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Short communication

Imipramine inhibits soluble enkephalin-degrading aminopeptidase activity in vitro

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Abstract

Considerable evidence has appeared recently connecting the mechanism of action of some antidepressant drugs with the inhibition of the enzymes responsible for enkephalin degradation. Imipramine in vitro inhibits the enkephalin-degrading aminopeptidase MII and interacts with the enzyme in a mixed competitive–noncompetitive manner. The present work shows that imipramine in vitro also inhibits reversibly soluble enkephalin-degrading aminopeptidase activity in rat brain. Kinetic analysis showed that this enzyme has two different binding sites for the drug, and that imipramine interacts with the enzyme in a mixed noncompetitive–acompetitive way. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Imipramine; Aminopeptidase; Enzyme inhibition

1. Introduction

The hypoalgesic effects of some tricyclic antidepressants have been reported to be sensitive to the opiate antagonist naloxone, suggesting an interaction between antidepressant drugs and the opioidergic system (Hummel et al., 1994). In this sense, enkephalins have been claimed to mediate pleasure, reward and emotions in rodents (Belluzzi and Stein, 1977). Other indirect evidence of the role of enkephalins in mental depression is the existence of changes in enkephalin-like inmunoreactivity in discrete brain regions after chronic antidepressive treatment (Staunton et al., 1982; De Felipe et al., 1985).

Inhibition of neuropeptide-degrading enzymes is a classically established mechanism for the physiological regulation of some peptidergic systems (Konkoy et al., 1996), and it can also be the mechanism of action of some drugs with central actions (Perrot et al., 1994, Maldonado et al., 1994). In this sense, some inhibitors of enkephalin-degrading peptidases have antidepressive effects (De Felipe et al., 1986; Tejedor-Real et al., 1995), and some antidepressant drugs increase enkephalin levels (De Felipe et al., 1985).

Recently, it has been demonstrated that imipramine in vitro inhibits the enkephalin-degrading enzyme aminopeptidase MII in rat brain (De Gandarias et al., 1997). Most of the known enkephalin-degrading enzyme inhibitors, such as tiorphan, kelatorphan, puromycin, bestatin, amastatin, etc., cause inhibition in a competitive way (Van Amsterdam et al., 1983; Bouboutou et al., 1984; Shimamura et al., 1983), and only imipramine and its active metabolite, desipramine, have been reported to interact in a mixed competitive–noncompetitive manner with the aminopeptidase MII (De Gandarias et al., 1997).

Thus, the aim of this work is to test whether imipramine is capable of directly inhibiting soluble enkephalin-degrading aminopeptidase activity, and to determine the type of interaction between the drug and the enzyme in vitro.

2. Materials and methods

We used 25 Sprague–Dawley male young–adult rats. After being anaesthetised with equithensin, the animals were perfused transcardially with saline solution, and the brains were quickly removed and frozen to dissect the brain cortex. The samples were homogenised in 3 ml

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hypotonic Buffer Tris HCl solution 10 mM, pH 7.4, and ultracentrifuged at $100\,000 \times g$ for 35 min. The resulting supernatant was used for the analysis of enzyme activity and proteins. All preparative steps were carried out at 4°C.

Only Tyr-aminopeptidases hydrolyse the artificial substrate Tyr-β-naphthylamide, so Tyr-aminopeptidase activity was spectrofluorimetrically measured at 345 nm (excitation) and 412 nm (emission), using Tyr-β-naphthylamide as substrate. Samples (10 μl) of the enzyme preparations were incubated 30 min at 37°C with 1 ml of phosphate buffer solution 100 mM, pH 7.4, containing 0.1 mg of bovine albumin, 0.1 mg of dithiothreitol and the different substrate concentrations. The reaction was stopped by the addition of 1 ml of acetate buffer solution 100 mM, pH 4.2.

To test if the drug has an effect in vitro on this enzyme, we used a concentration of 250 μM. This is the blood imipramine concentration measured in previous in vivo studies when imipramine was given in the commonly used dose of 30 mg/kg (De Felipe et al., 1986, 1989). To test if enzyme inhibition was reversible or not, we preincubated the enzyme preparations with imipramine. Later, the enzyme preparations were incubated with the substrate solution without the drug, being diluted from 250 to 2.5 μM. For the concentration–response experiments, enzyme preparations were divided into aliquots and preincubated 1 h at room temperature with the following concentrations of imipramine: 100 μM, 250 μM, 500 μM, 750 μM and 1000 μM. Results were fitted to the concentration–response equation:

$$F = 1/1 + \left(K_d + [D]\right)^{n_H}$$

where F = fraction of inhibition, $[D]$ = drug concentration, K_d = dissociation constant between the enzyme and the drug, and n_H = Hill coefficient.

To carry out the enzyme kinetic analysis, we incubated the enzyme preparations with the phosphate buffer solution plus increasing concentrations of substrate: 1, 2, 4, 8, 16 and 64 μM, with each substrate concentration being tested under control conditions and in the presence of 250, 500 and 750 μM of imipramine. To study the type of interaction between the enzyme and the drug, enzyme kinetics were calculated in the absence and in the presence of the drug by means of the Lineweaver–Burk plot:

$$1/V = \left(K_m/V_{max} * [S]\right) + \left(1/V_{max}\right)$$

where V = velocity of substrate degradation, V_{max} = maximum velocity of substrate degradation, $[S]$ = substrate concentration and K_m = Michaelis constant.

Enzyme activity is expressed as units of aminopeptidase (U.A.P.) per mg of protein. One unit of aminopeptidase activity is the amount of enzyme that hydrolyses one picomole of Tyr-β-naphthylamide per minute.

Data were compared by using the one-way analysis of variance (ANOVA), and multiple comparisons were made by using the Student *t*-test for paired data. Results are expressed as means ± S.E.M., and differences were considered statistically significant at $P < 0.05$. Curve fits were done by means of the nonlinear least-squares algorithm, and '*r*' values were in the range of 0.997 to 0.999.

3. Results

Incubation of the enzyme preparations with 250 μM of imipramine produced a reduction in soluble tyr-aminopeptidase activity (69.3 ± 1.2 vs. 55.4 ± 0.9 U.A.P./mg protein, $P < 0.01$). Drug washout showed that the aminopeptidase inhibition by imipramine was fully reversible (67.8 ± 1.2 U.A.P./mg protein). After that, we made a concentration-response curve in order to calculate the

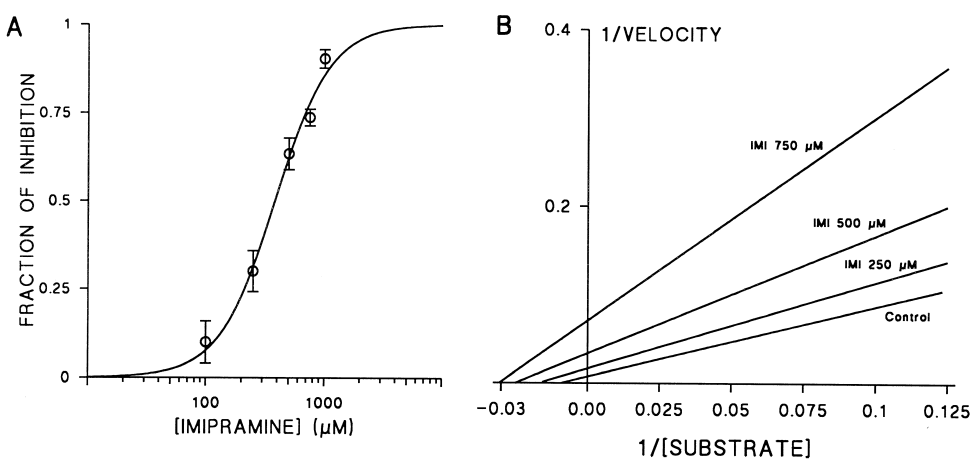

Fig. 1. (A) Concentration–response curve for the inhibition by imipramine of soluble tyr-aminopeptidase activity. Data points are means ± S.E.M. of twelve experiments. (B) Determination of the maximum degradation velocities (V_{max}) and Michaelis constants (K_m) for the aminopeptidase activity under control conditions and in the presence of imipramine 250, 500 and 750 μM, by means of the Lineweaver–Burk plot (highest X values do not appear in the plot but were included in the fit). Line fits do not cross one another in the positive range of Y.

Table 1
Maximum velocity of substrate degradation, V_{max} (μM/mg protein s), and Michaelis constant, K_m (μM), for aminopeptidase activity under control conditions and in the presence of several imipramine concentrations

AP	Control	Imipramine (μM)		
		250	500	750
V_{max}	284.5 ± 62.1	159.8 ± 22.2[a]	60.6 ± 11.6[b]	29.03 ± 3.8[b]
K_m	129.9 ± 21.9	85.3 ± 9.3[a]	44.8 ± 8.6[a]	32.7 ± 5.5[b]

Data were obtained by fitting the experimental values to a double-reciprocal plot. Data are means ± S.E.M. $n = 8$.
[a] $P < 0.05$.
[b] $P < 0.01$.

affinity of imipramine for the aminopeptidase. In Fig. 1A, pooled data from twelve experiments to determine the concentration dependence of inhibition of the aminopeptidase can be seen. A nonlinear least-squares fit of the concentration–response equation to the individual data points yielded an apparent K_d of 372 ± 16 μM and a Hill coefficient of 2.06 ± 0.07. This Hill coefficient close to 2 could suggest that each enzyme molecule was able to bind two imipramine molecules.

For finding out the type of interaction between the enzyme and imipramine, we studied enzyme kinetics in the absence and in the presence of the drug. The substrate concentrations used for the kinetic analysis were 1, 2, 4, 8, 16, 32 and 64 μM, each being tested alone or in the presence of 250, 500 or 750 μM of imipramine. First of all, it was deduced from control data that the enzyme follows classic Michaelis–Menten behaviour for a reaction between one enzyme and only one substrate, and no substrate or product inhibition was observed over the range of substrate concentrations studied (not shown).

To determine the enzyme kinetic parameters, we used the Lineweaver–Burk plot. Fig. 1B shows the extrapolation of the fits to the $Y = 0$ values. The aminopeptidase activity showed a clear and significant reduction in the V_{max} and in the K_m in the presence of imipramine (Table 1).

4. Discussion

Imipramine in vitro inhibits soluble enkephalin-degrading aminopeptidase activity in a concentration-dependent way. The binding between the drug and the enzyme molecule is fully reversible because the effects of the drug completely disappear on washout.

To find out about the type of interaction between aminopeptidase and drug molecules, we carried out an enzyme kinetic analysis, in which we used different imipramine concentrations. A concentration-dependent reduction in the degradation V_{max} and in K_m was seen in the presence of imipramine compared with the control values. These changes in the kinetic parameters, and the Hill coefficient of 2 may suggest a model of interaction with two binding sites for imipramine on the enzyme molecule, as has been described for the interaction between imipramine and desipramine with the membrane-bound aminopeptidase MII (De Gandarias et al., 1997). But in this case, imipramine interacted in a different manner with at least one of these binding sites. One binding site could be in the proteic structure where the drug would interact in a noncompetitive manner, decreasing the degradation V_{max}. The other binding site could be within the active centre, where the drug could interact with the enzyme and the substrate in an acompetitive way, decreasing K_m. Fig. 1B shows the classic picture of a mixed inhibition type between noncompetitive and acompetitive types.

These results suggest that the increase in enkephalin levels in the rat brain caused by antidepressants (De Felipe et al., 1985) could be at least partly due to the decrease in their degradation. This direct interaction between the enzyme and the drug molecule would be in accordance with the absence of effects of imipramine on the enzyme's activity in experiments carried out after in vivo treatments, because enzyme preparations from crude brain imply successive dilutions in different buffer solutions, yielding to the complete washout of the drug.

The inhibition of soluble aminopeptidase activity by imipramine could explain the analgesic effect of some antidepressant drugs. In this sense, it has been described that the antibiotic puromycin (a selective inhibitor of this enzyme) has naloxone-reversible antinociceptive activity (Chaillet et al., 1983). Our results reinforce the idea that the opioid system could be involved in the genesis and treatment of depressive illness (Tejedor-Real et al., 1995). However, further studies are needed to demonstrate clearly this relation in vivo, because of the high drug concentrations used in this work. Moreover, the role of neutro-endopeptidase in the genesis and possible treatment of depression needs to be studied.

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