THE EFFECT OF PUROMYCIN ON THE BIOLOGICAL ACTIVITY OF LEU-ENKEPHALIN

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Received 20 October 1978

1. Introduction

The endogenous pentapeptides, Leu-enkephalin (Tyr–Gly–Gly–Phe–Leu) and Met-enkephalin (Tyr–Gly–Gly–Phe–Met), bind to opiate receptors [1,2] and inhibit the electrically-evoked contractions of mouse vas deferens and guinea pig ileum [3]. The enkephalins are rapidly hydrolyzed by enzymatic activities present in brain and in serum; the peptide bond between the Tyr and the Gly comprises the preferential site of cleavage [4–6]. The rapid degradation probably accounts for the low and transient analgesic effect of the enkephalins [7]. The prevention of this hydrolysis is therefore necessary in order to study the interaction of the enkephalins with the opiate receptors.

The antibiotic puromycin known to inhibit brain arylamidase [8], is an effective inhibitor of the hydrolysis of enkephalin by brain homogenate [9,10]. Here we tested the specificity of the puromycin inhibitory activity on the degradation of enkephalin. In addition, we determined the effect of puromycin on the biological activities of enkephalin, such as, binding to brain opiate receptors and inhibition of the contractions induced by electrical stimulation of the guinea pig ileum.

2. Materials and methods

[*Tyrosyl*-3,5-^{3}H]Leu-enkephalin and [^{3}H]etorphine were obtained from Amersham Radiochemical Centre; puromycin and puromycin aminonucleoside from ICN Pharmaceuticals; and *p*-methoxy-L-phenylalanine from Sigma. Nitrous acid deaminated puromycin (hydroxypuromycin was prepared and purified as in [11]. Other materials and crude brain homogenate were obtained as in [6].

2.1. *Enzymatic hydrolysis of Leu-enkephalin*

Mixtures of labeled and unlabeled Leu-enkephalin (40 000 cpm, 0.1 μM final conc.) were incubated for 10 min at 30°C with the specified enzymatic activity in final vol. 100 μl. The respective incubation mixtures contained:

(a) 0.16 μg leucine aminopeptidase in 10 mM Tris–HCl (pH 7.5) and 2 mM $MgCl_2$;
(b) 3 μl rat serum in 10 mM Tris–HCl (pH 7.5);
(c) 1.7 μg crude brain homogenate in 10 mM Tris–HCl (pH 7.5) and 0.1 mg bovine serum albumin/ml. Reactions were stopped by immersing the tubes in a boiling water bath for 10 min. The liberation of free tyrosine during the reaction was assayed as described [6], utilizing columns with polystyrene beads (Porapak Q) to which enkephalin but not tyrosine is adsorbed.

2.2. *Binding of Leu-enkephalin and etorphine to crude brain homogenate*

Crude brain homogenate was preincubated for 30 min at 37°C (to destroy endogenous enkephalin). Aliquots of the homogenate (900 μg protein) were further incubated in final vol. 250 μl containing 0.18 M sucrose, 25 mM Tris–HCl (pH 7.5), and either 10 nM [*tyrosyl*-3,5-^{3}H]Leu-enkephalin (41 Ci/mmol) or 3 nM [^{3}H]etorphine (30 Ci/mmol). The samples were incubated for 10 min at 25°C (enkephalin) or for 30 min at 37°C (etorphine). Binding was assayed by filtration on 25 mm GF/B (Whatman) glass fiber filters. Non-specific binding, measured in the presence of 1 μM levorphanol was subtracted from the values obtained.

Elsevier/North-Holland Biomedical Press

2.3. *Guinea pig ileum preparation*

Ileum segments (2–3 cm long) were bathed at 37°C in Dulbecco modified Eagle's medium (GIBCO) containing 25 mM Hepes buffer (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid) pH 7.4, instead of $NaHCO_3$, and gassed with humified air. The ileum was stimulated by coaxial electrical stimulation with 1 ms pulses of 90 V at a frequency of 0.2 Hz. The contractions were recorded isotonically under a tension of 1 g using a Hewlett Packard 7DCDT-250 displacement transducer.

3. Results

Figures 1 and 2 demonstrate that puromycin is an effective inhibitor of the brain enzymatic activity which cleaves Leu-enkephalin at the Tyr–Gly peptide bond. Incubation of [*tyrosyl*-3,5-^{3}H]Leu-enkephalin with crude brain homogenate yielded free tyrosine with the concomitant disappearance of enkephalin as revealed by thin-layer chromatography of the reaction mixture (fig.1). Puromycin, at 0.1 mM, inhibited the hydrolysis of enkephalin almost completely.

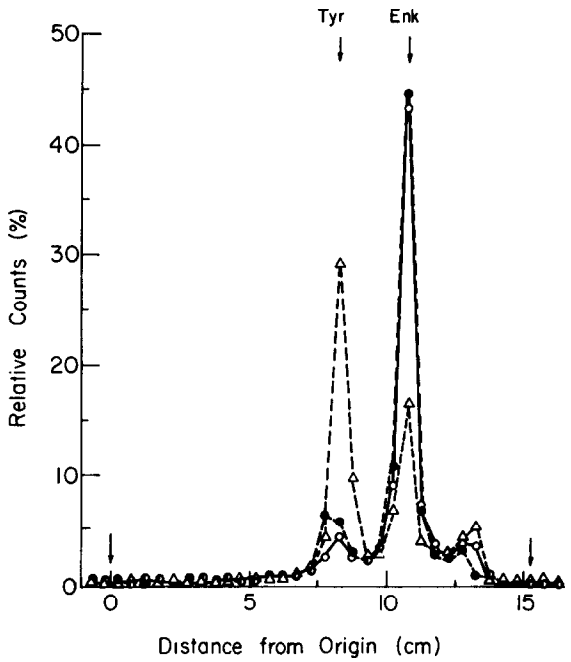

Fig.1. Thin-layer chromatography of labeled Leu-enkephalin incubated with rat brain homogenate in the presence and absence of puromycin. Incubation mixtures contained: 0.4 μM labeled enkephalin, 10 mM Tris–HCl (pH 7.4) and 10 μg brain homogenate/ml. Incubation was stopped by boiling. Samples (5 μl) were chromotographed on silica plates in *n*-butanol–acetic acid–water (4:1:1). Sections (0.5 cm) were counted for radioactivity. No incubation (o——o); 120 min incubation at 30°C without puromycin (△– – –△); 120 min incubation with 0.1 mM puromycin (•– – –•). Arrows show location of markers determined by fluorescamine spray as in [6].

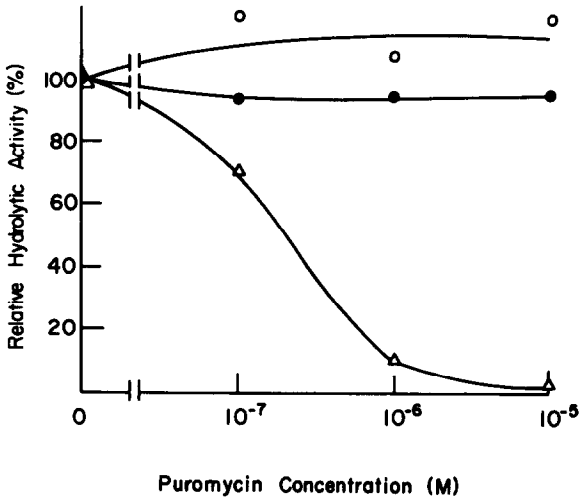

Fig.2. The effect of increasing concentrations of puromycin on the rate of hydrolysis of Leu-enkephalin by leucine aminopeptidase (o——o); rat serum (•——•); and rat brain homogenate (△——△). The amount of enkephalin hydrolyzed in the absence of puromycin was defined as 100 and was equivalent to 1.5, 2.8 and 1.5 pmol, respectively.

The effect of increasing concentrations of puromycin on the rate of enkephalin hydrolysis is shown in fig.2. The enzymatic activity in brain homogenate was found to be extremely sensitive to puromycin. Inhibition of 50% was observed with 0.2 μM puromycin (in the presence of 0.1 μM Leu-enkephalin). Complete inhibition (97–98%) was observed with 10 μM puromycin. Parallel experiments demonstrated that bacitracin, a known inhibitor of enkephalin hydrolysis, was less potent that puromycin. Bacitracin at 2 μM was required to inhibit enkephalin hydrolysis by 50% (in the presence of 0.1 μM Leu-enkephalin).

Interestingly, in contrast to the activity present in brain homogenate, the aminopeptidase activity present in rat serum as well as purified leucine amino-peptidase (both shown to cleave enkephalin at the

Table 1
Effect of puromycin and related compounds on the relative rate of hydrolysis of Leu-enkephalin by crude rat brain homogenate

Compound added	Relative hydrolysis
None	100.0
Puromycin, 10^{-4} M	2.6
Puromycin aminonucleoside, 10^{-4} M	99.9
p-Methoxy-L-phenylalanine, 10^{-4} M	85.9
Hydroxypuromycin, 10^{-4} M	99.3

Tyr–Gly peptide bond) were not affected by puromycin.

The capacity of puromycin to inhibit the hydrolysis of enkephalin by brain enzymatic activity was dependent on the integrity of the peptide bond between the aminonucleoside and the *p*-methoxy-L-phenylalanine moieties of the puromycin molecule. Neither the free puromycin aminonucleoside (puromycin without the *p*-methoxy-L-phenylalanine residue) nor the *p*-methoxy-phenylalanine were effective as inhibitors of the enzymatic activity in brain homogenates (table 1). The N-terminal amino group on the *p*-methoxy-L-phenylalanine moiety of puromycin was also found to be essential; since hydroxypuromycin, obtained by deamination of puromycin with nitrous acid, was ineffective as an inhibitor of enkephalin degradation.

Binding of labeled enkephalin to the opiate receptors of brain membranes was increased by the addition of puromycin to the incubation mixture. The potency of puromycin was especially evident with crude brain homogenates. Figure 3 demonstrates that the specific binding of enkephalin increased 15–20-fold upon the addition of 10–100 μM puromycin, reaching a spec. binding act. 45 fmol/mg protein. Binding of labeled etorphine, a non-hydrolyzable opiate alkaloid with high affinity for the opiate receptor, was not affected by puromycin and reached spec. act. 130 fmol/mg protein.

Puromycin inhibited the degradation of enkephalin by homogenates of guinea pig ileum. In the presence of 10 μM puromycin, the rate of hydrolysis was reduced by 80%. In another experiment, we tested the biological activity of enkephalin exerted upon the guinea pig ileum preparation in the presence and

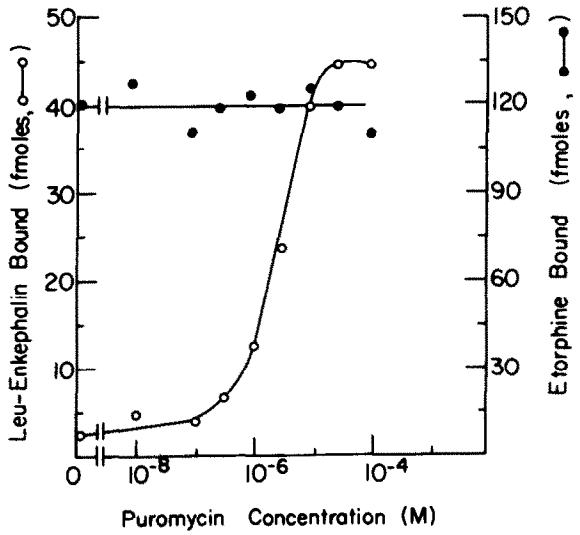

Fig.3. The effect of puromycin on the specific binding of etorphine or Leu-enkephalin to crude brain homogenate.

absence of puromycin (fig.4). The addition of 0.2 μM Leu-enkephalin to the electrically-stimulated guinea pig preparation in Dulbecco modified Eagle medium was found to completely abolish the electrically-induced contractions of the ileum (see fig.4A). Unlike the known effect of morphine, the inhibitory action of enkephalin on the contraction of the ileum was

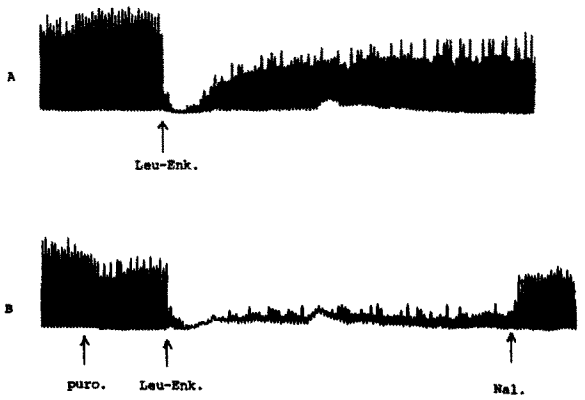

Fig.4. Prolongation by puromycin of the depressant effect of Leu-enkephalin on the electrically-induced contractions of the guinea pig ileum. Arrows mark the addition of 0.2 μM Leu-enkephalin, 0.1 mM puromycin and 1 μM naloxone, respectively.

found to be short-lived; the intensity of the contractions gradually increased and within a few minutes approached the original level. Puromycin alone, at 0.1 mM, inhibited the contraction very slightly. On the other hand, the presence of the antibiotic greatly extended the time during which enkephalin exerted its effect (fig.4B). This effect of enkephalin, both in the presence and absence of puromycin, could be reversed by either washing or by the addition of the antagonist naloxone. Thus, short treatments with puromycin did not detectably damage the potential of the ileum to contract electrically nor to be affected by the opiates.

4. Discussion

We have demonstrated that puromycin inhibits the degradation of enkephalin by brain as well as by guinea pig ileum homogenates. The integrity of the peptide bond in the puromycin molecule and the presence of the amino group of the *p*-methoxy-L-phenylalanine residue were found to be necessary requirements for this inhibition. The high potency of puromycin as an inhibitor might well be related to the resemblance of the *p*-methoxy-L-phenylalanine residue to the N-terminal region of enkephalin.

Bacitracin has been used by various laboratories to protect enkephalin against degradation and in particular, has been included in binding assays involving brain homogenates, in order to protect the peptide from enzymatic hydrolysis [2,12]. In the above work, puromycin was shown to be much more potent than bacitracin in preventing this degradation. In addition, puromycin at the effective concentrations did not interfere with the binding of etorphine to the opiate receptor, whereas, bacitracin was found to inhibit the binding of etorphine (Z.V., in preparation) as well as the binding of naloxone [12]. Puromycin, at 30 μM, enhanced the specific binding of Leu-enkephalin to the receptor of rat brain homogenates by 18-fold, reaching 45 fmol/mg protein. This value is 12-times that in [2] for the binding of Met-enkephalin to similar brain homogenates in the presence of bacitracin. Puromycin, therefore, should prove a useful reagent for protecting the enkephalins against hydrolysis and for studying their interaction with the opiate receptors.

Puromycin can be used to distinguish between the various enzymatic activities capable of hydrolyzing the Tyr–Gly peptide bond of enkephalin. Puromycin at 10 μM inhibited 97% of the hydrolytic activity present in rat brain homogenate. On the other hand, it did not inhibit the enzyme leucine aminopeptidase, as also found [8], nor did it inhibit the hydrolytic activity present in rat serum.

Puromycin and cycloheximide are known to inhibit protein synthesis and impair 'long term' memory [13,14]. We found that unlike puromycin, cycloheximide had no effect on the hydrolysis of enkephalin [9]. The results therefore suggest that the role of puromycin in blocking enkephalin hydrolysis is not directly related to the other effects mentioned. Recent experiments with various analogs of puromycin revealed that the capacity of puromycin to serve as a protein synthesis inhibitor can be distinguished from its capability to inhibit enkephalin degradation (Z.V., unpublished).

5. Conclusions

The antibiotic puromycin is an effective inhibitor of the degradation of Leu-enkephalin by enzymatic activities present in rat brain homogenates and guinea pig ileum. Puromycin does not inhibit the hydrolysis of enkephalin by either leucine aminopeptidase or rat serum. The protective effect of puromycin against the hydrolysis enhances the specific binding activity of Leu-enkephalin to the opiate receptors in brain homogenates by several fold. In addition, puromycin greatly prolongs the depressant effect of enkephalin on the electrically-induced contractions of the guinea pig ileum. The effect of puromycin in preventing enkephalin hydrolysis is apparently unrelated to its capacity to block protein synthesis and to impair long term memory.

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