Benzodiazepine analogues inhibit arachidonate-induced aggregation and thromboxane synthesis in human platelets

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- 1 Benzodiazepine analogues inhibit human platelet aggregation induced by arachidonate with an EC_{50} value of $0.68 \mu\text{M}$ for PK 11195, the most potent analogue used.
- 2 There was a highly significant correlation between the inhibition of arachidonate-induced aggregation and the affinity for the peripheral-type of benzodiazepine binding sites.
- 3 There was no significant correlation between the inhibition of the platelet activating factor (PAF)-induced aggregation and the binding to the peripheral-type of benzodiazepine binding sites.
- 4 The inhibition of platelet aggregation seems to result from the inhibition of arachidonic acid cyclo-oxygenation, since the synthesis of thromboxane and 12-hydroxy-heptadecatrienoic acid, both cyclo-oxygenase products, was reduced.
- 5 Our results suggest that peripheral-type of benzodiazepine binding sites on human platelets could be linked to cyclo-oxygenase.

Introduction

The central-type benzodiazepine receptor displays a high affinity binding for classical benzodiazepines. This receptor is linked to the γ -aminobutyric acid (GABA)-receptor-chloride-channel complex and it is responsible for many of the centrally-mediated effects of benzodiazepines. Besides this central-type receptor, peripheral-type binding sites for benzodiazepines have been identified both in the periphery and the central nervous system (Marangos *et al.*, 1982). Peripheral-type binding sites are distinct from the central-type receptors in their subcellular location and structural requirements (Anholt *et al.*, 1986). Porphyrins have been proposed as potential endogenous ligands of the peripheral-type sites located on mitochondria (Verma *et al.*, 1982).

The precise physiological function of the peripheral-type benzodiazepine binding sites is still not clear despite the fact that benzodiazepines exert many pharmacological activities at the periphery. Among these activities, some benzodiazepines inhibit platelet aggregation. Triazolam and alprazolam inhibit platelet activating factor (PAF)-induced aggregation (Kornecki *et al.*, 1984), and the inhibition of PAF binding to platelets has been proposed to explain the anti-aggregatory effect (Chesney *et al.*, 1987). This is however controversial for diazepam has been shown either to inhibit arachidonate-induced aggregation (Romstedt & Huzoor-Akbar, 1985) or to have no effect (Kornecki *et al.*, 1984).

Thus, despite the fact that benzodiazepines may alter platelet aggregation, several questions concerning their mechanism of action need to be answered (i) are the benzodiazepines acting in competition with PAF? (ii) are triazolobenzodiazepines the only active products? (iii) are the anti-aggregatory effects linked to the peripheral-type sites of benzodiazepines? In the present study, we compared the efficiency of a series of benzodiazepines for inhibiting PAF- or arachidonate-induced platelet aggregation and we attempted to correlate this inhibition with the affinity of those benzodiazepines for platelet peripheral binding sites.

Methods

Platelet preparation

Human platelets were obtained from healthy donors who had not taken any drug for at least ten days. Platelets were iso-

lated from plasma as previously described (Lagarde *et al.*, 1985) and resuspended in a Tyrode HEPES buffer (pH 7.4) without albumin.

Aggregation studies

Platelet aggregation was monitored by the turbidimetric method of Born (1962) with a Chrono-log dual aggregometer. The aggregation was induced by either arachidonic acid ($2.5 \mu\text{M}$) in ethanol (less than 0.5%), PAF ($0.4 \mu\text{M}$) or U 46619 ($0.1 \mu\text{M}$).

Binding studies

[³H]-Ro 4864, [³H]-PK 11195 and [³H]-diazepam have been used by different authors to measure the binding of these molecules. The use of [³H]-Ro 4864 or [³H]-PK 11195 provided grossly similar results (K_d in the range of 10 nM, non saturable binding at 10% of total binding) (Benavides *et al.*, 1984; Moingeon *et al.*, 1984; Weizman *et al.*, 1987), whereas the use of [³H]-diazepam led to contradictory results concerning the affinity (Wang *et al.*, 1980; Moingeon *et al.*, 1984; O'Beirne & Williams, 1984). This discrepancy probably comes from highly non saturable binding that we have found to occur with the latter ligand (results not shown). Therefore, we used [³H]-PK 11195 as the radioactive ligand in our study.

Platelet suspension ($200 \mu\text{l}$) was incubated in 1 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl and [³H]-PK 11195 ($0.01 \mu\text{M}$). After 30 min incubation at 4°C, the incubate was filtered on GF/C glass filters (Whatman), the filter was washed four times with 5 ml of cold buffer and the radioactivity on the filter was determined by liquid scintillation counting. Non saturable binding was measured in the presence of $100 \mu\text{M}$ cold PK 11195. We have checked that this value was the same as the value obtained in the absence of cold ligand with boiled platelet suspension.

Arachidonic acid metabolism

Platelets were incubated in the aggregometer for 3 min and [¹⁻¹⁴C]-arachidonic acid ($2.5 \mu\text{M}$) was added. The aggregation was monitored and the reaction was terminated after 4 min by addition of 1.2 ml ethanol. Lipids were extracted with 2.4 ml chloroform in the presence of 5×10^{-5} M butylated hydroxy-toluene as an anti-oxidant. The lipid extraction procedure was repeated once and the organic phase was concentrated by evaporation and spotted on silica gel plates (Merck 60G

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5721). The first elution with the mixture hexane/diethylether/acetic acid (60:40:1) allowed the separation of 12-hydroxy-heptadecatrienoic acid (HHT) (R_F 0.13), 12-hydroxy-eicosatetraenoic acid (12-HETE) (R_F 0.23) and arachidonic acid (R_F 0.43) from the origin. A second elution with diethylether/methanol/acetic acid (90:1:2) allowed the separation of thromboxane B_2 (R_F 0.27) from the origin (Lagarde *et al.*, 1985). The radioactivity of each spot was determined with a Berthold thin layer chromatography (t.l.c.) analyser.

Data analysis

IC_{50} was defined as the concentration of benzodiazepine analogue which induced a 50% inhibition of the [3H]-PK 11195 binding, with the concentration of radioactive ligand being $0.01 \mu M$. This value was determined by use of at least 4 concentrations of drug and the analysis was carried out with the Enzfitter programme from Elsevier Biosoft. The equation was taken as $\text{bound c.p.m.}_i = \text{c.p.m.}_0 \cdot IC_{50} / (IC_{50} + i)$ where c.p.m._0 is bound radioactivity without inhibitor, cpm_i is radioactivity in presence of inhibitor and i the inhibitor concentration. In an attempt to characterize further the binding, we substituted $(i)^h$ for i in the previous equation and determined this coefficient. The estimation of K_i from IC_{50} was determined by the method of Bennett (1978). EC_{50} was defined as the concentration of benzodiazepine analogue which induced 50% inhibition of the increased light transmission elicited by the aggregating agent (arachidonic acid or PAF). As the relationship relating the aggregation inhibition to the drug concentration is not known, the EC_{50} was obtained by successive assays of drug concentration until 50% inhibition was reached.

Drugs and materials

Ro 16-6028 (tert-butyl(S)-8-bromo-11,12,13,13a-tetrahydro-9-oxo-9H-imidazo[1,5-a]-pyrrolo[2,1-c][1,4]benzodiazepine-1-carboxylate), Ro 15-4513 (ethyl 8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate), Ro 15-1788, flumazenil (ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate) and midazolam (8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-a][1,4]benzodiazepine) were gifts from Hoffmann-la Roche, Basle, Switzerland.

CGS 8216 (2-phenylpyrazolo-[4,3-c]-quinolin-3 (5H)-one) was a gift from Ciba-Geigy, Basle, Switzerland.

RP 31264 and PK 11195 (1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline-carboxamide) were gifts from Rhone Poulenc, Vitry-s-Seine, France.

Alprazolam (8-chloro-1-methyl-6-phenyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine) and triazolam (8-chloro-6-(2-chlorophenyl)-1-methyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine) were gifts from Upjohn, Paris, France.

Ro 5-4864 (7-chloro-5-(4-chloro-phenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepine-2-one) and diazepam were from Fluka and Sigma laboratories respectively.

ACA 6 (3,3',4',5,7-penta-O-ethylquercetin) and MP 37 (3-carboxymethyl-3',4,5,7-tetra-O-ethylquercetin) were synthesized in our laboratory by Dr M. Picq.

[3H]-PK 11195 was from CEA, France (reference TMM-261, specific activity $2.44 \text{ MBq mmol}^{-1}$).

Results

The binding of [3H]-PK 11195 was studied in human platelets (Figure 1). Scatchard and Hill plots revealed straight lines indicating the occurrence of a single class of sites which do not interact. The values of K_d determined by the two different curves were 39 and 33 nm, respectively. The B_{max} value was 6.14 pmol/assay which corresponds to $41 \text{ fmol}/10^6$ platelets.

The 13 drugs that we tested exhibited various potencies in

Figure 1 [3H]-PK 11195 binding on human platelets. PK 11195 concentrations ranged from 2.4 to 200 nM. Each point was determined by use of 3 batches of blood platelets. (a) Scatchard analysis. The parameters were determined using the Enzfitter programme: $B_{\text{max}} = 6136 \pm 185 \text{ fmol/assay}$ ($41 \text{ fmol}/10^6$ platelets), $K_d = 39 \pm 3 \text{ nm}$. (b) Hill plot analysis. The parameters were determined using the Enzfitter programme: Hill coefficient = 0.93 ± 0.9 , $K_d = 33 \pm 7 \text{ nm}$.

inhibiting [3H]-PK 11195 binding (Table 1). These drugs were chosen to represent the different classes of benzodiazepine ligands, peripheral-type and central-type (including agonist, antagonist, partial inverse agonist). We also used two flavones (ACA6 and MP37) which exhibit inhibitory effects on platelet aggregation (Beretz & Cazenave, 1988). In addition, we have shown that these two flavones have some affinity for peripheral-type benzodiazepine sites (M. Picq and P. Fonlupt, unpublished results). The IC_{50} varied from $0.05 \mu M$ to values greater than $50 \mu M$. We confirmed that PK 11195, Ro 4864 and diazepam have the greatest affinities for the peripheral-type binding sites (IC_{50} : 0.05, 0.38, $0.70 \mu M$, respectively).

In an attempt to compare these values with the previously published ones (Wang *et al.*, 1980; Le Fur *et al.*, 1983; Benavides *et al.*, 1984) we calculated the corresponding K_i from IC_{50} values (Table 1). We found a slightly lower affinity for both PK 11195, Ro 4864 and diazepam (K_i : 39, 299, 551 nm, respectively) than those previously described (K_i : 4, 22, 194 nm, respectively). However, the relative K_i ratio (K_i of Ro 4864 or diazepam compared to K_i of PK 11195) was preserved. For the 13 products studied, the decreasing order of potency was: PK 11195 > Ro 4864 > diazepam > triazolam > alprazolam > midazolam > ACA 6 > Ro 16-6028 > Ro 19-4513 > MP 37 > suriclone > CGS 8216 > Ro 15-1788. When ligands of the peripheral-type benzodiazepine binding sites were added to platelet suspensions, a decrease in arachidonate-induced aggregation was observed (Figure 2). The potencies, in decreasing order, were PK 11195 > Ro 4864 > midazolam > diazepam (EC_{50} : 0.68, 2.4, 4.0, $8.5 \mu M$, respectively) as shown in Table 1.

Table 1 Inhibition of [^3H]-PK 11195 binding, and of arachidonic acid- and PAF-induced aggregation by various agents

Name	Type	n	Binding inhibition		Aggregation inhibition		PAF EC ₅₀ (μM)
			IC ₅₀ (μM)	h	Arachidonate K _i (nM)	EC ₅₀ (μM)	
PK 11195	peripheral	5	0.05 $\pm$ 0.02	(1.13)	39 $\pm$ 16	0.68 $\pm$ 0.19	0.14 $\pm$ 0.03
Ro 4864	peripheral	5	0.38 $\pm$ 0.09	(1.05)	299 $\pm$ 71	2.4 $\pm$ 0.6	3.3 $\pm$ 0.8
Diazepam	periph/central	5	0.70 $\pm$ 0.15	(0.97)	551 $\pm$ 118	8.5 $\pm$ 2.1	1.7 $\pm$ 0.4
Midazolam	agonist	3	2.28 $\pm$ 0.51	(1.08)	1795 $\pm$ 401	4.0 $\pm$ 1.2	nd
CGS 8216	antagonist	2	15.8 $\pm$ 3.43	(0.95)	12440 $\pm$ 2700	36 $\pm$ 15	nd
Ro 15-1788	antagonist	4			no inhibition at 38 μM		
Ro 19-4513	inverse agonist	3	5.3 $\pm$ 1.64	(1.01)	4173 $\pm$ 1291	36 $\pm$ 12	nd
Alprazolam	agonist	5	1.63 $\pm$ 0.35	(1.08)	1283 $\pm$ 273	14 $\pm$ 3	0.35 $\pm$ 0.07
Triazolam	agonist	3	1.14 $\pm$ 0.27	(0.93)	897 $\pm$ 212	15 $\pm$ 4	0.07 $\pm$ 0.02
Suriclone	agonist	2	11.0 $\pm$ 4.30	(0.95)	8661 $\pm$ 3385	21 $\pm$ 8	30 $\pm$ 15
Ro 16-6028	partial agonist	2	3.50 $\pm$ 0.68	(1.08)	2756 $\pm$ 535	35 $\pm$ 11	11 $\pm$ 4
MP 37	flavone	3	7.00 $\pm$ 1.86	(1.10)	5512 $\pm$ 1464	16 $\pm$ 5	nd
ACA 12	flavone	3	3.02 $\pm$ 0.60	(1.03)	2378 $\pm$ 472	12 $\pm$ 3	nd

The pharmacological profiles of these drugs have been reviewed by Gardner (1988).

Experiments and calculations were conducted as described in the Methods section where n is the number of different blood samples. Each was used for the binding, in triplicate, of [^3H]-PK 11195 in the presence of 4 different concentrations of each analogue. IC₅₀ is the concentration of drug which induced 50% inhibition of binding. The value reported in the table is the mean $\pm$ s.d. of values obtained in each individual experiment. The h value (determined as described in Methods) is given in parentheses. K_i values were calculated from IC₅₀ values according to Bennett (1978) ($K_i = \text{IC}_{50}/(1 + [\text{PK 11195}]/K_d)$). EC₅₀s were obtained by successive assays of drug concentration until 50% inhibition was reached. Values given in the table are the mean $\pm$ s.d. of values obtained in each individual experiment.

When log EC₅₀s were plotted versus log IC₅₀s a clear correlation appeared between these two groups of values ($\alpha < 0.001$) (Figure 3).

Benzodiazepines also inhibit PAF-induced aggregation, and we confirmed that triazolobenzodiazepines (triazolam and alprazolam) were potent inhibitors of PAF-induced aggregation (EC₅₀ 0.07, 0.35 μM , respectively) whereas the other analogues tested, except PK 11195 (EC₅₀ 0.14 μM), exhibited EC₅₀s greater than 1 μM (Table 1). However, when log EC₅₀s were plotted versus log IC₅₀s no significant correlation appeared (Figure 4).

Arachidonate is metabolized in platelets by lipoxygenase to 12-hydroxy-eicosatetraenoic acid (12-HETE) and by cyclooxygenase to mainly 12-hydroxy-heptadecatrienoic acid (HHT) and thromboxane (Tx) A₂, the latter interacting with platelet receptors to induce aggregation. As benzodiazepines counteracted the effect of arachidonate, a possible mechanism is the inhibition of thromboxane synthesis. We have measured the synthesis of TxB₂ (the stable metabolite of Tx A₂), HHT and 12-HETE, and the arachidonate incorporation into phospholipids, in the presence or absence of benzodiazepine analogues, at concentrations which inhibit aggregation. Concentrations were chosen for obtaining 5 to 100% inhibition. Figure 5 shows that the benzodiazepine analogues inhibited TxB₂ and HHT synthesis and that the inhibition of both products was correlated with the degree of inhibition. Moreover, we did not find any decrease of 12-HETE synthesis and

even a slight increase was observed in the presence of some benzodiazepine analogues. No relevant modification of arachidonate incorporation into phospholipids occurred (Table 2).

Another mechanism for explaining the inhibition of arachidonate-induced aggregation of platelets is the inhibition of prostaglandin H₂/thromboxane A₂ binding on their receptor sites. In the presence of PK 11195, the U-46619-induced aggregation (U-46619 is a stable TxA₂ analogue) was inhibited but the effect appeared for a concentration of PK 11195 more than 10 fold higher than that needed for inhibiting arachidonate-induced aggregation. Moreover, the inhibition of U-46619-induced aggregation was quite different from that induced by arachidonate. When induced by U-46619, the time course of aggregation was altered but not its extent (Figure 2). This result agrees with the fact that PK 11195, diazepam and Ro 4864 did not inhibit the binding of [^{125}I]-PTA-OH, a labelled analogue of thromboxane (unpublished results).

Discussion

The results of the present study show that when peripheral-type benzodiazepine sites were occupied by appropriate ligands, inhibition of arachidonate-induced aggregation occurred, which was presumably mediated by the inhibition of thromboxane synthesis.

Benzodiazepines bind to platelets of various species, rat,

Figure 2 Effect of PK 11195 on human platelet aggregation induced by arachidonic acid (a) or U-46619 (b). Concentrations used were 2.5 and 0.1 μM , respectively, of the aggregating agents.

Figure 3 Relationship between the inhibition of arachidonate-induced aggregation by benzodiazepine analogues and their affinity for the binding sites. The EC_{50} (μM) is the concentration of analogue which inhibited 50% of aggregation; IC_{50} (μM) is the concentration of analogue which inhibited 50% of $[^3H]$ -PK 11195 binding (0.01 μM). The line was determined by linear regression analysis; $n = 12$ (values corresponding to compound 2 are not available since it did not inhibit even at $10^{-4} M$), $r = 0.868$ (r limit for an $\alpha_{0.01}$ risk: 0.71), $\exp_{10}(\text{slope}) = 4.5$. The inset shows a linear regression analysis of drug ranks according to their potencies (Spearman's test). Abscissa scale: ranks for IC_{50} ; ordinates: ranks for EC_{50} . The higher the potency, the lower the rank. $n = 13$, $r = 0.89$, (r limit for an $\alpha_{0.01}$ risk: 0.68). 1 CGS 8216; 2 Ro 15-1788; 3 midazolam; 4 Ro 19-4513; 5 Ro 16-6028; 6 alprazolam; 7 triazolam; 8 diazepam; 9 Ro 4864; 10 suriclone; 11 PK 11195; 12 ACA 6; 13 MP 37.

mouse and man (Moingeon *et al.*, 1984; O'Beirne & Williams, 1984). Concerning the binding of benzodiazepine analogues to human platelets, our results are in accordance with previous studies reporting PK 11195, Ro 4864 and diazepam as being the most potent agents. This confirms that the affinity for the peripheral-type binding sites is totally different from the affinity for the central-type binding sites (Sieghart & Shuster, 1984; Le Fur *et al.*, 1983). As expected, because of the absence of GABA sites in platelets, we did not find any difference in the behaviour of agonists, antagonists or partial inverse agonists of central-type sites.

Some previous reports have shown that benzodiazepines are able to inhibit platelet aggregation. Kornecki *et al.* (1984)

Figure 4 Relationship between the inhibition of PAF-induced aggregation by benzodiazepine analogues and their affinity for the binding sites. EC_{50} (μM) is the concentration of analogue which inhibited 50% of aggregation; IC_{50} (μM) is the concentration of analogue which inhibited 50% of $[^3H]$ -PK 11195 binding (0.01 μM). The line was determined by linear regression analysis; $n = 7$, $r = 0.64$ (r limit for an $\alpha_{0.10}$ risk: 0.71). The inset shows the linear regression analysis of ranks as stated in Figure 3: $n = 7$, $r = 0.57$, (r limit for an $\alpha_{0.10}$ risk: 0.68). 5 Ro 16-6028; 6 alprazolam; 7 triazolam; 8 diazepam; 9 Ro 4864; 10 suriclone; 11 PK 11195.

Figure 5 Relationship between platelet aggregation and thromboxane (Tx) (a) or 12-hydroxyheptadecatrienoic acid (HHT) (b) synthesis in the presence of benzodiazepines. Aggregation was expressed as % of control; thromboxane and HHT as % of initial $[^{14}C]$ -arachidonate. 1 Control; 2 alprazolam 6.9 μM ; 3 diazepam 4 μM ; 4 alprazolam 13.9 μM ; Ro 4864 1.37 μM ; 6 diazepam 8 μM ; 7 PK 11195 0.6 μM ; 8 Ro 4864 3.4 μM ; 9 PK 11195 1.28 μM . Each point is the mean of 3 to 6 determinations; s.d. shown by bars. The line was determined by linear regression analysis; $n = 9$, $r = 0.90$ for (a), $r = 0.91$ for (b) (r limit for an $\alpha_{0.01}$ risk: 0.80).

showed that alprazolam and triazolam inhibited PAF-induced aggregation whereas diazepam did not. The latter result is questionable since a high arachidonate concentration (170 μM) was used (Kornecki *et al.*, 1984), which is far from the minimal concentration needed (2 μM). Other authors reported the inhibition of arachidonate-induced aggregation by diazepam or flurazepam (Romstedt & Huzoor-Akber, 1985). More recently, evidence was produced for a direct action of triazolobenzodiazepines on PAF binding sites whereas diazepam has no affinity for these sites (Kornecki *et al.*, 1984). In our study, we showed that the occupancy of peripheral-type benzodiazepine binding sites and the inhibition of arachidonate-induced aggregation are significantly correlated. Furthermore, there was no significant correlation between the binding on benzodiazepine sites and PAF-induced aggregation. We suggest that benzodiazepine sites are responsible for the inhibition of arachidonate-induced aggregation without any link to PAF binding sites. The inhibition of PAF-induced aggregation might then result from some structural feature, with the triazolo structure being the most efficient.

The mechanism by which peripheral-type benzodiazepine sites are coupled to platelet aggregation remains unclear. We suggest blockade of calcium influx may be involved because benzodiazepines have been described as inhibitors of calcium channels (Rampe & Triggle, 1986). However, Anwer *et al.*

Table 2 Metabolism of [^{14}C]-arachidonic acid in platelet suspensions: effect of ligands of peripheral-type benzodiazepine sites

	TxB_2	HHT	12-HETE	PL
Control	8.6 ± 1.7	10.9 ± 1.3	15.6 ± 0.8	13.9 ± 1.6
PK 11 195 $0.64 \mu\text{M}$	$2.9 \pm 0.2^*$	$3.9 \pm 2.0^*$	20.0 ± 10.9	13.8 ± 2.0
PK 11 195 $1.28 \mu\text{M}$	$2.1 \pm 0.2^*$	$1.7 \pm 0.1^*$	14.4 ± 1.6	11.0 ± 0.9
RO 4864 $1.7 \mu\text{M}$	$2.7 \pm 1.5^*$	$3.8 \pm 1.5^*$	20.6 ± 1.2	12.9 ± 0.7
RO 4864 $3.4 \mu\text{M}$	$3.0 \pm 1.1^*$	$2.2 \pm 1.3^*$	$22.9 \pm 0.2^*$	10.6 ± 0.2
Diazepam $4 \mu\text{M}$	7.7 ± 0.2	7.9 ± 1.2	22.5 ± 1.8	11.5 ± 0.7
Diazepam $8 \mu\text{M}$	$4.8 \pm 0.9^*$	$5.8 \pm 1.3^*$	$27.8 \pm 0.9^*$	13.5 ± 1.0
Alprazolam $6.9 \mu\text{M}$	8.2 ± 1.2	8.5 ± 0.8	19.1 ± 3.1	18.7 ± 0.9
Alprazolam $13.9 \mu\text{M}$	$5.5 \pm 1.4^*$	$6.7 \pm 0.8^*$	22.7 ± 2.2	14.5 ± 0.8

Radioactivity associated to thromboxane B_2 (TxB_2), 12-hydroxyheptadecatrienoic acid (HHT), 12-hydroxyeicosatetraenoic acid (12-HETE) and phospholipids (PL) was determined as described in the experimental section.

The results are expressed as % of initial radioactive [^{14}C]-arachidonic acid. Each value is the mean $\pm$ s.d. from 3 to 6 determinations.

As the distribution of the values was not known, statistical analysis of the results was carried out by using the Kruskal-Wallis test. When H value showed significant differences within the data, comparison of the mean values from control and each analogue concentration assays was performed by protected *t* test. * Statistically different from control with an α risk lower than 0.05.

(1988) reported the inhibition of ionomycin-induced aggregation by flurazepam in blocking the calcium entry suggesting that benzodiazepines act at a step proximal to the influx of calcium into platelets. In the present work, we studied the metabolism of arachidonate, in particular its oxygenation by platelet cyclo-oxygenase and lipoxygenase, and found that benzodiazepines inhibit the formation of cyclo-oxygenase products, TxB_2 and HHT. Indirect evidence has been previously reported with flurazepam and diazepam, which inhibited the formation of malondialdehyde (Romstedt & Huzoor-Akber, 1985). The link between benzodiazepine sites and cyclo-oxygenase remains unknown but a hypothesis is suggested by the work of Verma *et al.* (1987) who have shown that porphyrins are potent ligands for peripheral-type benzodiazepine sites. Since cyclo-oxygenase uses a porphyrin reversibly linked

to the enzyme as a cofactor (Kulmacz & Lands, 1984), we hypothesize that benzodiazepine might bind to the porphyrin site, thus inducing a loss of activity.

We conclude that our study indicates a possible link between peripheral-type benzodiazepine sites and cyclo-oxygenase in human platelets. This observation raises two questions: (i) are brain peripheral-type sites also linked to brain cyclo-oxygenase which is involved in cholinergic neurotransmission? and (ii) are benzodiazepines able to modify other porphyrin-associated enzymes? Answering these questions will probably be helpful in understanding the physiological role of peripheral-type benzodiazepine sites and in explaining the pharmacological effects of benzodiazepines at the periphery.

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(Received November 13, 1989)

Revised June 8, 1990

Accepted July 11, 1990)