlington Heights, IL, U.S.A.) and radioactivity was determined in a Beckman LS2800 β -radiation counter. Non-specific binding was determined by adding a large excess (100 μ M) of unlabelled PCP or TCP to the incubation mixtures (Vincent *et al.*, 1979). The trapped volume in this assay procedure, determined with [¹⁴C]-sucrose, amounted to 0.3 % of bound radioactivity and was not accounted for in the calculations. Binding data were calculated by Scatchard analysis using the LIGAND program (Munson & Rodbard, 1980).

RESULTS

Activation

Adrenaline (1 μ M), in the absence of PCP, caused a characteristic wave of platelet aggregation without prior shape change (Fig. 1). At very low concentrations of PCP (0.25–0.5 μ M) there was a prolongation of the lag phase to the onset of aggregation but, once it occurred, the rate and extent of aggregation were the

same as was observed in the absence of PCP. At higher PCP concentrations (1 μ M), aggregation was not seen to occur within the time limits of the experiment. However, PCP at concentrations as high as 200 μ M had no inhibitory effect on platelet aggregation induced by ADP (2.5 μ M), collagen (2.5 μ g/ml), α -thrombin (1 nM; 0.1 unit/ml) or arachidonate (50 μ M). Similar results were obtained with TCP: the inhibition was again characterized by a prolongation of the lag phase to the onset of aggregation but aggregation, once it occurred, was at the usual rate and to the usual extent (results not shown).

These anti-aggregatory effects of PCP were paralleled by its ability to inhibit the secretion of [³H]5-HT. PCP (1 μ M) reduced the adrenaline-induced release of [³H]5-HT from 75 ± 11 % to 15 ± 3 % (means $\pm$ S.E.M., $n = 5$) during the lag phase preceding the onset of aggregation.

PCP did not significantly affect cyclic AMP levels of either resting or adrenaline-stimulated platelets, regardless of whether or not adenylate cyclase was activated by prostaglandin I₂ (Table 1).

Ultrastructure

Control samples of PRP alone, or PRP with the same amount of ethanol used as a carrier for PCP, revealed the typical large aggregates in response to adrenaline (5.0 μ M). However, platelet samples combined with PCP prior to exposure to adrenaline revealed no aggregates and no ultrastructural changes indicative of activation (results not shown). PCP-treated platelets remained

Fig 1. Inhibition of adrenaline-induced platelet aggregation

Gel-filtered platelets with added Ca²⁺ (1 mM) and fibrinogen (1 mg/ml) were incubated for 10 min with various concentrations of PCP prior to the addition of adrenaline (final concn. 1 μ M). PCP concentrations were as follows: curve 1, 0 (control); curve 2, 0.25 μ M; curve 3, 0.5 μ M; curve 4, 1 μ M.

Table 1. Effects of PCP on cyclic AMP levels

Values given are means $\pm$ S.D. ($n = 3$) except for those marked with the asterisk, where $n = 2$. PGI₂, prostaglandin I₂.

[PCP] (μ M)...	Cyclic AMP (pmol/10 ⁸ platelets)			
	-PGI ₂		+PGI ₂ (1 μ M)	
	0	10	0	10
Control	1.4*	1.4*	87 $\pm$ 11	83 $\pm$ 11
+ Adrenaline (1 μ M)	1.2 $\pm$ 0.3	1.5 $\pm$ 0.2	34 $\pm$ 19	39 $\pm$ 9

Fig 2. Binding isotherm for [3H]PCP

The binding of [3H]PCP (35 nM) to washed human platelets was measured in the presence of increasing concentrations of unlabelled ligand. The binding curve shown is representative of separate experiments carried out in triplicate using platelets from eight different donors. The points are experimental values, while the line is the best fit to a model of two independent sites with non-specific binding. Ins: Scatchard plot of data.

discoid with well-defined granules and resembled in all respects unstimulated resting platelets.

Binding studies

Optimum conditions for the binding of [3H]PCP to platelets were similar to those found in rat synaptosomes (Vincent *et al.*, 1979), and were established by maintaining as high as possible the ratio of specific to non-specific binding in the presence of 100 μM unlabelled PCP. The binding was dependent on temperature, platelet number and pH. Under optimal conditions (pH 8.0; 4 °C; 4 × 10⁷ platelets/100 μl), the binding of [3H]PCP (35 nM) to platelets reached steady state in 5–10 min and, as in rat brain synaptosomes (Vincent *et al.*, 1979), about 60 % of the total was specifically bound, as shown by its rapid displacement in the presence of excess (100 μM) unlabelled PCP.

A representative 13-point binding isotherm for a low concentration (35 nM) of [3H]PCP in the presence of increasing concentrations of unlabelled PCP is shown in Fig. 2. Scatchard analysis (insert) using the LIGAND program showed that the best fit to the data was for a model of two types of independent binding sites with low non-specific binding. Co-analysis of all the data points from binding studies on platelets from eight different

Fig 3. Competition of [3H]PCP binding by inhibitors

The specific binding of [3H]PCP was measured in the presence of increasing concentrations of the various antagonists: (a), haloperidol (■), dexoxadrol (●) and levoxadrol (○). (b) (+)MK801 (○) and (-)MK801 (●). IC₅₀ values were determined graphically for 50 % displacement. Values given are the average of two determinations, each run in triplicate. Identical results were obtained with [3H]TCP.

donors carried out in triplicate demonstrated 3600 ± 1020 high-affinity binding sites per platelet with a K_d of 134 ± 33 nM, and (4.5 ± 1.1) × 10⁶ low-affinity binding sites with a K_d 47 ± 11 μM. The binding of [3H]PCP to platelets was unaffected by adrenaline or yohimbine at concentrations as high as 50 μM. Similar two-site binding was obtained with [3H]TCP. Binding data from all experiments are summarized in Table 2.

In the brain system, the absence of Na⁺ ions results in a 5-fold increase in the binding affinity of [3H]PCP, from 25 nM to 125 nM (Haring *et al.*, 1987a). In the platelet system, evaluation of PCP binding in N-methylglucamine buffer, in the absence of Na⁺, showed a 3–4-fold increase in affinity at the high-affinity site, from 134 nM to 38 ± 7 nM (1000 ± 420 sites/platelet), and a similar increase at the low-affinity site to 138 ± 35 μM and (16.5 ± 4.5) × 10⁶ sites (Table 2).

Stereoselectivity

Differentiation of true PCP binding sites in the brain from sigma opioid sites, which also bind PCP with high affinity, is

Table 2. Binding of [3H]PCP and [3H]TCP to platelets

n.d., not detected.

	High-affinity site		Low-affinity site		Non-specific binding
	K _{d1} (nM)	(sites/platelet)	K _{d2} (μM)	10 ⁻⁶ × (sites/platelet)	
[3H]PCP (n = 8)	134 ± 33	3600 ± 1020	47 ± 11	4.5 ± 1.1	0.024 ± 0.006
[3H]TCP (n = 3)	70 ± 17	1880 ± 470	18 ± 2	1.1 ± 0.1	0.025 ± 0.011
[3H]PCP (Na ⁺ -free; n = 7)	38 ± 7	1000 ± 420	138 ± 35	16.5 ± 4.5	0.013 ± 0.003
[3H]PCP (Metaphit-treated; n = 3)	n.d.	n.d.	81 ± 25	5.4 ± 2.1	0.015 ± 0.007

Fig 4. Inhibition of platelet aggregation by Metaphit

Metaphit (final concn. $5 \mu\text{M}$) was added to gel-filtered platelets (M), followed 1 min later by the agonist (A). Curve 1, $1 \mu\text{M}$ -adrenaline; curve 2, 1 nM -thrombin. For comparison, curve 3 shows the aggregation response to adrenaline ($1 \mu\text{M}$) in gel-filtered platelets that had not been treated with Metaphit.

based on the sensitivity of the latter to the neuroleptic drug haloperidol and the differing effects of stereoselective antagonists (Quirion *et al.*, 1987). The binding of [^3H]PCP (35 nM) to control platelets was unaffected by haloperidol at concentrations as high as $10 \mu\text{M}$ (Fig. 3) indicating that, in this respect, the platelet site resembles a true PCP site rather than a sigma opioid site. However, studies with stereoselective competitive inhibitors showed that dexoxadrol was able to displace [^3H]PCP bound to platelets only at high concentrations, and was only slightly more effective in this regard than levoxadrol. The IC_{50} values for the binding of these two inhibitors to the PCP binding site were $11 \mu\text{M}$ and $22 \mu\text{M}$ for dexoxadrol and levoxadrol respectively (Fig. 4). Similarly, (+)MK801 was slightly more effective at displacing [^3H]PCP from platelets than was (–)MK801, with IC_{50} values of $8 \mu\text{M}$ and $21 \mu\text{M}$ respectively.

Inactivation of the platelet PCP receptor

Metaphit is an irreversible acylating PCP analogue which blocks PCP binding sites in neuronal cells by forming covalent linkages to amino or thiol groups at the binding site (Rafferty *et al.*, 1985), and it causes PCP-like symptoms in rats (Contreras *et al.*, 1986). Platelets treated with Metaphit for 1 min prior to the addition of agonists did not aggregate normally in response to adrenaline, but responded normally to ADP, collagen and α -thrombin (Fig. 4).

High-affinity binding of PCP was not detectable in Metaphit-treated platelets, but the lower-affinity binding of PCP appeared to be unaffected (Table 2).

DISCUSSION

Adrenaline has unique properties as a platelet agonist insofar as it does not cause shape change and induces only localized increases in the cytoplasmic Ca^{2+} concentration (Salzman & Ware, 1988). It has been suggested that these localized changes in $[\text{Ca}^{2+}]$ may induce the exposure of fibrinogen receptors through an as-yet-uncharacterized second messenger system (Shattil *et al.*, 1989).

The present studies show that PCP and TCP can uniquely inhibit platelet activation induced by adrenaline, whereas no inhibitory effects were seen for platelet activation induced by

other common agonists such as thrombin, ADP, collagen or arachidonate; similar selective effects were seen with the covalently binding PCP analogue, Metaphit. The inhibition was also unusual in manifesting itself as a prolongation of the lag phase to the onset of aggregation, rather than, as is commonly seen, an inhibition in the rate and extent of aggregation. During this lag phase platelet ultrastructure was unchanged and platelets treated with adrenaline in the presence of PCP were indistinguishable from control platelets. The effects of PCP are not mediated by elevated cyclic AMP levels, and the involvement of cyclo-oxygenase products is unlikely since the inhibitory effects of PCP were observed using indomethacin-treated platelets.

The high-affinity PCP binding site of platelets appears to resemble most closely the true PCP binding site of the frontal cortex, rather than the sigma opioid sites of the hind brain and spinal cord. First, TCP, which is 500–1000 times more selective for true PCP receptors in the brain than for sigma opioid sites (Vignon *et al.*, 1983; Largent *et al.*, 1986), bound to the platelet receptor with similar high affinities. Secondly, the binding of PCP to the high-affinity platelet binding site was not inhibited by the neuroleptic drug haloperidol, which inhibits the binding of PCP to sigma opioid sites at low concentrations (Quirion *et al.*, 1987). Thirdly, under Na^+ -free conditions the binding affinity for PCP increased 3–4-fold from 134 nM to 38 nM , which is similar to the 5-fold increase from 125 nM to 25 nM seen in cortical synaptosomes (Vignon *et al.*, 1986; Haring *et al.*, 1987a,b). Fourthly, the acylating PCP analogue Metaphit selectively blocked the high-affinity binding of PCP to platelets, similar to its ability to block the high-affinity binding of PCP to rat synaptosomes (Rafferty *et al.*, 1985). The platelet receptor differed, however, from the PCP receptor of the frontal cortex insofar as it did not show the stereoselective inhibition by the two pairs of stereoselective inhibitors [levoxadrol/dexoxadrol and (+)/(–)MK801] that is seen in synaptosomes (Hampton *et al.*, 1982; Mendelsohn *et al.*, 1984). These results suggest that the high-affinity PCP binding site of platelets is a new type of binding site that is similar to, but not identical with, the PCP binding site of the frontal cortex.

The very low affinity at the second PCP binding site on platelets ($47 \mu\text{M}$), the large number of such sites (4.5×10^6) and the failure of Metaphit to block this low-affinity binding suggest that this binding does not represent a class of true binding sites, but may represent a compartment in the platelet membrane that can exchange rapidly with unlabelled PCP. Considering the relative hydrophobicity of PCP (Kamenka & Chicheportiche, 1988), these compartments may represent non-specific solubilization of PCP in platelet membrane lipids.

The mechanism of these selective inhibitory effects of PCP on adrenaline-induced platelet activation remains to be elucidated. PCP has been shown to inhibit 5-HT uptake into platelets (Arora & Meltzer, 1980) and synaptosomes (Smith *et al.*, 1977). In neuronal tissue, PCP has been shown to react with a variety of enzymes, receptors and ion channels (for review, see Domino & Kamenka, 1991). The inhibitory effect of PCP on platelet activation by adrenaline may, however, explain in part the protective effects of the drug in experimental stroke (Lawrence *et al.*, 1987). The accessibility of platelets may also facilitate the investigation of certain neuropathological conditions that affect PCP binding in brain, such as Alzheimer's disease and Huntington's disease (Greenamyre *et al.*, 1985; Maragos *et al.*, 1987; Young *et al.*, 1988). Finally, since changes in platelet function have been detected in various affective disorders including schizophrenia (Rehavi *et al.*, 1988), and since PCP exacerbates the symptoms of schizophrenia, abnormalities may be detectable in the interaction of PCP with platelets from schizophrenic patients.

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