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THE BENZODIAZEPINE RECEPTOR LIGANDS RO 5-4864 AND RO 15-1788 DO NOT BLOCK THE INHIBITION OF PAF-INDUCED PLATELET AGGREGATION SEEN WITH THE HETRAZEPINE WEB2086

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The hypothesis was tested that the hetrazepine WEB 2086 acts as an inhibitor of PAF-induced platelet aggregation via interaction with the platelet benzodiazepine receptor(BDZR). WEB 2086 is a potent inhibitor of rabbit platelet aggregation and ATP secretion induced by 370 nM PAF. The two BDZR ligands RO 5-4864 and RO 15-1788 (7-96 μM) are inactive as PAF antagonists. When platelets were pretreated with either BDZR ligand, and then exposed to various concentrations of WEB 2086, there was no alteration of the dose-response relationship of the hetrazepine on PAF-induced aggregation, as reflected by threshold concentration, ED50, or maximum inhibition seen with WEB 2086. Pretreatment of platelets with the BDZR ligands also failed to block the inhibitory action of WEB 2086 on PAF-induced ATP release. The data are consistent with the notion that WEB 2086 acts as a PAF antagonist through its action at a specific PAF receptor, and is dissociated from , and independent of , interaction with the benzodiazepine receptor. © 1989 Academic Press, Inc.

The hetrazepine WEB 2086 had been shown to be a potent competitive antagonist of Platelet-Activating Factor (PAF)-induced platelet aggregation and ATP secretion (1-4). There is evidence that certain other triazolobenzodiazepines may competitively inhibit binding of PAF to its platelet receptor (5). Since WEB 2086 is a derivative of the benzodiazepine class of compounds (2,3), and benzodiazepine receptors are present on platelet membranes (6,7),

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Abbreviations used are: ATP, adenosine triphosphate; BDZR, benzodiazepine receptor; DMSO, Dimethylsulfoxide; PAF, platelet activating factor (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphorylcholine); PRP, platelet rich plasma.

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it was speculated that the hetrazepine might manifest its anti-PAF activity by co-binding to the peripheral - type benzodiazepine receptor (BDZR) on the platelet membrane, thus hindering the affinity of PAF for its receptor.

If this were the case, one might view that if the platelet BDZR were occupied by a BDZR ligand, WEB 2086 might be unable to bind to this site, with the consequence the hetrazepine would not produce antagonism of PAF-induced platelet aggregation or ATP secretion. RO 5-4864 has been described as a specific peripheral-type BDZR ligand, while RO 15-1788 displays the properties of a specific CNS-type BDZR antagonist (8-11). We have utilized these two BDZR ligands to explore the possible interaction between the platelet PAF receptor and the BDZR. Specifically, experiments were performed to test the hypothesis that pretreatment of rabbit platelets with these two BDZR ligands would block the expected inhibitory effect of WEB 2086 on PAF-induced aggregation and ATP secretion.

Materials and Methods

Preparation of PRP

Blood was obtained from 2.5-3 kg white New Zealand rabbits of either sex anesthetized with sodium pentobarbital (20 mg/kg i.v.). Heparin (1000 I.U. i.v.) was administered 1 min. prior to exanguination; no additional anticoagulant was used. Blood was collected via puncture of the aortic arch using an 18 gauge needle and plastic syringe.

Blood was centrifuged in an IEC centrifuge at 200 x g for 10 min. at 20 C. Platelet rich plasma (PRP) was decanted and pooled. The PRP was placed in aggregometer tubes, capped, and equilibrated for 30 min before measurements were made.

Drugs and Chemicals

PAF acether was purchased from Sigma (St. Louis, MO) (2 μg/μl in chloroform). The solvent was evaporated and the PAF was diluted with deionized water to a concentration of 0.01 μg/μl. PAF solutions were kept frozen until use, at which time they were warmed to 4 C and vortexed. The solvent had no significant effect on platelet aggregation or ATP release.

Chronolume No. 395 luciferase/luciferin reagent was purchased from Chronolog Corp. (Havertown, PA). The reagent was dissolved in 1.25 ml of deionized water to yield a final concentration of 40 mg/ml. The reagent was kept frozen until use.

ATP was obtained from Calbiochem (La Jolla, CA). A 500 μM stock solution was prepared using deionized water. A ten-fold dilution yielded a working solution of 50 μM ATP. The solutions were kept frozen or at 4 C when used.

WEB 2086 (3-(4-(2-chlorophenyl)-9-methyl-6H-thieno (3,2-f) (1,2,4)-triazolo-(4,3-a) (1,4)-diazepine-2-yl)-1-(4-morpholinyl)-1-propanone) was obtained from Boehringer Ingelheim FRG. The substance was dissolved in 3 ml 0.1 N HCl, then neutralized with 2.7 ml 0.1 N NaOH. Deionized water was added to yield 25 ml of a 4 μg/μl stock solution. This was diluted further to make working solutions from 10^{-1} to 10^{-4} μg/μl as needed.

RO 5-4864 (7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,-4-benzo-diazepin-2-one) and RO 15-1788 (ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo [1,5-a] [1,4] benzodiazepine-3-carboxylate) were obtained from Hoffmann-LaRoche (Nutley, NJ). RO 15-1788 was dissolved in 20% (v/v) DMSO in water to a final concentration of 1μg/μl. RO 5-4864 was dissolved in propylene glycol.

Experimental Procedure

Platelet aggregation and ATP secretion were measured simultaneously in aliquots of PRP using a model 550 Lumi-Aggregation system (Chronolog Corp, Havertown, PA). Aggregation

was measured optically and ATP secretion was measured using luciferase reagent (4).

PRP was incubated with stirring at 37 C with the BDZ ligand for 2 min, and then incubated for one additional minute with different concentrations of WEB 2086. The PRP was then challenged with a 370 nM dose of PAF, which produced approximately 90 % of maximal aggregation in the absence of antagonists. Each determination was compared against an appropriate solvent-treated control to determine the percentage inhibition of maximum aggregation and ATP secretion caused by any dose of the antagonist. All studies were completed within 3 hours after preparation of PRP.

Dose-inhibition curves were plotted using Cricket Graph (Cricket Software, Philadelphia, PA) on an Apple Macintosh computer. ED_{50}'s (the dose producing 50% inhibition of control PAF response) were extrapolated graphically from the plotted curves.

Results and Discussion

When the PRP was pretreated (1 min.) with various concentrations of WEB 2086, in the absence of other agents, it produced a dose-dependent inhibition of PAF-induced platelet aggregation (Fig. 1). WEB 2086 also produced a dose-dependent inhibition of ATP release induced by 370 nM PAF (ED50 = 0.2 μM). This effect of WEB 2086 on aggregation and ATP secretion is similar to that previously reported in porcine platelets (4).

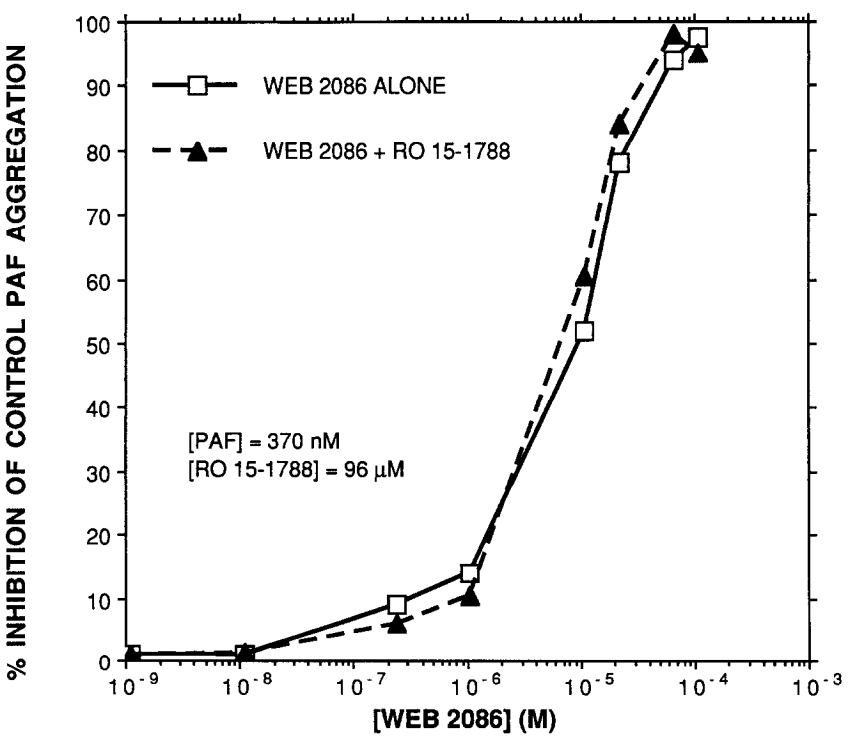

Fig. 1. Inhibition of PAF-induced platelet aggregation in rabbit PRP by the hetrazepine WEB 2086, alone (open squares), and following pre-treatment with the benzodiazepine ligand RO15-1788 (triangles). PRP was incubated with RO15-1788 for 2 min., followed by 1 min. treatment with various concentrations of WEB 2086 before challenge with 370 nM PAF. Each point is the mean of 2-5 determinations derived from 4 different samples of PRP.

When the PRP samples were pretreated with RO 15-1788 (7-96 μM) for two minutes, no attenuation of the PAF-induced responses was noted (data not shown). Pretreatment of platelets with RO 15-1788 (96 μM) for two minutes, followed by addition of various concentrations of WEB 2086, failed to alter the expected inhibitory effect of the hetrazepine on PAF-induced aggregation (Fig. 1). Similar results were obtained with RO 5-4864 (data not shown). Neither the slope of the WEB 2086 dose-response curve, the threshold concentration, ED50, nor maximum effect of the WEB 2086 were altered by pre-treatment with the BDZR ligands. Thus, under the conditions of our experiments, the findings indicate that there is no blockade or attenuation of the expected PAF inhibitory effect of WEB 2086 on PAF-induced aggregation or ATP secretion when rabbit platelets are concurrently exposed to the two BDZR ligands, RO 5-4864 and RO 15-1788.

These results are consistent with the report (12) that these two BDZR ligands also failed to block the inhibitory effect of the triazolobenzodiazepines, brotizolam and triazolam, on PAF-induced aggregation of human platelets. In IC_{50} concentrations exceeding 200 - 500 μM, the two BDZR ligands inhibited PAF-induced aggregation, although arachadonic acid-induced aggregation also was inhibited by RO 5-4864 (IC_{50} = 25μM), indicating a non-selective action (12). We have demonstrated (unpublished data) that diazepam (193-780 μM) also may produce a non-selective and non-competitive inhibiton of PAF and other agonist-induced aggregation of both rabbit and human PRP.

In summary, our results, coupled with reports relating to the structure-activity relations and receptor binding features of WEB 2086 and other triazolobenzodiazepines, indicate that there is a distinct PAF receptor that binds WEB 2086. The property of WEB 2086 as a competitive antagonist of PAF-induced platelet activation is mediated by specific affinity of this agent for this PAF receptor. This receptor affinity and inhibition of PAF effects appear to be independent of interaction with the platelet BDZR. Ongoing research is being directed to the question of which molecular features of the hetrazepine are essential for manifestation of its competitive PAF receptor antagonism .

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