Biochemical Pharmacology, Vol. 33, No. 10, pp. 1679–1681, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00
Pergamon Press Ltd.

SHORT COMMUNICATIONS

Analysis of the mechanism of the magnesium effect on the warfarin–albumin interaction

(*Received* 19 *July* 1983; *accepted* 28 *November* 1983)

In earlier studies [1, 2] it was found that the physiologically important ions Ca^{2+} and Cl^- influence the binding of warfarin and other drugs to albumin*. Ca^{2+} affects the binding of warfarin by altering the so-called N–B transition of albumin which takes place in the pH region 6 to 9. Although Cl^- also affects the binding of warfarin to albumin by influencing this conformational transition, its interference with the warfarin–albumin interaction is dominated by competition for the warfarin binding site. Mg^{2+} is another ion which is present in serum in a considerable amount. Therefore in this communication we report on the effect of Mg^{2+} on the binding of warfarin to albumin.

Pedersen [3] and Eatough *et al.* [4] found evidence that Ca^{2+} and Mg^{2+} share their binding sites on the albumin molecule. Therefore the effect of Mg^{2+} on the binding of warfarin to albumin can be expected to be similar to the effect of Ca^{2-}. To test whether this is, in fact, the case, we studied the effect of Mg^{2+} on the binding of warfarin to albumin by means of circular dichroism (CD) and equilibrium dialysis. CD has proven to be very useful for detecting alterations in the N⇄B equilibrium of albumin, when warfarin is used as a marker [5]. The effect of Mg^{2+} on the N–B transition under different conditions is shown in Fig. 1. The Ca^{2-} effect on the N–B transition was investigated under the same circumstances. Preliminary experiments at pH = 7.4, 8.4 and 9.0 showed that Ca^{2+} and Mg^{2+} do not have a qualitative effect on the induced CD spectrum of the warfarin–albumin complex; i.e. the maximum of the induced CD signal is found always around 310 nm (λ_{max}). From Fig. 1 it is clear that Mg^{2+} and Ca^{2+} cause a change in the θ_{obs}-pH profile, reflecting a shift in the N⇄B equilibrium of albumin. At the concentrations used, the effects of Mg^{2-} and Ca^{2+} are indistinguishable from each other. Due to the presence of Mg^{2+} or Ca^{2+} the pH_{50} (pH where 50% of the albumin is in the B conformation) shifts from 7.4 to 7.1 and the value of the Hill coefficient [6, 7] (a measure of the cooperative nature of the N–B transition of albumin) changes from 1.2 to 1.5.

We also investigated the effect of Mg^{2+} on the binding of warfarin to albumin at various pH's by means of equilibrium dialysis in the way described previously [1], see Fig. 2. To minimize a Donnan effect on the determination of free warfarin concentration (c_{free}) the experiments were performed in the presence of 100 mM Cl^-. The CD experiments were also carried out in the presence of 100 mM Cl^- in order to make the circumstances identical to those for the equilibrium dialysis experiments. We found that at pH = 6 Mg^{2+} has no significant effect on the binding of warfarin to albumin, whereas at pH = 9 this binding is decreased markedly by Mg^{2-}. These results suggest that Mg^{2-} displaces warfarin only when albumin is in the B conformation. If this is the case, then the magnitude of the displacing effect of Mg^{2+} will parallel the fraction of the protein occurring in the B conformation. In such a case a continuous increase in the magnitude of the Mg^{2+} effect on c_{free} with pH can be expected in the pH range 6–9. However, in Fig. 2 it is shown that such an effect occurs only for pH values > 7.8. At lower pH values Mg^{2+} has no significant

* Albumin here means human serum albumin.

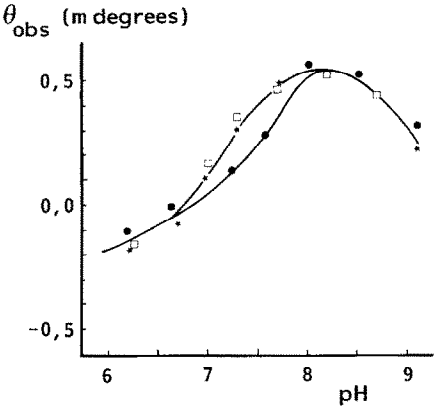


Fig. 1. θ_{obs} of warfarin–albumin complexes at 310 nm as a function of pH. Human serum albumin (lot number 307100, Biotest GmbH, Frankfurt a.M., G.F.R.) was deionized before use [1]. [Albumin] = 6×10^{-5} M and [warfarin] = 6×10^{-6} M. The pH was adjusted with M NaOH. θ_{obs} was measured as described previously [1]. Optical path length 20 mm. Temperature 25°C. [Cl^-] = 100 mM (○); [Cl^{-1}] = 100 mM, and Mg^{2+} = 10 mM (*); [Cl^-] = 100 mM, and [Ca^{2+}] = 5 mM (□).

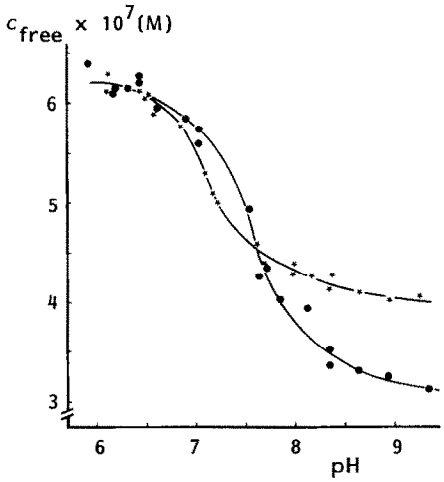


Fig. 2. Free warfarin concentrations as a function of pH. Dialysis data obtained as described previously [1]. Warfarin concentrations were measured with a liquid scintillation counter (Packard, model 2425) using [^{14}C]warfarin (Amersham, batch nr. 23). [Albumin] = 6×10^{-5} M, [warfarin] = 6×10^{-6} M and [Cl^-] = 100 mM. In the presence of 10 mM Mg^{2+} (*) and in the absence of Mg^{2+} (○). In the region pH = 8.2 – 9.3 borate buffers were used. Borate itself does not have any effect on the warfarin–albumin interaction [1]. At pH's lower than 8.2 only M NaOH was used to adjust the pH. Temperature 25°.

effect on c_{free}, whereas around pH = 7.2 in the presence of Mg^{2+} c_{free} is a little lower than in the absence of Mg^{2+}. These results therefore seem to contradict our suggestion that the displacement of warfarin by Mg^{2+} occurs only when the albumin is in the B conformation. However, we found (Fig. 1) that Mg^{2+} affects the N–B transition as well. For instance, an increase in the Hill coefficient can be noticed. An increase in the Hill coefficient implies that for pH values $< pH_{50}$ the N conformation is favoured. This effect explains why the curves in Fig. 2 coincide at pH < 6.8. At pH values $> pH_{50}$ an increase in the Hill coefficient implies a shift of the N⇄B equilibrium towards the B conformation. When Mg^{2+} displaces warfarin from the B conformation of albumin this effect at $pH > pH_{50}$ is more pronounced as a result of the increase in the Hill coefficient. The Mg^{2+} effect on the N–B transition of albumin also results in a shift of the pH_{50} to lower values. Depending on the pH region this results in different overall effects of Mg^{2+} on c_{free}. At relatively low pH, say pH < 7.5, where the fraction of albumin in the B conformation is small, a shift in the N⇄B equilibrium towards the B conformation due to the presence of Mg^{2+} will lead to a decrease in c_{free}, since in the presence of Mg^{2+} too the B conformation of albumin has a higher affinity for warfarin than has the N conformation. At higher pH, say pH > 7.8, where a considerable fraction of albumin is already in the B conformation, the Mg^{2+} effect on c_{free} because of a shift in the N⇄B equilibrium towards the B conformation is dominated by the fact that Mg^{2+} displaces warfarin from albumin in the B conformation. This is why the curves shown in Fig. 2 intersect. Therefore, at the physiological pH only a minor effect of Mg^{2+} on the concentration of free warfarin can be observed. However, in the case of drugs exerting a stronger effect on the N⇄B equilibrium than warfarin such as diazepam, giving a pH_{50} of about 6.0 [8], or oxyphenbutazon, giving a pH_{50} of about 8.1 [9], it is still a question whether a drug displacing effect is compensated for by a shift in the N⇄B equilibrium at pH = 7.4.

It should be noticed that on the alkaline side of pH = 8 a remarkable difference between the pH-dependences of c_{free} and θ_{obs} was found. In the pH region mentioned, albumin exists almost entirely in the B conformation and as far as the N–B transition is concerned, one expects only a small increase in θ_{obs} and a corresponding small decrease in c_{free} going from pH 8 to 9. The small decrease in c_{free} is actually found; however instead of the small increase in θ_{obs} a considerable decrease is found. This strong pH effect on the induced ellipticity of the warfarin–albumin complex is probably due to a conformational change in the immediate vicinity of the warfarin binding site. However, a significant effect on the stability of the warfarin complex is not exerted.

It will be interesting to know whether Mg^{2+} displaces warfarin from its binding site on the B conformation of albumin by a competitive mechanism. A double reciprocal plot ν_L^{-1} vs L_f^{-1} is frequently used [10, 11] to determine whether there is competition between ligands in their protein binding. The symbol ν_L is the number of molecules of ligand L bound per protein molecule and L_f is the free ligand concentration. This plot resembles the Lineweaver–Burk plot, which is used in enzyme kinetics. When the straight lines obtained using different concentrations of the binding inhibitor intersect on the ν_L^{-1} axis the conclusion is occasionally drawn [10] that the displacement of the ligand by the inhibitor is competitive. However, in the case of a non-competitive displacement the point where the straight lines intersect is also on the ν^{-1} axis, see Appendix. A different method is therefore needed to find out whether the mechanism involved in the displacement of warfarin by Mg^{2+} is a competitive one. Such a method follows directly from the general equation 3 given in the appendix and requires the value of the slope H of a ν^{-1} vs c_{free}^{-1} plot. At first, however, it has to be confirmed that under the experimental conditions used the double reciprocal plot is linear. In an earlier study [12] it was found that this is indeed the case. Accurate values of H can easily be obtained by determining c_{free} in plural at two extreme warfarin concentrations over the concentration range where the double reciprocal plot is linear. The slope H of a ν^{-1} vs c_{free}^{-1} plot (see Appendix and Fig. 3) will vary with the total Mg^{2+} concentration $[Mg^{2+}]$. It is pointed out in the appendix that a plot of H vs $[Mg^{2+}]$ will lead to a straight line with a positive slope when there is competition between Mg^{2+} and warfarin for one common binding site. It can be seen that the plot of H vs $[Mg^{2+}]$ is a curve which develops into a plateau at increasing $[Mg^{2+}]$. This means that a simple competitive mechanism between Mg^{2+} and warfarin in their binding to albumin can be excluded. The difference in charge and hydrophobicity of Mg^{2+} and (anionic) warfarin is also an argument against a common binding site. Displacement of warfarin by Mg^{2+} at high pH through a shift in the N⇄B equilibrium towards the N conformation can be ruled out since Mg^{2+} favours the B conformation. Therefore it is most likely that the origin of the warfarin displacing effect of Mg^{2+} at high pH is due to a local conformational change in the warfarin binding region on the albumin molecule. On the other hand one expects that such a conformational change influences λ_{max} of the induced CD spectrum and this is actually not found.

The warfarin displacing effect cannot be detected when the protein is in the N conformation. This could indicate that the affinity of albumin for Mg^{2+} is considerably lower when albumin is in the N conformation than when it is in the B conformation. A difference in the affinity of albumin in the two distinct conformational states for Mg^{2+} is in accordance with a calorimetric study [4] showing that the binding of Mg^{2+} to albumin increases with pH, unfortunately it is not possible to calculate a binding constant at different pH's from this study. It cannot be excluded, however, that in the N conformation the binding sites for Mg^{2+} and warfarin are farther apart than in the B conformation and therefore, that the binding of Mg^{2+} does not interfere with the binding of warfarin when the protein is in the N conformation.

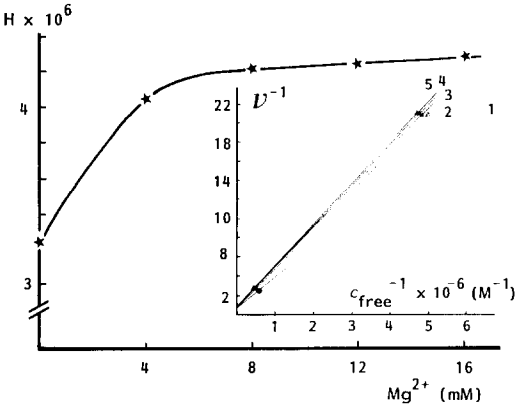


Fig. 3. Effect of Mg^{2+} on the slope of H of ν^{-1} vs c_{free}^{-1} plots as shown in the insert. Insert: plots of ν^{-1} vs c_{free}^{-1} in 100 mM Cl^-. [Albumin] = 6×10^{-5} M, pH adjusted with borate at pH = 9.0. In the absence of Mg^{2+} (1) and in the presence of Mg^{2+} in concentrations of 4(2), 8(3), 12 (4) and 16 (5) mM respectively. Each point is the mean of 5 determinations. The relative S.D, in c_{free} is 3%, resulting in a relative S.D. in H of 4%. Temperature 25°C. Under the experiment conditions the total Mg^{2+} concentration $[Mg^{2+}]$ is approximately equal to the free Mg^{2+} concentration. The intercept with the ν^{-1} axis is not significantly different from 1, which is in accordance with eqn 3 of the appendix.

Earlier [1] we found that Ca^{2+} does not displace warfarin from albumin, regardless of whether the protein is in the N or in the B conformation. The difference between the effects of Mg^{2+} and Ca^{2+} on the warfarin–albumin interaction is surprising because Mg^{2+} and Ca^{2+} should share their binding sites on the albumin molecule [3, 4].

In summary: to elucidate the mechanism of the Mg^{2+} effect on the warfarin–albumin interaction the experiments had to be done under non-physiological conditions. It is not possible to establish competitive displacement in protein binding using only the double reciprocal plot ν^{-1} vs L^{-1}. Mg^{2+} displaces warfarin from the B conformation of albumin by a local change in the warfarin binding region. At the physiological pH of 7.4 the effect of Mg^{2+} on the binding of warfarin to albumin can be neglected because the displacement of warfarin from albumin in the B conformation is compensated for by an effect of Mg^{2+} on the N⇄B equilibrium as can be reflected in a change in the Hill coefficient and the pH_{50} of the N–B transition. The Mg^{2+} effect on the binding of warfarin to albumin is significant only at pH > 8.

Acknowledgement—The authors would like to thank Dr. M. R. Egmond for his helpful discussions concerning the Appendix. They also would like to express their gratitude to the Biotest-Serum-Institut (Frankfurt am Main, G.F.R.) for the gifts of human serum albumin.

Department of Pharmaceutical Chemistry
Subfaculty of Pharmacy
State University of Utrecht
Catharijnesingel 60
3511 GH Utrecht
The Netherlands

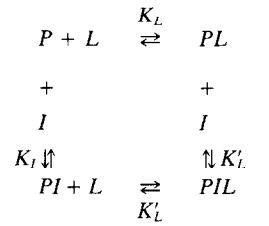
WILLEM F. VAN DER GIESEN*
JAAP WILTING

REFERENCES

1. J. Wilting, W. F. van der Giesen, L. H. M. Janssen, M. M. Weideman, M. Otagiri and J. H. Perrin, *J. biol. Chem.* **255**, 3032 (1980).
2. J. Wilting, B. J. 't Hart and J. J. de Gier, *Biochim. biophys. Acta* **626**, 291 (1980).
3. K. O. Pedersen, *Scand J. Clin. Lab. Invest.* **29**, 427 (1972).
4. D. J. Eatough, T. E. Jensen, L. D. Hansen, H. F. Loken and S. J. Rehfield, *Thermochim. Acta* **25**, 289 (1978).
5. J. Wilting, M. M. Weideman, A. C. J. Roomer and J. H. Perrin, *Biochim. biophys. Acta* **579**, 469 (1979).
6. A. Fersht, *Enzyme Structure and Mechanism*, p. 217. W. H. Freeman and Company, Reading and San Francisco (1977).
7. L. Stryer, *Biochemistry*, p. 68. W. H. Freeman and Company, San Francisco (1981).
8. J. Wilting, B. J. 't Hart and J. J. de Gier, *Biochim. biophys. Acta* **626**, 291 (1980).
9. J. H. M. Dröge, L. H. M. Janssen and J. Wilting, *Pharm. Weekbl. (Sci.)* **5**, 228 (1983).
10. E. Tsutsumi, T. Inaba, W. A. Makou and W. Kalow, *Biochem. Pharmac.* **24**, 1361 (1975).
11. U. Kragh-Hansen, *Pharmac. Rev.* **33**, 17 (1981).
12. W. F. van der Giesen, Thesis: Physicochemical behaviour of some 4-hydroxycoumarins, especially the binding of warfarin to human serum albumin, Utrecht, 1982.

Appendix

When a ligand (L) is bound by a protein (P) in the presence of a binding inhibitor (I) the reactions presented in scheme 1 have to be considered,



Scheme 1.

provided that P exists in one conformational state in the absence of L and I and that only one molecule of L and/or one molecule of I can be bound per molecule of P. The number of molecules L bound per molecule P (ν_L) at given concentrations is given by the following equation

$$\nu_L = \frac{[PL] + [PIL]}{[P] + [PL] + [PI] + [PIL]} \tag{1}$$

This equation can be rearranged in terms of binding constants and measurable concentrations. If the numerator and denominator of eqn 1 are divided by $[PL]$. I_f and if subsequently the binding constants K_L, K_I and K'_I are introduced, then the following equation will be obtained:

$$\nu_L = \frac{L_L.L_f(1 + K'_I.I_f)}{1 + K_I.I_f + K_L.L_f(1 + K'_I.I_f)} \tag{2}$$

where L_f and I_f are free concentrations of L and I respectively.

When ν_L is considered as a function of L_f, it is convenient to convert eqn 2 into eqn 3 to achieve a simple relationship between these parameters:

$$\nu_L^{-1} = \frac{1 + K_I I_f}{K_L\,(1 + K'_I.I_f)} \cdot L_f^{-1} + 1. \tag{3}$$

Plotting ν_L^{-1} versus L_f^{-1} then leads to straight lines with an intercept on the ν_L^{-1} axis at 1, regardless of whether all the reactions presented in scheme 1 are involved or not. An equation similar to eqn 3 can be derived when P has more than one binding site for I; however, when P has more than one binding site for L the derivation of such an equation is no longer possible.

Eqn. 3 can be written as:

$$\nu_L^{-1} = H.L_f^{-1} + 1 \tag{4}$$

where

$$H = \frac{1 + K_I.I_f}{K_L(1 + K'_I.I_f)} \tag{5}$$

The figure obtained when H is plotted vs I_f is indicative of the mechanism of the displacement. When $I_f \to 0$, H approaches K_L^{-1}, regardless of the values of K_I and K'_I. When $I_f \to \infty$, H approaches $K_I/K_L.K'_I$. For $K_I > K'_I$ the curve will have a positive slope, but for $K_I < K'_I$ it will have a negative slope. If $K_I = K'_I$, then the line obtained will be horizontal. In an extreme case such as $K'_I = 0$ a straight line with a positive slope is obtained. $K'_I = 0$ means a competition between I and L for a single common binding site. In that case K_L and K_I can be calculated.

After submitting the manuscript of this communication for publication a paper of U. Kragh-Hansen appeared in *Biochem. Pharmac.* **32**, 2679 (1983), dealing with the analysis of competitive binding at comparable concentrations of ligand, inhibitor and protein. On the contrary in our theoretical treatment of the effect of an inhibitor on ligand protein binding the inhibitor is present in excess and also other displacing mechanisms than the competitive one are taken into account.

* To whom all correspondence should be addressed.