formulas (Fig. 2c) and whose effects on HMT activity have not been reported to date. These drugs inhibited the enzyme activity as expected for the structure–inhibition relationship, and the mode of the inhibition was competitive with respect to histamine. Therefore, additional to our previous report [4], it may be concluded that the essential structure of the inhibitors affecting the HMT activity was CH_2—CH—N, except for famotidine. The inhibitory effect of famotidine was evidently competitive with respect to histamine (Fig. 1b–i); therefore, this effect was considered to be due to the inhibition of binding of the amine to the HMT molecule. Thithapandha and Cohn [7] have demonstrated that amodiaquine, chlorguanide and cycloguanil (antimalarials), whose chemical structures are quite different from our hypothetical one, significantly inhibit the activity of HMT derived from guinea pig brain. Taken together, we speculate here that the binding site of the HMT molecule for histamine may not be as definite as demonstrated in the present study; a more detailed molecular mechanism of the inhibitory effect that can explain this discrepancy should be examined.

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Vitamin K reductases in normal and in warfarin-resistant rats

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Vitamin K is required for the formation of gammacarboxyglutamic acid (Gla) residues in proteins. The Gla-residues are formed in a carboxylation reaction in which vitamin K hydroquinone (KH_2) is converted into an epoxide (KO). The conversion of vitamin K quinone (K) into KH_2 may be accomplished either by a dithiol-dependent reductase or by an NADH-dependent enzyme. The dithiol-dependent reductions of KO and K are extremely sensitive to the action of oral anticoagulants such as warfarin [1]. KH_2-dependent carboxylase activity as such can be assessed by reducing vitamin K into KH_2 in a chemical way before it is added to the reaction mixtures. The activity of the two K reductases, on the other hand, is frequently determined in an indirect way, namely by starting the carboxylation reaction with either K + DTT or with K + NADH. A direct measurement of the K reductases by establishing the production of KH_2 is less reliable because the hydroquinone is unstable and rapidly re-oxidized into its quinone form by traces of oxygen.

Materials and methods

Animals. Warfarin-resistant rats of the Scottish resistance strain (HS) were initially obtained from the Agricultural Science Service, Tolworth Laboratory (Tolworth/Surbiton, Surrey, U.K.). Warfarin-susceptible male Wistar rats were obtained from Winkelman (Borchen, F.R.G.). The animals entered the experiments at the age of 16 weeks and from that moment they were housed singly so that their water consumption could be checked. Warfarin treatment was performed by adding 5 mg/l of warfarin and 10 mM sodium phosphate buffer (pH 9.0) to the drinking water. Control animals received the same buffer without warfarin. The buffer was refreshed every day and the daily consumption was 25–30 ml. The intake of warfarin was routinely checked by measuring the serum levels with the method described by Thijssen *et al.* [2]. After 5 days the animals were sacrificed under ether anesthesia and the livers were excised for the preparation of microsomes [3].

Assays. Unless stated otherwise, the microsomal pellets were washed by repeated suspension and centrifugation (1 hr at 105,000 *g*): twice with buffer A (0.1 M NaCl, 50 mM Tris/HCl, pH 7.4) and once with 1 M NaCl in buffer A. The final microsomal pellet was resuspended in buffer A to a protein concentration of 40 mg/ml and stored at −80° until use. KH_2-dependent carboxylase was assayed in 0.25 ml reaction mixtures containing 4 mg of microsomal proteins, 0.5% (w/v) CHAPS, 4 mM FLEEL, 2 mM DTT, 0.1 M NaCl, 1 M $(NH_4)_2SO_4$, 50 mM Tris/HCl, pH 7.4, 0.4 mM KH_2 and 0.01 mCi $NaH^{14}CO_3$. Incubations were performed at 25° for 30 min and the reaction was terminated by adding 1 ml of 5% (w/v) trichloroacetic acid [3]. [K + DTT]-dependent carboxylation was measured under the same conditions but with K instead of KH_2. [K + NADH]-dependent carboxylation was detected by replacing KH_2 by K, and DTT by 2 mM NADH. Endogenous carboxylatable protein precursors were assayed by incubating the reaction mixtures for 1 hr at 25° in the absence of FLEEL and with KH_2 as a coenzyme. Protein concentrations were established according to Sedmak and Grossberg [4].

Results

The *in vitro* sensitivity for warfarin was measured in the hepatic [K + DTT]-dependent carboxylase assay, and the

warfarin-resistant animals were compared with normal Wistar rats. As shown in Fig. 1, the concentration curves of warfarin were closely similar in the two types of microsomes and the inhibitor concentrations required for 50% inhibition were 1–2 μM in both cases. Comparable results have been reported by Thijssen [5] for KO reductase.

The effect of *in vivo* warfarin treatment was measured as follows. Washed microsomes were prepared from the livers of four groups of 8 rats each. Two groups were formed by non-treated and warfarin-treated susceptible rats respectively, and the two other groups consisted of non-treated and warfarin-treated HS rats. All livers were processed and tested separately. The enzyme activities measured were: KH_2-dependent carboxylase, [K + DTT]-dependent carboxylase and [K + NADH]-dependent carboxylase and the results are summarized in Table 1. No difference was found between the warfarin serum levels of warfarin-treated Wistar and HS rats. The *in vivo* effect of the drug in susceptible animals was evident from the accumulation of carboxylatable precursor proteins in the microsomes (cf. columns 1 and 2). As was to be expected, a similar effect was not seen in resistant rats (columns 3 and 4). In susceptible, non-treated rats (column 1) the KH_2-stimulated $^{14}CO_2$ incorporation was much higher than

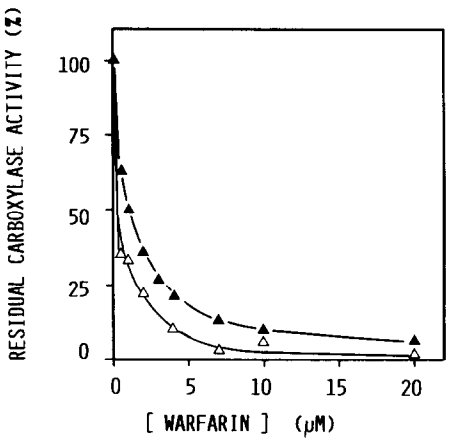

Fig. 1. Warfarin-inhibition of [K + DTT]-dependent carboxylase. Time-course experiments between 0 and 30 min were performed at each warfarin concentration and the initial carboxylation rate was calculated and expressed as a percentage of the non-inhibited reaction. 100% activity corresponds to 2430 dpm/min in susceptible rats (▲——▲) and to 2970 dpm/min in warfarin resistant rats (△——△).

the carboxylation in the presence of either K + DTT and K + NADH. This demonstrates that if K is used as a coenzyme, the reduction to KH_2 is the rate limiting step in the carboxylation reaction, at least under the conditions used in this assay. Therefore, the data obtained with K represent the respective reductase activities rather than the carboxylase activity *per se*. From the second column in Table 1 it appears that warfarin treatment of the animals not only leads to an accumulation of carboxylatable protein precursors in the microsomes, but also to an increased activity of both KH_2-dependent carboxylase and NADH-dependent K reductase. The fact that the DTT-dependent K reductase activity was strongly decreased indicates that, despite the extensive washing procedure, warfarin had either remained bound to the microsomal enzyme system or that the drug had inactivated the reductase in an as yet irreversible way. In warfarin-resistant rats (Table 1, columns 3 and 4) both the amount of [K + DTT]-stimulated carboxylase as well as the level of endogenous carboxylatable precursor proteins were unaffected by the warfarin treatment and the data were comparable with those obtained in non-treated susceptible rats. In this respect it should be mentioned that—like in the case of KO reductase [5]—also the DTT-dependent K reductase from warfarin-treated HS rats showed a strong increase of enzymatic activity during the subsequent washing cycles which are included in the procedure we normally use for the preparation of microsomes. This was demonstrated in an experiment in which resistant and susceptible animals were treated with warfarin. Microsomes were prepared from the various livers and samples were taken before washing and after each subsequent washing cycle. As is shown in Fig. 2 the enzymatic activity in the microsomes from susceptible animals remained low throughout the washing procedure, whereas the activity in microsomes from resistant rats increased after each subsequent step until it was comparable with that of non-treated animals. From Table 1 it becomes also clear that as compared to normal Wistar rats, the HS rats are characterized by high levels of KH_2-dependent carboxylase and NADH-dependent K reductase. The levels of these two enzyme activities are closely similar to those found in warfarin-treated normal rats.

Discussion

Most studies up till now concerning warfarin resistance in rats have been performed with animals of the Welsh resistant strain. It has been demonstrated [1] that the resistance in these animals originates from a strongly reduced inhibitory effect of warfarin on the dithiol-dependent reductase activities (both, KO and K reductase).

A second warfarin-resistant strain, which had developed in Scotland independent of the Welsh strain, was described

Table 1. Effects of warfarin treatment on vitamin K-dependent enzymes in the liver

	$^{14}CO_2$ incorporated (dpm × 10^{-3}) in microsomes from:			
	warfarin-susceptible rats		warfarin-resistant rats	
Enzyme activity	non-treated	warfarin-treated	non-treated	warfarin-treated
F L E E L carboxylation				
+ KH_2	165 (±21)	292 (±36)	284 (±34)	329 (±36)
+ K + DTT	82 (±8)	14 (±4)	98 (±10)	95 (±8)
+ K + NADH	8.3 (±2.5)	22.2 (±2.2)	23.7 (±3.6)	25.2 (±4.7)
Protein carboxylation				
+ KH_2	0.8 (±0.2)	5.5 (±0.5)	1.4 (±0.3)	1.8 (±0.3)

Peptide carboxylation was performed in the presence of 4 mM F L E E L, protein carboxylation was measured in the absence of the pentapeptide. Blanc values (no vitamin K and no reductant added) ranged between 200 and 500 dpm and were subtracted. The data represent the means (±SEM) of eight individual rats.

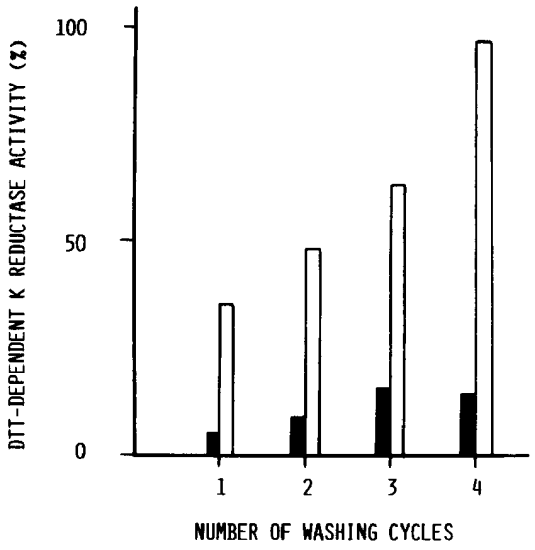

Fig. 2. Effect of washing of microsomes. Warfarin-treated normal (closed bars) and warfarin-resistant (open bars) rats were used for the preparation of microsomes. Step 1 represents the non-washed resolubilized microsomes. Two washing cycles with a low-salt buffer (steps 2 and 3) were followed by a high-salt wash (step 4) and the [K + DTT]-dependent carboxylase activity was measured and expressed as a percentage of that in non-treated animals. 100% activity corresponds to 2570 dpm/min in susceptible rats and to 3033 dpm/min in resistant rats.

by Greaves and Ayres [6] and it was demonstrated by Thijssen [5] that in HS rats *in vitro* sensitivity of KO reductase for warfarin inhibition was comparable to that in normal rats but that the inhibitory effect was reversible. Here we report that warfarin treatment of HS rats also decreases the DTT-dependent K reductase activity but that—like KO reductase [3]—the enzyme can be re-activated by an extensive washing procedure. This is in contrast to the situation in susceptible rats, in which warfarin seems to be tightly linked to the dithiol-dependent reductases in such a way that the drug cannot be removed. From these results we conclude that, although the mutation causing warfarin resistance seems to be different from that in Welsh resistant strains, also in HS rats both dithiol-dependent enzyme activities (KO reductase and K reductase) are similarly affected. This strongly supports the hypothesis, that the two dithiol-dependent reductase activities are exerted by only one enzyme.

It was also found that warfarin treatment of susceptible rats induces an increase of the KH_2-dependent carboxylase and of the warfarin-insensitive NADH-dependent K reductase activity. By this adaptation all enzymes required for the warfarin-insensitive pathway of carboxylation are mobilized to substantially higher levels, probably leading to a more efficient utilization of the resources of vitamin K quinone. Because KO reductase cannot be bypassed in a comparable way, it follows that the quinone will only be used once, and that the epoxide cannot be recycled anymore.

In the HS rats, the microsomal level of the enzymes involved in the [K + NADH]-dependent carboxylation was found to be permanently high, and independent of the treatment of the animals with vitamin K antagonists. Although it cannot be concluded as yet, it seems at least feasible that the mutation in the dithiol-dependent reductase causing warfarin resistance, also induces an increase of these enzymes via a mechanism which is comparable with that seen in susceptible rats during warfarin treatment. If this abnormal enzyme distribution contributes to the warfarin resistance remains to be seen, however.

Conclusions

Livers from warfarin-resistant rats of the Scottish strain contain high levels of KH_2-dependent carboxylase and NADH-dependent K reductase. The level of dithiol-dependent K reductase is normal. The latter enzyme is sensitive for warfarin but its binding to the drug is reversible, whereas in susceptible rats it is not.

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