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Identification of the site of interaction of the dihydropyridine channel blockers nitrendipine and azidopine with the calcium-channel α_1 subunit

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The dihydropyridine binding site of the rabbit skeletal muscle calcium channel α_1 subunit was identified using tritiated azidopine and nitrendipine as ligands. The purified receptor complex was incubated either with azidopine or nitrendipine at an α_1 subunit to ligand ratio of 1:1. The samples were then irradiated by a 200 W UV lamp. The ligands were only incorporated into the α_1 subunit, which was isolated by size exclusion chromatography and digested either by trypsin (azidopine) or endoproteinase Asp-N (nitrendipine). Each digest contained two radioactive peptides, which were isolated and sequenced. The azidopine peptides were identical with amino acids 13-18 (minor peak) and 1428-1437 (major peak) of the primary sequence of the skeletal muscle α_1 subunit. The nitrendipine peptides were identical with amino acids 1390-1399 (major peak) and 1410-1420 (minor peak). The sequence from amino acids 1390 to 1437 is identical in the α_1 subunits of skeletal, cardiac and smooth muscle and follows directly repeat IVS6. These results indicate that dihydropyridines bind to an area that is located at the putative cytosolic domain of the calcium channel.

Key words: channel blocker/charge carrier/skeletal muscle/voltage-dependent ion channel

Introduction

The voltage-activated calcium channels are a heterogeneous population of membrane-spanning proteins that play a key role in the signal transduction of many excitable cells. L-type channels have been studied intensely in cardiac, smooth and skeletal muscle (Bean, 1989; Trautwein and Hescheler, 1990). These channels are blocked by dihydropyridines (DHPs), which bind to the transmembrane spanning or intracellular region of the channel as shown by single channel analysis (Kokubun and Reuter, 1984; Hess *et al.*, 1984; Reuter *et al.*, 1985). The calcium conducting unit of the channel is the α_1 subunit of the calcium channel blocker receptor (CaCB receptor), which has been purified from skeletal muscle together with the other subunits (Borsotto *et al.*, 1985; Flockerzi *et al.*, 1986; Sieber *et al.*, 1987; Takahashi *et al.*, 1987; Vaghy *et al.*, 1987; Leung *et al.*, 1988) and has been cloned from skeletal (Tanabe *et al.*, 1987; Ellis *et al.*, 1988), cardiac (Mikami *et al.*, 1989) and smooth muscle (Biel *et al.*, 1990). Injection of the cDNAs of these α_1 subunits into oocytes (Mikami *et al.*, 1989; Biel *et al.*, 1990) or myotubes of dysgenic mice (Tanabe *et al.*, 1988) induces 'L-type' calcium current, which is blocked by DHPs.

The DHPs bind specifically to the α_1 subunit of the membrane-bound (Ferry *et al.*, 1984; Galizzi *et al.*, 1986) or purified calcium channel with nanomolar affinity (Glossmann *et al.*, 1987; Sieber *et al.*, 1987; Takahashi *et al.*, 1987; Vaghy *et al.*, 1987). Structure-activity studies (Triggle *et al.*, 1989) and molecular modelling (Höltje and Marrer, 1988) of the 2,6-dimethyl-1,4-DHPs suggested that the substituent at the C-4 phenyl ring, the carboxymethylester at C-3 and the 1,4-dihydropyridine ring are in close contact with the channel protein and are of major importance for potent antagonistic activity. Nitrendipine contains a nitrophenyl ring at C-4 and attaches covalently, most likely through the nitro group (Ebel *et al.*, 1978) to the α_1 subunit of the calcium channel and other proteins (Campbell *et al.*, 1984; Ichida *et al.*, 1989) upon irradiation. Azidopine (Glossmann *et al.*, 1987), a DHP photoaffinity analogue, has an *p*-azidobenzoyl substitution at the end of the C-5 side chain. The distance between the azido group and the 1,4-DHP ring is between 8.43 to 14.54 Å (Glossmann *et al.*, 1987). Therefore, azidopine should label an area within 15 Å of the true DHP binding site, whereas nitrendipine will be incorporated directly into amino acid side chains at the DHP binding site. It is shown that both compounds label peptides immediately following repeat IVS6 of the skeletal muscle calcium channel α_1 subunit.

Results

In initial experiments the stability of three tritium-labeled DHPs, namely azidopine, nitrendipine and PN 200-110, was tested. PN 200-110 was useless as a peptide marker since the tritium counts were rapidly lost at pH 2.1 and 6.5 from PN 200-110 covalently attached to the CaCB receptor. In contrast, the tritium label of azidopine was stable for at least 4 h at pH 7.4 and 6.5 and decreased slowly at pH 2.1 (Figure 1). The time-dependent decrease in tritium-labeled protein could indicate that the azidophenyl group reacts after UV irradiation with its protein to form a covalent, but under acidic conditions reversible, bond. The tritium label of nitrendipine was rapidly lost at pH 2.1, decreased slowly at pH 6.5, i.e. by 25% in 4 h at 20°C, and was stable at pH 7.4. The lower stability of the nitrendipine label at acidic pHs was expected since the carboxymethylester at C-5 is tritiated. This ester bond hydrolyzes rapidly at acidic pH. However, the relative stability of the tritium carboxymethylester at pH 6.5 suggested that nitrendipine can be used as peptide marker if peptide isolation is carried out at pH 6.5. A slow hydrolysis of the methylester occurs during peptide

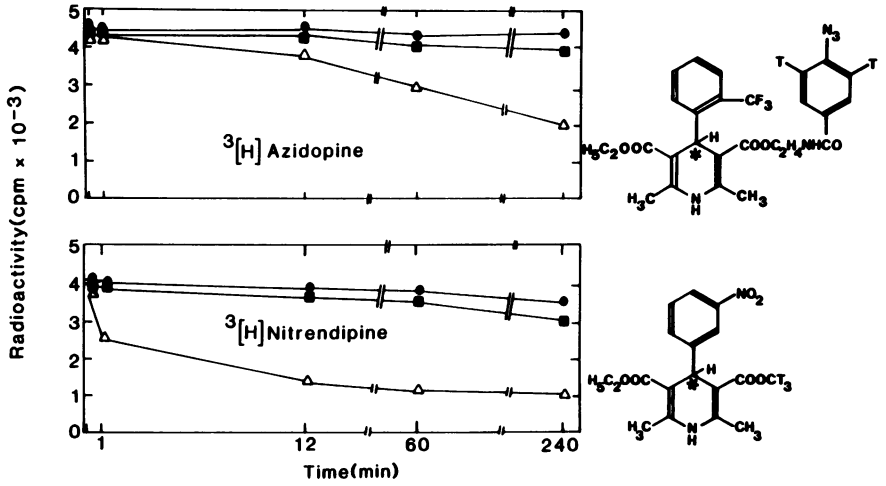

Fig. 1. Stability of the DHP-labeled CaCB receptor was tested as described in Materials and methods at pH 7.4 (●), 6.5 (■) and 2.1 (△). The 'T' in the structures on the right side stands for tritium.

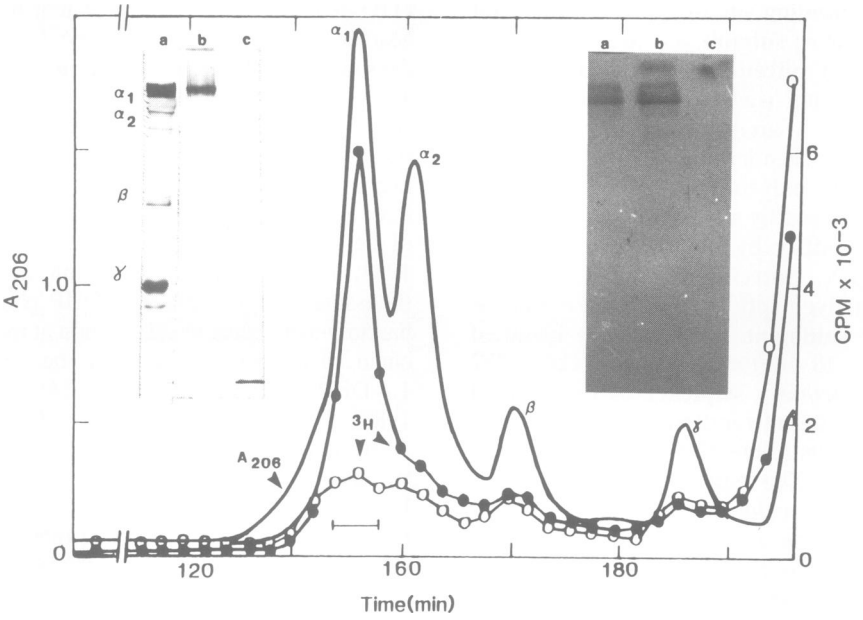

Fig. 2. Separation of the CaCB receptor subunits by gel filtration. The CaCB receptor was labeled by nitrendipine in the absence (●) and presence (○) of an 1000-fold excess of ±PN 200-110. The subunits of the CaCB receptor elute in the order α_1, α_2, β and γ and were monitored at A_{206}. The inset at the left side shows a silver-stained SDS gel of the nitrendipine-labeled CaCB receptor (**lane a**, 3 μg), the isolated α_1 subunit (**lane b**, 3 μg) and the digested α_1 subunit (**lane c**, 3 μg). The inset on the right side shows the corresponding fluorogram.

isolation but this does not affect significantly the retention times of labeled peptides on reversed-phase columns since hydrolysis of this ester does not change significantly the hydrophobicity of the nitrendipine-modified peptide.

Azidopine and nitrendipine label specifically the α_1 subunit of the CaCB receptor as shown previously (Gallizi *et al.*, 1986; Glossmann *et al.*, 1987; Sieber *et al.*, 1987; Takahashi *et al.*, 1987) and in Figure 2. Specific label was not associated with the α_2, β and γ subunit of the CaCB receptor. The specific labeling of the high-affinity DHP site was achieved by the use of stoichiometric concentrations of ligand and α_1 subunit. The amount of covalently bound ligand was determined directly before the digestion of the isolated α_1 subunit. Azidopine and nitrendipine labeled 9 and 4.6% of the DHP binding sites respectively.

Tryptic digestion of the azidopine-labeled α_1 subunit yielded one major radioactive peak which contained 30.5% of the applied radioactivity (Figure 3a). A further radioactive peak was not eluted when the column was washed with 90% isopropanol. The minor peak following peak A in Figure 3(a) gave no distinctive peak upon rechromatography. Rechromatography of peak A yielded peptides A1, A2 and A3, which contained 20, 48 and 12% of the applied radioactivity (Figure 3b). Peptides A1 and A2 had the sequences given in Table I, whereas the amount of peptide A3 was too low to determine unequivocally the amino acid sequence. The amount of peptide (see Table I) estimated from the specific radioactivity of the peptides and the recovery of the amino acids after Edman degradation (see Table I) agreed well and suggested that peptide A2 was the major peptide labeled by azidopine.

The nitrendipine-labeled α_1 subunit was digested by

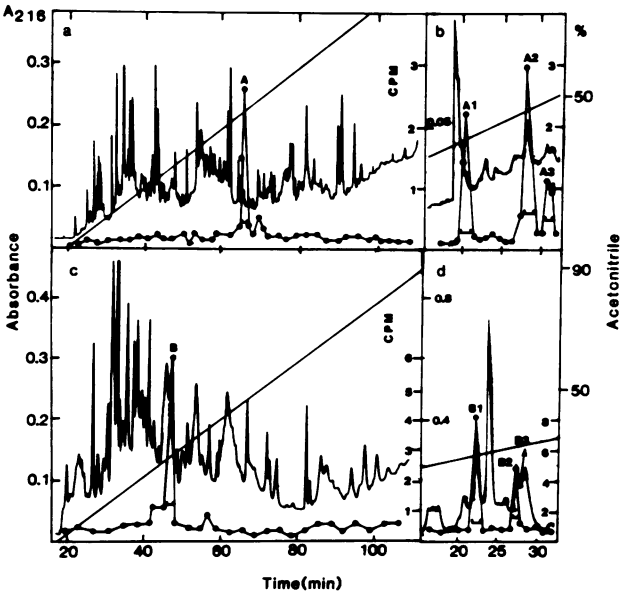

Fig. 3. HPLC profile of the azidopine (**a,b**) and nitrendipine (**c,d**) labeled peptides The amount of digested α_1 subunit applied in (a) and (c) was 1 mg and 2.8 mg respectively. The radioactivity (●) was determined in every fraction in 5 μl aliquots. The total recovery of applied radioactivity was 71 and 73.5% in (a) and (c) respectively. The radioactive fractions were pooled and applied to a second column (b,d). Pooled fractions are indicated by a bar (⊢). Note that the peptide B3 in (d) is the shoulder after peptide B2. The radioactivity is given on the right, inside axis as c.p.m. × 10^{-4} per 1 μl aliquot.

Table I. Amino acid sequences of the peptides labeled by azidopine and nitrendipine

No.	Amino acid sequences	Position	Ligand (pmol)	Peptide (pmol)
Azidopine-labeled peptides				
A1	KKQPK	13–18	15	10
A2	RIQPPLGFGK	1428–1437	40	35
Nitrendipine-labeled peptides				
B1	DWSILGPHHL	1390–1399	120	500
B2	DPEAKGRIKHL	1410–1420	40	25
	TAKLKV	717–722		50
B3	DPEAKGRIKHL	1410–1420	4	<5
	TAKLKV	717–722		35

Ligand refers to the amount of radioactivity applied to the polybrene filter. Peptide refers to the amount of peptide calculated from the individual yield of each cycle. For further details see Materials and methods and text.

endoproteinase Asp-N. The separation of the resulting peptides gave one radioactive peak B, which contained 35.6% of the applied radioactivity (Figure 3c). Again, the elution of the column with 90% isopropanol yielded no further radioactive peak. Rechromatography of peak B yielded the radioactive peptides B1 and B2, which contained 54 and 18% of the applied radioactivity (Figure 3d). Peptide B1 gave a single sequence and was recovered at a high yield (Table I). Edman degradation of peptide B2 gave two sequences (Table I), one of which was unusual for the protease that was used. The elution profile shown in Figure 3(d) indicates that the radioactive peptide B2 was contaminated by peptide B3. Sequencing of peptide B3 yielded the major sequence 'TAKLKV' contaminated with a very small

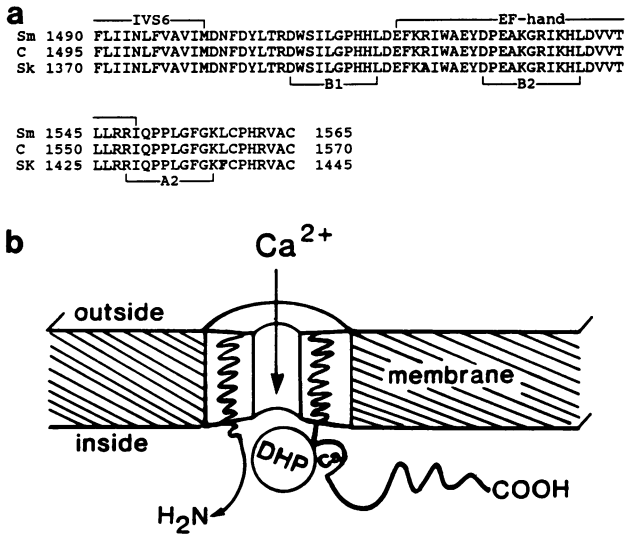

Fig. 4. (**a**) Localization of the azidopine (A1) and nitrendipine (B1, B2) labeled peptides on the deduced primary sequence of the smooth (Sm), cardiac (C) and skeletal (Sk) muscle CaCB receptor α_1 subunit; IVS6 and EF-hand indicate the position of the putative transmembrane sequence IVS6 and the putative calcium-binding site. (**b**) A schematic diagram of the calcium channel α_1 subunit showing the localization of the DHP-binding site.

amount of the other peptide of B2, suggesting that the nitrendipine-labeled peptide was the peptide starting with 'DPE. . .'. The amount of radioactivity recovered in peptide B1 was lower than the amount of the peptide itself. This discrepancy was not unexpected since initial experiments (Figure 1) showed that tritiated nitrendipine hydrolyses slowly at the pH of 6.5 that was used. Therefore, peptide B1 contains the major sequence labeled by nitrendipine in the intact α_1 subunit.

Discussion

The two major peptides labeled by azidopine and nitrendipine are between amino acids (aa) 1390 and 1437 of the deduced sequence of the calcium channel α_1 subunit (Tanabe *et al.*, 1987). The peptides are located directly behind repeat IVS6 on the putative cytosolic domain of the calcium channel (Figure 4). Four additional observations support that these peptides are part of the DHP binding site. (i) The sequence between aa 1390 and 1437 is conserved in the deduced sequence of the cardiac (Mikami *et al.*, 1989) and smooth muscle (Biel *et al.*, 1990) α_1 subunit. It contains only an exchange at aa 1404 and 1438 (Figure 4). (ii) The binding of DHPs to the calcium channel requires the presence of micro-to millimolar calcium (Glossmann *et al.*, 1982; Gould *et al.*, 1983; Luchowski *et al.*, 1984; Glossmann and Ferry, 1985; Ptasienski *et al.*, 1985; Ruth *et al.*, 1985). The sequence 1401–1429 contains a putative calcium binding site (Babitch, 1990). The occupation of this site by calcium could be necessary to induce the correct folding of the DHP-binding domain. (iii) Single channel recording showed (Hess *et al.*, 1984; Reuter *et al.*, 1985) that nitrendipine approaches the calcium channel through the membrane lipid bilayer. Its blocking effect may be caused by binding either to the transmembrane or the cytosolic domain of the channel. (iv) The charged DHPs, amlodipine (Burges *et al.*, 1985) and tiamdipine (Kwon *et al.*, 1990) have a long duration of

action, slow onset and offset kinetics in intact tissue, but have an affinity comparable to that of nifedipine in membrane fragments. These charged DHPs enter the membrane bilayer rapidly (Mason *et al.*, 1989) from the extracellular side but exit slowly at the cytosolic side of the membrane.

These observations are in good agreement with a localization of the DHP site at the cytosolic domain of the calcium channel. Several amino acids that are far apart on the primary sequence contribute to the structure of the ligand-binding sites of the sodium channel (Tejedor and Catterall, 1988) and the nicotinic acetylcholine receptor (Giraudat *et al.*, 1986, 1987; Oberthür *et al.*, 1986; Dennis *et al.*, 1988). Analogy with these amino acids suggests, therefore, that the DHP-binding domain of the calcium channel is composed of additional amino acids that may be located far away from peptide B1. One possible candidate is the amino terminus which contains the minor peptide A1 labeled by azidopine. Other parts of the channel, such as the cytosolic region of the transmembrane domain, may also contribute to the binding site. It will be of interest to identify the structural component of the channel that is responsible for inactivation of the channel and induction of high-affinity DHP binding (Bean, 1984).

Materials and methods

[^{3}H]Azidopine (53 Ci/mmol) and [^{3}H]nitrendipine (84 Ci/mmol) were purchased from Amersham and Du Pont–New England Nuclear respectively. Trypsin and endoproteinase Asp-N (both sequencing grade) were from Boehringer-Mannheim. The HPLC size exclusion columns were from Pharmacia LKB. The reversed-phase columns including the C4 microbore were from Macherey-Nagel and the C8 RP column was from Merck.

Purification of the skeletal muscle CaCB receptor

The CaCB receptor was purified from rabbit skeletal muscle as described (Sieber *et al.*, 1987; Schneider *et al.*, 1990). The pure CaCB receptor was stored at −70°C. Silver-stained SDS gels (see Figure 2) indicated an average purity of 93% and the presence of the known subunits of the CaCB receptor (Hofmann *et al.*, 1990).

Stability of the tritium label of the covalently labeled CaCB receptor

The CaCB receptor was photolabeled as described below. Radioactive compounds that were not incorporated into the protein were displaced by an 1000-fold excess of ± PN 200-110 added after photolysis. Thereafter, the CaCB receptor was incubated either in 10 mM HEPES/NaOH, pH 7.4, or in 10 mM ammonium acetate, pH 6.5, or in 0.1% TFA, pH 2.1, at room temperature for different times. The incubation was stopped by adding 'PEG stop solution' as described by Flockerzi *et al.* (1986) and Schneider *et al.* (1990).

Photolabeling of the CaCB receptor and isolation of the α_1 subunit

The pure CaCB receptor (11.5 mg for azidopine and 18.2 mg for nitrendipine), purified from the back and leg muscles of 120 rabbits, was incubated at a concentration of 0.2 mg/ml for 90 min in the dark at 4°C with azidopine and nitrendipine as described (Sieber *et al.*, 1987). The respective radioactive ligand was added in a concentration equimolar with the α_1 subunit. Photolysis was carried out on ice in 8 ml portions in a 8 cm Petri dish (level 2 mm) with a 200 W UV lamp (260–320 nm) for 5 min (azidopine) and 15 min (nitrendipine). The 20-fold concentrated (Schneider *et al.*, 1990) CaCB receptor was denaturated for 30 min at 20°C in the presence of 1% SDS and stored in aliquots at −70°C. The subunits of the labeled CaCB receptor were separated by size exclusion chromatography (Sieber *et al.*, 1987) on an Ultropac TSK G 3000 SW column connected to a TSK G 4000 SW SEC column in 50 mM sodium phosphate, pH 7.0, containing 0.1% SDS (Figure 2). Satisfactory separation of the subunits was achieved only with loads up to 0.6 mg CaCB receptor per run. The fractions containing the α_1 subunit and specific radioactivity were pooled and stored at −70°C.

Carboxymethylation, digestion and isolation of labeled peptides

Carboxymethylation was carried out at 37°C (Dalbon *et al.*, 1988) and the SDS content of the sample was decreased by dialysis against 50 mM sodium phosphate, pH 7.2, 6 M urea and 80 g/l Dowex AG 1×2 (Tanabe *et al.*, 1987). Dialysis was carried out for 1 h at 20°C and continued for 15 h at 4°C. The dialysis was continued further at 4°C against 10 mM ammonium hydrogen carbonate, pH 8.0, for 80 h with frequent buffer changes. The azidopine-labeled α_1 subunit was digested with trypsin at a protein to enzyme ratio of 100:1 at 37°C for 16 h. The α_1 subunit labeled with nitrendipine was digested with endoproteinase Asp-N at a protein to enzyme ratio of 200:1 at 37°C for 20 h. The azidopine-labeled α_1 subunit was concentrated in a Speed Vac Lyophilizator. The azidopine-labeled digest was applied in two portions of 1 mg on a LiChroCART 250-4 column filled with LiChrospher 100 RP-8 5 μm (Merck). The column was eluted with buffer A (10 mM ammonium acetate, pH 6.5) at a flow rate of 0.75 ml/min using a linear acetonitrile gradient from 0 to 90% within 90 min. The nitrendipine-labeled digest was loaded in two portions of 2.8 mg on a 250/8/4 Nucleosil 300-5 C4 column (Macherey-Nagel). The same buffers were used for elution using a flow rate of 1 ml/min. The radioactive fractions were pooled. The azidopine- and nitrendipine-labeled peptides were rechromatographed on a 11 × 4 mm Nucleosil 300-5 C4 column (Macherey-Nagel) and on a Microbore 125 × 6 × 2 Nucleosil 300-7 C4 (Macherey-Nagel) respectively. The azidopine peptides were eluted with a linear acetonitrile gradient from 0 to 54% in 60 min. The nitrendipine peptides were eluted with a linear acetonitrile gradient from 18 to 36% in 40 min. The radioactive peptides were pooled and lyophilized to dryness (azidopine) or to a volume of 100 μl (nitrendipine). The amino acid sequences were determined by an Applied Biosystems 470 A gas-phase sequenator connected on line to a PTH Analyzer 120 A from Applied Biosystems. The polybrene filters were routinely initially flushed with 100% TFA to decrease contaminations which obscured detection of picomolar amounts of PTH amino acids. This treatment removed >80% of the tritium incorporated into the peptides. The amount of peptide was calculated from a non-linear regression curve using the individual yield of each cycle (for further details see Swiderek *et al.*, 1988).

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