Detection of the tripeptide Tyr-Gly-Gly, a putative enkephalin metabolite in brain, using a sensitive radioimmunoassay

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A sensitive and specific radioimmunoassay has been developed for YGG. The tripeptide was previously derivatised with *p*-benzoquinone to prepare the immunogen and the ^{125}I tracer as well as in samples submitted to the RIA. The sensitivity is about 1 nM as compared with 8000 nM for underivatised YGG. Measurable amounts of endogenous YGG immunoreactivity, co-eluting in HPLC with authentic YGG, were detected in mouse striatal extracts.

Enkephalin metabolism *Tyr-Gly-Gly* *Radioimmunoassay*

1. INTRODUCTION

The enkephalins (YGGFM or YGGFL) are substrates for a variety of peptidases but only two enzyme activities appear to be involved in their physiological inactivation [1]: an aminopeptidase activity, sensitive to bestatin [2], responsible for the cleavage of the YG bond and 'enkephalinase' (EC 3.4.24.11) [3], inhibited by Thiorphan [4] and responsible for the cleavage of the GF bond. Hence the YGG fragment constitutes one of the characteristic reaction products of enkephalinase and its tissue level might potentially constitute an index of opioid peptide release. We have developed a RIA for this tripeptide to explore this possibility.

2. MATERIALS AND METHODS

Chemicals were from the following sources: synthetic peptides from Bachem, Bubendorf, Switzerland; 1,4-benzoquinone, puriss. grade, from Fluka AG, Switzerland; polyethylene glycol 6000 from Touzart et Matignon, Paris; organic solvents, analytical grade, from Merck, Darmstadt and other chemicals from Sigma, USA. [^{3}H]Tyr-Gly-Gly was prepared by hydrolysis of [^{3}H](Met^5)enkephalin (Amersham, England) using a highly purified preparation of kidney enkephalinase [5] and isolated by polystyrene bead column chromatography [6]. Its radioactive purity was checked by HPLC [5].

2.1. *Immunogen preparation*

The tripeptide YGG was cross-linked to bovine serum albumin (BSA) using 1,4-benzoquinone (Bzq) in two steps [7]: 8 mg BSA dissolved in 2 ml of 0.1 M phosphate buffer, pH 6, containing 0.15 M NaCl, were treated with 15 mg Bzq dissolved in 0.5 ml EtOH. The solution was kept for 1 h at room temperature, then filtered through a Sephadex G-25 column (1.5 × 5 cm) equilibrated in 0.15 M NaCl with which elution was thereafter performed. The first coloured peak (3.5 ml) corresponding to Bzq-BSA was mixed with 12 mg YGG dissolved in 0.5 ml of 0.5 M carbonate buffer, pH 9. After 24 h at room temperature, the reaction was stopped by addition of 0.5 ml of 1 M lysine, pH 7.5, and the mixture was dialysed against 0.15 M NaCl. Using [^{3}H]YGG as tracer, it was estimated that approx. 30 molecules of YGG

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were conjugated to each BSA molecule, i.e. 150 μg/mg BSA.

2.2. *Antiserum production*

The YGG-Bzq-BSA conjugate (4 mg/animal) was emulsified in complete Freund's adjuvant and injected intradermally at multiple sites along the back of 3 rabbits. Booster immunisations (2 then 1 mg) with incomplete Freund's adjuvant were administered at approximately two weekly intervals, until antibodies of sufficient titre and affinity were generated.

2.3. *Preparation of tissue extracts*

Mouse striata were homogenised in 30 vols of 0.4 N $HClO_4$. After centrifugation (15000 $\times$ $g \cdot$min), clear supernatants were adjusted to pH 6 and stored at -20°C until used. After thawing and centrifugation (15000 $\times$ $g \cdot$min), 1 ml of the clear extract was chromatographed on a column of bonded SiO_2 (Sep-Pak C_{18}, Waters). The column was washed with 0.5 ml H_2O and YGG eluted with 1.5 ml MeOH, the recovery being of 80%, as determined using [^{3}H]YGG. The eluates were dried under reduced pressure, redissolved in 0.2 ml H_2O and submitted to HPLC on a C_{18} μBondapak column. Elution was performed with H_2O at a rate of 1.5 ml/min and 1.5 ml fractions, collected during 20 min, were lyophilised. Residues were redissolved in 0.1 ml H_2O.

2.4. *Derivatisation of samples with p-benzoquinone*

To 0.1 ml samples of tissue extracts or solutions of peptide standards were added 75 μg Bzq dissolved in 0.1 ml EtOH and 0.05 ml of 0.08 M phosphate buffer, pH 6. After 18 h at 20°C, the excess of Bzq was removed by extraction into 0.7 ml $CHCl_3$, and aliquots of the aqueous phase used for the RIA.

2.5. *Preparation of the tracer*

To 1 mg (3.4 μmol) of YGG and 10^5 dpm of [^{3}H]YGG dissolved in 0.4 ml of 0.1 M phosphate buffer, pH 6, were added 6 mg (56 μmol) of Bzq, dissolved in 0.1 ml EtOH and the mixture left at room temperature for 1 h. The excess of Bzq was removed by extraction into $CHCl_3$ and the aqueous phase spotted onto a silica gel plate and submitted to TLC, using *n*BuOH/AcOH/H_2O (4 : 1 : 1) as solvent. Bzq-YGG (R_F = 0.58), clearly separated from YGG (R_F = 0.28), and representing about 50% of the total (evaluated as described in legend of fig.1) was extracted from the plate. To 385 μg (1 μmol) of Bzq-YGG dissolved in 0.1 M carbonate buffer, pH 9, were added 885 μg (3 μmol) of YGG in a final volume of 0.5 ml; the solution was left at room temperature for 18 h and, then submitted to TLC as above. The new spot (R_F = 0.10), presumably corresponding to YGG-Bzq-YGG, was extracted into 0.05 M phosphate buffer, pH 7.4, to obtain a 0.5 mg/ml solution. 20 μl of this solution were treated with 1 mCi of $Na^{125}I$ (spec. act. 2000 Ci/mmol, Amersham, England) dissolved in 10 μl in a tube coated with iodogen [8]. After à 20 min reaction time, the formed ^{125}I-YGG-Bzq-YGG was isolated by TLC in the system described above (R_F = 0.19).

2.6. *Radioimmunoassay procedure*

The diluent for reagents was 0.1% BSA made up in 50 mM phosphate buffer, pH 7.4. Standard and sample curves were run in triplicate.

Derivatised materials (0.1 ml), i.e. tissue extracts of pure peptides, were mixed with the ^{125}I tracer and diluted antiserum in a final volume of 0.3 ml. The mixture was incubated either for 2 h at 37°C or for 15–18 h at 4°C, then kept in an ice-bath for 30 min. After addition of 0.1 ml of 0.5% bovine γ-globulin solution and 0.5 ml of 17% polyethylene glycol solution, the tubes were vortexed and centrifuged at 3000 $\times$ g for 20 min. The supernatants were discarded and pellets counted in a γ counter.

Results were expressed as percent of displacement of bound ^{125}I tracer as a function of displacer concentration and IC_{50} values determined.

3. RESULTS AND DISCUSSION

A sensitive RIA for such a short peptide as YGG could be developed owing to its derivatisation with Bzq, according to a procedure previously used to attach covalently antibodies to enzymes [7]. This method was presently applied to raise antibodies against YGG in rabbits, to obtain a suitable ^{125}I tracer and to pretreat tissue samples to increase the assay sensitivity.

Thus, whereas the alternative tracer ^{125}I-YGG shows no significant binding to antisera, the tracer

^{125}I-YGG-Bzq-YGG allowed the selection of an antiserum giving a B_0 value of 25% at a dilution of 1/18000.

In addition, derivatisation of YGG by Bzq decreased its IC_{50} value from 8000 to 1 nM (fig.2), allowing the detection of about 10 pg of YGG per assay. However, a reliable RIA requires that derivatisation should be performed in a quantitative and reproducible manner. To meet this requirement, the experimental conditions for Bzq coupling to YGG (time, pH, reagent concentrations) were studied, taking into account the interference of endogenous reacting compounds in tissue samples. Under the selected conditions, the reaction was complete after 18 h (fig.1) and a tracer amount of [^{3}H]YGG, added to a tissue extract, coupled to the extent of 92 ± 10% (mean ± SE of 5 experiments).

In spite of the small size of YGG, limited cross-reactivities with parent peptides like YG, YG-NH_2, FGG, MGG or the enkephalins, were found (fig.2). The very low cross-reaction of Y (<0.01%) is of particular interest in view of the relatively high tissue level of this amino acid.

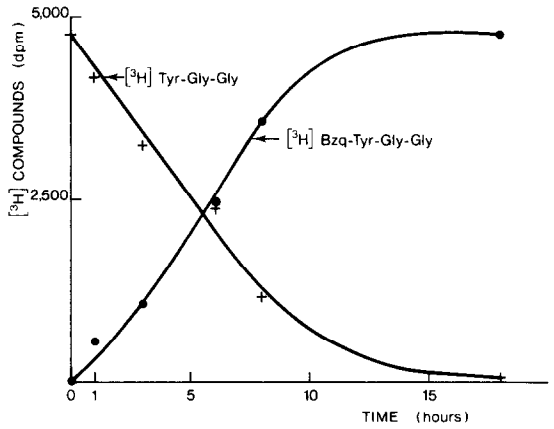

Fig.1. Derivatisation of Tyr-Gly-Gly with *p*-benzoquinone. To YGG (10 ng) and [^{3}H]YGG (10^5 dpm i.e. 300 pg) dissolved in 0.1 ml H_2O, were added 75 μg Bzq dissolved in 0.1 ml EtOH and 0.05 ml phosphate buffer (0.08 M, pH 6) and the reaction developed at 20°C for the indicated duration. After removal of the excess of Bzq by $CHCl_3$ extraction, aliquots were submitted to silica gel TLC using *n*BuOH/AcOH/H_2O (4:1:1). Radioactivity of the spots was evaluated with a scanner (Berthold, FRG). R_F values: 0.28 (YGG) and 0.58 (Bzq-YGG).

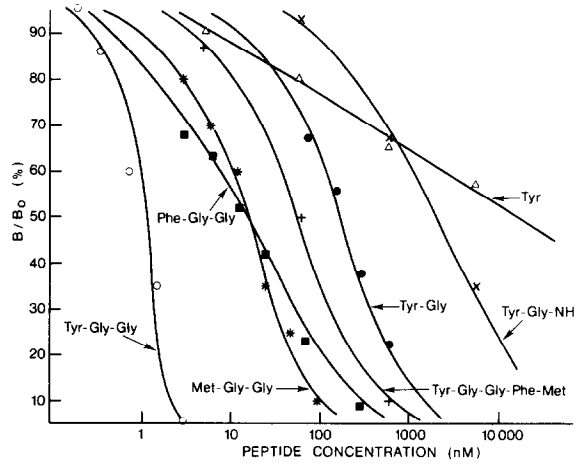

Fig.2. Cross-reactions of antibodies raised against Tyr-Gly-Gly with various compounds. Tyr and all peptides were prederivatised with Bzq as described in section 2 and incubated in the presence of 10 pM ^{125}I tracer and the antiserum (1/18000). The IC_{50} value of non-derivatised YGG was 8000 nM (not shown).

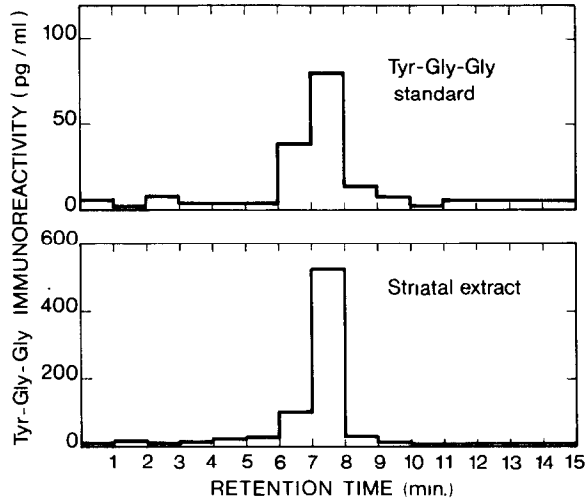

Fig.3. HPLC elution pattern of a Tyr-Gly-Gly standard and Tyr-Gly-Gly immunoreactivity from an extract of mouse striatum. Samples (0.2 ml) were injected into a C_{18} μBondapak column, elution performed with pure water at a rate of 1.5 ml/min and fractions radioimmunoassayed. Retention times were 5 min (Y), 7 min (YG), 8 min (YGG), >20 min (enkephalins). The recovery of YGG-immunoreactivity in tubes 6–8 was about 95% of the injected material.

When the RIA was applied to a mouse striatal extract, displacement of the bound tracer, paralleling that of authentic YGG, was observed (not shown). Furthermore, the immunoreactivity co-eluted with authentic YGG on a HPLC column (fig.3).

From this experiment a striatal level of YGG of 4.3 pmol/mg protein could be calculated, representing about 1/10 that of enkephalins [9]. A variety of other data support the idea that YGG is in a dynamic state in brain and largely arises from endogenous enkephalin breakdown. For instance, the regional distribution of YGG parallels to a large extent that of fragments of the proenkephalin molecule; YGG recovery, mainly in soluble subcellular fractions, is consistent with its extraneuronal localisation; finally its level is rapidly and markedly altered by inhibition of peptidases known to be responsible for endogenous enkephalin metabolism ([10]; in preparation).

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