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Rubimetide, humanin, and MMK1 exert anxiolytic-like activities via the formyl peptide receptor 2 in mice followed by the successive activation of DP_1, A_{2A}, and $GABA_A$ receptors

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ABSTRACT

Rubimetide (Met-Arg-Trp), which had been isolated as an antihypertensive peptide from an enzymatic digest of spinach ribulose-bisphosphate carboxylase/oxygenase (Rubisco), showed anxiolytic-like activity prostaglandin (PG) D_2-dependent manner in the elevated plus-maze test after administration at a dose of 0.1 mg/kg (ip.) or 1 mg/kg (p.o.) in male mice of *ddY* strain. In this study, we found that rubimetide has weak affinities for the FPR1 and FPR2, subtypes of formyl peptide receptor (FPR). The anxiolytic-like activity of rubimetide (0.1 mg/kg, ip.) was blocked by WRW4, an antagonist of FPR2, but not by Boc-FLFLF, an antagonist of FPR1, suggesting that the anxiolytic-like activity was mediated by the FPR2. Humanin, an endogenous agonist peptide of the FPR2, exerted an anxiolytic-like activity after intracerebroventricular (icv) administration, which was also blocked by WRW4. MMK1, a synthetic agonist peptide of the FPR2, also exerted anxiolytic-like activity. Thus, FPR2 proved to mediate anxiolytic-like effect as the first example of central effect exerted by FPR agonists. As well as the anxiolytic-like activity of rubimetide, that of MMK1 was blocked by BW A868C, an antagonist of the DP_1-receptor. Furthermore, anxiolytic-like activity of rubimetide was blocked by SCH58251 and bicuculline, antagonists for adenosine A_{2A} and $GABA_A$ receptors, respectively. From these results, it is concluded that the anxiolytic-like activities of rubimetide and typical agonist peptides of the FPR2 were mediated successively by the PGD_2-DP_1 receptor, adenosine-A_{2A} receptor, and GABA-$GABA_A$ receptor systems downstream of the FPR2.

1. Introduction

Rubimetide (Met-Arg-Trp) has been isolated from an enzymatic digest of spinich ribulose-bisphosphate carboxylase/oxygenase (Rubisco) as an inhibitory peptide for angiotensin I-converting enzyme (ACE) [21]. However, its antihypertensive effect was mainly mediated by the prostaglandin (PG) D_2-DP_1 receptor-dependent vasorelaxation rather than ACE inhibition [25]. We found previously that rubimetide exhibited anxiolytic-like activity in the elevated plus-maze test as evaluated by% of time spent in open arms or by% of entries into open arms at a dose of 0.1 mg/kg (ip.) or 1 mg/kg (p.o.) in mice [26]. Rubimetide had no effect on locomotive activity since it did not modify total entry number into open and closed arms. The anxiolytic-like activity of rubimetide was mediated by PGD_2 and the DP_1 receptor downstream of an unidentified receptor, since it was blocked by BW A868C, a DP_1 antagonist [26]. Based on these results we have demonstrated for the first time that PGD_2 itself has anxiolytic-like activity via the DP_1 receptor [27]. Furthermore, the anxiolytic-like activity of PGD_2 was blocked by SCH58261 and bicuculline, antagonists for the adenosine A_{2A} and $GABA_A$ receptors, respectively, but not by flumazenil, an antagonist for the benzodiazepine binding site [27]. From these results, it was concluded that anxiolytic-like activity of PGD_2 was mediated by successive activation of adenosine-A_{2A} receptor, and GABA-$GABA_A$ receptor systems downstream of the DP_1 receptor, which is essentially the same as its sleep induction mechanism [7,10,13,14,20]. In this study, we aimed to identify the receptor to

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which rubimetide binds upstream of the PGD_2-DP_1 receptor system.

Formyl peptides are a group of peptides carrying formyl-Met at the amino terminus, of which a typical example is formyl-Met-Leu-Phe (fMLF). Formyl peptides induce various biological effects such as chemotaxis, phagocytosis, and inflammation via formyl peptide receptors (FPRs) [22]. Some peptides having Met residues at their aminino terminus such as Met-Leu-Phe show weak affinity for FPR even if it is not formylated [16]. We previously isolated soymetide-13 (MITLAIPVNKPGR) from a trypsin digest of soy protein based on a stimulatory effect for phagocytosis by human polymorphonuclear leukocyte (PMNL) [19]. It was derived from soy β-conglycinin α' subunit, and proved to be the first FPR1 agonist peptide derived from exogenous protein. Then, we chose FPRs as a candidate of the receptor of rubimetide since it also has a Met residue at the amino terminus.

There are at least 3 subtypes, FPR1, FPR2 (or FPRL1) and FPR3 (or FPRL2) or their homologues in the human and mouse FPRs [22]. As for the distribution of FPR subtypes in the central nerve system, functional FPR1 and FPR2 have been detected in human astrocytoma cell lines [9], and mRNA encoding FPR2 has been detected in mouse brain [18].

2. Materials and methods

2.1. Animals

Four-week-old male ddY mice were obtained from SLC (Shizuoka, Japan). All animals were housed in a temperature-controlled room (23 °C) on a 12 h light–dark cycle with lights on at 07:00. All animals had free access to food pellets and water. All experiments were approved by the Kyoto University Ethics Committee for Animal Research Use. All animals were euthanized by an overdose of anesthesia drugs after the experiment.

2.2. Reagents

Rubimetide was synthesized by an Fmoc strategy. Humanin was obtained from Peptide Research Institute Inc. (Osaka, Japan). Boc-FLFLF was obtained from Bachem AG (Bubendorf, Switzerland). WRW4 (Trp-Arg-Trp-Trp-Trp-Trp), MMK1, BW A868C, bicuculline, and SCH58261, were obtained from Tocris Bioscience (Bristol, UK).

2.3. Elevated plus-maze test

The elevated plus-maze test was performed as described previously [26]. Four arms (24 cm long × 5 cm wide) were placed 50 cm above the ground. Two opposite arms were delimited by acrylic vertical walls (13 cm high, closed arms), whereas the other two, opposite arms had unprotected edges (open arms). A mouse was placed in the center of the maze facing an open arm and observed for 5 min to measure the cumulative time and frequency of entries into the open and closed arms. Arm entry was defined as the entry of four paws into an arm. Open-arm entry time (time spent in open arms) was expressed as a percentage of the total entry time (% of time), and the number of open-arm entries was expressed as a percentage of the number of total entries (% of visit). In this system, diazepam (1 mg/kg, i.p.) exhibited significant anxiolytic-like activity. Rubimetide dissolved in saline was administered ip. 30 min before the test. For the test after icv. administration, samples dissolved in artificial cerebrospinal fluid (ACSF) were injected to the third cerebroventricule 20 min before the test in a volume of 4 μ liters. Antagonists were co-administered with samples. Suitable dosage of WRW4 (10 mg/kg ip, and 0.5 nmol/mice icv) to block the anxiolytic-like activities of FPR2 agonists without affecting basal anxiety level was determined by preliminary experiments. Suitable doses of BW A868C, SCH58261, and bicuculline to block the anxiolytic-like activities of PG-D2, adenosine, and GABA, respectively, have been determined in our previous studies using *ddY* mice [26,27]. The total number of visits to the closed and open arms, and the cumulative time spent in the open and closed arms were measured on a monitor through a video camera system. Data were checked by observers who were unaware of the experimental groups.

Table 1
Affinities of rubimetide and typical peptides for FPRS.

Peptides	Ki (μM)	
	FPR1	FPR2
fMLF	0.00045	0.47
WKYMVm	0.00034	0.00001
MRW (rubimetide)	106	266

2.4. Receptor binding assay

Receptor binding assay for FPR1 was performed as follows [4]. Membrane preparation of CHO cells stably expressing human FPR1 were incubated with 0.3 nM [^{3}H] fMLF in 25 mM HEPES buffer, pH 7.4, containing 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 0.2% BSA for 60 min at 25C. 1 μM unlabelled fMLF was used as a non-specific ligand. After incubation, unbound tracer was removed by filtration on Whatman GF/C discs, followed by extensive washing with ice-cold buffer, and bound label was counted.

Receptor binding assay for FPR2 was performed as follows [8]. Membrane preparation of CHO cells stably expressing human FPR2 were incubated with 0.025 nM [^{125}I]WKYMVm in 50 mM HEPES buffer, pH 7.4, containing 100 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 2 mM $CaCl_2$, and 0.5% BSA for 90 min at 25C. 1 μM unlabelled WKYMVm was used as a non-specific ligand. After incubation, unbound tracer was removed as described above, and bound label was counted.

2.5. Statistical analysis

All values are expressed as the means ± S.E.M. Analysis of variance (ANOVA) followed by Fisher's test was used to assess differences among groups. *P*-values less than 0.05 were considered significant.

3. Results

3.1. Identification of the receptor mediating anxiolytic-like activity of rubimetide

Affinities of rubimetide for human FPR1 and FPR2 were measured by radioligand binding assay (Table 1). It showed weak affinities for both FPR1 (Ki = 106 μM) and FPR2 (Ki = 266 μM). Ki value of fMLF for the FPR1 was 0.45 nM while that of WKYMVm for the FPR2 was 0.01 nM under these conditions.

At a dose of 0.1 mg/kg ip., rubimetide exhibited an anxiolytic-like activity in the elevated plus-maze test [26]. Then, we investigated the effect of selective antagonists of individual receptors on the anxiolytic-like activity of rubimetide. WRW4 (10 mg/kg, ip.), an FPR2 antagonist [15], blocked the anxiolytic-like activity of rubimetide (Fig. 1-A). However, Boc-FLFLF (2.0 mg/kg, ip.), an FPR1 antagonist [17], did not block the anxiolytic-like effect as shown in Fig. 1-B. Recently, we confirmed that Boc-FLFLF given at the same dosage could block an anorexigenic effect of fMLP given ip. (unpublished results). These suggest that the anxiolytic-like activity of rubimetide is mediated by FPR2.