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The Inhibitory Effect of Calumenin on the Vitamin K-dependent γ-Carboxylation System

CHARACTERIZATION OF THE SYSTEM IN NORMAL AND WARFARIN-RESISTANT RATS*

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Nadeem Wajih‡, David C. Sane‡, Susan M. Hutson§, and Reidar Wallin‡¶

From the Departments of ‡Internal Medicine and §Biochemistry, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157

The vitamin K-dependent γ-carboxylation system is responsible for post-translational modification of vitamin K-dependent proteins, converting them to Gla-containing proteins. The system consists of integral membrane proteins located in the endoplasmic reticulum membrane and includes the γ-carboxylase and the warfarin-sensitive enzyme vitamin K_1 2,3-epoxide reductase (VKOR), which provides γ-carboxylase with reduced vitamin K_1 cofactor. In this work, an *in vitro* γ-carboxylation system was designed and used to understand how VKOR and γ-carboxylase work together as a system and to identify factors that can regulate the activity of the system. Results are presented that demonstrate that the endoplasmic reticulum chaperone protein calumenin is associated with γ-carboxylase and inhibits its activity. Silencing of the calumenin gene with siRNA resulted in a 5-fold increase in γ-carboxylase activity. The results provide the first identification of a protein that can regulate the activity of the γ-carboxylation system. The propeptides of vitamin K-dependent proteins stimulate γ-carboxylase activity. Here we show that the factor X and prothrombin propeptides do not increase reduced vitamin K_1 cofactor production by VKOR in the system where VKOR is the rate-limiting step for γ-carboxylation (Wallin, R., Sane, D. C., and Hutson, S. M. (2002) *Thromb. Res.* 108, 221–226). These findings put calumenin in a central position concerning regulation of γ-carboxylation of vitamin K-dependent proteins. Reduced vitamin K_1 cofactor transfer between VKOR and γ-carboxylase is shown to be significantly impaired in the *in vitro* γ-carboxylation system prepared from warfarin-resistant rats. Furthermore, the sequence of the 18-kDa subunit 1 of the VKOR enzyme complex (Rost, S., Fregin, A., Ivaskeviclus, V., Conzelmann, E., Hortnagel, K., Pelz, H-J., Lappegard K., Seifried, E., Scharrer, I., Tuddenham, E. G. D., Muller, C. R., Storm, T. M., and Oldennburg, J. (2004) *Nature* 427, 537–541) was found to be identical in the two rat strains. This finding supports the notion that different forms of genetic warfarin resistance exist.

The vitamin K-dependent γ-carboxylation system is a multicomponent system of integral membrane proteins and lipids located in the endoplasmic reticulum (ER)[1] (1, 2). The system modifies vitamin K-dependent proteins post-translationally by adding an extra carboxyl group onto the γ-carbon of specific Glu residues located normally in the N-terminal part of the proteins (3). This modification converts Glu residues to Gla residues, which bind calcium (4). The calcium binding is essential for allowing the proteins to fulfill their physiological functions (5). In the blood coagulation system, seven proteins produced by the liver (prothrombin, factors VII, IX, and X, protein S, protein C, and protein Z) are dependent on the modification (3). Vitamin K-dependent proteins are also made outside the liver. They include the bone proteins osteocalcin and matrix Gla protein (6), the Axl ligand Gas6 (7), and four putative membrane proteins PRGP1, PRGP2, TmG3, and TmG4 (8, 9), some of which are located in the brain (9).

Activity measurements have shown that the vitamin K-dependent γ-carboxylation system is present in most cells and tissues isolated from an eukaryotic organism (10). This indicates that vitamin K-dependent proteins play vital roles in a variety of physiological processes. One example is the functioning of matrix Gla protein as a binding protein for bone morphogenetic protein-2 (11), a mechanism that may be involved in regulation of the growth factor activity of bone morphogenetic protein-2. Certain bleeding disorders (12) and pathological calcification of the arterial wall (13) have been associated with impaired vitamin K-dependent γ-carboxylation of proteins (14). In order to understand the underlying pathology caused by impaired γ-carboxylation, it is necessary to understand the γ-carboxylation system at the molecular level.

The vitamin K-dependent γ-carboxylation system consists of 1) the vitamin K-dependent γ-carboxylase, which requires the reduced hydroquinone form of vitamin K_1 (vit.K_1H_2) as cofactor, and 2) the warfarin-sensitive enzyme vitamin K_1 2,3-epoxide reductase (VKOR), which produces the cofactor (15). Concomitant with γ-carboxylation, the hydroquinone is converted to the metabolite vitamin K_1 2,3-epoxide, which is reduced back to the vit.K_1H_2 cofactor by VKOR (15). This interconversion of vitamin K metabolites is known as the vitamin K cycle (16). Of all of the components that constitute the γ-carboxylation system, only the γ-carboxylase has been purified, cloned, and characterized extensively (17). The γ-carboxylase is a 94-kDa protein with five putative transmembrane domains spanning the ER membrane (18). VKOR, on the other hand, appears to be an enzyme complex embedded in the ER membrane, where more than one protein is needed for expression of warfarin-

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¶ To whom all correspondence should be addressed: Dept. of Internal Medicine, Wake Forest University School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157. Tel.: 336-716-6166; Fax: 336-716-9821; E-mail: rwallin@wfubmc.edu.

[1] The abbreviations used are: ER, endoplasmic reticulum; vit.K_1H_2, reduced hydroquinone form of vitamin K_1; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; siRNA, small interfering RNA; RIPA, radioimmune precipitation assay; VKOR, vitamin K_1 2,3-epoxide reductase.

sensitive vitamin K_1 2,3-epoxide reducing activity (2). Recently, an 18-kDa hydrophobic ER membrane protein has been identified that appears to be a subunit of the VKOR protein-lipid membrane complex (19).

In our efforts to understand the VKOR enzyme complex and warfarin resistance, we compared kinetically the complex in normal rats and warfarin-resistant rats and used 4-azido-^{3}H-warfarin alcohol for labeling and identification of the warfarin binding component of VKOR (1). From experiments with partially purified preparations of VKOR from normal and warfarin-resistant rats, we demonstrated that the ER chaperone protein calumenin became labeled radioactively (1). Additional experiments showed that 1) calumenin was overexpressed in the liver of rats from our warfarin-resistant colony, 2) the protein sequence of calumenin in resistant rats and normal rats were identical, and 3) the recombinant protein inhibited VKOR activity and at the same time made VKOR more resistant to inhibition by warfarin (1). We concluded from these experiments that overexpression of calumenin in liver may represent one form of genetic warfarin resistance seen in the rat.

Most biochemical data on VKOR have been derived from experiments with crude liver microsomes. Consistent findings from these experiments have been 1) genetic warfarin resistance, attributed to VKOR, is associated with low VKOR activity in liver, and 2) resistant rats require high intake of vitamin K to avoid bleeding (20–22). Collectively, these observations have led to the consensus that warfarin resistance is linked to a genetically altered VKOR enzyme (16). Recently, this has been verified in humans and rats that are genetically resistant to warfarin (19). However, work with our colony of warfarin-resistant rats questions this model as the only mechanism for genetic resistance, since we have been able to produce γ-carboxylation systems from normal and warfarin-resistant rats that are kinetically indistinguishable. Thus, the mechanism(s) responsible for the poorly functioning γ-carboxylation system in our colony of warfarin-resistant rats could not be the result of dysfunctional VKOR and γ-carboxylase enzymes. In order to explore this mechanism(s) and also to expand our understanding of the γ-carboxylation system, we have undertaken further studies on the γ-carboxylation system in normal and warfarin-resistant rats. Here we demonstrate that calumenin is an inhibitory protein of γ-carboxylase and is associated with γ-carboxylase in the ER. In addition, we demonstrate that transfer of vit.K_1H_2 cofactor from VKOR to γ-carboxylase is impaired in our colony of warfarin-resistant rats, which may be a contributing factor to the poorly functioning γ-carboxylation systems normally observed in warfarin-resistant rats. Furthermore, we found the sequence of the 18-kDa VKOR subunit 1 (19) to be identical in normal and warfarin-resistant rats.

EXPERIMENTAL PROCEDURES

Materials

The γ-carboxylase peptide substrate FLEEL was from Sigma. The factor X propeptide ESLFIRREQANNILARVTRA and the prothrombin propeptide HVFLAPQQARSLLQRVRRA were synthesized by BACHEM Inc. (Torrance, CA).

The γ-carboxylase peptide SRRWKDHADMLKQY (residues 434–447 of the γ-carboxylase sequence) (23) was synthesized by the Protein Core Laboratory at Wake Forest University School of Medicine (Winston-Salem, NC). All peptides were more than 99.5% pure. CHAPS, vitamin K_1, warfarin, dithiothreitol, and the protease inhibitor mixture for use with mammalian cells and tissue extracts were from Sigma. Vit.K_1H_2 was prepared as described by Sadowski *et al.* (24), and vitamin K_1 2,3-epoxide prepared as described by Tishler *et al.* (25). $NaH^{14}CO_3$ (specific activity 54 mCi·mmol) was obtained from ICN Pharmaceuticals (Chicago, IL). The mammalian expression vector pcDNA3.1/Zeo$^-$ (Invitrogen) containing the calumenin cDNA was prepared as described (1). Recombinant calumenin was expressed in *E. coli* and purified as described by our laboratory (1). Antibodies to recombinant calumenin were raised in rabbits using Freund's adjuvant as approved by the Animal Care and Use Committee at the Wake Forest University School of Medicine. Rabbit antibodies to the γ-carboxylase peptide SRRWKDHADMLKQY were obtained by using a conjugate of the peptide and KLH as antigen. The peptide was coupled to KLH using the Inject carboxyl-reactive antibody production and purification kit with mcKLH from Pierce. All antibodies were affinity-purified on Sepharose column resins with the respective antigen attached as ligand. Stocks of affinity-purified antibodies were stored in 50% glycerol/water at −85 °C. Affinity-purified antibodies were attached to agarose beads using the Profound™ co-immunoprecipitation kit from Pierce. Antibodies to cyclophilin B and α-tubulin were from Affinity Bioreagents (Golden, CO) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively.

siRNA Design and Preparation

siRNAs directed against the calumenin RNA sequence (CALU-NM_001219) were synthesized by Dharmacon, Inc. (Lafayette, CO) using the Dharmacon *Smart* technology. A sophisticated algorithm was used to analyze the calumenin mRNA sequence, and four *Smart*-selected siRNA duplexes were synthesized and provided in a single pool for use in experiments aimed at silencing the calumenin gene. A human cyclophilin B siRNA duplex was provided as the positive control. The negative control consisted of four pooled nonspecific siRNA duplexes with an average GC content of 33%.

Cellular Transfection with siRNA

HEK293 cells were cultured in 10-cm dishes in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. siRNAs were combined with Lipofectin (Invitrogen) and transfected according to the protocol provided by Dharmacon, Inc. In preliminary experiments, transfected cells were harvested at times 0, 48, and 76 h and tested for content of cyclophilin B and calumenin proteins by Western blotting. Cells were incubated with the calumenin siRNA *Smart* pool for 72 h to determine its effect on γ-carboxylase activity. Cells were prepared for assays and Western blotting as described below.

Cellular Transfection with Calumenin c-DNA

γ-Carboxylase Assays—Transient transfection of COS-1 cells with the calumenin-pcDNA3.1/Zeo$^-$ expression vector was carried out with the FuGene 6 transfection system from Roche Applied Science. Forty-eight h after transfection, cells were harvested in phosphate-buffered saline and suspended in 250 mM phosphate, 0.5 M KCl, 20% glycerol, 0.75% CHAPS, pH 7.85 (buffer D) containing 10 μl of the Sigma protease inhibitor mixture per ml. Final protein concentration was 2 mg/ml.

Immunoprecipitations—Cells were transfected and harvested as described above and extracted with the modified RIPA buffer 50 mM Tris, 1% Nonidet P-40, 0.25% deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 7.4, containing 1 μg/ml each of the protease inhibitors aprotinin, leupeptin, and pepstatin as recommended by Upstate Biotechnology, Inc. (Lake Placid, NY).

Immunoprecipitation

Agarose beads with affinity-purified anti-calumenin antibodies were added to RIPA buffer extracts of transfected cells, and immunoprecipitation was carried out overnight at 4 °C on a rotating shaker. Removal of unbound proteins and wash of the agarose beads in spin cup columns were carried out as described in the instruction for use of the Profound™ co-immunoprecipitation kit from Pierce. Release of calumenin antigen and co-immunoprecipitating proteins bound to the anti-calumenin-agarose beads was accomplished with the pH 2.8 elution buffer included with the kit. Three protein fractions resulting from three consecutive spins of the cups with the pH 2.8 buffer were collected for analysis. Capturing of calumenin and associated proteins was also accomplished by open column chromatography of the RIPA buffer extracts on columns packed with the anti-calumenin-agarose beads. The column was washed with RIPA buffer containing 0.4 M NaCl before bound proteins were eluted with 4 M urea, 0.5 M NaCl in 0.1 M sodium acetate buffer, pH 4.0.

Animals

Male Sprague Dawley rats weighing 250–300 g were purchased from Zivic Miller Laboratories, Inc. (Pittsburgh, PA). A breeding colony of rats that are genetically resistant to warfarin is maintained at Wake Forest University School of Medicine. The colony originated at the University of Wisconsin in 1967, and rats were screened for warfarin resistance as described (20). All rats were housed, fed, and used for

experiments as approved by the Animal Care and Use Committee at Wake Forest University School of Medicine. Liver microsomes and partially purified preparations of VKOR were prepared according to procedures published by our laboratory (1, 26).

Extraction of Microsomes

For removal of microsomal luminal and peripherally bound membrane proteins, microsomes obtained from 4 g of rat liver were suspended in 8 ml of 100 mM Na_2CO_3, 1.2 M KCl, 0.025% deoxycholate, pH 11.5, with a Potter Elvehjelm glass homogenizer and centrifuged at 100,000 × *g* for 45 min (27). The pellet was resuspended in 8 ml of 50 mM Tris base with the same homogenizer and centrifuged a second time at 100,000 × *g* for 45 min. Pellets (extracted microsomes) were stored at −85 °C until used for experiments.

SDS-PAGE and Western Blotting

Prior to SDS-PAGE, proteins in buffer D-solubilized microsomes were precipitated with cold acetone (−20 °C) by mixing 1 part of a protein solution with 5 parts of acetone. The mixture was left overnight at −20 °C, and precipitated protein was harvested by centrifugation. The precipitated protein was washed consecutively with 5 ml of cold 10% trichloroacetic acid (4 °C) and 5 ml of cold ether/ethanol (1:1; v/v) (−20 °C). The protein pellet from the ether/ethanol wash was dried by N_2 aspiration and dissolved in SDS-PAGE running buffer containing 2% mercaptoethanol. Cell proteins in RIPA buffer were mixed with SDS-PAGE running buffer prior to electrophoresis. Western blotting of proteins transferred to polyvinylidene difluoride membranes was carried out as described (1).

Reverse Transcriptase-PCR and DNA Sequencing

Five μg of total RNA from livers of normal and warfarin-resistant rat was used for cDNA synthesis using oligo(dT) according to the manufacturer's recommendation (Invitrogen). The following primers were used to produce a cDNA corresponding to the ortholog on chromosome 1 in the rat representing the human gene on chromosome 16 identified by Rost *et al.* (19) to express the 18-kDa warfarin-sensitive subunit 1 of the VKOR enzyme complex. Primer design was based on the rat mRNA sequence with accession number XM 219366. The primer for the sense strand was 5′-TGT CGA CAT GGG CAC CAC CTG GAG-3′, which covered positions 14–37 bp in the published sequence. The antisense primer was 5′-ATG AGG TGG GAC CTC AGG GCT TTT TG-3′, which covered positions 494–519 bp. Thirty-five cycles of PCR were performed with the following conditions: denaturation at 94 °C for 30 s, annealing for 1 min at 60 °C, and then extension for 2 min at 72 °C. At the end of 35 cycles, the reaction was extended for 7 min at 72 °C. The PCR products were purified using the Qiagen PCR purification kit and separated on a 1.2% EtBr-stained agarose gel. The purified PCR product was then ligated into the TA cloning vector pCR 2.1 (Invitrogen). Positive clones were identified by EcoRI digestion and sequenced on both strands using M13 reverse and forward primers.

Enzyme Assays

Warfarin-sensitive VKOR activity was measured as described (28) by estimating the percentage conversion of vitamin K_1 2,3-epoxide to vitamin K_1. The vitamin and the epoxide were separated on a reversed phase C18 column in 100% methanol and quantified against external standards. γ-Carboxylase activity was assayed as described (29) as $^{14}CO_2$ incorporation into the synthetic peptide FLEEL. The reaction was either triggered by adding chemically reduced vitamin K_1H_2 (100 μg/ml) to the assay mixture or triggered by VKOR-produced reduced vitamin in a reaction mixture containing 40 μM vitamin K_1 2,3-epoxide and 8 mM dithiothreitol. Both assays were carried out as described (29) with saturating FLEEL concentration for the reactions.

RESULTS

Inhibition of γ-Carboxylase Activity by Calumenin—In a previous publication (1), we showed that calumenin inhibits VKOR and VKOR supported γ-carboxylase activity. The observed inhibition of VKOR-supported γ-carboxylase activity could have reflected a reduction in delivery of vit.K_1H_2 cofactor to γ-carboxylase, but similar results could also have been obtained if calumenin inhibits γ-carboxylase activity. To test this hypothesis, we expressed calumenin in COS-1 cells and measured γ-carboxylase activity in transiently transfected cells. As shown in Fig. 1*A*, specific γ-carboxylase activity was reduced

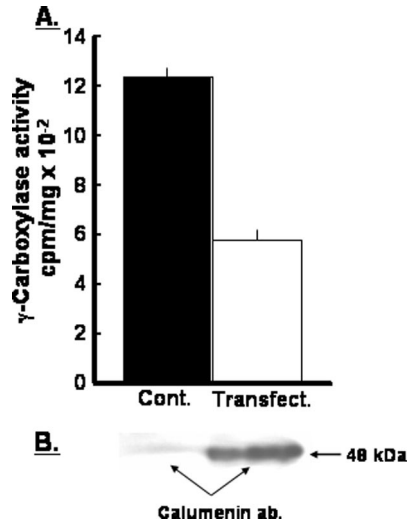

FIG. 1. **γ-Carboxylase activity in COS-1 cells transfected with the calumenin cDNA construct.** Cells were cultured and transfected transiently with the calumenin-pcDNA3.1/Zeo$^-$ cDNA construct for 48 h as described under "Experimental Procedures." *A*, enzyme activity was measured as $^{14}CO_2$ incorporation into the γ-carboxylase peptide substrate FLEEL. The activity was triggered by the addition of chemically reduced vitamin K_1H_2 to the test system (see "Experimental Procedures"). Measured activities represented by the *bars* are the average of three parallel incubations, and S.D. values are shown. *B*, Western blotting of cell proteins present in the assay samples with calumenin antibodies. Each lane were adjusted to contain 25 μg of cell protein.

53% in transfected cells (*Transfect.*) compared with the control (*Cont.*). Fig. 1*B* shows a Western blot of cell proteins present in the test system. Our affinity-purified calumenin antibodies recognized calumenin in control cells and transfected cells. However, a significantly higher concentration of calumenin was present in transiently transfected cells, supporting the hypothesis that calumenin does interfere with γ-carboxylase and inhibits its enzymatic activity. Additional evidence supporting this hypothesis was obtained from experiments with rat liver microsomes where we physically removed calumenin by carrying crude microsomes through an extraction procedure. Fig. 2, *C* and *D*, shows that calumenin was removed from microsomal vesicles when microsomes from warfarin-resistant rats were extracted sequentially with 1) the deoxycholate-containing carbonate buffer, pH 11.5 (*Carb.-Doc-pH11.5 ext.*) and 2) the 50 mM Tris base solution (*Tris base ext.*) (see "Experimental Procedures"). The proteins remaining in the microsomal vesicles after extraction are shown in Fig. 2*C*, *lane 4*. These proteins represent integral proteins of the ER membrane (27). Fig. 2*D* shows a Western blot of the proteins shown in Fig. 2*C*. Calumenin antibodies recognized calumenin in the deoxycholate-containing carbonate buffer, pH 11.5, and the 50 mM Tris base extracts (*lanes 2* and *3*) but failed to recognize calumenin among the proteins present in the extracted vesicles (*lane 4*). The extraction procedure did not remove γ-carboxylase and VKOR from the microsomal membrane, since no activity of these enzymes could be detected in the two extracts (data not shown).

As shown in Fig. 2, *A* and *B*, both VKOR and γ-carboxylase activities are significantly lower in microsomes (*Mic.*) from warfarin-resistant rats (*R*) compared with microsomes from normal rats (*N*). Fig. 2*A* also shows that VKOR in microsomes from warfarin-resistant rats is less sensitive to warfarin (*W*) inhibition (compare *Mic.*; *R* and *R* + *W*) than VKOR in microsomes from normal (*N*) rats (compare *Mic.*; *N* and *N* + *W*). However, when extracted microsomes (*Ext.-Mic.*) were tested for VKOR and γ-carboxylase activities, both specific activities

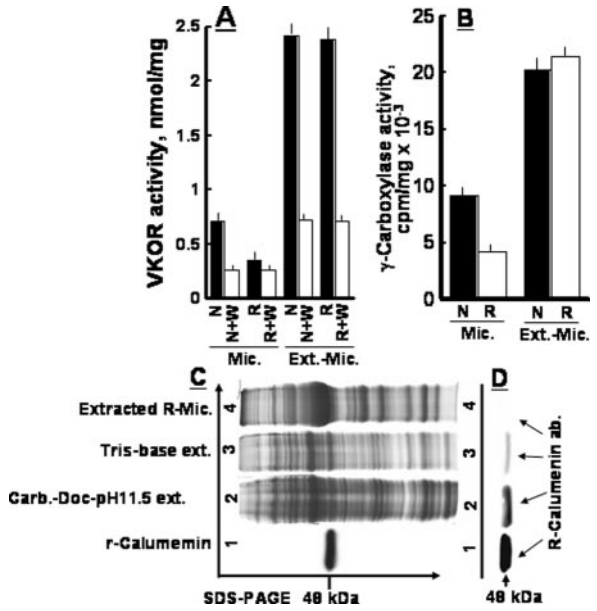

FIG. 2. **VKOR and γ-carboxylase activities in microsomal membrane vesicles devoid of luminal and peripherally bound proteins.** Rat liver microsomes from control rats (*N*) and warfarin-resistant rats (*R*) were isolated and extracted sequentially with a carbonate-deoxycholate, pH 11.5-containing buffer (*Carb.-Doc-pH11.5*) and 50 mM Tris base (*Tris-base*) as described under "Experimental Procedures." *C*, a Coomassie Blue-stained SDS-PAGE gel with recombinant calumenin (*lane 1*) and proteins in the deoxycholate-containing carbonate buffer, pH 11.5 (*Carb.-Doc-pH11.5 ext.*) (*lane 2*), and the 50 mM Tris-base (*lane 3*) extracts of microsomes from warfarin-resistant rats. The remaining membrane proteins in the microsomal vesicles are shown in *lane 4*. *D*, a Western blot of the proteins in *C*, *lanes 1–4*, with anti-calumenin antibodies. *A* and *B*, VKOR and γ-carboxylase activities measured in microsomes (*Mic.*) from control (*N*) and warfarin-resistant (*R*) rats and extracted microsomal vesicles (*Ext.-Mic.*) from control and warfarin-resistant rats. *Black* and *open bars* represent control samples and samples containing 10 μM warfarin, respectively. Enzyme activities were measured as described under "Experimental Procedures." γ-Carboxylase activity was triggered with chemically reduced vitamin K_1H_2. Each *bar* represents the average of three parallel incubations, and S.D. values are shown.

were found to be not significantly different in warfarin-resistant and normal rats (see *A* and *B*, *Ext.-Mic.*). The extraction procedure also produced microsomal vesicles from resistant and normal rats with VKOR activities that were equally sensitive to inhibition by warfarin (*A*, *Ext.-Mic.*). In summary, these results demonstrate that we could prepare γ-carboxylation systems from warfarin-resistant and normal rats with indistinguishable VKOR and γ-carboxylase activities and that the observed increase in these activities coincided with the removal of calumenin from the test system.

Calumenin siRNA Inhibits Synthesis of Calumenin and Stimulates γ-Carboxylase Activity—In this experiment, we used the highly specific technology of siRNA to directly demonstrate an effect of calumenin on γ-carboxylase activity. Fig. 3*A* shows that knockdown of the positive control protein cyclophilin B was lower at 72 h than at 48 h. Scanning of the immunoreactive protein bands showed integrated band intensities that were 49% (48 h) and 30% (72 h) of the control (100%) (see Fig. 3*C*). No significant change in α-tubulin cell concentration was measured, indicating that the cells were viable for 72 h. Based on these data, we elected to transfect the cells with calumenin siRNA for 72 h. As shown in Fig. 3, *B* and *D*, after 72 h calumenin was reduced 75% in cells transfected with calumanin siRNA (*Calumenin siRNA*) compared with the control (*Control siRNA*). Thus, the calumenin *Smart* siRNA pool had silenced the calumenin gene significantly, and its effect on γ-carboxylase activity could be determined. These results are shown in Fig. 4. Compared with the control (*Control siRNA*), specific γ-carboxylase activity triggered with chemically reduced vitamin K_1H_2 had increased 5-fold in cells transfected with calumenin siRNA (*Calumenin siRNA*). This result added strong support to the hypothesis that calumenin is an inhibitor of the γ-carboxylation system.

Calumenin Is Physically Associated with γ-Carboxylase in the ER—Calumenin is a chaperone for ER proteins and has been shown by electron microscopy to be associated with ER membrane proteins (30). Since we could demonstrate effects of calumenin on γ-carboxylase activity, we designed experiments to find out whether a physical association could be demonstrated between calumenin and γ-carboxylase. Affinity-purified anti-calumenin antibodies covalently linked to agarose beads were used for immunoprecipitation of calumenin and proteins associated with calumenin in the RIPA buffer extract obtained from COS-1 cells transfected transiently with the calumenin cDNA construct. γ-Carboxylase activity could be measured in these cell extracts (data not shown), which showed that the modified RIPA buffer (see "Experimental Procedures") used for extraction released γ-carboxylase from the ER membrane. Fig. 5*A* shows Western blots with anti-calumenin and anti-γ-carboxylase antibodies of proteins present in the wash and the three consecutively collected protein fractions eluted from the beads with the pH 2.8 elution buffer. Calumenin antibodies identified calumenin in the wash and in the second and third eluted fractions (Fig. 5*A*, *Calumenin abs.*). γ-Carboxylase was identified in the wash and the third eluted fraction (*A*, anti-*γ-carboxylase abs.*), suggesting that γ-carboxylase was attached to calumenin that was bound to the beads via the immobilized anti-calumenin antibodies. Control beads with rabbit IgG retained neither calumenin nor γ-carboxylase (data not shown). We also carried out immunoaffinity chromatography of the RIPA buffer extract using an open column packed with the beads. Fig. 5*B* shows an SDS-PAGE image of Coomassie Blue-stained proteins (*Coomassie stain*) retained by the column resin and eluted from the column with 4 M urea, 0.5 M NaCl, pH 4.0, and Western blots of these proteins with anti-calumenin (*Calumanin abs.*) and γ-carboxylase antibodies (*γ-carboxylase abs.*), respectively. The antibodies identified calumenin and γ-carboxylase in the retained fraction. When this experiment was carried out with a column packed with beads attached to rabbit IgG, the column retained neither calumenin nor γ-carboxylase (data not shown). Several other proteins were also retained by the anti-calumenin affinity column (see Fig. 5*B*, *Coomassie stain*). Some of these proteins are likely to represent additional ER proteins to which calumenin binds as a chaperone but could also represent additional proteins needed for assembly of the γ-carboxylation system as a supramolecular protein complex.

Cofactor Transfer between VKOR and γ-Carboxylase in Normal and Warfarin-resistant Rats—As shown in Fig. 2, extraction of liver microsomes produced γ-carboxylation systems from normal and warfarin-resistant rats that were indistinguishable when characterized by their individual VKOR and γ-carboxylase activities. If the systems in the two rats strains behaved identically, we expected VKOR-supported γ-carboxylase activity to also be the same in the extracted microsomes from the two rat strains. Fig. 6 shows FLEEL carboxylation by γ-carboxylase when the vitamin K_1H_2 cofactor was provided by VKOR as a result of vitamin K_1 2,3-epoxide reduction. As shown, the specific activity in the lipid-protein-detergent vesicles from normal rats was 2.5-fold higher than this activity measured in the vesicles from warfarin-resistant rats at saturating concentrations of FLEEL for the reaction. This result was reproduced in three separate experiments with three dif-

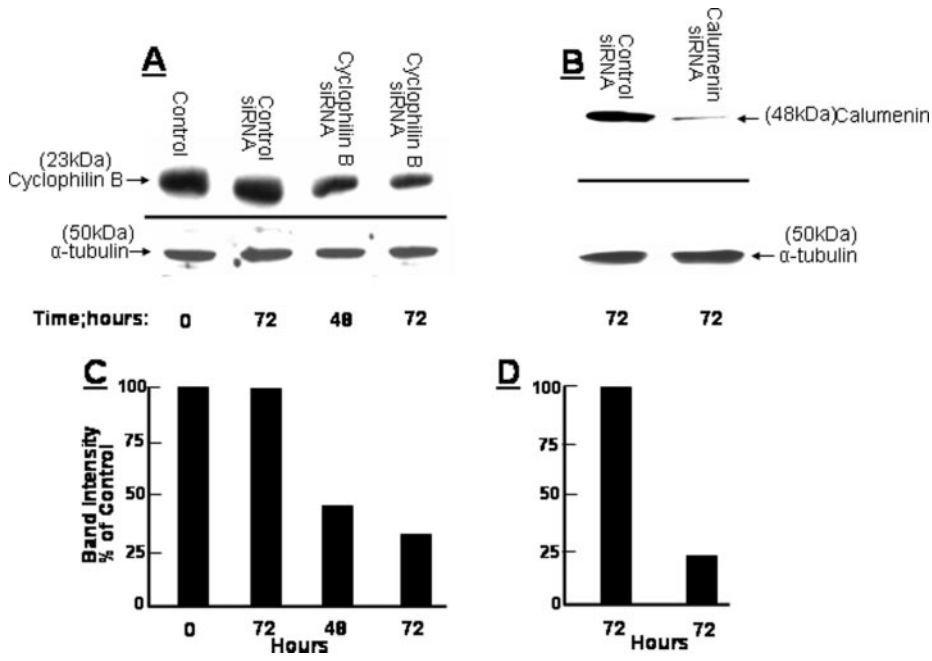

FIG. 3. **Calumenin siRNA reduces calumenin protein in HEK293 cells.** HEK293 cells were cultured and transfected with siRNAs as described under "Experimental Procedures." *A*, a control experiment with siRNA specific for cyclophilin B. Cells were harvested at times 0, 48, and 72 h, and cellular proteins were processed for SDS-PAGE and Western blotting (see "Experimental Procedures"). *Lanes* labeled *Control* and *Control siRNA* have proteins from cells transfected with Lipofectin only and nonspecific siRNA, respectively. *Lanes* labeled *Cyclophilin B* have proteins from cells transfected with cyclophilin B siRNA. Each lane was adjusted to contain equal amounts of protein (25 μg) and probed with antibodies against cyclophylin B and α-tubulin, respectively. *B*, a 72-h transfection experiment with control siRNA (*Control siRNA*) and the *Smart* pool of calumenin siRNA (*Calumenin siRNA*). Each lane contains 25 μg of protein and was probed with anti-calumenin and α-tubulin antibodies, respectively. *C* and *D*, integrated band intensities of the cyclophilin B and anti-calumenin immunoreactive bands as a percentage of controls (100%).

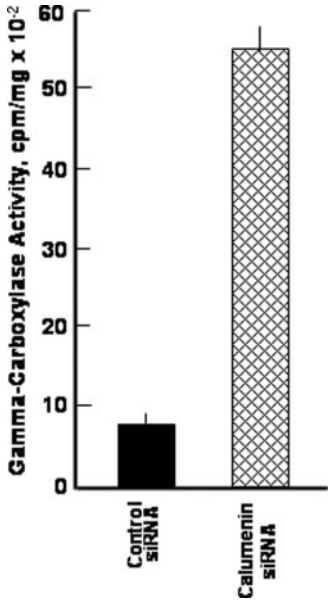

FIG. 4. **Calumenin siRNA enhances γ-carboxylase activity in HEK293 cells.** Cells transfected with control siRNA (*Control siRNA*) and the calumenin *Smart* pool of siRNA (*Calumenin siRNA*) were harvested after 72 h and prepared for measurements of γ-carboxylase activity triggered with chemically reduced vitamin K_1H_2 as described under "Experimental Procedures." Specific γ-carboxylase activities in control and calumenin siRNA-transfected cells are shown. Each measurement is the average of three parallel incubations, and S.D. values are shown.

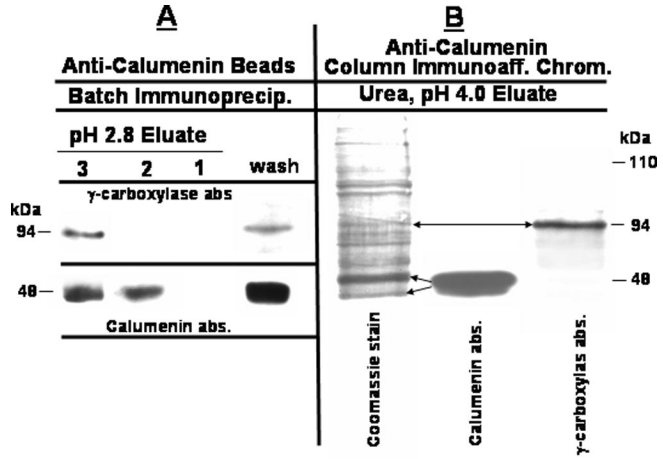

FIG. 5. **Calumenin is associated with γ-carboxylase in the ER.** COS-1 cells transfected with the calumenin-pcDNA3.1/Zeo⁻ expression vector were harvested after 48 h and extracted with RIPA buffer for immunoprecipitation of calumenin and proteins associated with calumenin using anti-calumenin-agarose beads as described under "Experimental Procedures." For batch immunoprecipitation (*A*, *Batch Immunoprecip.*), beads were washed in spin cups, and proteins bound to the beads were eluted with the pH 2.8 buffer as described under "Experimental Procedures." The wash fraction and three fractions from the pH 2.8 eluate (fractions 1–3) were collected consecutively. The proteins in each fraction were subjected to SDS-PAGE and Western blotting with γ-carboxylase (*γ-carboxylase abs.*) and anti-calumenin (*Calumenin abs.*) antibodies, respectively (see *A*). *B*, results from immunoaffinity chromatography of the RIPA buffer extract on a column packed with the anti-calumenin-agarose beads. The column was washed with RIPA buffer containing 0.4 M NaCl before proteins bound to the beads were eluted with the 4 M urea, 0.5 NaCl, pH 4.0, buffer as described under "Experimental Procedures." *B*, the Coomassie Blue-stained proteins (*Coomassie stain*) in the urea fraction and their reaction when subjected to Western blotting with anti-calumenin (*Calumenin abs.*) and γ-carboxylase (*γ-carboxylase abs.*) antibodies, respectively.

ferent preparations of extracted microsomes from normal and warfarin-resistant rats and revealed a significant difference in the γ-carboxylation systems present in normal rats and our warfarin-resistant rats at the level of cofactor transfer in the two systems.

Communication between VKOR and γ-Carboxylase within the γ-Carboxylation System—We have shown previously that VKOR is the rate-limiting step in the γ-carboxylation system (29). Thus, in order to cope with a demand for increased output of γ-carboxylated proteins by the system, increased cofactor production by VKOR is needed. Since the propeptides of vitamin K-dependent proteins stimulate γ-carboxylase activity (31), we asked whether the propeptides could produce a signal within the γ-carboxylation system that would result in increased cofactor production by VKOR. For these experiments, we elected to use the factor X propeptide, since this propeptide has been shown to have the greatest affinity for γ-carboxylase (31). We also investigated the effect of the prothrombin propeptide. We used extracted microsomes from normal rat livers as

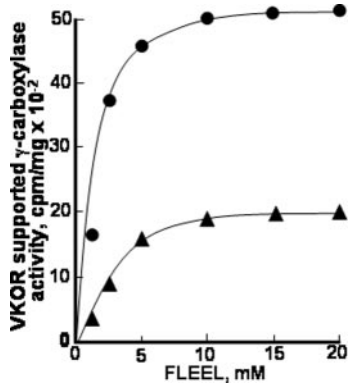

FIG. 6. **VKOR-supported γ-carboxylase activity in normal and warfarin-resistant rats.** Microsomal vesicles extracted with the carbonate-deoxycholate, pH 11.5, buffer and 50 mM Tris base (see "Experimental Procedures") were suspended in buffer D and used for γ-carboxylase assays. Proteins were adjusted to the same concentration (5.4 mg/ml) in both preparations, which had the same γ-carboxylase activity when triggered with chemically reduced vitamin K_1H_2 (22,500 ± 990 cpm/mg, $n = 3$). VKOR activities were also insignificantly different in the two preparations (2.3 ± 0.1 nmol/mg, $n = 3$). Shown is γ-carboxylase activity measured with increasing FLEEL concentration present in the test system. Activity was triggered with vitamin K_1H_2 produced by VKOR at saturating dithiothreitol and vitamin K_1 2,3-epoxide concentrations for the reaction (see "Experimental Procedures"). Each data point is the average of three parallel incubations differing by <5%. *Filled circles*, vesicles from normal rats. *Filled triangles*, vesicles from warfarin-resistant rats.

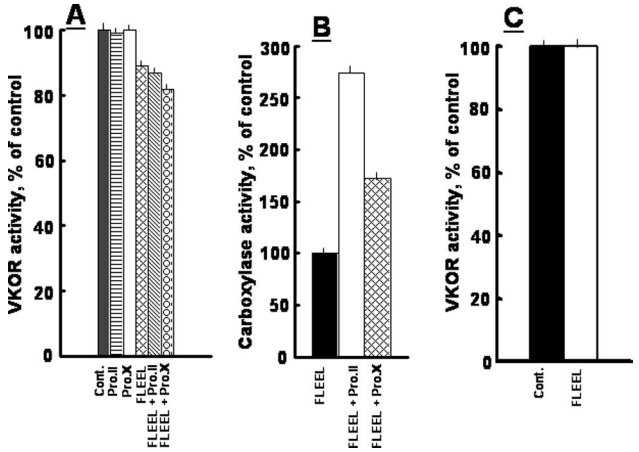

FIG. 7. **The propeptides of factor X and prothrombin do not stimulate vitamin K_1H_2 cofactor production by VKOR in an *in vitro* γ-carboxylation system.** Extracted microsomal vesicles from normal rats were prepared as described under "Experimental Procedures" and suspended in buffer D for γ-carboxylase and VKOR activity measurements. *B*, γ-carboxylase activity triggered with chemically reduced vitamin K_1H_2 in the absence (*FLEEL*) and presence of 10 μM of the prothrombin (*FLEEL + Pro.II*) and the factor X propeptides (*FLEEL + Pro.X*), respectively. Activities are shown as percentage of the control (FLEEL, 100%), which contained no propeptide. *A*, VKOR activity in the presence of 10 μM of the prothrombin propeptide (*Pro.II*), 10 μM of the factor X propeptide (*Pro.X*), 40 mM FLEEL (*FLEEL*), 10 μM prothrombin propeptide plus 40 mM FLEEL (*FLEEL + Pro.II*) and 10 μM factor X propeptide plus 40 mM FLEEL (*FLEEL + Pro.X*), respectively. VKOR activity is shown as the percentage of the control (100%) that contained no propeptide and FLEEL (*Cont.*). *C*, VKOR activity in a partially purified preparation of VKOR in the absence (*Cont.*) and presence of 40 mM FLEEL (*FLEEL*). All activities are the average of three parallel incubations. S.D. values are indicated on the *bars*.

the test system. As shown in Fig. 7*B*, both propeptides stimulated γ-carboxylase activity. Surprisingly, we found that the prothrombin propeptide, when used in this test system, had the greatest stimulating effect on γ-carboxylase activity. Fig. 7*A* shows that these propeptides, when present in the test system, did not affect VKOR activity. However, when present together

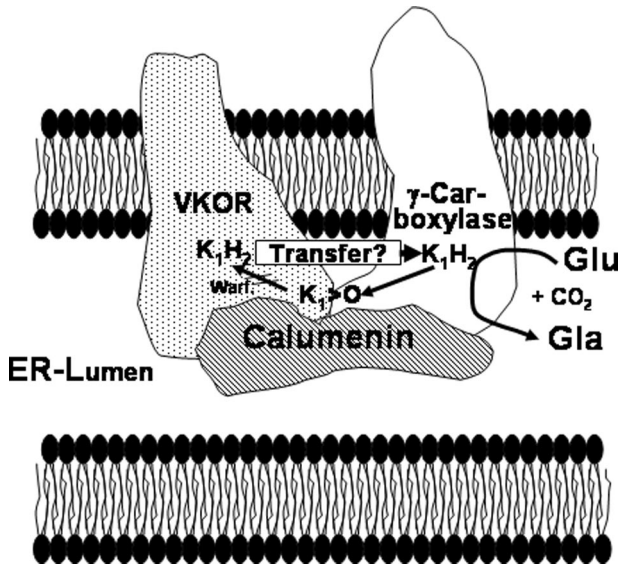

FIG. 8. **A putative molecular model of the γ-carboxylation system.** VKOR and γ-carboxylase are parts of an enzyme complex in the ER lipid bilayer. Calumenin binds to γ-carboxylase as an inhibitory chaperone and also affects the activity and warfarin sensitivity of VKOR.

with the γ-carboxylase substrate FLEEL in the VKOR test system, a small reduction in VKOR activity was measured. This result could be expected, since the addition of FLEEL to the VKOR test system would turn on γ-carboxylation of FLEEL and production of vitamin K_1 2.3-epoxide (29), resulting in an apparent decline in measured VKOR activity (see "Experimental Procedures"). To prove that FLEEL does not have an effect on VKOR activity, we added FLEEL to a partially purified preparation of VKOR that is devoid of γ-carboxylase activity (1). As shown in Fig. 7*C*, FLEEL had no effect on VKOR activity. The data argue against the existence of a propeptide-mediated mechanism within the γ-carboxylation system that would enhance cofactor production by the system and put calumenin in a central position as a regulatory protein of vitamin K-dependent γ-carboxylation.

Sequencing of the 18-kDa Putative VKOR Enzyme from Livers of Normal and Warfarin-resistant Rats—We sequenced the rat cDNA corresponding to the human gene gi:13124769 proposed by Rost *et al.* (19) to be the warfarin-sensitive subunit 1 of the VKOR enzyme complex. The results reveled complete identify between the protein sequence in normal and warfarin-resistant rats (data not shown).

DISCUSSION

In this work, we have identified, for the first time, a protein that can regulate the activity of the γ-carboxylation system. This conclusion is based on data that include 1) inhibition of γ-carboxylase activity by transient transfection with a calumenin cDNA construct, 2) silencing of the calumenin gene by *Smart* siRNA, and 3) a proteomic approach that demonstrates the existence of protein-protein interactions between γ-carboxylase and calumenin. In a previous paper (1), we showed that calumenin inhibits VKOR activity and protects the enzyme from warfarin inhibition. Together, these data indicate that calumenin plays a role in regulation and performance of the γ-carboxylation system and thus biosynthesis of functional vitamin K-dependent proteins.

Calumenin belongs to the CREC subfamily of Ca^{2+}-binding proteins where an EF-hand motif binds the metal (32). The CREC proteins are found in the secretory pathway, and most of the proteins have been shown to have chaperone functions (32).

All proteins have C-terminal retrieval sequences (HDEL, HDEF, and HEEF) for retention either in the ER or the Golgi apparatus (32). In contrast to other EF-hand family members like calmodulin and parvalbumin (33), the CREC proteins show weak Ca^{2+} binding with association constants in the millimolar range (34). It is known that EF-hand proteins with strong affinity for Ca^{2+} expose hydrophobic amino acids upon metal binding, which is responsible for binding of these proteins to their targeted partner proteins (38). Whether or not Ca^{2+} binding by the CREC proteins is important for their protein-protein interactions is an unsettled issue that needs to be addressed in order to understand the interactions between calumenin and VKOR and γ-carboxylase, respectively. EDTA stimulates VKOR activity when present in the test system.[2] This finding may indicate that metals do play a role in binding.

Calumenin is a water-soluble acidic protein (1, 32). Consistent with electron microscopy studies of the ER membrane (30) and our previous demonstration that calumenin is strongly associated with lipid-detergent micelles derived from the ER membrane (1) is the notion that calumenin, despite being a hydrophilic protein, is associated with ER membrane proteins. As a chaperone, we propose that calumenin has an important regulatory function of the γ-carboxylation system.

Our previous (1) and current results suggest that calumenin targets both VKOR and γ-carboxylase of the γ-carboxylation system and is responsible for the genetic warfarin resistance in our colony of warfarin-resistant rats. Different forms of genetic warfarin resistance has been described for rats (36, 37). Our finding that the 18-kDa subunits 1 of the VKOR enzyme complex in normal and warfarin-resistant rats have identical sequences supports our previous hypothesis that overexpression of calumenin in liver represents one form of genetic warfarin resistance. Rost *et al.* (19) have identified mutations in the subunit that coincide with warfarin-resistant phenotypic humans and rats. However, the relationship between the mutations and the phenotypes is complex and will require more work to clarify the various types of resistance at the molecular level.

Extraction of microsomal vesicles allowed us to produce calumenin-deficient γ-carboxylation systems from warfarin-resistant and normal rats that were indistinguishable with respect to their individual VKOR and γ-carboxylase activities. On the other hand, the ability of VKOR to provide γ-carboxylase with reduced vit.K_1H_2 cofactor was found to be significantly impaired in the system prepared from our colony of warfarin-resistant rats. We propose that the result reflects impaired transfer of reduced vit.K_1H_2 cofactor from VKOR to γ-carboxylase within the γ-carboxylation system. The ER lumen harbors proteins in an oxidative environment (38). Since vit.K_1H_2 is sensitive to oxidation, transfer of reduced cofactor between VKOR and γ-carboxylase in the ER membrane must be protected from oxidation. Therefore, as depicted by the putative model shown in Fig. 8, we propose that VKOR and γ-carboxylase reside close to each other in the ER membrane and constitute an enzyme complex that is structurally organized for maximum efficiency as a γ-carboxylation system. The lipid bilayer plays an essential role in this organization as we know from phospholipase A2 experiments that the complex will be destroyed by treatment with the lipase.[2] If our results are representative for the system *in vivo*, the impaired vit.K_1H_2 cofactor transfer in our resistant rats could explain the need for these rats to increase their intake of vitamin K in order to prevent internal bleeding. Our kinetic analyses of the γ-carboxylation system (29) show that γ-carboxylase has a large capacity to γ-carboxylate its substrates. Since VKOR is the rate-limiting step in production of functional coagulation factors, increased delivery of cofactor to γ-carboxylase would be a way to overcome suppression of the system. Indeed, when high concentrations of vitamin K_1 are present in liver, increased vit.K_1H_2 cofactor production can be furnished by the alternative warfarin-insensitive vitamin K-reducing pathway in liver (39), which probably rescues the poorly functioning γ-carboxylation system.

Biological systems commonly have feedback systems that regulate the need for products produced by the systems. Therefore, we hypothesized that that a regulatory mechanism may exist that can increase synthesis of functional fully γ-carboxylated vitamin K-dependent proteins when needed. We have shown that calcified lesions in the arterial wall contain elevated concentrations of under-γ-carboxylated matrix Gla protein that is nonfunctional as a binding protein for the growth factor bone morphogenetic protein-2 (11). Since bone morphogenetic protein-2 is a potent bone-forming growth factor (41), enhanced production of fully γ-carboxylated matrix Gla protein could potentially have prevented the pathology. The propeptides of newly synthesized precursors of vitamin K-dependent proteins activate γ-carboxylase (31, 41). However, the results obtained from experiments with our *in vitro* γ-carboxylation test system indicate that the propeptides do not stimulate the γ-carboxylation system to produce more vit.K_1H_2 cofactor that is rate-limiting for γ-carboxylation. On the other hand, silencing of the calumenin gene up-regulates the capacity of the system, which potentially could produce more fully γ-carboxylated functional proteins, a mechanism that may have therapeutic use in prevention of disease processes such as ectopic calcifications (42).

[2] N. Wajih, D. C. Sane, S. M. Hutson, and R. Wallin, unpublished data.

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The Inhibitory Effect of Calumenin on the Vitamin K-dependent γ-Carboxylation System: CHARACTERIZATION OF THE SYSTEM IN NORMAL AND WARFARIN-RESISTANT RATS

Nadeem Wajih, David C. Sane, Susan M. Hutson and Reidar Wallin

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