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INHIBITION OF CALMODULIN-ACTIVATED CYCLIC NUCLEOTIDE PHOSPHODIESTERASE: MULTIPLE BINDING-SITES FOR TRICYCLIC DRUGS ON CALMODULIN

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Abstract—A cyclic nucleotide phosphodiesterase from guinea-pig heart is activated by calmodulin in the presence of calcium ions. Activation was measured over a range of calmodulin concentrations, and is antagonised by several tricyclic psychotropic drugs including trifluoperazine, imipramine, chlorpromazine and amitriptyline. When the concentration of amitriptyline was increased, its apparent inhibition constant for binding to calmodulin decreased. This was due in part to binding of amitriptyline to glass surfaces; but after correction for this the discrepancy was still significant. It is proposed that this is due to two sites on calmodulin for amitriptyline, with binding to either site being sufficient to prevent calmodulin from activating phosphodiesterase.

Several mammalian tissues contain a calcium-activated isoenzyme of cyclic nucleotide phosphodiesterase, and the protein calmodulin is required to mediate this activation [1–3]. A number of tricyclic psychotropic drugs can inhibit calmodulin-activated phosphodiesterase [4] and can bind to calmodulin directly [5, 6] with a stoichiometry of two or more molecules of drug per molecule of calmodulin. Calmodulin when complexed with the drug is unable to activate phosphodiesterase or several other calmodulin-dependent systems such as erythrocyte calcium-activated ATPase [7].

We here report the effects of varying the calmodulin concentration on the activation of guinea-pig heart phosphodiesterase, and the antagonism of this by several tricyclic drugs. For one of these, amitriptyline, the concentration of drug was also varied and the apparent affinity of calmodulin for amitriptyline was found to increase as the concentration of drug was raised. This was due in part to binding of amitriptyline to glass surfaces: after correction for this, the remaining variation in the affinity for calmodulin was still significant, and might be due to two sites on calmodulin for amitriptyline.

MATERIALS AND METHODS

Guinea-pig hearts were homogenised in 40 mM Tris–HCl pH 7.5. The 48,000 *g* supernatant was chromatographed on DEAE–cellulose (DE 52) in the buffer described by Thompson *et al.* [8], eluting with a gradient of 0–0.5 M NaCl. The first peak of activity contained most of the calmodulin-activated enzyme, and these fractions were pooled and concentrated by ultrafiltration. Activity in this fraction (Fraction I) was found to give no activation with $CaCl_2$ unless calmodulin was added; the same was true for the crude supernatant (which was used in some of the experiments) although the degree of activation was lower.

In assays of cyclic nucleotide phosphodiesterase [9], recoveries of nucleosides were measured by including 0.1 μM [^{14}C]adenosine or [^{14}C]guanosine. Substrates were 1 μM [^{3}H]-cAMP or [^{3}H]-cGMP. Assays were in duplicate or triplicate and rates are expressed as percentage of substrate converted to product in 20 min. Inhibitors were found not to interfere with the 5′-nucleotidase reaction. Radiolabelled compounds were from the Radiochemical Centre, Amersham. Pure calmodulin, prepared from phosphorylase kinase, was a gift from Dr. P. Cohen (Dundee).

The concentrations of amitriptyline in solution, under conditions of the phosphodiesterase assay, were measured spectrophotometrically at 240 nm. Glass test-tubes with 0.2 ml of 40 mM Tris–HCl buffer, pH 7.5 and amitriptyline were incubated at

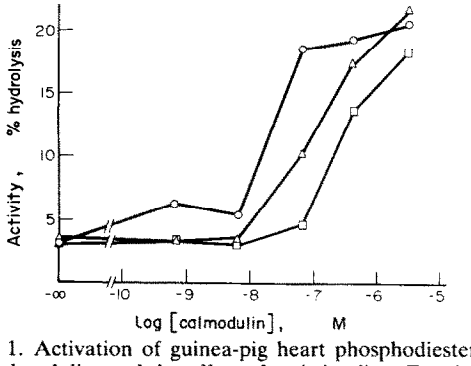

Fig. 1. Activation of guinea-pig heart phosphodiesterase by calmodulin, and the effect of amitriptyline. Fraction I, from DEAE–cellulose chromatography, was used, assayed in 0.2 ml of 40 mM Tris–HCl/100 μM EGTA/150 μM $CaCl_2$, pH 7.5, at 30° with incubations of 20 min. Both calmodulin and Ca^{2+} were necessary for activation. ○, no amitriptyline; △, +26 μM amitriptyline; □, +65 μM amitriptyline (corrected for binding to glass).

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30° for 10 min and then aliquots removed and diluted in Tris buffer in a semi-micro quartz cuvette. Determinations were made in triplicate.

RESULTS AND DISCUSSION

Calmodulin activated heart phosphodiesterase (Fig. 1) in a concentration-dependent manner. Amitriptyline shifted the activation curves to higher calmodulin concentrations but did not significantly alter the maximum activation obtained. Other tricyclic psychotropic drugs were similarly tested at one concentration of drug but with a range of calmodulin concentrations, and the apparent inhibition constants are presented in Table 1. The apparent K_i-values obtained are lower than the IC_{50}-values obtained using fixed concentrations of calmodulin, as anticipated by others [10, 11]; they are similar to the dissociation constants determined directly by measuring calcium-dependent binding to calmodulin, which were 1 μM for trifluoperazine [5] and 5 μM for chlorpromazine [6]. There is no obvious relation between these apparent K_i-values and neuroleptic or antidepressant efficacy, as also noted by others [10].

With amitriptyline, the estimated K_i-values varied considerably, and appeared to decrease as the concentration of amitriptyline was increased. The concentration of amitriptyline in solution was determined spectrophotometrically at 240 nm [12]. At the lower concentrations of amitriptyline used, the measured concentration of amitriptyline was much lower than expected (Fig. 2), presumably due to loss of amitriptyline due to binding to glass surfaces.

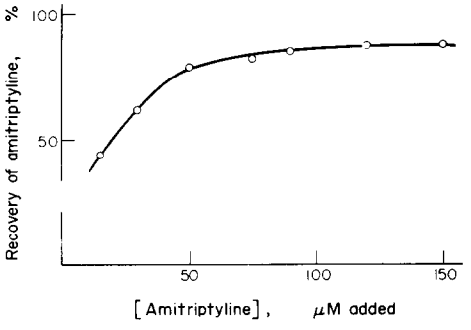

Fig. 2. Loss of amitriptyline from solution. Glass test-tubes were set up with amitriptyline in 0.2 ml of 40 mM Tris–HCl buffer pH 7.5, and aliquots were pipetted directly into quartz cuvettes containing more of the same buffer.

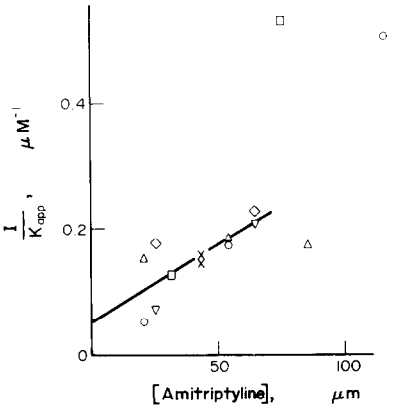

Fig. 3. Variation of apparent inhibition constant with amitriptyline concentration. Concentrations were corrected for binding to glass. Different symbols denote different experiments. Conditions were as in the legend to Fig. 1 except as follows: × and □ crude supernatant (not Fraction I) was the source of enzyme; ▽ cGMP (not cAMP) was the substrate.

After correction of the values of amitriptyline concentration to allow for binding to the walls of the assay tubes the effect was still significant (see Fig. 3). A plausible mechanism for this is suggested by the reports that calmodulin can bind more than one molecule of tricyclic drug: calmodulin is reported to bind two molecules of trifluoperazine strongly (K_D = 1 μM) in a calcium-dependent manner, and a further 24 much more weakly (K_D = 5 mM) which are calcium-independent [5]. The binding of two molecules of trifluoperazine is required to maximally

Table 1. Apparent affinity of calmodulin for tricyclics

Compound	Nominal Concentration (μM)	Apparent K_i (μM)	IC_{50} and {Reference} (μM)
Trifluoperazine	5	1.05	10 {4}
Chlorpromazine	20	1.5	42 {4} 6 {10}
Amitriptyline	30 – 120	2 – 26	130 {4}
Imipramine	50	14	-
Desipramine	50	8	125 {4}

Apparent K_i values were estimated from the right-shifts of activation-curves at 50% activation, as in Fig. 1. Concentrations and values of apparent K_i are nominal, i.e. based on the amount of tricyclic added, uncorrected for binding to glassware. For amitriptyline, estimates of free drug were made; the corrected concentration range of amitriptyline was 21–117 μM, and of the apparent K_i was 1.9–18 μM. The IC_{50} values were reported for bovine phosphodiesterase at pH 8.0 and with fixed concentrations of calmodulin: in ref. 4, 400 μM cAMP and 10 units of calmodulin were used (calmodulin protein not stated); in ref. 10, 10 μM cAMP and 100 ng/ml calmodulin were used.

affect the environments of the metal atoms as shown by n.m.r. spectroscopy [13]. For chlorpromazine, three strong calcium-dependent sites were reported ($K_D = 5\ \mu M$) and approximately 17 weaker sites ($K_D = 130\ \mu M$) [6]. The stoichiometry of amitriptyline binding has not been reported, but this compound can displace calmodulin-bound [^{3}H]trifluoperazine [6]; this and the close structural resemblances of amitriptyline, trifluoperazine and chlorpromazine suggest that amitriptyline is also likely to have multiple binding-sites.

$$\underset{\text{(active)}}{\text{CDR}} \overset{K_1}{\rightleftharpoons} \underset{\text{(inactive)}}{\text{CDR-A}} \overset{K_2}{\rightleftharpoons} \underset{\text{(inactive)}}{\text{CDR-A}_2} \overset{K_3}{\rightleftharpoons} \underset{\text{(inactive)}}{\text{CDR-A}_3} \rightleftharpoons \text{(etc.)}$$

where CDR is calmodulin complexed with Ca^{2+}; A is amitriptyline; and K_1, K_2, K_3, etc., are the operational dissociation constants i.e.

$$K_n = \frac{[\text{CDR-A}_{n-1}][\text{A}]}{[\text{CDR-A}_n]}$$

If, as depicted above, only free calmodulin–Ca^{2+} (without any amitriptyline bound) is active, then the apparent inhibition constant, K_{app} (assuming equilibrium between complexes) is given by:

$$\frac{1}{K_{app}} = \frac{1}{K_1} + \frac{A}{K_1K_2} + \frac{A^2}{K_1K_2K_3} \cdots + \frac{A^n}{K_1K_2K_3 \ldots K_{n+1}} \cdots$$

Therefore a plot of $1/K_{app}$ versus the amitriptyline concentration would give, if only two molecules of amitriptyline were bound, a straight line of positive intercept ($1/K_{app}$) and positive slope ($1/K_1K_2$). However, if three or more molecules of amitryptyline can bind and inhibit calmodulin's activity, a higher-order curve with increasing gradient will be produced. The data for a number of experiments are plotted in Fig. 3. The line is an unweighted least squares fit to all the points where less than 70 μM amitriptyline was present. If only one amitriptyline binding-site gave inhibition, then the line would be horizontal: in fact the gradient is positive ($P < 0.01$); if all the points are included, $P < 0.002$. With the considerable experimental scatter, a straight line seems sufficient to describe the data, indicating two binding-sites. From the line drawn, the intercept gives $K_1 = 18\ \mu M$ and $K_2 = 23\ \mu M$. The 95 per cent confidence limits for K_1 are between 13 and 33 μM: and for K_2, between 14 and 60 μM.

The extent of inhibition will be highly sensitive to changes in the concentration of amitriptyline as a direct result of multiple binding: this would be expected of any multiple binding inhibitor, and has been observed qualitatively for trifluoperazine [11]. It does not require positive co-operativity between binding-sites, and will be expected even when K_2 is larger than K_1.

This appears to be the first demonstration of the multiple-binding of inhibitors affecting the function of calmodulin. Since the mechanism of calmodulin activation, and sensitivity to inhibition, may be different for different enzymes [14], other calmodulin-activated processes may not show the same responses to multiple binding inhibitors. However, erythrocyte calcium–calmodulin-activated ATPase may be similar to phosphodiesterase in this respect: Gietzen *et al.* [7] determined IC_{50} values for trifluoperazine and penfluridol at three concentrations of calmodulin; when recalculated, their data suggest that more than one site for these drugs on calmodulin might be involved in the inhibition.

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