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Calcium- and calmodulin-antagonism of elnadipine derivatives: comparative SAR

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Summary — The Ca^{2+}-antagonistic properties of elnadipine derivatives have been quantified by means of binding experiments in bovine cerebral cortex membranes using ^{3}H-nitrendipine. Competition experiments have shown a 308-fold concentration range of K_i-values (2.9×10^{-10} to 8.9×10^{-8}) for elnadipine derivatives and a eudismic ratio for elnadipine and its (+)-enantiomer of 448. Calmodulin (CaM)-antagonistic properties have been measured in the test system of CaM-stimulated phosphodiesterase. The concentration range of IC_{50} values only amounts to 9 (5×10^{-7} to 4.5×10^{-6} M). In contrast to Ca^{2+}-antagonism, enantioselectivity of CaM-inhibition by elnadipine is negligible. CaM-antagonistic potency of elnadipine derivatives is diminished by a factor of 10 to 5000 as compared to their Ca^{2+}-antagonistic potency. The following conclusions regarding structural determinants of Ca^{2+}- and CaM-antagonistic properties can be made: lipophilic substituents of the oxadiazole in 5-position of the dihydropyridine (DHP) ring increase the CaM-antagonistic and decrease the Ca^{2+}-antagonistic potency; correspondingly the unsubstituted compound is the strongest Ca^{2+}- and the weakest CaM-antagonist; 1,3,4-oxadiazole substitution is superior to 1,2,4-oxadiazole as regards Ca^{2+}- and CaM-antagonistic potency.

Ca-antagonists / elnadipine / SAR / calmodulin

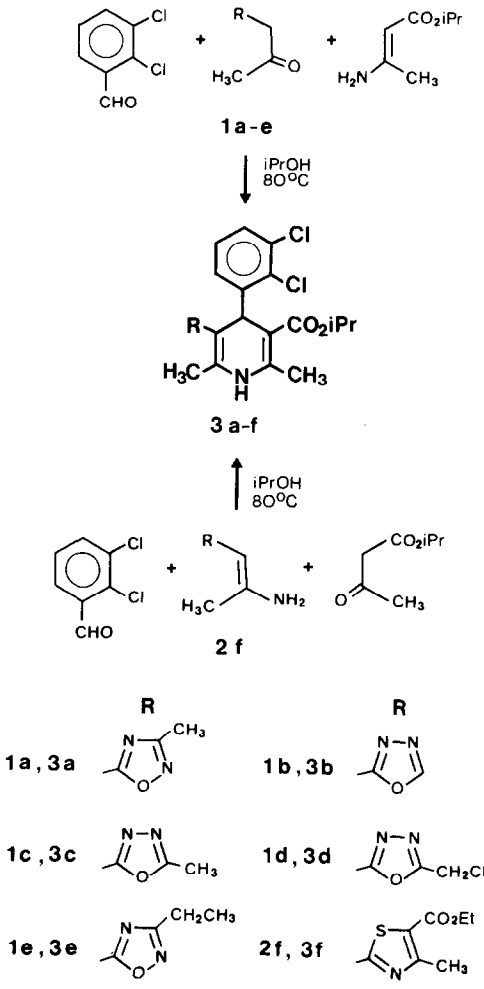
Introduction

Felodipine was the first dihydropyridine (DHP)-type Ca^{2+}-antagonist (CaA) identified as an inhibitor of calmodulin (CaM) [1]. As a result, several authors have investigated the potential importance of CaM inhibition by DHP as an additional molecular mode of their vasodilatory action [2–7]. A large number of detailed studies on structure–activity relationships (SAR) of the Ca^{2+}-antagonistic properties of DHP are available [8–10]. Corresponding data on SAR regarding the CaM-antagonistic properties of DHP are rather limited [2]. The aim of the present paper was to make a comparative SAR study for the Ca^{2+}- and the CaM-antagonistic properties of a series of DHP derivatives characterized by varied heterocyclic substitution in the 5-position of the DHP ring (scheme 1). In addition, the enantioselectivity of both actions was determined.

Chemistry

The synthesis of the racemic 1.4-dihydropyridines **3a-f** is outlined in scheme 1. It is a variation of the Hantzsch synthesis in which the alkyl acetoacetate or the aminocrotonate have been replaced by the acetonyl heterocycles **1a–e** [11, 12] or the aminopropenyl thiazole **2f**. The 1.4-dihydropyridines **3a–f** were obtained in 22–71% yield by heating equimolar amounts of the starting materials in isopropanol at reflux temperature for several hours. Condensation of aminocrotonic thioamide with ethyl 1-chloroacetoacetate in the presence of triethylamine yielded the 2-aminopropenylthiazole derivative **2f**.

The synthesis of the enantiomeric 1.4-dihydropyridines **8a** + **b** is shown in scheme 2. The chiral aminocrotonates **5R** + **S** were prepared from ethyl (R)- or (S)-lactate by reaction with diketene and aminolysis of the resulting acetoacetates **4R** + **S** with excess ammonia in ethanol. The condensation of the 1.3.4-oxadiazole **1b** with 2.3-dichlorbenzaldehyde in the presence of catalytic amounts of piperidine gave the benzylidene derivative **6** as a mixture of *cis* and *trans* isomers. The pure (S, R) diastereomeric 1.4-dihydropyridine **7a** was obtained in 57% yield by the cyclocondensation of the (S) enantiomer **5R** with **6** in dimethylsulfoxide. This main diastereomer could be separated from the minor (S, S) diastereomer by recrystallisation from ethanol. Starting from the (R)

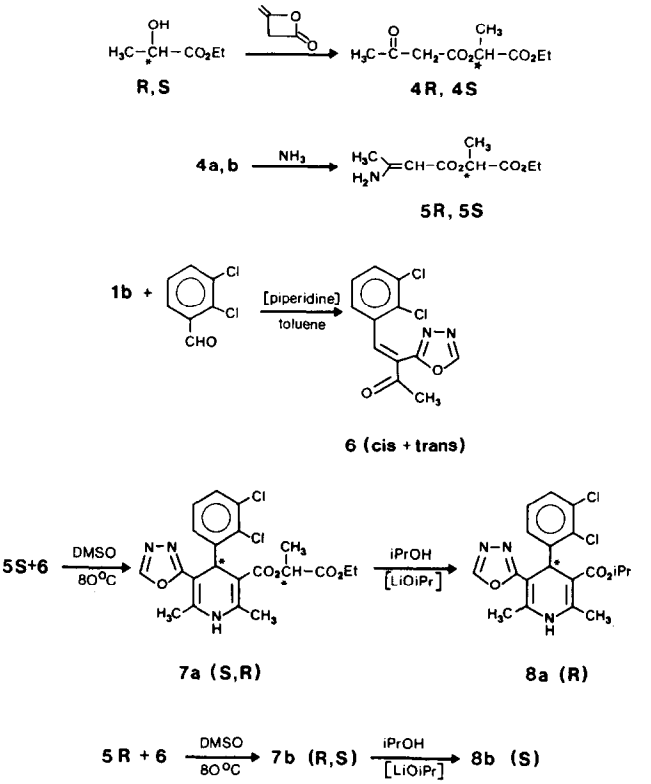


Scheme 1.

enantiomer **5R** and **6** the (R, S) diastereomer **7b** was formed by the same procedure. The diastereomeric excess (de) of this synthesis was 30%. From these diastereomers **7a** and **7b** the enantiomers **8a** and **8b** were obtained by transesterification with isopropanol in the presence of lithiumisopropylate at 80°C. No racemization was observed under these conditions. The absolute configuration of the (–) enantiomer **8b** (= elnadipine) was determined by X-ray analysis (Paulus EF, 1985; Angewandte Physik Hoechst AG, unpublished results) as the (S)-enantiomer.

Results

Structure–activity analyses of dihydropyridine derivatives have been almost exclusively focused on the prime action exerted by these compounds: an



Scheme 2.

inhibition of calcium currents through L-type calcium channels. Increasing experimental evidence on additional effects of dihydropyridines indicates calmodulin inhibition as a topic of major interest. SAR-analyses of the Ca-antagonistic properties of DHP revealed 2 critical moieties: 1) properties and positioning of substituents at the phenyl ring in 4-position of the DHP-ring; 2) substitution pattern in 3- and 5-position of the DHP ring.

SAR analyses of the CaM-antagonistic properties of DHP are almost completely lacking. Regarding aromatic substitution, halogens have been shown to increase potency; influence of substituent positioning is marginal. No SAR analyses have been made – at least to our knowledge – for alkyl DHP carboxylates. Thus, using elnadipine as a lead, we made a comparative SAR study for the Ca^{2+}- as well as the CaM-antagonistic properties of elnadipine derivatives characterized by differing substitution at 5-position of the DHP ring.

The characteristic biological action of the test compounds in binding experiments and in the calmodulin-PDE assay for the compound **3c** is shown in figure 1. Biological data and lipophilicity values are

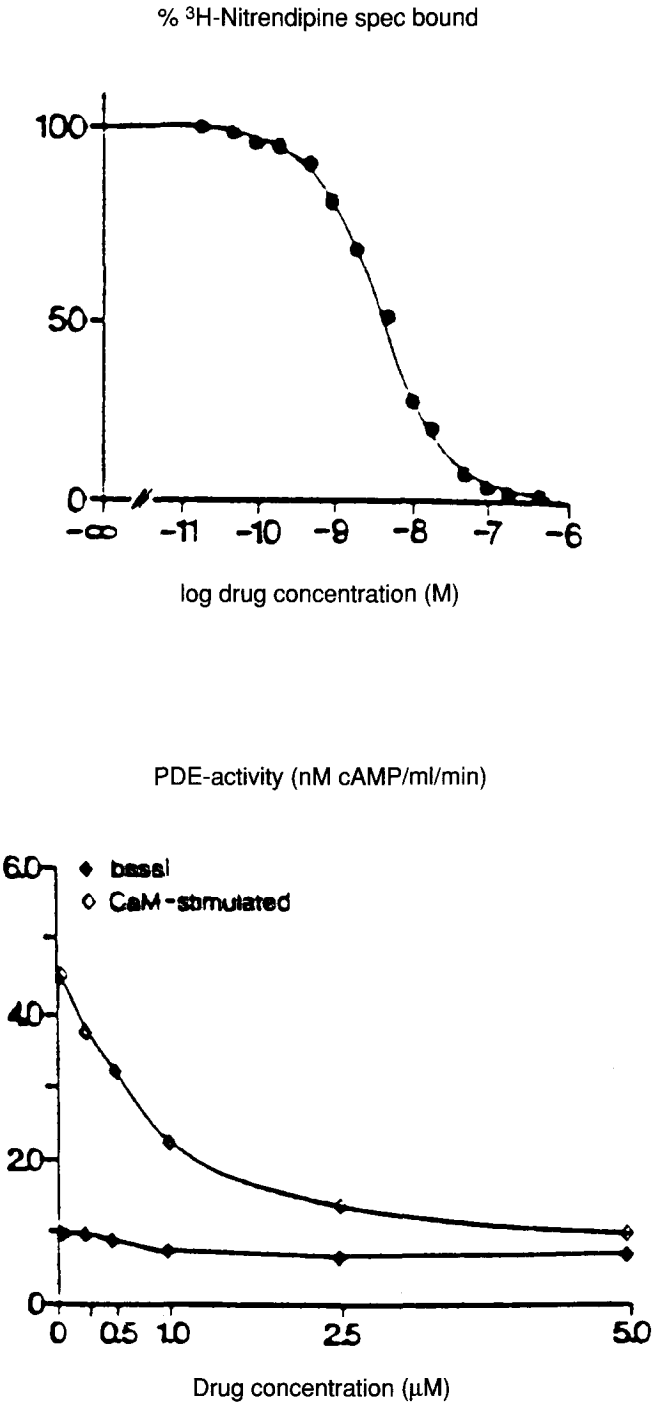


Fig 1. Typical Ca^{2+}- and CaM-antagonistic properties of elnadipine derivatives as shown here for compound **3c**.

summarized in table I for the total set of test compounds. Binding experiments for quantifying Ca^{2+}-antagonistic properties were performed in bovine cerebral cortex membranes using tritiated nitrendipine. Competition experiments have shown a 308-fold concentration range of K_i-values (2.9×10^{-10} to 8.9×10^{-8} M). Ca^{2+}-antagonistic potency almost exclusively resides in the levorotatory isomer as demonstrated by a eudismic ratio (S/R) of 448.

The rank order of Ca^{2+}-antagonistic potency of the racemates is given in figure 2. As can be derived from this ranking, 1,3,4-oxadiazole substitution is superior to 1,2,4-oxadiazole or 1,4-thiazole. Substitution of the 1,3,4-oxadiazole ring is detrimental.

CaM-antagonistic potency of the same set of derivatives is summarized in table I. For this action, enantioselectivity is lacking and the concentration range of IC_{50} values only yields a factor of about 10 between the strongest and the weakest compound. Ranking of the racemic compounds shown in figure 2 again indicates a superiority of the 1,3,4-oxadiazole ring, as also found for Ca^{2+}-antagonistic properties.

Figure 2 also demonstrates that lipophilic substitution of the oxadiazole increases CaM-antagonistic potency, while Ca^{2+}-antagonistic activity is diminished. Potency factors between Ca^{2+}- and CaM-antagonistic properties vary markedly: **3c** is a 168-fold stronger Ca^{2+}-antagonist than CaM-antagonist, while this factor is only 12 in the case of **3f**.

The lack of interdependence between the 2 biological actions compared in this study is also demonstrated by their differing correlations with drug lipophilicity. While Ca^{2+}-antagonistic potency exhibits a significant inverse correlation with R_M (fig 3), no such correlation is found for CaM-antagonistic potency (fig 4).

Discussion

Ca^{2+}-antagonists of the dihydropyridine type were long viewed as selective inhibitors of potential dependent Ca^{2+}-channels which exhibit additional biological action – if at all – only at pharmacologically irrelevant concentrations. Since then, several effects of dihydropyridines have been reported which are not related to channel blockade, but might contribute to a refined molecular interpretation of the mode of action of dihydropyridines. To mention a few of the reported additional features of dihydropyridine-like Ca^{2+}-antagonists: 1), a stimulation of Na^+, K^+-ATPase by nimodipine and nitrendipine [13]; and 2), an inhibition of both the basal and the Ca^{2+}-CaM-stimulated phosphodiesterase activity [14].

Boström *et al* were the first to discover the CaM-antagonistic properties of DHP-type CaA felodipine [1]. Afterwards several authors dealt with the putative importance of CaM-antagonism for the pharmacological, *ie* the vasodilatory properties of Ca^{2+}-antagonists. Taken together, the CaM hypothesis is exper-

Table I. Physicochemical (R_M) and biological data of the test compounds. The right column gives the factor by which the Ca^{2+}-antagonistic properties of a compound surmount its CaM-antagonistic properties.

Compound	R_M	*CaM inhibition* IC_{50} *(μM)*	*PDE inhibition* IC_{50} *(μM)*	*Binding inhibition* IC_{50} *(nM)*	K_i *(nM)*	*n H*	*CaM/ binding*
3b	0.135	4.5 ± 0.71	7.5	1.18 ± 0.10	0.65 ± 0.08	1.07 ± 0.02	3814
8b	0.135	2.8 ± 0.43	5.7	0.52 ± 0.03	0.29 ± 0.03	1.10 ± 0.04	5354
8a	0.135	3.5 ± 0.55	8.7	271 ± 24.0	130 ± 15.0	1.07 ± 0.01	13
3c	0.155	0.8 ± 0.09	–	4.76 ± 0.52	2.44 ± 0.20	1.07 ± 0.03	168
3d	0.213	0.5 ± 0.06	–	10.0 ± 2.10	5.10 ± 0.92	1.04 ± 0.02	52
3e	0.363	3.5 ± 0.22	–	52.3 ± 17.3	27.4 ± 1.00	1.00 ± 0.03	67
3a	0.353	4.2 ± 0.27	13	30.8 ± 5.00	15.7 ± 2.40	0.96 ± 0.02	136
3f	0.488	2.1 ± 0.22	–	175 ± 23.0	89.2 ± 7.80	1.14 ± 0.04	12

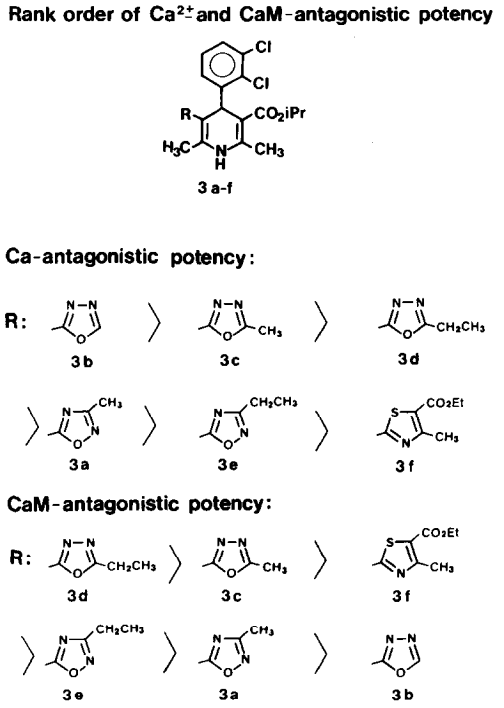


Fig 2. Ranking of the Ca^{2+}-(left column) and the CaM-(right column) antagonistic potencies of racemic elnadipine derivatives. Only the varying substituents in 5-position of the dihydropyridine ring are shown.

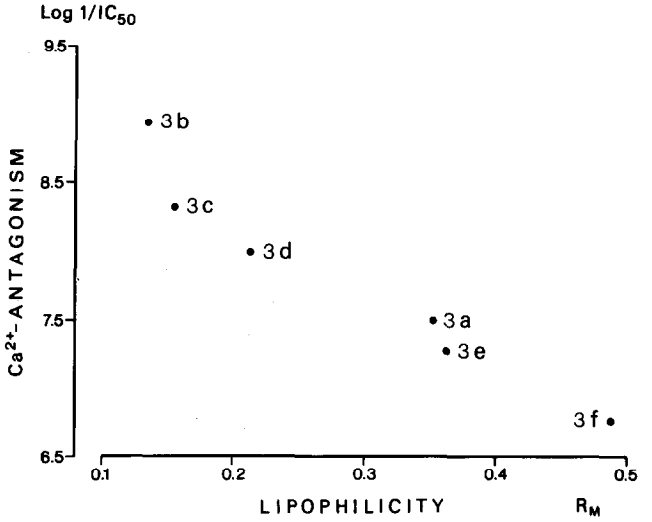


Fig 3. Correlation of the Ca^{2+}-antagonistic potency of elnadipine derivatives with drug lipophilicity (R_M) revealing a significant inverse correlation.

imentally evidenced by: 1), a remarkably high lipophilicity of most CaA; 2), a pronounced intracellular enrichment of several CaA [15, 16]; 3), binding of diphenylalkylamine- and DHP-like CaA to CaM [16, 17]; 4), an inhibition of CaM-stimulated phosphodiesterase and myosin light chain kinase [18, 19]; 5), a corresponding allosteric regulation of the DHP binding by diltiazem [20, 21] and gallopamil to both Ca^{2+}-channels and CaM; and 6), a significant correlation between CaM-binding of CaM-antagonists and their affinities to the DHP binding site [22].

The main argument against the CaM hypothesis is the pronounced discrepancy between the calcium- and the calmodulin-antagonistic potencies of Ca^{2+}-antagonists, *ie* dihydropyridines. The factor by which *eg* nifedipine is a stronger Ca^{2+}- than CaM-antagonist approximates 2000 [23]. But such simple derivatives of potency factors might be misleading, especially in terms of the complex interaction pattern of ligands with calmodulin. Calmodulin's ability to activate target proteins might depend on the degree of

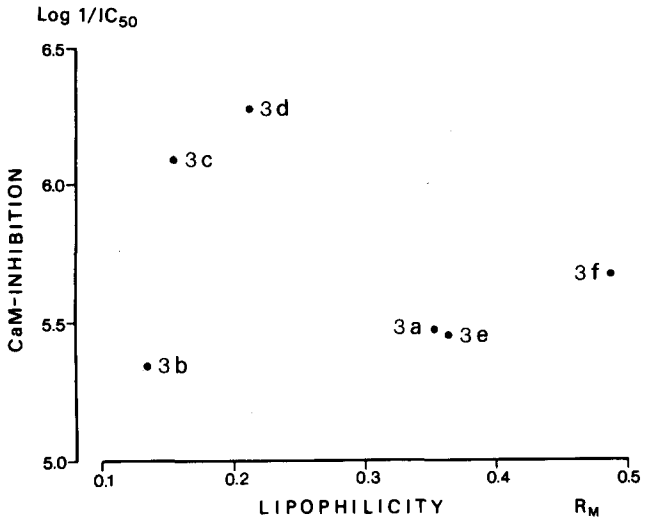


Fig 4. Correlation of the CaM-antagonistic potency of elnadipine derivatives with drug lipophilicity (R_M).

occupancy of both its Ca^{2+}- as well as its ligand binding sites [20, 21]. Occupation of one of the CaM binding sites by various ligands can drastically increase the binding constant for DHP to other sites; such a mechanism could reduce the selectivity ratio if it occurs *in vivo*. Thus, CaM-inhibition remains a molecular mechanism putatively contributing to the pharmacological profile of DHP-like CaA.

Comparative structure–activity studies for the Ca^{2+}- and the CaM-antagonistic component of dihydropyridine actions would allow a rational approach to optimizing or eliminating the corresponding components. From a historical point of view it is understandable that structure–activity studies have primarily focused on the Ca^{2+}-antagonistic properties of DHP. Results of such studies have been described elsewhere [8–10]. These studies have elucidated 2 main regions of the DHP molecule adequate for proper drug manipulation, *ie* the substitution pattern of the phenyl ring in 4-position and the substitution of 3- and 5-position of the DHP ring.

So far, structure–activity studies of the CaM antagonistic properties of DHP have rarely been performed. Roufogalis *et al* [24] studied the importance of phenyl ring substitution for CaM-antagonism indicating an increase of potency by halogen substitution which in contrast to Ca^{2+}-antagonistic properties of DHP does not depend on substituent positioning. Corresponding results have been reported by Walsh *et al* [2] indicating an equieffective inhibition of phosphodiesterase and myosin light chain kinase by felodipine and its *p*-chloroanalogue. This is in sharp contrast to Ca^{2+}-antagonistic properties, where *p*-substitution drastically diminishes potency. For alkyl DHP carboxylates, no structure–activity studies on their CaM-inhibitory properties have been performed.

The present paper focused on a comparative SAR study of Ca^{2+}- and CaM-antagonistic properties of a set of dihydropyridine derivatives characterized by variations in the 5-position of the dihydropyridine nucleus. Concerning CaM-antagonism, data summarized here indicate a superiority of 1.3.4-oxadiazole-substitution over the corresponding 1.2.4-heterocycle. Alkyl substitution (ethyl > methyl) is advantageous.

Comparison of these results with the corresponding Ca^{2+}-antagonistic properties of the same set of compounds clearly demonstrates differing structure–activity relationships for the Ca^{2+}- and the CaM-antagonistic properties of these derivatives. This is substantiated by the pronounced differences in the enantioselectivity of the Ca^{2+}- and CaM-antagonistic action. As expected from the literature [25] in the case of elnadipine also, a profound enantioselectivity of Ca^{2+}-antagonistic actions was observed. On the contrary, no significant differences in the CaM-antagonistic properties of the enantiomers were observed.

In conclusion, our investigations demonstrate that the Ca^{2+}- and the CaM-antagonistic properties of dihydropyridines seem to be distinct and separable drug properties allowing rational structural manipulations with respect to pure Ca^{2+}-antagonists, pure CaM-antagonists or hybrid molecules enclosing both pharmacological properties. Satisfactory purity of Ca^{2+}-antagonistic properties seems to be found with elnadipine, exhibiting a potency ratio of more than 5000. Oxadiazole substitution with even more lipophilic substituents is hypothesized to allow the development of at least preferential CaM-inhibitors. Last, but not least, hybrid molecules exhibiting both Ca^{2+}- as well as CaM-antagonism might be achieved by developing compounds containing a non-substituted 1.3.4-oxadiazole in the 5-position of the DHP ring and adequately lipophilic ester substituents in the 3-position.

Taken together, structural manipulation of dihydropyridines as described here appears to provide a rational approach for directing the pharmacological profile of dihydropyridines regarding their Ca^{2+}- and CaM-antagonistic properties.

Experimental protocols

Chemistry

2.3-Dichlorobenzaldehyde was purchased from Bayer AG. Isopropyl aminocrotonate was prepared from isopropyl acetoacetate according to Cope [26]. The acetonyl heterocycles **1a–e** were prepared according to Kübel [11, 12]. Ethyl (S)-lactate was purchased from Aldrich and ethyl (R)-lactate was prepared from (R)-lactic acid by the method of Gerrard *et al* [27]. Aminocrotonic thioamide (3-imino-thiobutyramide) was

prepared from 3-iminobutyronitrile by the method of Adams and Salck [28]. Melting points, determined with the Büchi apparatus SMP-20, are uncorrected. Analyses indicated by the symbols of the elements were within ± 0.4% of the theoretical values. ^{1}H NMR spectra were recorded on an 80 MHz spectrometer (type FT 80 (Varian)) using tetramethylsilane as internal reference and Eu $(tfc)_3$ and Eu $(hfc)_3$ as chiral shift reagent. Chemical shifts and relative integrals of the peak areas agreed with those expected for the assigned structures. Optical rotations were measured on the polarimeter Polartronic D (Schmidt and Haensch, Berlin) at room temperature.

2-(2-Aminopropen-1-yl)-4-methyl-5-ethoxycarbonyl-thiazole ***2f***

The mixture of 34.8 g (0.3 mol) of aminocrotonic thioamide, 49.5 g (0.3 mol) of ethyl 2-chloroacetoacetate, 45 g (0.45 mol) of triethylamine and 150 ml of ethanol was heated to reflux for 20 min. Upon cooling to room temperature and adding 250 ml of water, a solid precipitated which was filtered off and recrystallized from isopropanol. Yield 38.3 g (56%) mp 98–100°C. Anal $C_{10}H_{14}N_2O_2S$ (C, H, N, O, S). ^{1}H NMR ($CDCl_3$) δ: 1.43 (t, 3H); 2.04 (s, 3H); 2.77 (s, 3H); 4.40 (q, 2H); 5.31 (s, 1H); 6.98 (m, 2H).

Synthesis of 1.4-dihydropyridines ***3a–e***

The mixture of 0.01 mol 2.3-dichlorobenzaldehyde, 0.01 mol isopropyl aminocrotonate, 0.01 mol of the acetonyl heterocycles **1a–e** and 20 ml of isopropanol was heated to reflux for 15 h. The solvent was evaporated and the residue recrystallized from ethyl acetate (**3a**), diethylether (**3b**, **3c**), diisopropylether (**3d**) and *n*-hexane (**3e**).

Isopropyl-1.4-dihydro-2.6-dimethyl-4-(2.3-dichlorophenyl)-5-(3-methyl-1.2.4-oxadiazol-5-yl)-pyridine-3-carboxylate ***3a***

Yield 1.0 g (24%). mp 194–196°C. Anal $C_{20}H_{21}Cl_2N_3O_3$ (C, H, Cl, N, O). ^{1}H NMR ($CDCl_3$) δ: 1.15 (q, 6H); 2.26 (s, 3H); 2.28 (s, 3H); 2.29 (s, 3H); 5.00 (m, 1H); 5.62 (s, 1H); 6.60 (s, 1H); 7.0–7.3 (m, 3H).

Isopropyl-1.4-dihydro-2.6-dimethyl-4-(2.3-dichlorophenyl)-5-(1.3.4-oxadiazol-2-yl)-pyridine-3-carboxylate ***3b***

Yield 1.8 g (45%). mp 166–168°C. Anal $C_{19}H_{19}Cl_2N_3O_3$ (C, H, Cl, N, O). ^{1}H NMR ($CDCl_3$) δ: 1.03–1.42 (q, 6H); 2.38 (d, 6H); 5.00 (m, 1H); 5.61 (s, 1H); 6.52 (s, 1H); 7.05–7.55 (m, 3H); 8.37 (s, 1H).

Isopropyl-1.4-dihydro-2.6-dimethyl-4-(2.3-dichlorophenyl)-5-(5-methyl-1.3.4-oxadiazol-2-yl)-pyridine-3-carboxylate ***3c***

Yield 3.0 g (71%). mp 196–198°C. Anal $C_{20}H_{21}Cl_2N_3O_3$ (C, H, Cl, N, O). ^{1}H NMR ($CDCl_3$) δ: 0.90–1.35 (q, 6H); 2.25 (s, 3H); 2.30 (s, 3H); 2.42 (s, 3H); 4.93 (m, 1H); 5.24 (s, 1H); 6.85–7.40 (m, 3H).

Isopropyl-1.4-dihydro-2.6-dimethyl-4-(2.3-dichlorophenyl)-5-(5-ethyl-1.3.4-oxadiazol-2-yl)-pyridine-3-carboxylate ***3d***

Yield 2.7 g (63%). mp 161–163°C. Anal $C_{21}H_{23}Cl_2N_3O_3$ (C, H, Cl, N, O). ^{1}H NMR ($CDCl_3$) δ: 1.00–1.45 (q, 6H); 1.47 (t, 3H); 2.36 (s, 3H); 2.42 (s, 3H); 2.97 (q, 2H); 5.00 (m, 1H); 5.59 (s, 1H); 6.94–7.55 (m, 3H).

Isopropyl-1.4-dihydro-2.6-dimethyl-4-(2.3-dichlorophenyl)-5-(3-ethyl-1.2.4-oxadiazol-5-yl)-pyridine-3-carboxylate ***3e***

Yield 2.6 g (59%). mp 167–169°C. Anal $C_{21}H_{23}Cl_2N_3O_3$ (C, H, Cl, N, O). ^{1}H NMR ($CDCl_3$) δ: 1.87–2.25 (q, 6H); 1.20 (t, 3H); 2.22 (d, 6H); 2.61 (q, 2H); 4.92 (m, 1H); 5.99 (s, 1H); 6.49 (s, 1H); 6.80–7.43 (m, 3H).

Isopropyl-1.4-dihydro-2.6-dimethyl-4-(2.3-dichlorophenyl)-5-(4-methyl-5-ethoxycarbonyl-thiazol-2-yl)-pyridine-3-carboxylate ***5f***

The mixture of 1.75 g (0.01 mol) 2.3-dichlorobenzaldehyde, 2.3 g (0.01 mol) **2f**, 1.44 g (0.01 mol) isopropyl acetoacetate and 30 ml of isopropanol was heated to reflux for 5 h. The solvent was evaporated and the residue treated with a 1:1-mixture of diethylether and *n*-hexane. A solid precipitated that was filtered off and recrystallized from isopropanol. Yield 1.1 g (22%). mp 167–170°C. Anal $C_{24}H_{26}Cl_2N_2O_4S$ (C, H, Cl, N, O, S). ^{1}H NMR ($CDCl_3$) δ: 1.00–1.35 (q, 6H); 1.36 (t, 3H); 2.20 (s, 3H); 2.35 (s, 3H); 2.69 (s, 3H); 4.35 (q, 2H); 5.00 (m, 1H); 5.63 (s, 1H); 6.05 (s, 1H); 7.05–7.70 (m, 3H).

(R)-(1-Ethoxycarbonyl-ethyl)-acetoacetate ***4R*** *and (S)-(1-Ethoxycarbonyl-ethyl)-acetoacetate* ***4S***

To the mixture of 118 g (1 mol) of ethyl (*R*)- or (*S*)-lactate, 5 ml of triethylamine and 300 ml of dichloromethane 84 g (1 mol) of diketene was added drop by drop keeping the mixture at boiling point. After complete addition the solution was stirred for 15 h, the solvents were evaporated and the oily residue was purified by distillation. Yield 163 g (81%). bp 140–145°C/26 mmHg. α_D = + 35° (dichloromethane, c = 2.0) for **4R** and α_D = – 33° (dichloromethane, c = 2.0) for **4S**. Anal $C_9H_{14}O_5$ (C, H, O). ^{1}H NMR ($CDCl_3$) δ: 1.23 (t, 3H); 1.50 (d, 3H); 2.29 (s, 3H); 3.48 (s, 2H); 4.19 (q, 2H); 5.08 (q, 1H).

(R)-(1-Ethoxycarbonyl-ethyl)-aminocrotonate ***5R*** *and (S)-(1-Ethoxycarbonyl-ethyl)-aminocrotonate* ***5S***

The mixture of 80 g (0.39 mol) **4R** or **4S**, 20 g (1.17 mol) of ammonia and 250 ml of ethanol was stirred at room temperature for 20 h. After evaporation of the solvent and excess ammonia, the oily residue was purified by distillation. Yield 53 g (68%). bp 130–135°C/2 mmHg. α_D = + 75° (dichloromethane, c = 2.0) for **4R** and α_D = – 77.5° (dichloromethane, c = 2.0) for **4S**. Anal $C_9H_{15}NO_4$ (C, H, N, O). ^{1}H NMR ($CDCl_3$) δ: 1.37 (t, 3H); 1.52 (d, 3H); 2.05 (s, 3H); 4.22 (q, 2H); 4.53 (s, 1H); 5.00 (q, 1H).

2-(1-(2.3-Dichlorobenzylidene)-acetonyl)-1.3.4-oxadiazol ***6*** *(cis/trans-mixture)*

The mixture of 70 g (0.56 mol) **1b**, 98 g (0.56 mol) of 2.3-dichloro-benzaldehyde, 300 ml of toluene and 5 ml of piperidine was heated to reflux using a water separator. When no further water separated, the solvent was evaporated and the residue recrystallized from isopropanol. Yield 98 g (62%). mp 98–100°C. Anal $C_{12}H_8Cl_2N_2O_2$ (C, H, Cl, N, O). ^{1}H NMR ($CDCl_3$) δ: 2.42 (s, 0.9H); 2.58 (s, 2.1H); 6.80–7.38 (m, 3H); 7.78 (s, 0.3H); 8.25 (s, 0.7H); 8.45 (s, 0.7H); 8.51 (s, 0.3H).

(S)-(1-Ethoxycarbonyl-ethyl)-(R)-1.4-dihydro-2.6-dimethyl-4-(2.3-dichlorophenyl)-5-(1.3.4-oxadiazol-2-yl)-pyridine-3-carboxylate ***7a*** *and (R)-(1-ethoxycarbonyl-ethyl)-(S)-1.4-dihydro-2.6-dimethyl-4-(2.3-dichlorophenyl)-5-(1.3.4-oxadiazol-2-yl)-pyridine-3-carboxylate* ***7b***

The mixture of 30 g (0.106 mol) of **6**, and 21.3 g (0.106 mol) **5S**, or (for the synthesis of **7b**) of **5R**, and 100 ml of dimethylsulfoxide was heated at 80°C for 15 h. By cooling to room temperature and adding 300 ml of water, a solid separated which was filtered off and recrystallized from 500 ml ethanol. Yield 28 g (57%). mp 219–221°C. α_D = + 98° (dichloromethane, c = 2.0) for **7a** and α_D = – 100° (dichloromethane, c = 2.0) for **7b**. Anal $C_{21}H_{21}Cl_2N_3O_5$ (C, H, Cl, N, O). ^{1}H NMR ($CDCl_3$) δ: 1.25 (t, 3H); 1.57 (d, 3H); 2.54 (s, 6H); 4.21 (q, 2H); 5.28 (q, 1H); 5.78 (s, 1H); 6.10 (m, 1H); 7.20–7.80 (m, 3H); 8.5 (s, 1H).

Isopropyl-(R)-1.4-dihydro-2.6-dimethyl-4-(2.3-dichlorophenyl)-5-(1.3.4-oxadiazol-2-yl)-pyridine-3-carboxylate ***8a*** *and Isopropyl-(S)-1.4-dihydro-2.6-dimethyl-4-(2.3-dichlorophenyl)-5-(1.3.4-oxadiazol-2-yl)-pyridine-3-carboxylate* ***8b***

10 g (0.021 mol) of **7a**, or (for the synthesis of **8b**) of **7b**, were added to the solution of 0.15 g lithium (0.021 mol) in 100 ml of isopropanol. The resulting mixture was heated to reflux for 20 h and, after concentration to a volume of about 30 ml under reduced pressure, was poured into 150 ml of ice water. A solid separated which was filtered off and recrystallized from a 6:4 mixture of ethanol:water. Yield 5.5 g (64%). mp 199–201°C. Anal α_D = + 90° (dichloromethane, c = 2.0) for **8a** and α_D = – 90° (dichloromethane, c = 3.8) for **8b**. Anal $C_{19}H_{19}Cl_2N_3O_3$ (C, H, Cl, N, O). ^{1}H NMR ($CDCl_3$) δ: 1.00–1.45 (q, 6H); 2.46 (s, 6H); 5.10 (m, 1H); 5.71 (s, 1H); 6.00 (m, 1H); 7.20–7.70 (m, 3H); 8.42 (s, 1H).

Biological tests

Binding studies

Membrane preparation. Bovine cerebral cortices were homogenized in Tris–HCl (50 mmol/l, pH 7.4 at room temperature) with a glass teflon potter. The wet weight to volume ratio was 1:50. The membrane suspension was centrifuged twice at 48 000 *g* for 10 min. The final crude pellet was resuspended in Tris–HCl at a protein concentration of 2–3 mg/ml and immediately stored at – 77°C until use. Protein content of tissue homogenates was determined according to the method of Lowry *et al* [29] using bovine serum albumin as standard.

Binding assay. Equilibrium binding assays were carried out at 25°C in a final volume of 0.2 ml containing 50 mmol/l Tris–HCl and 0.6–0.8 mg/ml membrane protein. [^{3}H]-Nitrendipine (2.59–3.22 TBq/mmol, New England Nuclear, Dreieich, Germany) and the non-labeled ligands were added at a concentration of 0.3–0.4 nmol/l and at the concentrations indicated in the figures, respectively. The samples were incubated for 60 min and rapidly filtered by vacuum through Whatman glass fiber filters (GF/C) using a cell harvester (Scatron, Norderstedt, Germany); the filters were rinsed with 20 ml ice-cold Tris–HCl, dried and counted for radioactivity by liquid scintillation counting. All values obtained were the mean of triplicate samples. Specific [^{3}H]-nitrendipine bound was defined as the difference between [^{3}H]-nitrendipine binding in the absence and presence of 0.5 µmol/l nifedipine (Sigma, Deisenhofen, Germany). In all procedures involving 1.4-dihydropyridines, these compounds were protected against light. Affinity constants obtained from competition experiments were calculated using an iterative nonlinear least squares fitting procedure (GIP: Giessener Iterations Program). The binding data are given as mean value ± SEM of *n* independent experiments.

Calmodulin-PDE assay

PDE activity was measured in a 2-step procedure: [^{3}H]-cAMP was first hydrolyzed by PDE to [^{3}H]-5-AMP. [^{3}H]-5-AMP was then converted to [^{3}H]-adenosine and inorganic phosphate by 5-nucleotidase. The first step was started by adding 50 µl PDE (0.5 mU) to the incubation mixture and terminated after 10 min by adding 50 µl 0.1 N HCl. The second step was initiated by adding 50 µl 5-nucleotidase and stopped by cooling in ice water. The tritiated adenosine formed was separated from the nucleotides by elution on QAE–Sephadex A-25 columns with 2 ml ammonium formiate 30 mmol/l. Counting was by liquid scintillation spectrometry. Experiments were performed at 30°C. The inhibition of enzyme activity by increasing concentrations of test compounds was measured in the presence (CaM-stimulated activity) and in the absence (basal activity) of CaM. Data given are means (± SD) of 5 independent experiments performed in triplicate.

Determination of lipophilicity

Lipophilicity data of test compounds (R_M-values) were measured by the aid of reversed phase thin layer chromatography as described by Mannhold *et al* [23].

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