A novel 1,4-dihydropyridine-binding site on mitochondrial membranes from guinea-pig heart, liver and kidney

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The 1,4-dihydropyridine ($\pm$)-[^3H]nitrendipine reversibly binds to mitochondrial preparations from guinea-pig heart with a dissociation constant (K_d) of 593 ± 77 nM and a maximum density of binding sites (B_{max}) of 1.75 ± 0.27 nmol/mg of protein. This low-affinity high-capacity 1,4-dihydropyridine-binding site does not discriminate between the enantiomers of nitrendipine and is also found in mitochondrial membranes from guinea-pig liver (K_d 586 ± 91 nM; B_{max} 0.36 ± 0.04 nmol/mg of protein) and kidney (K_d 657 ± 149 nM; B_{max} 0.56 ± 0.12 nmol/mg of protein). Phenylalkylamines (e.g. verapamil) inhibit ($\pm$)-[^3H]nitrendipine binding with micromolar inhibition constants, but the benzothiazepine D-*cis*-diltiazem, a potent Ca^{2+} -channel blocker, is without effect. The binding is heat-stable, shows a V-shaped pH-dependence with a minimum around pH 7.0, and is strongly dependent on ionic strength in the incubation medium. The cations $\text{La}^{3+} > \text{Cd}^{2+} \gg \text{Co}^{2+} > \text{Ca}^{2+} \gg \text{Ba}^{2+} > \text{Mg}^{2+} > \text{Li}^+ > \text{Na}^+$ and the anions $\text{NO}_3^- > \text{Cl}^- \gg \text{F}^-$ stimulate the binding, whereas $\text{PO}_4^{3-} > \text{SO}_4^{2-}$ slightly inhibit it. The low-affinity ($\pm$)-[^3H]nitrendipine-binding site located on the mitochondrial inner membrane is biochemically and pharmacologically different from the 1,4-dihydropyridine-receptor domain of the L-type Ca^{2+} channel. Furthermore, it is not identical with any of the low-affinity 1,4-dihydropyridine-binding sites described so far.

INTRODUCTION

Some 1,4-dihydropyridines [e.g. ($\pm$)-nitrendipine, ($\pm$)-nimodipine, (–)-azidopine] bind to one of three drug-receptor domains associated with the L-type Ca^{2+} channel having dissociation constants in the nanomolar and subnanomolar range [for reviews, see Triggle & Janis (1987), Vaghy *et al.* (1987a) and Glossmann *et al.* (1985)]. In radiolabelled form these compounds and structurally related analogues have been extensively used in the solubilization and purification of the Ca^{2+} -channel protein (Borsoatto *et al.*, 1984; Flockerzi *et al.*, 1986; Curtis & Catterall, 1984; Striessnig *et al.*, 1986). The photoincorporation of (–)-[^3H]azidopine (Ferry *et al.*, 1984) and of [^3H]PN 200-110 (Galizzi *et al.*, 1986) into crude membrane preparations as well as into the purified Ca^{2+} -channel protein (Striessnig *et al.*, 1986; Vaghy *et al.*, 1987b) has led to the identification of the α_1 -subunit of the L-type Ca^{2+} channel from skeletal muscle (Glossmann *et al.*, 1987; Takahashi *et al.*, 1987). Recently, the cDNA sequence of this Ca^{2+} -antagonist-receptor-carrying channel polypeptide was reported (Tanabe *et al.*, 1987). The nanomolar dissociation constants of the 1,4-DHPs in depolarized membrane fragments shift to the micromolar or submicromolar range upon polarization of the preparation, e.g. in intact heart cells (Kokubun *et al.*, 1986). This membrane-potential-induced low-affinity state of the L-type Ca^{2+} channel (Sanguinetti & Kass, 1984; Bean, 1984) is not to be confused with low-affinity 1,4-DHP-binding sites which have been also found in completely depolarized

crude membrane preparations (Bellemann *et al.*, 1981, 1982; Glossmann & Ferry, 1983; Kenessey *et al.*, 1984; Vaghy *et al.*, 1985; Striessnig *et al.*, 1985; Ferry *et al.*, 1987). 1,4-DHPs bind to these sites with K_d values in the submicromolar range; their density surpasses that of the L-type- Ca^{2+} -channel-associated 1,4-DHP receptors by several orders of magnitude.

These low-affinity 1,4-DHP-binding sites have been characterized with the help of reversible 1,4-DHP binding (Bellemann *et al.*, 1981, 1982; Glossmann & Ferry, 1983; Vaghy *et al.*, 1985; Ferry *et al.*, 1987; Striessnig *et al.*, 1985; Ruth *et al.*, 1985), radioligand binding-inhibition by nitrendipine or nifedipine (Cantor *et al.*, 1984) or functional assays (Vaghy *et al.*, 1982; Schaechtele *et al.*, 1987). Recently, in addition to a 165 kDa protein associated with the L-type Ca^{2+} channel, Ferry *et al.* (1987) photolabelled 39 kDa and 45 kDa polypeptides with (–)-[^3H]azidopine in crude heart membranes. These low- M_r polypeptides were suggested to be the structural equivalent to low-affinity 1,4-DHP-binding sites which did not bind ($\pm$)-Bay K 8644, a well-known Ca^{2+} -channel activator. Correspondingly (–)-[^3H]nitrendipine (Campbell *et al.*, 1984) or a [^{125}I]iodinated 1,4-dihydropyridine (Sarmiento *et al.*, 1986) photolabels polypeptides with molecular masses in the 30–40 kDa range in a variety of tissues, including heart. With the aid of ($\pm$)-[^3H]nitrendipine we have now physically separated a class of low-affinity 1,4-DHP-binding sites from Ca^{2+} -channel-associated receptors in guinea-pig heart by means of differential and sucrose-gradient centrifugation. We demonstrate below that these low-affinity sites are

Abbreviations used: B_{max} , maximum density of binding sites; D600, methoxyverapamil; D888, desmethoxyverapamil; 1,4-DHP, 1,4-dihydropyridine; DMSO, dimethyl sulphoxide; h , Hill coefficient; k_{+1} , association rate constant; k_{-1} , dissociation rate constant; K_d , dissociation constant; n , number of experiments; NBTG, nitrobenzylthioguanosine; 1,4-DHP ester, 1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)pyridine-3,5-dicarboxylic acid 3-ethyl 5-(2-aminoethyl) ester; PEG, poly(ethylene glycol); PMSF, phenylmethanesulphonyl fluoride; $t_{1/2}$, half-life of the receptor-ligand complex; IC_{50} , concentration causing half-maximal inhibition.

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associated with the mitochondrial inner membrane and are also found on purified mitochondria from liver and kidney. The latter tissues, in contrast with the heart, did not contain 1,4-DHP receptors coupled to the L-type Ca^{2+} channel.

MATERIALS AND METHODS

Materials

Sources of unlabelled calcium-channel ligands were previously given (Glossmann & Ferry, 1985; Striessnig *et al.*, 1985; Ferry *et al.*, 1987). The optical enantiomers of the calcium-channel agonist Bay K 8644, as well as most of the unlabelled 1,4-dihydropyridines, were from Bayer A.G., Wuppertal, Germany. $(\pm)$ - ^3H -Nitrendipine (81.1 Ci/mmol) was from New England Nuclear, Vienna, Austria, and 5-hydroxy[G- ^3H]tryptamine (20 Ci/mmol) was from Amersham International, Vienna, Austria. All other chemicals were obtained from Merck, Darmstadt, Germany, or Sigma, Munich, Germany, and were of the highest grade available.

Membrane preparation

For sucrose-density-gradient centrifugation, membrane vesicles were prepared from guinea-pig hearts and separated on a 20–40%-(w/w)-sucrose gradient by isopycnic centrifugation at 100 000 *g* for 15 h at 2 °C as described by Vetter *et al.* (1982).

Preparation of mitochondria and differential centrifugation were done as described by Matlib *et al.* (1984) and Reinhart & Bygrave (1981), with the following modifications. Trimmed ventricles were homogenized with the aid of a Teflon pestle (three bouts of three strokes at 1500 rev./min; all steps were performed at 2 °C) in buffer A containing 250 mM-sucrose, 10 mM-Tris/Hepes, pH 7.4 at 2 °C, 1 mM-Tris-EGTA and 0.1 mM-PMSF. Alternatively, buffer B, containing 180 mM-KCl, 1 mM-EGTA and 0.5% (w/v) bovine serum albumin, pH 7.4 at 2 °C, was used with the same results. The homogenate was centrifuged at 1000 *g* for 5 min. The rotor (r_{av} , 10.8 cm) was always operated at 2 °C. The pellet ('1P') was usually discarded and the supernatant ('1S') spun down at 8000 *g* for 10 min and washed once in buffer A. The resulting pellet was taken up in buffer C, which contained 50 mM-Tris/HCl and 0.1 mM-PMSF pH 7.4 at 2 °C, washed two times in buffer C, and sedimented at 39 000 *g* for 10 min. This final washed pellet was termed '8P', contained the highest specific activity of the mitochondrial enzyme marker, and was used in the binding assays. The combined supernatants from the 8000 *g* run ('8S') were usually discarded. For localization experiments, they were spun down at 39 000 *g* for 10 min and washed twice in buffer C ('39P'), leaving combined supernatants, termed '39S'.

The submitochondrial localization of $(\pm)$ - ^3H -nitrendipine-binding sites was determined in the 8P fraction obtained from guinea-pig liver, either by swelling and subsequent sonication of the mitochondria, exactly as described by Sottocasa *et al.* (1967) or by selective digitonin solubilization as described by Anholt *et al.* (1986).

Binding experiments

Unless indicated otherwise, binding of $(\pm)$ - ^3H -nitrendipine was assayed as follows: membranes (3–22 μg of protein) were incubated with 1.1–3.4 nM- $(\pm)$ - ^3H -nitrendipine in a 0.25 ml volume of buffer C

supplemented with 500 mM-NaCl for 60 min at 37 °C, pH 7.4. 'High-affinity binding' was defined as the difference between total binding and binding in presence of 10 μM - $(\pm)$ -Bay K 8644 (see Ferry *et al.*, 1987); binding of $(\pm)$ - ^3H -nitrendipine to low-affinity sites was measured in the presence of 10 μM - $(\pm)$ -Bay K 8644, with 10 μM - $(\pm)$ -nicardipine as non-specific-binding definition. Saturable binding was linear up to 0.12 mg/ml (results not shown). Because of the very high ratio of low- to high-affinity binding [$> 8:1$ at a total $(\pm)$ - ^3H -nitrendipine concentration of about 1.5 nM] in saturation and competition experiments the high-affinity $(\pm)$ - ^3H -nitrendipine-binding sites were not blocked by $(\pm)$ -Bay K 8644 as had been necessary in crude heart membranes (Ferry *et al.*, 1987). In pH-dependence-determination experiments, buffer C supplemented with 500 mM-NaCl was used in which 50 mM-Tris was replaced by 100 mM-BisTris; the desired pH was obtained by adjustment with HCl at 37 °C. For saturation analysis the specific activity was lowered to 0.76–0.86 Ci/mmol (in four experiments down to 0.008 Ci/mmol) by addition of unlabelled $(\pm)$ -nitrendipine (or its enantiomers) with overlapping points as described by Glossmann & Ferry (1985). Binding experiments with $(+)$ - ^3H -PN 200-110 were performed with 3 μM - $(\pm)$ -Bay K 8644 as non-specific-binding control as described by Glossmann & Ferry (1985). Binding experiments with $(-)$ - ^{125}I -iodocyanopindolol were performed as described by Bonacker & Glossmann (1981) with 0.1 mM- $(-)$ -isoprenaline as a non-specific-binding definition. Bound and free ligand were separated by filtration over glass-fibre filters as described by Glossmann & Ferry (1985). Briefly, the incubation mixture was diluted with 3.5 ml of ice-cold PEG (10 mM-MgCl₂/MgCl₂/10 mM-Tris/HCl, pH 7.4) buffer and passed through Whatman GF/C filters under negative pressure, followed by two subsequent washes with 3.5 ml of PEG buffer. Throughout the text below the term 'binding' refers to *specific* binding of the respective drug.

To assess chemical stability of $(\pm)$ - ^3H -nitrendipine in the binding experiments, 1 nM- $(\pm)$ - ^3H -nitrendipine was incubated with 100 μg of mitochondrial protein in 0.25 ml of buffer C supplemented with 500 mM-NaCl for 6 h at 37 °C. Bound ligand was separated from unbound ligand by centrifugation and extracted with ethanol. T.l.c. analysis of ethanol-extracted mitochondria-bound $(\pm)$ - ^3H -nitrendipine using a silica-gel support with a chloroform/acetone (95:5, v/v; 25 °C, 2 h) mobile phase showed that more than 95% of the bound radioactivity co-migrated with unlabelled $(\pm)$ -nitrendipine or $(\pm)$ - ^3H -nitrendipine incubated for 6 h at 37 °C in the absence of membranes.

Drugs were prepared from 10 mM stock solutions in DMSO. As DMSO proved to be an inhibitor of the low-affinity $(\pm)$ - ^3H -nitrendipine-binding site with an IC_{50} value of $4.7 \pm 0.6\%$ (v/v) (mean $\pm$ asymptotic S.D., $n = 3$), its concentration was kept below 0.1% whenever possible.

Unless indicated otherwise, protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Determination of succinate dehydrogenase and monoamine oxidase

Succinate dehydrogenase (EC 1.3.99.1) was assayed as described by King (1967). The determination of

monoamine oxidase (EC 1.4.3.4) activity was performed as described by Fowler & Strolin Benedetti (1983), with 5-hydroxy[G-³H]tryptamine as substrate and 100 μ M-pargyline as blank definition (Lenzen *et al.*, 1983).

Data analysis

Data from equilibrium binding and kinetic experiments were analysed with the LIGAND program written by Munson & Rodbard (1980). Binding-competition experiments were analysed as described previously (Glossmann & Ferry, 1985).

RESULTS

Subcellular localization of low-affinity ($\pm$)-[³H]nitrendipine binding sites

When guinea-pig hearts were homogenized and the crude membranes separated on a 20–40 %-(w/w)-sucrose gradient as described by Vetter *et al.* (1982), the low-affinity ($\pm$)-[³H]nitrendipine binding co-sedimented with the activity of succinate dehydrogenase (EC 1.3.99.1), an enzyme located exclusively in the mitochondrial inner membrane (see Fig. 1). On the other hand, ($\pm$)-Bay K 8644-inhibitable (+)-[³H]PN 200-110 binding, highly selective for Ca²⁺-channel-linked 1,4-dihydropyridine receptors (Ferry *et al.*, 1987), as well as (–)-[¹²⁵I]iodocyanopindolol binding as a probe for plasma-membral β -adrenoceptors, were found in much lighter fractions. Thus sarcolemmal and mitochondrial membranes could be clearly separated, with the low-affinity ($\pm$)-[³H]nitrendipine-binding sites located in the mitochondrial fraction.

Having established the almost exclusive location of the low-affinity binding sites on mitochondria, we tried to enrich them by differential centrifugation, as the sucrose gradient technique was too time-consuming and the recoveries were low. Fig. 2 shows that tissues, when homogenized under iso-osmotic conditions, yielded a pellet after an 8000 *g* centrifugation step ('8P') which had both the highest specific activity of succinate dehydrogenase and the highest density of low-affinity ($\pm$)-[³H]nitrendipine-binding sites. Conversely, the highest density of binding sites for (+)-[³H]PN 200-110 and (–)-[¹²⁵I]iodocyanopindolol together with high-affinity ($\pm$)-[³H]nitrendipine-binding sites was found in the pellet which sedimented at 39 000 *g* ('39P'). Therefore the '8P' pellet (which additionally contained the smallest specific sarcolemmal-marker activity of all fractions) was used in all further experiments. It is referred to as 'mitochondrial membranes'.

When mitochondria from the guinea-pig liver 8P fraction were subjected to short hypo-osmotic shock followed by brief sonication (as detailed by Sottocasa *et al.*, 1967), three different fractions could be separated on a discontinuous sucrose gradient: (a) a soluble fraction at 0.45 M-sucrose, (b) a band at the 0.76/1.32 M-sucrose interface ('light' fraction) and (c) a pellet below the 1.32 M-sucrose layer ('heavy' fraction). As can be seen in Fig. 3, the heavy fraction contained the highest specific activity of both succinate dehydrogenase and low-affinity ($\pm$)-[³H]nitrendipine binding, whereas the light fraction was enriched in monoamine oxidase (EC 1.4.3.4) activity, a marker for the mitochondrial outer membrane. Accordingly, when guinea-pig liver mitochondria (8P) were digitonin-solubilized at different digitonin/protein

Fig. 1. Sucrose-density-gradient profile of low-affinity ($\pm$)-[³H]-nitrendipine binding in comparison to sarcolemmal and mitochondrial markers

Cardiac membrane vesicles were prepared as described in the Materials and methods section and centrifuged for 15 h at 2 °C in a 20–40 %-(w/w)-sucrose gradient at 100 000 *g*. In all, 16 fractions of 2 ml each were recovered. A 100 μ l portion of each fraction, containing 3–17 μ g of protein [determined as described by Bradford (1976)] was incubated in a total volume of 0.25 ml (buffer C supplemented with 500 mM-NaCl) with 2.6 nM-($\pm$)-[³H]-nitrendipine in presence of 3 μ M-($\pm$)-Bay K 8644, or in 0.25 ml of buffer C with 0.56 nM-(+)-[³H]PN 200-110. (–)-[¹²⁵I]iodocyanopindolol at 2.71 pM was used for binding experiments, and succinate dehydrogenase (SCDH) activity [expressed in decrease of absorbance (– Δ A) in the photometric assay] was determined as indicated in the Materials and methods section. (a) Low-affinity ($\pm$)-[³H]-nitrendipine binding (●) and (+)-[³H]PN 200-110 binding (▲); (b) succinate dehydrogenase activity (●) and (–)-[¹²⁵I]iodocyanopindolol binding (▲); (c) protein yield (■) and sucrose concentration (▼).

Fig. 2. Differential centrifugation profile of low- and high-affinity $(\pm)$ - $[^3\text{H}]$ nitrendipine binding in comparison with sarcolemmal and mitochondrial markers

Portions (5–23 μg protein) of the different fractions were incubated with either 2.0–2.5 nM $(\pm)$ - $[^3\text{H}]$ nitrendipine, 1.4 nM $(+)$ - $[^3\text{H}]$ PN 200-110, or 19.1–20.4 pM $(-)$ - $[^{125}\text{I}]$ iodocyanopindolol. Assay conditions, as well as the definition of high- and low-affinity $(\pm)$ - $[^3\text{H}]$ nitrendipine binding, are given in the Materials and methods section. Values for binding experiments are given as means $\pm$ S.E.M. for three independent experiments performed in duplicate. Succinate dehydrogenase (SCDH) activity (expressed in μmol of succinate metabolized/min per mg of protein) was determined by using 5.6–12.4 μg of protein (means $\pm$ S.E.M.; $n = 6$). Estimated protein recoveries in the 8000 g and 39000 g steps were 108 and 98 % respectively. Recovery of total binding or activity (= specific binding/activity $\times$ total protein) for each parameter was 107 ± 19 % in the 39000 g step. The terms 1P, 1S, 8P, 8S, 39P and 39S are defined in the text; HOM is homogenate.

ratios, monoamine oxidase activity was completely solubilized at 1 mg of digitonin/mg of protein, whereas both low-affinity $(\pm)$ - $[^3\text{H}]$ nitrendipine binding as well as succinate dehydrogenase activity remained in the pelletable fraction.

Kinetics of low-affinity $(\pm)$ - $[^3\text{H}]$ nitrendipine binding

$(\pm)$ - $[^3\text{H}]$ Nitrendipine binding to mitochondrial membranes in presence of 10 μM $(\pm)$ -Bay K 8644 reached equilibrium well before 120 min at 37 °C. Addition of 10 μM $(\pm)$ -nicardipine leads to a monophasic decay of the ligand-binding-site complex (for an example, see Fig. 4). The calculated association rate constant, k_{+1} , was $4.49 \pm 1.18 \times 10^{-4} \text{ M}^{-1} \cdot \text{min}^{-1}$ (mean $\pm$ S.E.M., $n = 5$), the dissociation rate constant, k_{-1} , $0.016 \pm 0.004 \text{ min}^{-1}$ ($n = 7$). From these k_{+1} and k_{-1} values, a kinetic dissociation constant of 357 nM could be calculated. This value

corresponded well to the K_d determined by equilibrium-saturation analysis (see below). When $(\pm)$ - $[^3\text{H}]$ -nitrendipine binding was monitored up to 6 h, a small increase could be observed (31 % of the value at 120 min; results not shown). The slow time course of this process excluded any interference with the kinetic experiments described above. The process is slower at 37 °C than at 56 °C and is most likely explained by a temperature-dependent increase in binding affinity (see below).

Equilibrium $(\pm)$ - $[^3\text{H}]$ nitrendipine binding to mitochondrial membranes from heart, liver and kidney

Equilibrium binding parameters of $(\pm)$ - $[^3\text{H}]$ nitrendipine for mitochondrial membranes from different guinea-pig organs are as follows: a K_d of $593 \pm 77 \text{ nM}$ and a B_{max} of $1.75 \pm 0.27 \text{ nmol/mg}$ of protein in heart (mean $\pm$ S.E.M., $n = 18$), a K_d of $586 \pm 91 \text{ nM}$

Fig. 3. Submitochondrial localization of low-affinity (±)-[³H]-nitrendipine binding

Portions (3.5–33.3 μg of protein) of the different fractions obtained by swelling and sonication of guinea-pig liver mitochondria were incubated with 1.5–2.2 nM-(±)-[³H]-nitrendipine to assess low-affinity binding (three different experiments performed in triplicate). Additionally, the fractions were tested for succinate dehydrogenase (SCDH; eight determinations) and monoamine oxidase activity (MAO; six determinations) (see the Materials and methods for details). 8P, mitochondria from differential centrifugation as starting material; A, soluble mitochondrial fraction; B, light mitochondrial fraction; C, heavy mitochondrial fraction. Values shown are means ± S.E.M.

and a $B_{\max}$ of 0.36 ± 0.04 nmol/mg of protein in liver ($n = 11$), and a K_d of 657 ± 149 nM and a $B_{\max}$ of 0.56 ± 0.12 nmol/mg of protein in kidney ($n = 8$). Fig. 5 shows an example of a saturation analysis with

(±)-[³H]nitrendipine and heart mitochondrial membranes. Lowering the temperature from 37 °C to 2 °C decreased the binding capacity of low-affinity (±)-[³H]nitrendipine-binding sites, whereas the K_d was only marginally changed (see the legend to Fig. 5).

Differential heat-sensitivity of low- and high-affinity (±)-[³H]nitrendipine binding

Fig. 6 shows the changes in low- and high-affinity (±)-[³H]nitrendipine binding when the mitochondrial fractions are kept at 56 °C for the times indicated before the binding assay. Whereas residual high-affinity binding of (±)-[³H]nitrendipine (originating from plasma-membrane contamination) was almost completely abolished (> 95 %) after 10 min at 56 °C, low-affinity site-bound (±)-[³H]nitrendipine actually increased. This increase could be attributed to an approx. 2-fold increase in affinity of the binding sites for (±)-[³H]nitrendipine (results not shown).

pH- and ion-dependence of low-affinity (±)-[³H]nitrendipine binding

Low-affinity (±)-[³H]nitrendipine binding displayed a V-shaped pH dependence with a minimum around pH 7.0 (Fig. 7).

The cation-dependence of low-affinity (±)-[³H]-nitrendipine binding is shown in Fig. 8(a) and Table 1. In buffer C, (±)-[³H]nitrendipine binding was 0.045 ± 0.004 pmol/mg of protein (mean ± S.E.M., $n = 5$) at a total ligand concentration of 1.1–3.4 nM. Enhancement of binding was saturable by (in order of decreasing potency) $\text{La}^{3+} > \text{Cd}^{2+} \gg \text{Co}^{2+} > \text{Ca}^{2+} \gg \text{Ba}^{2+}$, whereas $\text{Mg}^{2+} > \text{Li}^+ > \text{Na}^+ = \text{K}^+$ increased the binding, without reaching saturation, up to the highest concentrations employed.

The anions $\text{NO}_3^- > \text{Cl}^-$ also increased (±)-[³H]-nitrendipine binding (Fig. 8b, Table 1). F^- , SO_4^{2-} , and PO_4^{3-} were without effect in buffer C. However, when tested in buffer C supplemented with 500 mM-NaCl (as used for standard low-affinity binding measurements), $\text{PO}_4^{3-} > \text{SO}_4^{2-}$ slightly decreased (±)-[³H]nitrendipine binding, whereas F^- marginally increased it. NO_3^- stimulation in buffer C supplemented with 500 mM-NaCl did not exceed the maximum values attained in buffer C alone (Table 1).

Thus the stimulation of (±)-[³H]nitrendipine binding by Na^+ and NO_3^- was not additive.

Low-affinity (±)-[³H]nitrendipine binding was not enhanced by raising osmolarity alone, e.g. by addition of 1 M-sucrose to buffer C (results not shown).

Pharmacological profile of low-affinity (±)-[³H]nitrendipine binding

(±)-[³H]Nitrendipine displacement experiments using Ca^{2+} -channel drugs from three main chemical classes were performed on mitochondrial membranes from heart (Table 2), liver and kidney.

Apart from PN 200-110, none of the tested drugs displayed any stereoselectivity in the guinea-pig heart. The enantiomers of nitrendipine inhibited (±)-[³H]-nitrendipine binding with the same IC_{50} value (Table 2). Other 1,4-dihydropyridine Ca^{2+} -channel drugs were effective inhibitors, with IC_{50} values in the same range as the dissociation constant of (±)-nitrendipine. The following 1,4-DHP structure-activity relationship can be inferred. (1) Esterification of both the 3-carboxy and the

Fig. 4. Association and dissociation kinetics of the $(\pm)$ - ^3H nitrendipine low-affinity binding site in cardiac mitochondrial membranes

(a) Association kinetics. A $14.2 \mu\text{g}$ portion of protein (1.71 nmol of binding sites/mg of protein) was incubated with 1.81 nM $(\pm)$ - ^3H nitrendipine in 0.25 ml of buffer C supplemented with 500 mM -NaCl up to 120 min at 37°C in the presence of $10 \mu\text{M}$ $(\pm)$ -Bay K 8644. (b) Plot of $(1/L_0 - R_0) \cdot \ln[(L_0 - B_t)/(R_0 - B_t)]$ versus incubation time, where L_0 and R_0 are the total concentrations of ligand and binding site respectively, and B_t is the concentration of binding-site-ligand complex at time t . The slope of the line, determined by linear regression, yielded an association rate constant, k_{+1} , of $3.384 \times 10^4 \text{ M}^{-1} \cdot \text{min}^{-1}$ (0 – 10 min , $r = -0.999$). (c) Dissociation kinetics. After 120 min incubation with $(\pm)$ - ^3H nitrendipine, dissociation of bound ligand (B_0) was initiated by addition of $10 \mu\text{M}$ unlabelled $(\pm)$ -nitrendipine and bound ligand measured for the indicated times (B_t). (d) Semilogarithmic plot of $\ln(B_t/B_0)$ versus time. The slope was determined by linear regression (0 – 60 min) and yielded a dissociation rate constant, k_{-1} , of 0.0296 min^{-1} , corresponding to a receptor-ligand-complex half-life, $t_{1/2}$, of 23.4 min . The K_d calculated from k_{-1}/k_{+1} was 874 nM . Values shown are means of duplicate determinations.

Fig. 5. Saturation isotherm of $(\pm)$ - ^3H nitrendipine binding to cardiac mitochondrial membranes at 37 and 2°C

A portion ($14.4 \mu\text{g}$ of protein) was incubated with 60 – 1500 nM $(\pm)$ - ^3H nitrendipine at a specific radioactivity of 0.76 – 0.86 Ci/mmol for 120 min at 37°C ($\bullet$) or for 240 min at 2°C ($\blacksquare$). (b) The line drawn was calculated with the parameters computed from the Hofstee transformation (a), which revealed a K_d of 499 nM and a B_{max} of 1.98 nmol/mg of protein for the 37°C saturation isotherm (linear regression; $r = -0.995$; values shown are means for two pooled experiments performed in duplicate). The 2°C saturation isotherm yielded a K_d of 259 nM and a B_{max} of 0.136 nmol/mg of protein ($r = -0.997$). Values are means for a single experiment performed in duplicate.

Fig. 6. Differential heat-sensitivity of low- and high-affinity $(\pm)$ - ^3H nitrendipine binding to cardiac mitochondrial membranes

A portion (5.4 – $16.1 \mu\text{g}$) of protein from the mitochondrial fraction (8P) was heated at 56°C in an assay volume of 0.1 ml of buffer C for the times indicated. Subsequent binding assays were performed in a total volume of 0.25 ml of buffer C supplemented with 500 mM -NaCl for 60 min at 37°C using 1.21 – 1.61 nM $(\pm)$ - ^3H nitrendipine, and high- ($\blacktriangle$) and low- ($\bullet$) affinity binding was determined as described in the text. Values are given as normalized means $\pm$ S.E.M. of three experiments performed in triplicate.

5-carboxy group is essential for binding to the mitochondrial low-affinity 1,4-DHP site; the 5-nitro-substituted compounds Bay K 8644 and the enantiomers (*R*)- and (*S*)-202-791 did not inhibit at concentrations

reaching their solubility limits ($20 \mu\text{M}$); the 1,4-DHP Bay M 5579 with only one esterified carboxy group was also ineffective over the tested concentration range. (2) The chain length of the substituted esters is the most

Fig. 7. pH-dependence of low-affinity (±)-[³H]nitrendipine binding to cardiac mitochondrial membranes

A portion (17.1 µg) of membrane protein in an assay volume of 0.25 ml was incubated with 3.2 nM-(±)-[³H]-nitrendipine at 37 °C for 60 min in Bistris buffer (see the Materials and methods section). Values shown are means ± S.E.M. of six determinations from two independent experiments.

important determinant of binding potency (e.g. nitrendipine versus nifedipine, niludipine versus nimodipine). Isopropyl groups are preferred over isobutyl groups (Bay e 6927 versus nisoldipine). An ester with a 2-aminoethyl group is highly unfavourable for binding (1,4-DHP ester). (3) 7-Bromo-substitution at the benzoxadiazole 4-residue somewhat decreases affinity but is not detrimental at all for binding (PN 200-110 versus VO 2605).

The phenylalkylamines were much less potent inhibitors compared with the 1,4-DHPs by a factor of 100 (Table 2). Again, none of the tested phenylalkylamines displayed any stereoselectivity. Our limited data on phenylalkylamine structure-activity relationships show that (1) both benzene rings are essential for binding (D619 was ineffective up to 100 µM) and that (2) the number of methoxy groups at one of the two benzene rings does not affect potency (D888, verapamil, D600).

The benzothiazepines D- and L-*cis*-diltiazem did not inhibit (±)-[³H]nitrendipine binding in concentrations up to 100 µM.

Comparative studies on mitochondrial membranes from liver and kidney showed a similar inhibition profile, despite small differences in potencies. An exception were the enantiomers of PN200-110, which did not display stereoselectivity in the two organs. We obtained IC₅₀ values of 1800 nM for (+)-PN200-110 and 2200 nM for

Table 1. Effects of cations and anions on the low-affinity binding of (±)-[³H]nitrendipine to heart mitochondrial membranes

EC₅₀ (concentration causing half-maximal effect) values are only given for saturable-binding stimulation (means ± asymptotic S.D.). For all other experiments (*) the average binding at 500 mM is given. *h* is the Hill coefficient, *n* is the number of experiments. Basal (±)-[³H]nitrendipine bound was 0.045 ± 0.004 pmol/mg of protein in buffer C alone (mean ± S.E.M., *n* = 5).

Ion	Maximally bound (±)-[³ H]nitrendipine (pmol/mg of protein)	EC ₅₀ (mM)	<i>h</i>	<i>n</i>
Cations (chloride salts)				
Control	0.045	—	—	5
La ³⁺	1.88	3.3 ± 1.8	1.08 ± 0.60	2
Cd ²⁺	1.77	10.1 ± 1.0	1.80 ± 0.46	3
Co ²⁺	2.03	49.7 ± 17.4	1.74 ± 0.77	2
Ca ²⁺	2.77	52.5 ± 2.7	1.93 ± 0.16	3
Ba ²⁺	3.33	104 ± 14	1.74 ± 0.40	2
Mg ²⁺	2.98*	—	—	2
Li ⁺	2.51*	—	—	2
Na ⁺	1.27*	—	—	3
K ⁺	1.26*	—	—	3
Anions (sodium salts)				
Control	0.045	—	—	5
NO ₃ ⁻	2.70*	—	—	2
Cl ⁻	1.27*	—	—	3
F ⁻	0.28*	—	—	2
SO ₄ ²⁻	0.17*	—	—	2
PO ₄ ³⁻	0.03*	—	—	3
Anions in the presence of 500 mM-NaCl				
Control	0.66	—	—	1
NO ₃ ⁻	1.84*	—	—	1
F ⁻	0.96*	—	—	1
SO ₄ ²⁻	0.40*	—	—	1
PO ₄ ³⁻	0.28*	—	—	1

Table 2. Inhibition constants of various 1,4-DHPs, phenylalkylamines and benzothiazepines for ($\pm$)-[3 H]nitrendipine binding to cardiac mitochondrial membranes

A 3–22 μ g portion of protein from cardiac mitochondrial membranes was incubated in 0.25 ml of buffer C supplemented with 500 mM-NaCl and with 1.1–3.4 nM ($\pm$)-[3 H]nitrendipine at 37 °C for 60 min. Values are means $\pm$ asymptotic s.d., h is the Hill coefficient and n is the number of experiments, each performed in duplicate. The percentage of maximal inhibition is given as a percentage of specific control binding at the highest concentration of drug tested. Note that nitrendipine did not display any stereoselectivity. Abbreviation: n.e., not effective up to the highest concentration indicated.

Compound	IC ₅₀ (nM)	Maximal inhibition (%)	h	n
1,4-DHPs				
Niludipine	316 $\pm$ 21	100	1.43 $\pm$ 0.11	3
(+)-PN 200-110	322 $\pm$ 43	100	0.93 $\pm$ 0.10	3
(-)-PN 200-110	1160 $\pm$ 78	100	1.00 $\pm$ 0.06	3
(+)-Nitrendipine	409 $\pm$ 34	100	1.41 $\pm$ 0.15	8
(-)-Nitrendipine	600 $\pm$ 68	100	1.23 $\pm$ 0.15	6
($\pm$)-Nitrendipine	730 $\pm$ 61	100	1.47 $\pm$ 0.12	5
(+)-Bay e 6927	541 $\pm$ 40	100	1.44 $\pm$ 0.13	3
(-)-Bay e 6927	362 $\pm$ 52	100	1.29 $\pm$ 0.19	3
(+)-Nimodipine	431 $\pm$ 91	94	1.14 $\pm$ 0.24	2
(-)-Nimodipine	507 $\pm$ 71	91	1.24 $\pm$ 0.19	3
(+)-Nicardipine	493 $\pm$ 80	98	1.21 $\pm$ 0.19	3
(-)-Nicardipine	375 $\pm$ 24	100	1.47 $\pm$ 0.11	5
VO 2605	832 $\pm$ 138	97	1.17 $\pm$ 0.22	3
(+)-Azidopine	1342 $\pm$ 412	100	0.88 $\pm$ 0.26	3
(-)-Azidopine	834 $\pm$ 154	100	1.11 $\pm$ 0.22	3
Nifedipine	3815 $\pm$ 893	100	1.02 $\pm$ 0.17	3
Nisoldipine	4125 $\pm$ 1510	100	0.91 $\pm$ 0.18	3
($\pm$)-1,4-DHP	> 20000	—	—	4
(+)-Bay K 8644	> 20000	—	—	4
(-)-Bay K 8644	> 20000	—	—	4
(+)(S)-202-791	n.e. (10 μ M)	—	—	2
(-)(R)-202-791	n.e. (10 μ M)	—	—	2
Bay M 5579	n.e. (10 μ M)	—	—	2
Phenylalkylamines				
(+)-D600	20055 $\pm$ 2290	100	1.14 $\pm$ 0.13	3
(-)-D600	21750 $\pm$ 2200	100	1.05 $\pm$ 0.10	3
(+)-D888	57750 $\pm$ 9960	100	0.71 $\pm$ 0.07	4
(-)-D888	50160 $\pm$ 23070	100	0.75 $\pm$ 0.16	4
(+)-Verapamil	121400 $\pm$ 39500	100	0.85 $\pm$ 0.15	4
(-)-Verapamil	79820 $\pm$ 21920	100	0.92 $\pm$ 0.16	5
($\pm$)-D619	n.e. (100 μ M)	—	—	2
Benzothiazepines				
D-cis-Diltiazem	n.e. (100 μ M)	—	—	3
L-cis-Diltiazem	n.e. (100 μ M)	—	—	3
Miscellaneous				
EGTA	n.e. (10 mM)	—	—	2
NBTG	n.e. (0.1 mM)	—	—	2
Amiloride	n.e. (0.1 mM)	—	—	2
Doxorubicin	n.e. (0.1 mM)	—	—	2
Vanadate	n.e. (0.1 mM)	—	—	2
Ouabain	n.e. (0.1 mM)	—	—	2
Ro5-4864	n.e. (10 μ M)	—	—	2

(-)-PN200-110 in the liver, and 480 nM for (+)-PN200-110 and 713 nM (-)-PN200-110 in the kidney. Renal and hepatic mitochondrial preparations did not show any high-affinity 1,4-dihydropyridine binding, e.g. with (+)-PN200-110 as radioligand and 3 μ M-($\pm$)-Bay K 8644 as a non-specific-binding control (results not shown).

DISCUSSION

The possibility that the low-affinity ($\pm$)-[3 H]-nitrendipine-binding sites in heart or other organs

simply represent a fraction of Ca²⁺ channels with decreased affinity for Ca²⁺-channel drugs can be excluded by the following results. (1) Different fractionation techniques, i.e. sucrose-density-gradient centrifugation or differential centrifugation, separate low-affinity binding sites from the Ca²⁺-channel-drug-binding sites and the β -adrenoceptors in heart. (2) The low-affinity binding sites were found in mitochondria of liver and kidney, where no high-affinity Ca²⁺-channel-related receptors are detected. (3) After disruption of liver mitochondria by either swelling or digitonin treatment, the low-affinity binding sites co-purify with succinate dehydrogenase, a

Fig. 8. Ion-dependence of (±)-[³H]nitrendipine binding to cardiac mitochondrial membranes

A portion (14.8–27.4 µg) of membrane protein in 0.25 ml was incubated with 2.4–4.6 nM (±)-[³H]nitrendipine at 37 °C for 60 min. (a) Effects of the bivalent cations Cd²⁺ (■), Ca²⁺ (●), and Mg²⁺ (▲) on (±)-[³H]nitrendipine binding. (b) Effects of the anions NO₃⁻ (▲), Cl⁻ (●), SO₄²⁻ (▼), and PO₄³⁻ (■). Means are of two or three determinations performed in duplicate.

marker for the mitochondrial *inner* membrane, and are separated from monoamine oxidase, a marker for the mitochondrial *outer* membrane. (4) The density of the low-affinity nitrendipine-binding sites in guinea-pig heart mitochondria (1.8 nmol/mg of protein) by far exceeds the density of L-type Ca²⁺ channels in hearts from a variety of species, including the guinea pig, in crude as well as more purified preparations (0.3–1.6 pmol/mg of protein; Bellemann *et al.*, 1981; Garcia *et al.*, 1986; Ferry *et al.*, 1987). This caused considerable problems in photoaffinity and affinity-labelling studies (for a review, see Glossmann *et al.*, 1987). (5) Raising the temperature to 56 °C for 10 min completely inactivates the Ca²⁺-channel-linked (±)-[³H]nitrendipine-binding sites, whereas low-affinity binding is actually *increased*, in agreement with earlier studies on crude membranes (Glossmann & Ferry, 1983; Vaghy *et al.*, 1985). (6) The pH-dependence of low-affinity nitrendipine binding is opposite to that of 1,4-dihydropyridine binding to the L-type Ca²⁺ channel (Glossmann *et al.*, 1984). (7) The cations La³⁺ and Co²⁺ enhance low-affinity binding, while inhibiting 1,4-DHP binding to the L-type Ca²⁺ channel (Glossmann *et al.*, 1982; Glossmann & Ferry, 1983). On the other hand, the Ca²⁺ chelator EGTA does not abolish low-affinity (±)-[³H]nitrendipine binding, which is in clear contrast with its effect on the L-type Ca²⁺ channel (Gould *et al.*, 1982; Glossmann *et al.*, 1982; Luchowski *et al.*, 1984). (8) (±)-[³H]Nitrendipine binding-inhibition experiments with the mitochondrial site using Ca²⁺-channel drugs from the three main chemical classes show that the steric as well as other structural requirements are fundamentally different from those for the L-type Ca²⁺ channel (Table 1 of Striessnig *et al.*, 1985). The complete absence of (±)-[³H]nitrendipine binding regulation by *D-cis*-diltiazem is noteworthy. Potent Ca²⁺-channel drugs, i.e. (–)-Bay K 8644 and the enantiomers of 202-791, did not show any effect on mitochondrial low-affinity (±)-[³H]nitrendipine binding. Conversely, VO 2605, a 7-bromo-substituted PN200-110 (Ferry & Glossmann, 1984) had an IC₅₀ value of 832 nM. The substitution at this position is detrimental for Ca²⁺-channel binding (Triggle *et al.*, 1980).

The beneficial (or toxic) effects of Ca²⁺-channel drugs cannot be solely explained by their inhibition of the Ca²⁺-channel activity (Zsoter & Church, 1983). During oral therapy, 28 % of the low-affinity (±)-[³H]nitrendipine-binding sites could be occupied, rendering them likely candidates for the as-yet-unexplained Ca²⁺-channel-drug effects [assumptions: peak plasma concentration 70 ng/ml (Raemisch & Sommer, 1984), equal distribution, *K*_d 500 nM, intracellular accumulation (Pang & Sperelakis, 1983, 1984; Lüllmann, 1987) not considered].

Seven distinct candidates for low-affinity 1,4-DHP-binding sites have been reported up to now: (1) the nucleoside transporter (Striessnig *et al.*, 1985; Ruth *et al.*, 1985), (2) the Na⁺/Ca²⁺ exchanger (Vaghy *et al.*, 1982), (3) the Na⁺/K⁺-ATPase (Pan & Janis, 1984), (4) the sarcolemmal Ca²⁺-ATPase (David-Dufilho *et al.*, 1984), (5) the peripheral benzodiazepine receptor (Cantor *et al.*, 1984), (6) the calmodulin-dependent cyclic AMP phosphodiesterase (Schaechtle *et al.*, 1987) and (7) the multi-drug-resistance-related membrane glycoprotein gp150-180 (Cornwell *et al.*, 1987; Safa *et al.*, 1987). Of these seven candidates, only the Na⁺/Ca²⁺ exchanger (for a review, see Carafoli, 1982) and the peripheral benzodiazepine receptor (Anholt *et al.*, 1986) have a reported mitochondrial location; the cyclic AMP phosphodiesterase is not even located in membranes (Nair, 1966). None of the tested functional inhibitors of these candidates inhibited (±)-[³H]nitrendipine binding.

In conclusion, the low-affinity (±)-[³H]nitrendipine binding site described here is located in the mitochondrial inner membrane and is clearly distinct from both the 1,4-dihydropyridine-receptor domain of the L-type Ca²⁺ channel as well as all other candidates for low-affinity 1,4-dihydropyridine binding reported so far.

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