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Further studies on the pharmacological profile of the neuropeptide S receptor antagonist SHA 68

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ABSTRACT

Neuropeptide S (NPS) regulates various biological functions by selectively activating the NPS receptor (NPSR). Previous studies demonstrated that the non-peptide molecule SHA 68 acts as a selective NPSR antagonist. In the present study the pharmacological profile of SHA 68 has been further investigated in vitro and in vivo. In cells expressing the mouse NPSR SHA 68 was inactive per se up to 10 μM while it antagonized NPS-stimulated calcium mobilization in a competitive manner showing a pA_2 value of 8.06. In the 10–50 mg/kg range of doses, SHA 68 counteracted the stimulant effects elicited by NPS, but not those of caffeine, in mouse locomotor activity experiments. In the mouse righting reflex assay SHA 68 fully prevented the arousal-promoting action of the peptide. The anxiolytic-like effects of NPS were slightly reduced by SHA 68 in the mouse open field, fully prevented in the rat elevated plus maze and partially antagonized in the rat defensive burying paradigm. Finally, SHA 68 was found poorly active in antagonizing the NPS inhibitory effect on palatable food intake in rats. In all assays SHA 68 did not produce any effect per se. In conclusion, the present study demonstrated that SHA 68 behaves as a selective NPSR antagonist that can be used to characterize the in vivo actions of NPS. However the usefulness of this research tool is limited by its poor pharmacokinetic properties.

1. Introduction

Neuropeptide S (NPS, human sequence SFRNGVGTGMKKTSFQRAK) is the endogenous ligand of the 7TM receptor NPSR [39,45]. In cells expressing the recombinant NPSR, NPS displayed high affinity and stimulated calcium mobilization and cAMP accumulation suggesting Gq and Gs coupling for the NPSR [35,45]. In vitro, in brain tissue preparations, NPS has been reported to control neuronal electrophysiological properties [19,25] and neurotransmitter release [34]. In vivo, in rodents, NPS has been shown to control several biological functions including wakefulness [6,37,45], locomotor activity [8,15,30,37,41,45], stress and anxiety [22,30,37,44,45], food intake and gastrointestinal functions [3,9,11,17,41], memory processes [18,19,25,27], drug abuse [2,7,24,30], and nociception [23]. To deeply investigate these NPS-sensitive biological functions and to identify the therapeutic potential of drugs interacting with NPSR, potent and selective ligands are required (for a recent review in this field see [16]).

SHA 68, i.e. the racemic mixture (9R/S)-3-oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide has been identified by Takeda researchers [12] and characterized pharmacologically in vitro and in vivo by Okamura et al. [28]. In radioligand ([^{125}I][Tyr^{10}]NPS) binding experiments performed in HEK293$_{hNPSR}$ cells SHA 68 displayed high affinity (pKi 7.3). In calcium mobilization experiments SHA 68 was inactive per se while antagonizing NPS stimulatory effects in a concentration-dependent and competitive manner. Similar high pA_2 values were obtained with SHA 68 at Ile107 (7.6) and Asn107 (7.8) hNPSR isoforms expressed in HEK293 cells. SHA 68 appeared to be selective for NPSR since it did not affect signaling at 14 unrelated G-protein-coupled receptors [28]. In vivo in mice SHA 68 reached pharmacologically relevant levels in plasma and brain after i.p. administration. Despite this, SHA 68 (50 mg/kg i.p.) was only able to partially counteract NPS-induced stimulation

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of locomotor activity [28]. In a separate study [19], bilateral intra-amygdala administrations of SHA 68 exerted functionally opposing responses compared to NPS: in fact the peptide induced anxiolytic-like effects in the open field test while anxiogenic-like effects were measured in response to SHA 68. Finally, NPS treatment attenuated MK-801-induced vacuolization in the rat retrosplenial cortex and this protective effect of NPS could be blocked by systemic administration of SHA 68 [29].

In the present study the pharmacological profile of SHA 68 was further investigated in vitro in calcium mobilization experiments performed on HEK293 cells expressing the mouse NPSR and in vivo in a rather large panel of assays sensitive to NPS including locomotor activity (LA), righting reflex (RR) and open field behavior (OF) in mice, and elevated plus maze (EPM), defensive burying (DB) and palatable food intake (PFI) paradigms in rats.

2. Materials and methods

2.1. Cell culture and calcium mobilization experiments

$HEK293_{mNPSR}$ were generated as previously described [35] and maintained in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, hygromycin B (100 mg/l), and cultured at 37 °C in 5% CO_2 humidified air. $HEK293_{mNPSR}$ cells were seeded at a density of 50,000 cells/well into poly-D-lysine coated 96-well black, clear-bottom plates.

The following day, the cells were incubated with medium supplemented with 2.5 mM probenecid, 3 μM of the calcium sensitive fluorescent dye Fluo-4 AM and 0.01% pluronic acid, for 30 min at 37 °C. After that time the loading solution was aspirated and 100 μl/well of assay buffer (Hank's Balanced Salt Solution; HBSS) supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2.5 mM probenecid and 500 μM Brilliant Black (Aldrich) was added. Concentrated solutions (1 mM) of NPS were made in bidistilled water and kept at −20 °C. SHA 68 was dissolved in 10% Tween 80 solution in saline. Serial dilutions were carried out in HBSS/HEPES (20 mM) buffer (containing 0.02% bovine serum albumin fraction V). After placing both plates (cell culture and master plate) into the fluorometric imaging plate reader FlexStation II (Molecular Devices, Sunnyvale, CA), fluorescence changes were measured. On-line additions were carried out in a volume of 50 μl/well. To facilitate drug diffusion into the wells in antagonist type experiments, the present studies were performed at 37 °C and three cycles of mixing (25 μl from each well moved up and down 3 times) were performed immediately after antagonist injection to the wells. Inhibition response curves were determined against the stimulatory effect of 30 nM NPS. SHA 68 was injected into the wells 24 min before adding NPS.

2.2. Animals

All experimental procedures adopted for in vivo studies complied with the standards of the European Communities Council directives (86/609/EEC) and national regulations (D.L. 116/92). Male Swiss mice (30–38 g, Morini RE, Italy) and male Wistar rats (200 g for DB and EPM studies and 300 g for PFI studies, Charles River MI, Italy) were used. They were housed in Plexiglas® cages (Tecniplast, MN, Italy), under standard conditions (22 °C, 55% humidity, 12 h light–dark cycle, lights on 7.00 am) with food (mice: MIL, standard diet Morini RE, Italy, rats: 4RF, Mucedola, Settimo Milanese, MI, Italy) and water *ad libitum* for at least 5 days before experiments began. Each animal was used only once, with the exception of PFI studies. NPS was given intracerebroventricularly (i.c.v.), while SHA 68 was injected intraperitoneally (i.p.) 10 min before NPS. In mice, i.c.v. injections (2 μl per mouse) were given under light (just sufficient to produce a loss of the righting reflex) isoflurane anesthesia, into the left ventricle according to the procedure described by [21] and routinely adopted in our laboratory [36]. For i.c.v. injections in rats, stainless-steel guide cannula (23 ga) (Plastic One, Roanoke, VA, USA) were stereotaxically implanted in the right lateral ventricle, to a depth of 0.5 mm above the ventricle (coordinates were taken from the rat brain atlas [32] and adjusted for the animal body weight), under ketamine plus xylazine anesthesia (115 + 2 mg/kg i.p.; Farmaceutici Gellini, Aprilia, Italy and Bayer, Milan, Italy, respectively) and fixed in place with acrylic dental cement and one skull screw. A removable plug was kept in place except during the drug injections. In rats i.c.v. injections were in a volume of 5 μl. After the end of the experiment, rats were i.c.v. injected with 5 μl of Evans Blue and sacrificed under anesthesia. The correct placement of the cannula was ascertained by inspection of dye diffusion in the right lateral ventricle. All procedures were randomized across test groups.

2.3. Mouse locomotor activity

Experiments were performed during the light cycle (between 09.00 and 13.00) according to [15]. Naïve mice were treated i.c.v. 5 min before the beginning of the test and LA was recorded for 60 min. For these experiments the ANY-maze video tracking system was used (Ugo Basile, application version 4.52c Beta). Mice were positioned in a square plastic cage (40 cm × 40 cm), one mouse per cage. Four mice were monitored in parallel. Mouse horizontal activity was monitored by a camera while vertical activity was measured by an infrared beam array. The parameters measured were cumulative distance traveled (total distance in m that the animal traveled during the test), immobility time (the animal is considered immobile when 90% of it remains in the same place for a minimum of 2.5 s), and the number of rearings (the number of beam breaks due to vertical movements). Previous studies performed under the present experimental conditions demonstrated that NPS-stimulated LA in a dose-dependent manner [15]; from these studies the dose of 0.1 nmol was selected as the lower dose inducing statistically significant effects. SHA 68 (10 and 50 mg/kg, i.p.) was administered 10 min before NPS (0.1 nmol, i.c.v.). In a separate series of experiments, to investigate the selectivity of action of SHA 68, the NPSR antagonist (at 50 mg/kg, 10 min pretreatment) was tested against the stimulatory effect of caffeine (20 mg/kg, i.p.). Caffeine was administered 5 min before recording LA.

2.4. Mouse righting reflex recovery

This assay was performed according to the procedures previously described in detail [37]. Briefly, mice were given an i.p. injection of diazepam (15 mg/kg). When the animals lost the RR, they were placed in a plastic cage and the time was recorded by an expert observer blind to drug treatments. Animals were judged to have regained the RR response when they could right themselves three times within 30 s. Sleep time is defined as the amount of time between the loss and regaining of the RR and was rounded to the nearest minute. Previous studies performed under the present experimental conditions demonstrated that NPS facilitated RR recovery in a dose-dependent manner [37]; from these studies the dose 0.1 nmol was selected as the lower dose inducing statistically significant effects. SHA 68 (10 and 50 mg/kg, i.p.) was administered 10 min before NPS, and the peptide was given i.c.v. (0.1 nmol) 5 min before the injection of diazepam. In a separate series of experiments in order to investigate in detail the possible effects of the NPSR antagonist on benzodiazepine-induced sleep, SHA 68 (50 mg/kg, i.p.) was injected 10 min before the administration of different doses (5, 10, and 15 mg/kg) of diazepam.