Interaction between calmodulin and five different spin-labelled chlorophenothiazines

Jean Luc OLIVIER, Dominique RAINTEAU, Gilbert BEREZIAT and Claude WOLF
 Biochimie, CHU Saint-Antoine, 27 rue Chaligny, 75571 Paris Cédex 12, France

The binding to purified calmodulin of five spin-labelled derivatives of chlorophenothiazine was investigated by e.s.r. spectrometry and by the antagonizing potency on the calmodulin-dependent activation of myosin light chain kinase. The results of a comparative study and the influence of pH and ionic strength on the binding support the occurrence of an electrostatic binding involving the terminal amino group of the side-chain of the chlorophenothiazine. These results are discussed in relation to the specificity of the interaction that holds the antipsychotic drug–calmodulin complex together.

INTRODUCTION

The interaction between calmodulin and numerous small hydrophobic molecules (drugs or fluorescent probes) is well illustrated in the literature. It is a calcium-dependent binding which is claimed to antagonize the interaction of calmodulin with the target enzyme *in vivo* (Saitoh *et al.*, 1985). It has also a pharmacological relevance since brain calmodulin could bind the phenothiazine antipsychotics (Levin & Weiss, 1976).

The specificity of the interaction is still very controversial since very different hydrophobic molecules bind to calmodulin with a low dissociation constant ($K_d < 10^{-6}$ M).

Roufogalis (1981) has pointed out the absence of correlation between the ability to bind equally well to calmodulin for various chlorinated isomers of phenothiazine and their respective antipsychotic potency. With various chloronaphthalene sulphonamides (Tanaka *et al.*, 1982) a very simple relationship is described between octanol/water partition and binding to calmodulin. Finally, a 'non-specific hydrophobic binding' concept has emerged (Bereza *et al.*, 1982).

Among the numerous studies published, some have devoted special attention to the fact that the ligands with a very high affinity are basic.

As a matter of fact, the binding of the positively charged nitrogen of the piperazine moiety of trifluoperazine to glutamic side chains of a troponin C fragment, homologous to calmodulin, is hypothesized in the detailed model proposed by Gariépy & Hodges (1983). Besides, the inactivation of chlorpromazine by ring sulfoxidation is accompanied by a change of the conformation of the positively charged side chain, as seen by X-ray crystallography (Dahl *et al.*, 1982). The induced circular dichroism of trifluoperazine bound to calmodulin is reduced by increasing the ionic strength (Shimizu & Hatano, 1984). These three observations support an electrostatic binding between calmodulin and the side chain of the antipsychotic drug. The interaction of quaternary phenothiazine salts with calmodulin has been recently documented by Prozialeck (1984). In this report the fact that the binding of non-dissociable methiodide salt is not pH-dependent, whereas the binding of the hydrochloride salt decreases at pH values at which the drug would be non-ionized, indicates that it is the cationic

species that binds to calmodulin. This interaction, in addition to the hydrophobic binding of the phenothiazine ring, could reinforce the specificity of the drug action.

To test this hypothesis, five different spin-labelled derivatives of 2-chlorophenothiazine have been tested. Their side-chains are varied in length and in the charge of the terminal amino group. The present paper is published in addition to the previous study carried out with only one of the present spin-labels (Rainteau *et al.*, 1984). However, the refinement of the spectroscopic data processing and the various probes available presently allow a comparative study.

MATERIALS AND METHODS

Calmodulin and spin-label I (Fig. 1) were prepared according to Rainteau *et al.* (1984).

Spin-labels II, III, IV and V (Fig. 1) were prepared in the laboratory of Dr. S. Pécar (Department of Pharmacy, University of Ljubljana, Yugoslavia) according to Kikelj *et al.* (1983). To calmodulin (10^{-4} M), the spin-label was added as portions of a concentrated methanolic solution (10^{-2} M). The lyophilized calmodulin was freshly dissolved in 0.5 ml of acetate buffer (10^{-2} M, pH 6.0) in the presence of calcium (CaCl_2 , 8×10^{-4} M). For the study of the binding as a function of pH, Tris/maleate buffer was preferred. Concentrations of calmodulin and phenothiazine were checked at 278 or 258 nm respectively.

The settings for the e.s.r. spectrometer (Bruker D-200) were the following: temperature, 22 °C; microwave (band-X) irradiation at 20 mW; central field, 3442.7 G (0.34427 T); modulation field, 1.6 G (0.16 mT). A 100 G (10 mT) sweep width was recorded and digitalized as a 400 data (12 byte) block. Digital spectra were treated according to the following procedure: (1) base line correction, (2) correction of the mid-peak position by right- or left-shifting. To calculate the dissociation constant (K_d) and the number of binding sites (N) the following procedure was applied. (1) From the spectrum recorded in the presence of calmodulin, the free component of the spectrum (recorded in the absence of calmodulin) was subtracted. The resulting spectrum is the 'bound' spectrum. At pH 6.0, the high-field resonance of the freely rotating probe was clearly resolved and the reversion of this peak was selected as the end-point of the

Fig. 1. Chemical structures of the spin-labelled 2-chlorophenothiazines used in the present study

subtraction. (2). The 'total' and the 'bound' spectra were integrated twice. The ratio of 'bound' absorbance/'total' absorbance gives the percentage of bound probe for the eight to ten different concentrations of spin probe. (3) A linear regression of $1/\text{bound}$ versus $1/\text{free}$ gives N and K_d :

$$1/\text{Bound} = \frac{K_d}{[\text{calmodulin}] \cdot N \cdot \text{free}} + \frac{1}{[\text{calmodulin}] \cdot N}$$

For the determination of the inhibition by spin-labelled phenothiazines of the calmodulin-dependent activation of myosin light chain kinase, the assay of activity was carried out according to Le Peuch *et al.* (1979). This was performed at pH 7.5 and 6.4 in the absence of spin-label (activation = 100%) or in the presence of various spin-label concentrations. The 50% inhibition concentrations (IC_{50}) were estimated for the various probes at the two different pH values.

RESULTS

Fig. 1 shows the formulae of the various spin-labelled 2-chlorophenothiazines used. Spin-label I retains a tetramethylpyrrolidinoyl as the reporter group. The other probes are labeled with the tetramethylpiperidinoyl group. The double bond in the reporter group of I gives opportunity for an electronic delocalization with the conjugated carbonyl and, through the amide linkage, with the nitrogen free doublet of the side chain. Moreover there is a steric hindrance between one of the dimethyl

Fig. 2. E.s.r. spectrum of spin-label V (35×10^{-6} M) is recorded in the presence of calmodulin (10^{-4} M) under the standard conditions described in the Materials and methods section

From the total spectrum, the free component (high-field resonance peak denoted 'F') is subtracted to obtain the bound spectrum. The area under the integrated spectrum (broken line) gives the total and bound concentration of spin probe.

groups of the reporter ring and the carbonyl. As a result, the rotation of the tetramethylpyrrolidinoyl of I is partially hindered. For the other spin-labels the reporter group can freely rotate relatively to the amino group. The distance between the amino group of the side chain and the phenothiazine ring is varied from two methylene groups (II) to three methylene groups (I, III, IV and V). These distances (0.5–0.6 nm) are shorter than the distance required (0.6–1.0 nm) for the interaction of trifluoperazine in the troponin C model (Gariépy & Hodges, 1983).

The basicity of the terminal amino group is different due to the various substituents on the nitrogen. According to chemical data (Edsall & Wyman, 1958) we obtain the following decreasing order of basicity: piperazine (spin-label V) > trimethylamine (spin-labels II and III) > cyanamine (spin-label IV) > amide (spin-label I). Spin-label I could even be a donor for H-bonding, i.e. slightly acidic.

Spin-label IV is apolar under the present experimental conditions and it is poorly water-soluble. Special care was taken above 35×10^{-6} M to quantify the spectra obtained with IV since they are broadened by spin exchange. Fig. 2 gives an example of spectrum for the spin-label V (35×10^{-6} M) in the presence of calmodulin (10^{-4} M) and calcium (8×10^{-4} M). The high-field resonance for unbound spin probe (denoted by 'F' on the total spectrum) is resolved under the actual experimental conditions and it allows the subtraction of a 'free' spectrum

Table 1. Binding of the different spin probes to calmodulin in the presence of calcium

The concentration of bound probe and the rotational time are calculated from the spectra, after subtraction of the unbound component, at calmodulin (10^{-4} M) and for eight to ten various concentrations of spin probe.

Spin label	$10^6 \times$ Dissociation constant at pH 6.0 (M)	Number of binding sites	Rotational correlation time (ns)
III	6.3 ± 1.5	1.8	29.7 ± 1.6
II	10.4 ± 2.0	1.0	33.4 ± 1.4
V	7.6 ± 2.0	0.9	9.4 ± 0.8
IV	40.0 ± 8.0	2.0	16.4 ± 2.3
I	22.0 ± 4.0	1.1	27.9 ± 3.0

(spin-label tumbling freely in the absence of protein). The 'bound' spectrum is obtained and the area under the integrated spectrum (broken line) is used to estimate the relative concentration of bound/total spin probe. As described in the Materials and methods section the procedure is applied for eight to ten different spin-label concentrations and the binding parameters are given in Table 1. Rotational correlation times are also calculated from the 'bound' spectra (Freed & Fraenkel, 1963). In Table 1 the spin probes are listed according to the polarity of the terminal amino group. The three basic spin-labels (V, II and III) display the highest affinity, whereas the apolar spin-label IV has less affinity. The difference between the three basic spin probes (V, II and III) is not significant (S.E.M. 2×10^{-6} M for two experiments). However the differences between the basic spin probes and the neutral probes IV and I are found with reliability. The dissociation constant for spin-label I is significantly higher than the value previously published (Rainteau *et al.*, 1984). This seems related to the different buffering condition used in this study (acetate, 10 mM, pH 6.0,

Fig. 3. Influence of the ionic strength on the binding of spin-label III (50×10^{-6} M) to calmodulin (10^{-4} M)

KCl was added to the standard buffer (acetate, 10 mM, pH 6.0).

Fig. 4. Influence of pH on the binding of spin-label III (basic) or spin-label IV (apolar) (50×10^{-6} M) to calmodulin (10^{-4} M)

The pH of the Tris/maleate buffer was adjusted with the appropriate amount of Tris base and was checked in the presence of the protein. □, spin-label III; ■, spin-label IV.

instead of Tris/HCl, 10 mM, pH 7.4). As for spin-label I, the binding of the other probes is modified when the acetate buffer is substituted by Tris buffer (results not shown). The determination of the number of binding sites does not discriminate between $N = 1$ or $N = 2$. Particularly, there is no correlation between the number of binding sites and the length of the chain. In contrast with previously published results (Giédroc *et al.*, 1985) the binding of the phenothiazine is not apparently co-operative and the $1/\text{bound}$ versus $1/\text{free}$ plots are linear with $r^2 > 0.98$ up to 10^{-1} M. The motion of the reporter group for the basic spin-labels II and III is slow compared with the long-side-chain spin-label V and with the neutral-side-chain spin-label IV. The immobilization of the reporter group is a combination on one hand of the distance between the reporter group and the phenothiazine ring and, on the other hand, it is sensitive to the charge of the terminal amino group. The rotational correlation time of spin-label I is slow but, as described above, its rotation is hindered by a special chemical condition.

We infer from these results, obtained with a variety of spin probes under the same physicochemical conditions, that the basic spin-labels could interact with a negatively charged group on calmodulin. To investigate this possible electrostatic binding spin-labels III (basic) and IV (neutral) have been studied for the influence of the ionic strength and of the pH on the binding. An increase of the ionic strength (Fig. 3) reduces the binding of spin-label III from 80% (acetate, 10 mM) to 63% (KCl added, 150 mM). The influence of the addition of NaCl has been similarly described for the binding of spin-label I (Rainteau *et al.*, 1984). When the pH decreases (Fig. 4) between 6.5 and 6.0, there is a sharp increase from 50% to 80% of bound spin-label III. Simultaneously (Fig. 5) the rotational correlation time is doubled. On the contrary, the binding of the neutral spin probe IV remains unchanged for this range of pH. The influence of the pH on the binding of I (Rainteau *et al.*, 1984) displays an apparent continuous decrease between pH 9 and pH 5.

Fig. 5. Influence of pH on the rotational correlation time of spin-label III bound to calmodulin

The rotational correlation time is estimated according to Freed & Fraenkel (1963) from the bound spectrum.

Table 2. Inhibition by the spin-labelled phenothiazines of the calmodulin-dependent activation of myosin light chain kinase

The concentration which gives a 50% reversal of the enzyme activation (IC_{50}) is determined at pH 7.5 or 6.4.

Spin label	$10^6 \times IC_{50}$ (M)	
	pH 7.5	pH 6.4
III	14.0 ± 1.0	5.2 ± 0.5
II	18.0 ± 1.0	10.1 ± 0.8
V	5.0 ± 0.5	1.3 ± 0.2
IV	31.0 ± 3.0	33.0 ± 3.0
I	6.5 ± 0.6	7.9 ± 0.7

Below pH 4.5, the calmodulin precipitates and all types of probes (III, IV or I) are released (results not shown).

The correlation between the binding of the spin probes to purified calmodulin and the efficacy to antagonize the calmodulin activation of myosin light chain kinase is shown in Table 2.

The basic spin-labels V, II and III are the most efficient inhibitors, in agreement with their high affinity for purified calmodulin (Table 1). Taking into account the standard deviation of the assay, the piperazine derivative V is significantly more efficient than the other basic probes II and III. This is particularly clear at acidic pH. Then the pH influences the inhibitory potency of the basic probes. On the contrary, the pH does not change the IC_{50} for the neutral spin probe IV. The behaviour of spin-label I is apparently more complex. First, it has an high antagonizing potency comparable with the potency of basic probes. Secondly, spin-label I displays a reverse tendency, compared with the basic spin probes, to increase its antagonizing activity at alkaline pH. As it will be discussed further the slightly acidic (H^+ donor) behaviour of the amidogroup of I could be the basis for this behaviour. As a matter of fact, the reinforcement of

the binding of I at alkaline pH has been already observed by Rainteau *et al.* (1984) by e.s.r.

DISCUSSION

The structural requirements for binding to calmodulin of phenothiazines were postulated for an homologous sequence of troponin C (Gariépy & Hodges, 1983). They coincide with the pharmacological data (Weiss *et al.*, 1980). One point of attachment is a hydrophobic interaction between lipophilic portions of the drug and non-polar regions of calmodulin. At the other point of attachment, an electrostatic interaction occurs between a positively charged amino group on the drug and a negatively charged acidic residue on calmodulin. The nitrogen, protonated at physiological pH, should be coupled to the hydrophobic region by a chain of at least three carbon atoms according to Gariépy & Hodges (1983) and Weiss *et al.* (1980). Studies on the pH dependence of the binding of trifluoperazine to calmodulin (Weiss *et al.*, 1980) show that binding increases markedly above pH 4.5, reaches a maximum at pH 5.0 and remains constant up to pH 8.0. Above pH 8.0 the binding decreases markedly. Since the isoelectric point of calmodulin is about 4.5 and the pK_a of trifluoperazine is 8.1, this suggests an electrostatic interaction between the negatively charged calmodulin and the positively charged phenothiazine. On the other hand, if a selected group of phenothiazines and related antipsychotics is considered (Weiss *et al.*, 1982) an excellent correlation exists between calmodulin inhibition and hydrophobicity. The affinity of chloronaphthalene sulphonamides for calmodulin correlates also with their hydrophobicity (Tanaka *et al.*, 1982), even when derivatives with various alkyl side-chains are studied. This is apparently in contrast with the assessment that the length of the side-chain is relevant for the ability of a drug to inhibit calmodulin.

Detailed studies on the hydrophobic moieties of calmodulin antagonists are currently available and structure-activity relationships suggest that hydrophobicity is required but that the specificity for this point of attachment is low (Roufogalis, 1981). On the contrary, few systematic data concern the substituents on the amino-group of the side-chain of antipsychotics. For this reason, in the present study the interest is focused on the charge and on the position of the terminal side-chain amino group.

First, we confirm that a positively charged amino group reinforces the interaction. Apolar antagonists (like IV) have less affinity for calmodulin than have basic compounds. The pH dependence of the binding for polarizable spin probes suggests also that the binding becomes more tight at acidic pH when the protonation of the amino group is fulfilled. This is observed for the binding parameters derived from e.s.r. as well as for myosin light chain kinase antagonism.

However we wish to point out the difficulty in assessing a really accurate concentration for non-water-soluble antagonists. This is to say that if one uses these non-soluble antagonists above the critical micellar concentration, the compounds partition between the binding to calmodulin and the binding to micelles and then the affinity will be underestimated. This point has been already discussed for the binding of the apolar peptide haemolysin to calmodulin (Cox *et al.*, 1985). E.s.r. makes the aggregation of the antagonist observable thanks to the

broadening of the spectrum by spin exchange. Thus it prevents some misinterpretation of the results. Self-association of chlorpromazine to form a micelle which could interact with calmodulin is a possible explanation for the apparent positive co-operativity as discussed by Gièdroc *et al.* (1985). This could also explain why, at relatively modest concentrations of chlorpromazine, e.g. 20×10^{-6} M, 1 mol of calmodulin is able to bind as much as 5 mol of the drug. But if this should have happened here we would have observed a decrease in the e.s.r. peaks. This has been only observed in the present study with the neutral spin probe **IV** above 35×10^{-6} M. For the other probes tested under our experimental conditions, no aggregation is detected by a broadening of the e.s.r. lines up to 2×10^{-4} M and the number of binding sites lies between one and two per molecule of calmodulin. The protonation of the basic spin-label **III** occurs under the present experimental condition, in the presence of calmodulin, around pH 6.2 and for the protonated drug the binding is reinforced. Spin-label **IV** cannot be protonated and its binding remains less avid and constant. The binding of spin-label **I** could be considered with the possible occurrence of H-bonding of the amido group with a negative acceptor on calmodulin. The spin label **I** displays an acidic behaviour, which gives very different optimal conditions for the binding to calmodulin compared with the basic spin-probes (**II**, **III** and **V**). The binding of **I** decreases when the pH becomes acidic, in accord with previous results (Rainteau *et al.*, 1984) and this is confirmed by a slight increase of the antagonism of the myosin light chain kinase activation at alkaline pH (Table 2). We assume that the polarization of the amido group is slightly decreased when pH becomes acidic, which supports a decreased binding to calmodulin.

It is noticeable that only the comparison between the binding of a neutral or a basic probe at various pH values allows to attribute the reinforcement of binding to be due to a change in the polarity of the probe and not to a change in the calmodulin conformation with pH (Fig. 4).

The stereospecificity of the interaction is illustrated by the differences of binding for spin-labels **II** and **III**. At acidic pH (6.0 or 6.4) an additional methylene group in the side-chain modifies only slightly the binding

measured by e.s.r. (Table 1) but it reinforces significantly the antagonism of myosin light chain kinase activation (Table 2). At pH 7.5, where the charge of the amino group is decreased, the position of the terminal amino group is less critical and the inhibitory potency of **II** is close to that of **III**.

Lengthening of the side-chain by a piperazine moiety (**V**) increases again the inhibitory potency in the enzymic assay but the binding parameters estimated by e.s.r. are not significantly changed compared with other basic probes. This suggests a special antagonizing role for the piperazine at the interface of calmodulin with its target enzyme.

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