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ScienceDirect
Regulatory Peptides 144 (2007) 10–16
REGULATORY PEPTIDES
www.elsevier.com/locate/regpep

A novel, simple bioactivity assay for relaxin based on inhibition of platelet aggregation

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Received 15 February 2007; received in revised form 12 April 2007; accepted 8 May 2007
Available online 21 May 2007

Abstract

In humans, the relaxin hormone family includes H1, H2 and H3 isoforms and insulin-like peptides 3 to 6. The ever-increasing interest in relaxin as potential new drug requires reliable methods to compare bioactivity of different relaxins. The existing bioassays include *in vivo* or *ex vivo* methods evaluating the organ-specific responses to relaxin and *in vitro* methods based on measurement of cAMP increase in relaxin receptor-bearing cells. We previously demonstrated that relaxin dose-dependently inhibits platelet aggregation. On this basis, we have developed a simple, reliable bioassay for relaxin used to compare purified porcine relaxin, assumed as reference standard, with two recombinant human H2 relaxins, H3 relaxin, insulin-like peptides 3 and 5.

Pre-incubation of platelets with relaxins (3, 10, 30,100, 300 ng/ml; 10 min.) caused the inhibition of ADP-induced platelet aggregation. Within the 10–100 ng/ml range, porcine relaxin showed the highest effects and a nearly linear dose–response correlation. Lower peptide concentrations were ineffective, as were insulin-like peptides 3 and 5 at any concentration assayed. Platelet inhibition was mediated by specific RXFP1 relaxin receptor and cGMP, whose intracellular levels dose-dependently increased upon relaxin. For comparison, we stimulated THP-1 cells, a relaxin receptor-bearing cell line, with porcine relaxin, human H2 and H3 relaxins at the above concentrations (15 min.). We observed a dose-related increase of intracellular cAMP similar to the trend of platelet inhibition. Insulin like peptide 5 was ineffective. In conclusion, this study shows that inhibition of platelet aggregation may be used to assess bioactivity of relaxin preparations for experimental and clinical purposes.

Keywords: Relaxin; Platelets; THP-1 cells; Cyclic nucleotides; Bioassay

1. Introduction

Relaxin (RLX) belongs to a newly defined hormone family, termed the relaxin peptide family, that includes three different RLXs, H1, H2 and H3, as well as insulin-like peptide (INSL)3, INSL4, INSL5 and INSL6 [1,2]. In humans, 3 separate genes have been found and designated *RLN1*, *RLN2* and *RLN3* [1,2]. The peptide encoded by the *RLN2* gene, H2 RLX, is the major circulating form and is produced mainly in the *corpus luteum* [3]. Circulating RLX accounts for most of the known biological effects of the hormone in humans and experimental animals. They act on the female reproductive system as well as on non-reproductive targets, including the cardiovascular system and the connective tissues [2,4–7].

RLX is the ligand for two leucine-rich repeat-containing G-protein coupled receptors (LGRs), LGR7 and LGR8 [8], now classified as relaxin family peptide receptors 1 and 2 (RXFP1 and RXFP2), respectively [9]. RXFP1 is the main and most specific H2 RLX receptor, but it is also able to bind to H1 and H3 RLX, although with less affinity. On the other hand, RXFP2 chiefly binds INSL3 and also H1 and H2 RLX, but with less affinity [8,10]. These receptors have been found on most if not all RLX target tissues and cells and are abundantly expressed in the reproductive, nervous, renal and cardiovascular systems [8]. More recently other G-protein coupled receptors for peptides of

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0167-0115/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.regpep.2007.05.004

the relaxin family have been discovered and termed RXFP3 (formerly GPCR135) and RXFP4 (formerly GPCR142) [9]. RXFP3 binds with high affinity H3 RLX [11] while RXFP4 binds H3 RLX [12] and also INSL5 [13].

Many of the milestone studies on the biological effects of RLX had been carried out using highly purified, natural RLX, extracted mainly from sow ovaries [14]. Characterisation of the *RLN2* gene sequence has also allowed the production of synthetic human RLX by recombinant DNA technology [15]. Availability of recombinant H2 RLX, besides giving a further momentum to RLX research, has made clinical trials possible of RLX in promoting cervical ripening in women with delayed delivery [16] and in the treatment of scleroderma [17].

In view of the ever-increasing interest in RLX as a potential new drug, for instance in fibrosis, reproductive dysfunctions and cardiovascular diseases [2,6,7,18–20], it is extremely important to develop reliable methods to compare the biological activity of different RLXs. In 1995 we described a specific effect of RLX on the inhibition of human and rabbit platelet aggregation [21]. The current study shows that inhibition of platelet aggregation may be the basis for a simple, rapid and cheap bioassay for RLX with several advantages over the currently used bioassays.

2. Materials and methods

2.1. Relaxins and related peptides

The following peptides of the RLX family were used for bioactivity comparison: i) highly purified luteal porcine RLX (pRLX, 2500–3000 U/mg) used as reference standard, prepared according to Sherwood and O'Byrne [14] and kindly donated by Dr O.D. Sherwood, University of Illinois at Urbana-Champaign (Urbana, IL, USA); ii) recombinant human H2 RLX from Phoenix (cat. #035-62, Phoenix Europe GmbH, Karlsruhe, Germany); iii) recombinant human H2 RLX (batch B917056/1/1), formerly used for clinical trials in scleroderma patients, prepared by Boehringer-Ingelheim for Connetics Co. (Palo Alto, CA); iv) recombinant human H3 RLX (Phoenix, cat. #035-36); v) recombinant human INSL3 (Phoenix, cat. #035-27); vi) recombinant human INSL5 (Phoenix, cat. #035-61). Stock solutions of the different RLX preparations (500 μg/ml) were used. For H2 RLX (Phoenix), H3 RLX, INSL3 and INSL5 the stock solution was 20 μg in 500 μl ethanol 95%, according to the manufacturer's instruction. They were thawed immediately prior the experiment. Before doing the working dilutions, protein content was measured by a Micro BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as standard. RLX solutions were kept in silicon-coated test tubes to prevent RLX adhering to the walls.

2.2. Isolation of human platelets

Freshly drawn blood samples were collected from healthy volunteers who gave explicit, informed consent to their enrolment in this study. None of them consumed any cyclooxygenase inhibitor within 20 days prior to entering the study. About 20 ml of blood were taken from each subject, using sodium citrate (3.8%, 1:10 v/v) as anticoagulant. Platelet-rich plasma was obtained by centrifugation of the whole blood at 250 *g* for 15 min at 20 °C. Samples of platelet-rich plasma were directly used for the aggregation studies after adjustment of platelet count to 3×10^8 per ml with platelet-poor plasma.

2.3. In vitro culture of THP-1 cells

Human monocytic THP-1 cells were purchased at the European Collection of Cell Cultures (ECACC, Salisbury, UK) and cultured in suspension in RPMI medium containing 10% foetal calf serum (Sigma, Milan, Italy), 250 U/ml penicillin G and 250 μg/ml streptomycin, in a 5% CO_2 atmosphere at 37 °C.

2.4. Platelet aggregation assay

Samples of platelet-rich plasma were incubated for 10 min at 37 °C in the absence or presence of 3, 10, 30, 100 and 300 ng/ml of each RLX and INSL preparation. After incubation, aliquots of the samples were placed in a four-channel aggregometer (Aggrecoder PA 3210, Daiichi, Kagaku Co. Ltd, Kyoto, Japan) at 37 °C with continuous stirring and, finally, stimulated with ADP (3 μM). These concentrations and incubation times of RLX and ADP were chosen basing on the previously reported studies on the effects of pRLX on platelets [21]. Before performing the experiments, the aggregometer was calibrated by assuming the OD of a suspension of platelet-rich plasma as 100 and the OD of a suspension of platelet-poor plasma as 0. For each platelet preparation, the dose of ADP was adjusted to obtain submaximal aggregation (mean percent values in the different preparations: 87 ± 6 SEM). OD was monitored continuously for 5 min after ADP addition and the peak values were selected to calculate the percent inhibition of aggregation [4,21]. The intra-assay variability of the studied parameters was assessed by repeating the assay 3 times on the same platelet sample for each hormone dilution and the inter-individual variability was assessed by repeating the assay in 4 independent experiments using platelets from different donors.

2.5. Assay for cGMP on platelets

Intracellular cGMP has been shown to mediate RLX-induced inhibition of platelet aggregation through a nitric oxide-dependent mechanism [21]. Therefore, we measured cGMP levels in platelets exposed to the noted RLXs. Samples of platelet-rich plasma, 500 μl each, containing about 10^9 platelets were incubated for 10 min at 37 °C in the absence or presence of 3, 10, 30, 100 and 300 ng/ml of pRLX, H2 RLXs and H3 RLX. According to Bani et al. [21], the phosphodiesterase inhibitor isobutyl-methylxanthine (IBMX, 100 μM) was added to the samples 5 min before the hormones to prevent cGMP catabolism. On incubation with the different agents, the platelet samples were added with 500 μl of 5% trichloroacetic acid, centrifuged, and trichloroacetic acid extracted with 0.5 M tri-*n*-octylamine dissolved in 1,1,2-trichlorotrifluoroethane. Finally, the samples were acetylated with acetic anhydride and the amounts of cGMP in the aqueous phase were measured by ELISA. The values are expressed as pmol cGMP/mg of