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MOLECULAR PHARMACOLOGY, 41:298-307

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[^{35}S]Sadopine, a Novel High Affinity, High Specific Activity, L-Type Ca^{2+} Channel Probe: Characterization of Two Equipotent Diastereomers with Opposite Allosteric Properties

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Received August 14, 1991; Accepted November 13, 1991

SUMMARY

A novel ^{35}S-labeled dihydropyridine (DHP), 1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-pyridine-3,5-dicarboxyl-3-[2-(*N-tert*-butoxycarbonyl-L-[^{35}S]methionyl)-aminoethyl]-ester-5-ethyl ester, ([^{35}S]sadopine) (800–1400 Ci/mmol), the respective (+)- and (−)-diastereomers, and unlabeled (±)-, (−)-, and (+)-sadopine were synthesized. [^{35}S]Sadopine is an excellent high affinity, high specific activity radioligand to label selectively the DHP receptor of L-type Ca^{2+} channels in tissue sections as well as in membrane fragments. Both diastereomers bind to the DHP receptors in a saturable and reversible manner, with equal, subnanomolar, dissociation constants. Despite their similar affinities, (+)- and (−)-sadopine differ with respect to their kinetic properties [the association and dissociation rate constants are 10-fold higher for (+)-[^{35}S]sadopine at 22°] and their allosteric modulation of the phenylalkylamine or benzothiazepine binding domain. (+)-Sadopine is a negative but (−)-sadopine a positive allosteric modulator of (−)-[*N-methyl*-^{3}H]LU49888 or (+)-*cis*-[^{3}H] diltiazem binding at 30°. Both diastereomers act as L-type Ca^{2+} channel blockers in cardiac and smooth muscle cells. Computer-based analysis of the electrostatic potentials of the two diastereomers and calculation of the interaction energies with a hypothetical DHP receptor model predicted not only the similar affinities of (+)- and (−)-sadopine but also their Ca^{2+} channel-blocking effects. The temperature-dependent allosteric differences between the diastereomers suggest that two distinct conformational states of the DHP receptor are stabilized *in vitro*, both corresponding to a nonconducting state of the channel. Our data indicate that access to the DHP receptor site, but not binding affinity, is a function of the opposite stereochemistry of the sadopine diastereomers. Therefore, labeled and unlabeled (+)- and (−)-sadopine will be useful probes to further characterize the molecular basis of DHP-Ca^{2+} channel interaction and the pharmacological and physiological significance of the different allosteric conformations of the channel induced by Ca^{2+} channel-active drugs.

Much of our knowledge concerning the structure, function, and tissue distribution of DHP-sensitive (L-type) Ca^{2+} channels has come from their specific, high affinity interaction with different chemical classes of organic Ca^{2+} channel drugs (1, 2). These drugs interact with distinct binding domains located on the α_1 subunit of the channel complex (1, 3). Drug-binding domains selective for DHPs, phenylalkylamines, and benzothiazepines [e.g. (+)-*cis*-diltiazem] are the most well characterized ones, but several others have been recently described (for reviews, see Refs. 1, 4, and 5). Equilibrium and kinetic binding studies eventually led to an allosteric receptor model, in which the distinct drug receptors are coupled to one another and to binding sites for divalent cations via positive or negative heterotropic allosteric mechanisms (1, 5). DHP Ca^{2+} channel activators (agonists), like the *S*-enantiomers of 202-791 and BAY K 8644 (6), are exclusively negative allosteric modulators of phenylalkylamine binding to purified L-type Ca^{2+} channels from skeletal muscle and of benzothiazepine binding to membrane-bound channels (1, 5). In contrast, all DHP antagonists so far tested are positive allosteric modulators. It was, therefore,

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ABBREVIATIONS: DHP, dihydropyridine; BAY K 8644, methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate; B_{max}, maximum density of binding sites; *t*-Boc, tertiary butoxycarbonyl; EGTA, ethyleneglycol bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid; K_D, dissociation constant; LU49888, 5-(3-azidophenethyl)-methylamino-2-(3,4,5-trimethoxyphenyl)-2-isopropylvaleronitrile; MEP, molecular electrostatic potential; PN200-110, isopropyl 4-(1,2,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-methoxycarbonylpyridine-3-carboxylate; 202-791, isopropyl 4-(1,2,3-benzoxydiazo-4-yl-1,4-dihydro-2,6-dimethyl-5-nitro-3-pyridinecarboxylate; T-tubule, transverse tubule; TLC, thin layer chromatography.

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proposed that DHP agonists and antagonists can be differentiated *in vitro* by virtue of their allosteric properties (1, 5, 7, 8).

In this study, we report the synthesis and pharmacological characterization of two diastereomers of a novel ^{35}S-labeled DHP, termed sadopine. In radiolabeled form, sadopine is a highly suitable, high resolution, high specific activity, L-type Ca^{2+} channel probe for autoradiographic studies. In addition, the two labeled and unlabeled diastereomers, (+)-sadopine and (−)-sadopine, reveal unique kinetic and equilibrium binding properties, with temperature-dependent, opposite, and reciprocal allosteric effects on the phenylalkylamine and the benzothiazepine binding domain. Our findings will be discussed in terms of existing models for the DHP receptor of L-type Ca^{2+} channels. Preliminary results of this work have been reported elsewhere (9).

Experimental Procedures

Materials. The (+)-enantiomer, (−)-enantiomer, and the racemate of the amino precursor 1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridine carboxylic acid 2-(aminoethyl)ethyl ester hydrochloride were synthesized in our laboratories (10). The sources for other reagents were as follows: *t*-Boc-L-[^{35}S]methionine-*N*-hydroxysuccinimidyl ester (800–1400 Ci/mmol), (+)-[^{3}H]PN200-110 (85 Ci/mmol), (−)-[^{3}H]desmethoxyverapamil (78 Ci/mmol), and (+)-*cis*-[^{3}H]diltiazem (146 Ci/mmol), Amersham, Little Chalfont, UK; polyethylene glycol 6000 and unlabeled *t*-Boc-L-methionine-*N*-hydroxysuccinimidyl ester, Serva (Heidelberg, FRG); elastase and bovine serum albumin (essentially fatty acid free), Sigma (Munich, FRG); collagenase, Worthington; and 0.2-mm silica gel 60 TLC plates, Merck (Darmstadt, FRG). [*N-methyl*-^{3}H]LU49888 (85 Ci/mmol) was kindly provided from Knoll AG (Ludwigshafen, FRG). All other chemicals used were from sources described previously (11–13).

Synthesis of two unlabeled sadopine diastereomers. Of the four theoretically existing diastereomers of sadopine [1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl-pyridine-3,5-dicarboxyl-3-[2-(*N-tert*-butoxycarbonyl-L-methionyl)-aminoethyl]-ester-5-ethyl ester], (+)-sadopine and (−)-sadopine were synthesized. These optical configurations refer to the asymmetric carbon atom of the DHP molecule; the chiral center of the methionine residue was always in the L-configuration. The free base of the respective amino precursors was prepared by extracting 4.0 mg (10 μmol) of the hydrochloride salt in 0.2 ml of 1 M NaOH with 2 × 0.2 ml of ethyl acetate. *t*-Boc-L-methionine-*N*-hydroxysuccinimidyl ester (0.35 mg; 1 μmol), dissolved in 0.05 ml of toluene, was added to the extracts. The solvent was evaporated to dryness under a gentle stream of nitrogen at room temperature, and the residue was dissolved in 0.07 ml of ice-cold ethanol, followed by the addition of 0.03 ml of 0.1 M boric acid/NaOH, pH 8.5. After incubation for 60 min on ice, the reaction mixture was separated by TLC on 0.2-mm silica gel 60 plates developed in acetic acid ethyl ester/diethylether (30:70; v/v). Sadopine migrated with a R_F value of 0.52 ± 0.05 (three experiments), whereas the unreacted precursor remained at the origin. Reaction yields were >85%. The products were eluted from the plate with ethanol. Sadopine concentrations in the eluate were determined by spectrophotometry (absorbance at 358 nm, using the racemic amino precursor as a standard). Sadopine was stored protected from light in ethanol at −25° and was stable for at least 4 weeks.

Synthesis of two ^{35}S-labeled sadopine diastereomers. (+)-[^{35}S] Sadopine and (−)-[^{35}S]sadopine were synthesized according to the protocol described above, using 0.25 mCi (0.25 nmol) of *t*-Boc-L-[^{35}S] methionine-*N*-hydroxysuccinimidyl ester (800–1400 Ci/mmol) and 0.05 mg (120 nmol) of the respective amino precursor. The reaction product was separated by TLC as described above or by high performance liquid chromatography on a Shandon ODS Hypersil column [0.46 × 12.5 cm, 5-μm particle size; isocratic elution at a flow rate of 1.5 ml/min with acetonitrile/H_2O (50%/50%, v/v)]. Of the radioactivity used, 35% ± 12% (three experiments) was recovered as radiolabeled sadopine. The radiochemical purity of the labeled diastereomers was >95%, as determined by TLC in three different systems.

Binding studies. Isolation of partially purified rabbit skeletal muscle T-tubule membranes and guinea pig hippocampus membranes and purification of skeletal muscle Ca^{2+} channels were described earlier in detail (8, 11). All binding studies with particulate membranes were performed in 50 mM Tris·HCl, pH 7.4, 0.1 mM phenylmethylsulfonyl fluoride, in a final assay volume of 0.25 ml, unless indicated otherwise. For equilibrium binding experiments with rabbit skeletal muscle T-tubule membranes, the incubation times were 40 min at 37°, 60 min at 22°, or 360 min at 2° for (+)-[^{35}S]sadopine and 60 min at 37°, 180 min at 22°, or 840 min at 2° for (±)-[^{35}S]sadopine as well as (−)-[^{35}S] sadopine.

Receptor-bound radioactivity was separated from unbound ligand by filtration with ice-cold 10% (w/v) polyethylene glycol 6000, 10 mM Tris·HCl, pH 7.4, 10 mM $MgCl_2$ (filtration buffer), over Whatman GF/C filters. For purified skeletal muscle L-type Ca^{2+} channels, receptor-bound radioactivity was precipitated in the presence of 0.5 mg of bovine serum albumin and 0.5 mg of γ-globulin, by dilution in ice-cold filtration buffer, 3 min before separation as described above. Nonspecific binding was defined in the presence of 1 μM (±)-PN200-110 [[^{35}S] sadopine and (+)-[^{3}H]PN200-110], 3 μM (−)-desmethoxyverapamil [(−)-[^{3}H]desmethoxyverapamil or [*N-methyl*-^{3}H]LU49888], or 10 μM (+)-*cis*-diltiazem [(+)-*cis*-[^{3}H]diltiazem] and was subtracted from total binding to yield specific binding. Experimental conditions for labeling of the phenylalkylamine and benzothiazepine binding domain (see also Ref. 13) are given in the legends.

Drug dilution. Serial dilutions of unlabeled compounds were prepared in dimethylsulfoxide, as described previously (14), to prevent nonspecific adsorbtion of the drugs to plastic and glass surfaces. A final dimethylsulfoxide concentration of 1% (v/v) was never exceeded. This concentration does not affect the binding of Ca^{2+} channel ligands (14).

Binding data analysis and protein determination. Specific binding data were computer-fitted, by nonlinear methods, to the general dose-response equation (concentration-response curves) (15) or a rectangular hyperbola (saturation experiments). IC_{50} or EC_{50} values are the drug concentration causing 50% inhibition or stimulation, respectively. Inhibition is defined as $100 \cdot (B_o - B)/B_o$, where B is the specific binding in the presence and B_o in the absence of added drug. Stimulation is defined as $100 \times B/B_o$. Kinetic constants were calculated as described (11, 13). Protein concentrations were determined according to the method of Lowry *et al.* (16) (membranes) or Bradford (17) (purified skeletal muscle Ca^{2+} channels).

Electrophysiological experiments. Rabbit ear artery smooth muscle cells and cardiomyocytes of neonatal (2–4-day-old) Wistar rats were prepared as described elsewhere (18, 19). Electrophysiological measurements were performed using the whole-cell configuration of the patch-clamp technique. Electrodes were made from Kwik-fill borosilicate capillaries (World Precision Instruments, New Haven, CT) and had resistances between 2 and 4 MΩ. The following internal pipette solutions were used (concentrations in mM): $CsCl_2$, 110; EGTA, 10; tetraethylammonium, 20; $MgCl_2$, 1; ATP, 5; cAMP, 0.1; Na-HEPES, 10, pH 7.4 (cardiomyocytes), and NaCl, 126; $MgSO_4$, 3.2; NaH_2PO_4, 1; EGTA, 2; glucose, 11.2; ATP, 2; tetraethylammonium, 10; Na-HEPES, pH 7.2 (smooth muscle cells). All experiments were carried out in high-Ba^{2+} external solution (in mM: $BaCl_2$, 110; Na-HEPES, 10, buffered to pH 7.4). All recordings were made at 22°. Cells were clamped at −60 mV (ear artery smooth muscle cells) or −45 mV (cardiomyocytes), and 100-msec test depolarizations were applied every 5 sec. Whole-cell inward Ba^{2+} current evoked by voltage pulses was recorded under control conditions or after a 3-min incubation with the Ca^{2+} channel drugs.

Isolated rabbit aortic smooth muscle strips. The effects of Ca^{2+}

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antagonists on smooth muscle contraction were determined according to a standard protocol (13). Cumulative contraction-response curves to K^+ in 10-mM steps from 3 to 53 mM were constructed. Strips were relaxed to base-line levels by washing for 20 min in 3 mM K^+ between each experiment. Strips giving reproducible contraction after three cumulative contraction curves were preincubated for 20 min with sadopine or vehicle (control) before drug effects were measured in additional contraction-response curves.

Autoradiography. Autoradiographic experiments were performed as described previously (11), using 20-μm-thick, slide-mounted, guinea pig brain sections. Nonspecific labeling was determined after preincubation of slices for 30 min with 1 μM (±)-PN200-110. Autoradiograms were obtained by exposure of dried slices to LKB Ultrofilm for 4–10 days at −80°.

Computer modeling. A detailed description of computer modeling of different DHPs has been published elsewhere (20). The molecular structures of (+)- and (−)-sadopine were constructed using a molecular modeling software package of SYBYL (TRIPOS Associates Inc., St. Louis, MO), followed by analysis for sterically allowed low energy conformations. Molecular energies were calculated using the force field algorithm of SYBYL. The MEPs were calculated for the geometry-optimized structures using the POTENTIAL option of SYBYL. Calculations were made for a grid with an interval of 1 Å. Calculated potentials of the sadopine diastereomers and other well known DHP agonists and antagonists were analyzed using SIMPOT. SIMPOT is a program that can identify potential differencies between two groups of molecules, using a simple algorithm (20). DHP-induced MEP changes were calculated with a tryptophan molecule serving as the MEP sensor of the receptor.

Interaction energies of Ca^{2+} antagonists were calculated for an extended DHP receptor model that allows interaction of the DHP with two additional binding sites of the receptor. Binding site 1 is assumed to interact with the negatively charged substituent of the phenyl ring via electrostatic forces, binding site 2 with the alkyl ester groups via van der Waal interactions. Twenty-five possible combinations obtained from a set of five different amino acids (tryptophan, phenylalanine, isoleucine, lysine, and glutamate, representing aromatic, aliphatic, basic, and acidic residues) were analyzed (20). Only a model with a lysine and a phenylalanine (or tryptophan) representing binding sites 1 and 2, respectively, were found to yield interaction energies for various DHPs that correlate with their pharmacological potencies (20). The lysine/phenylalanine model was used for all calculations.

Results

[^{35}S]Sadopine, a new, high specific activity, high resolution probe for autoradiographic studies. In order to develop a DHP with high specific activity for high sensitivity labeling of L-type Ca^{2+} channel-associated DHP receptors, we introduced *t*-Boc-L-[^{35}S]methionine in position 3 of the substituted pyridine ring (Fig. 1A). Introduction of bulky substituents in this position has previously been shown to yield derivatives with high affinity and selectivity for the DHP receptor, e.g., the tritiated phenylazide [^{3}H]azidopine (1). Similar to [^{3}H]azidopine, racemic [^{35}S]sadopine binds with subnanomolar affinity to L-type Ca^{2+} channels in guinea pig hippocampus (see legend to Fig. 1B). The distribution of [^{35}S]sadopine-labeled L-type Ca^{2+} channels is identical to those reported previously for (+)-[^{3}H]PN200-110-, [^{3}H]nitrendipine-, or [^{125}I]iodipine-labeled channels (5, 11), with highest densities of specific labeling in the molecular layer of the gyrus dendatus and the cerebral cortex (Fig. 1). [^{35}S]Sadopine has a 15–20 times higher specific activity than tritiated Ca^{2+} channel ligands, requiring significantly shorter exposure times. It offers the known advantages of the higher resolution and the longer half-life of a ^{35}S-label, compared with ^{125}I.

Opposite allosteric and kinetic properties of the sadopine diastereomers. We expected one of the diastereomers in the racemic mixture to possess less pharmacological activity, as found for other DHP enantiomers (7, 11). To identify the more potent diastereomer, we synthesized the respective unlabeled derivatives. Their affinities were analyzed in competition studies with (+)-[^{3}H]PN200-110-labeled guinea pig hippocampus (see legend to Fig. 1B) and skeletal muscle L-type Ca^{2+} channels. In skeletal muscle, (+)-sadopine and (−)-sadopine concentration-dependently inhibited (+)-[^{3}H]PN200-110 binding to the DHP receptor, with pseudo-Hill slopes close to unity, as expected for a simple competitive interaction. Surprisingly, we found no significant difference for the two diastereomers [K_i values of 0.456 ± 0.08 nM for (−)-sadopine and 0.566 ± 0.09 nM for (+)-sadopine in skeletal muscle; means ± standard deviations, three experiments].

Because the DHP receptor is allosterically coupled to other drug receptor domains on the channel α_1 subunit (1, 5), we studied the effects of the two enantiomers on (−)-[^{3}H]desmethoxyverapamil-labeled phenylalkylamine and (+)-*cis*-[^{3}H]diltiazem-labeled benzothiazepine receptors in skeletal muscle membranes (Figs. 2 and 3). At 30°, (−)-sadopine allosterically stimulated the binding of (+)-*cis*-[^{3}H]diltiazem to membrane-bound channels (Fig. 2A) and that of (−)-[*N-methyl*-^{3}H]LU49888 to purified Ca^{2+} channels (Fig. 3D), with nanomolar EC_{50} values. In contrast, (+)-sadopine was an allosteric inhibitor under identical conditions. The allosteric effects on (+)-*cis*-[^{3}H]diltiazem binding were investigated in more detail in kinetic and saturation experiments. Stimulation of (+)-*cis*-[^{3}H]diltiazem binding by (−)-sadopine could be explained by a decrease in the dissociation rate (Fig. 2D) and an increase in the apparent number of high affinity binding sites (Fig. 2C). In contrast, (+)-sadopine increased the dissociation rate constant and decreased the B_{max} for (+)-*cis*-[^{3}H]diltiazem. The stimulatory effect of (−)-sadopine was temperature dependent and was reversed to an inhibitory effect at 2° (Fig. 2B).

Phenylalkylamine binding to membrane-bound Ca^{2+} channels was inhibited efficiently only by the (+)-enantiomer (IC_{50} = 8.36 ± 2.34 nM, n_H = 0.94 ± 0.11, 100% maximal inhibition, three experiments), whereas (−)-sadopine caused an incomplete and biphasic inhibition, with <40% inhibition at 10 μM (Fig. 3C). The racemic mixture showed an intermediate effect, demonstrating that the allosteric effect of one diastereomer can be reversed by its optical antipode. At the moment we do not understand the biphasic allosteric effect of (−)-sadopine. One possible explanation is a dualistic action of the (−)-enantiomer on the dissociation of the labeled phenylalkylamine, resulting in a decrease of dissociation in the lower concentration range but in an increase at higher concentrations. Such concentration-dependent effects on dissociation can be observed for DHP receptors. At a concentration of 1 μM, (−)-desmethoxyverapamil decreases (+)-[^{3}H]PN200-110 dissociation from skeletal muscle Ca^{2+} channels (21) but accelerates dissociation at higher concentrations.[3]

To confirm the identical affinity of the sadopine diastereomers and to test whether their opposite allosteric effects were reciprocal, we synthesized the labeled compounds with identical

[3] J. Striessnig and H. Glossmann, unpublished observations.

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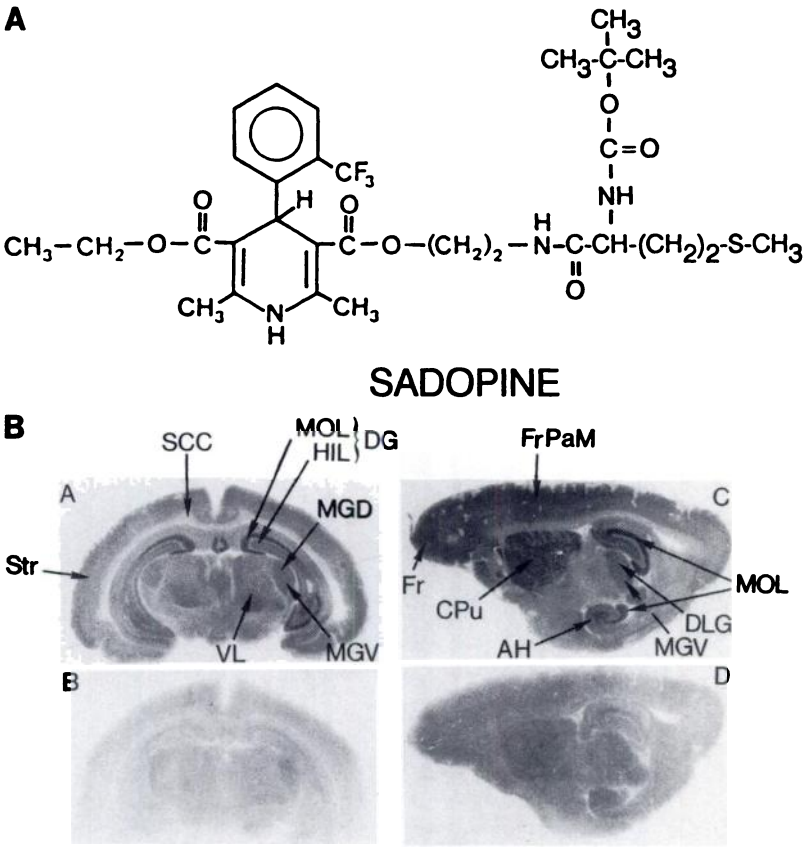

Fig. 1. A, Chemical structure of sadopine. B, Autoradiographic distribution of DHP-sensitive L-type Ca^{2+} channels in guinea pig brain labeled with [^{35}S]sadopine. Frontal (A and B) and sagittal (C and D) guinea pig brain sections at the level of the hippocampal formation were incubated for 90 min at 22° with 52 pM [^{35}S]sadopine, after preincubation (see Experimental Procedures) in the absence (A and C) or presence (B and D) of 1 μM (±)-PN200-110. *AH*, Ammons horn; *CPu*, caudate putamen; *DLG*, dorsal lateral geniculate nucleus; *Fr*, frontal cortex; *FrPaM*, frontoparietal cortex, motor area; *HIL*, hilus of the dentate gyrus; *MGD*, mediate geniculate nucleus, pars dorsalis; *MGV*, mediate geniculate nucleus, pars ventralis; *MOL*, molecular layer of the dentate gyrus; *SCC*, splenium of the corpus callosum; *Str*, striate cortex; *VL*, ventrolateral thalamic nucleus. The following K_i values (in pM) were determined for the unlabeled sadopine diastereomers in (+)-[^{3}H]PN200-110-labeled (130–340 pM ligand concentration) guinea pig hippocampus membranes (0.08 mg/ml membrane protein): (±)-sadopine, 524.0 ± 245; (+)-sadopine, 416 ± 251; (−)-sadopine, 502 ± 188 (two experiments).

specific activities. In saturation experiments, K_D values of 0.57 ± 0.24 nM [(−)-[^{35}S]sadopine] and 0.64 ± 0.31 nM [(+)-[^{35}S]sadopine] (three experiments) were obtained for skeletal muscle L-type Ca^{2+} channels (Fig. 4A). These were in excellent agreement with the K_i values of the unlabeled drugs obtained in displacement experiments (see Table 2). In three independent experiments, a lower B_{max} (1.31 ± 0.36 pmol/mg of membrane protein) was found for (+)-[^{35}S]sadopine, compared with (−)-[^{35}S]sadopine (2.21 ± 0.23 pmol/mg of protein), indicating that (+)-[^{35}S]sadopine stabilizes a smaller fraction of DHP receptors in a high affinity state than does (−)-[^{35}S]sadopine. These B_{max} values were similar to those reported for [^{3}H]nifedipine and [^{3}H]BAY K 8644 (7, 11) but lower than, for example, those for (−)-[^{3}H]azidopine and (+)-[^{3}H]PN200-110 (11, 22).

The most striking difference between the binding properties of the two diastereomers was found in kinetic experiments. The dissociation kinetics of both diastereosiomers were extremely temperature dependent and about 2 orders of magnitude faster at 37° than at 2° (Table 1). At all temperatures tested, the dissociation rates of the (+)-[^{35}S]sadopine-receptor complexes were 5–10 times faster than those for (−)-[^{35}S]sadopine-labeled channels (Fig. 4, B and C). If the association rates of the two sadopine diasteroisomers are similar and binding affinity is mainly determined by k_{-1}, one would expect a higher K_D for (+)-[^{35}S]sadopine than for (−)-[^{35}S]sadopine. Because equal affinities for the two diastereomers were found, their association rate constants must be different. This is indeed the case, as shown in Table 1. The association rate constants were again highly temperature dependent for both ligands, and (+)-[^{35}S]sadopine associated faster than did (−)-[^{35}S]sadopine (Table 1).

We next investigated whether the opposite allosteric regulation of the benzothiazepine or phenylalkylamine receptor domains by the sadopine diastereomers was reciprocal. Unlabeled (+)-*cis*-diltiazem allosterically stimulated (−)-[^{35}S]sadopine binding, whereas (+)-[^{35}S]sadopine binding was inhibited under identical assay conditions (Fig. 5A). Racemic [^{35}S]sadopine binding reflected the net effect of (+)-*cis*-diltiazem on the binding of the two labeled diastereomers in the racemic mixture. Stimulation of (−)-[^{35}S]sadopine binding was strongly dependent on temperature and reversed to an inhibitory effect at low temperatures (Fig. 5, B and C). As shown above, (+)-sadopine was inhibitory for (−)-[^{3}H]desmethoxyverapamil binding. The reciprocal coupling was demonstrated by experiments in which unlabeled (−)-desmethoxyverapamil was an effective inhibitor of (+)-[^{35}S]sadopine but not of (−)-[^{35}S]sadopine binding (Fig. 3, A and B).

Taken together, our data show that (+)-sadopine-labeled DHP receptors are coupled exclusively in a negative allosteric manner to the phenylalkylamine and benzothiazepine receptors, whereas (−)-sadopine can also act as a positive allosteric regulator, depending on temperature.

Functioning of both sadopine diastereomers as Ca^{2+} channel blockers. To test the functional properties of the two enantiomers, we investigated their effects on K^+-induced smooth muscle contraction as well as on Ba^{2+} currents through L-type Ca^{2+} channels in isolated smooth muscle cells and cardiomyocytes. Both diastereomers blocked depolarization-induced smooth muscle contractions with similar (nanomolar) IC_{50} values (Fig. 6, E and F; Table 2). This inhibition is due to a block of voltage-dependent L-type Ca^{2+} channels, as shown by whole-cell voltage-clamp experiments with isolated smooth

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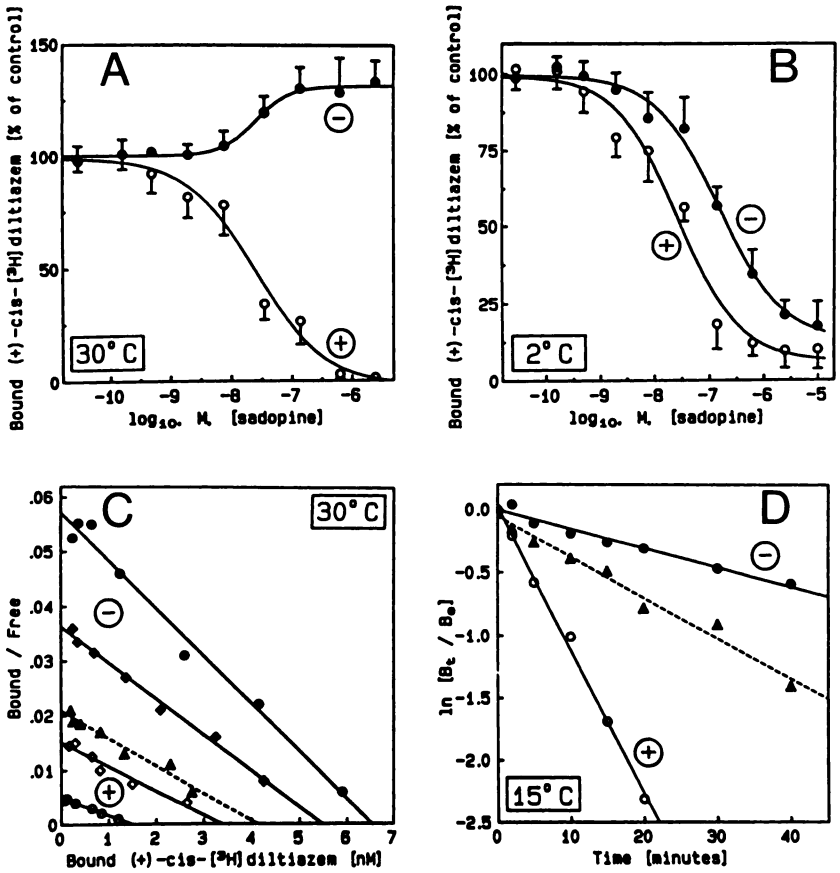

Fig. 2. Allosteric interaction of the sadopine diastereomers with the benzothiazepine binding domain. A, Effects of increasing concentrations of (+)-sadopine (○) and (−)-sadopine (●) on (+)-*cis*-[^{3}H]diltiazem binding. Skeletal muscle Ca^{2+} channels (0.145–0.416 mg/ml membrane protein) were incubated with 1.87–3.56 nM (+)-*cis*-[^{3}H]diltiazem in the absence or presence of the indicated concentrations of the sadopine diastereomers at 30°. The concentration of bound radioactivity in the absence of sadopine (control = 100%) is given in parenthesis. The following binding parameters were obtained: ● (43.2 pM), EC_{50} = 25.70 ± 3.9 nM, n_H = 1.54 ± 0.39, maximal stimulation = 131.5 ± 9.8%; ○ (41.8 pM), IC_{50} = 25.64 ± 1.6 nM, n_H = 0.79 ± 0.22. B, As described above but at 2°: ● (115.4 pM), IC_{50} = 154.8 ± 21.4 nM, n_H = 0.74 ± 0.11; ○ (111.3 pM), IC_{50} = 26.91 ± 3.64 nM, n_H = 0.76 ± 0.19. C, Effects of the sadopine diastereomers on (+)-*cis*-[^{3}H]diltiazem equilibrium binding parameters. Equlibrium binding data in the Scatchard transformation. Skeletal muscle Ca^{2+} channels (0.369 mg/ml membrane protein) were incubated with 2.52–502.5 nM (+)-*cis*-[^{3}H]diltiazem in the absence (control) or presence of (+)- or (−)-sadopine. ▲, Control (K_D = 186.8 nM, B_{max} = 4.11 nM, r = 0.97); ◆, 10 nM (−)-sadopine present (K_D = 150.47 nM, B_{max} = 5.47 nM, r = 0.98); ●, 100 nM (−)-sadopine present (K_D = 114.2 nM, B_{max} = 6.53 nM, r = 0.98); ◇, 10 nM (+)-sadopine present (K_D = 222.9 nM, B_{max} = 3.37 nM, r = 0.98); ○, 30 nM (+)-sadopine present (K_D = 298.2 nM, B_{max} = 1.50 nM, r = 0.99). D, Dissociation kinetics of the benzothiazepine receptor. Data shown are means from duplicate determinations. B_o is the concentration of bound (+)-*cis*-[^{3}H]diltiazem at equilibrium. B_t is the concentration after initiation of the dissociation at time t. Skeletal muscle Ca^{2+} channels (0.249 mg/ml membrane protein) were labeled with 2.41 nM (+)-*cis*-[^{3}H]diltiazem for 240 min at 15°. Dissociation (time zero) was induced by addition of 10 μM (+)-*cis*-diltiazem (▲) (K_{-1} = 0.0324 min^{-1}, r = 0.98) or by addition of 10 μM (+)-*cis*-diltiazem plus 1 μM (−)-sadopine (●) (K_{-1} = 0.0153 min^{-1}, r = 0.98) or by simultaneous addition of 10 μM (+)-*cis*-diltiazem plus 1 μM (+)-sadopine (○) (K_{-1} = 0.114 min^{-1}, r = 0.99).

muscle cells (Fig. 6, C and D) and isolated cardiac myocytes (Fig. 6, A and B). I-V curves were generated by depolarizing cells from a constant holding potential (ear artery smooth muscle cells, −60 mV; cardiomyocytes, −45 mV) to different test potentials. Maximal Ba^{2+} inward currents were observed after depolarization to 20–30 mV. The inward currents were blocked to a similar extent by 1 μM (+)- and (−)-sadopine over the whole voltage range tested, without significant changes in the peak of the I-V curve. The effect of both enantiomers on steady state inactivation of the Ca^{2+} channel current was investigated in ear artery smooth muscle cells. As typically observed for DHP antagonists, both enantiomers (1 μM) shifted the midpoint of the steady state inactivation curve to more hyperpolarizing potentials [(+)-sadopine, 22.0 ± 2.0 mV; (−)-sadopine, 23.7 ± 1.5 mV; means ± standard deviations, three experiments).

Prediction of the binding properties of sadopine to a hypothetical computer model of the DHP receptor. Investigation of the MEPs of DHP Ca^{2+} channel drugs has previously led to the discovery of a defined segment of space where agonists and antagonists possess MEPs with opposite sign (the MEP of agonists is negative and the MEP of antagonists is positive). Interaction of the DHPs with tryptophan, serving as a simplified MEP sensor of the receptor in our model, allowed us to calculate the effective MEP changes induced by the DHPs. Both sadopine diastereomers were found to enhance the MEP of the MEP sensor (Table 2), an intrinsic property found for all DHP antagonists investigated. In contrast, all DHP agonists shift the sensor MEP in a negative direction (20). Addition of two additional binding sites for the DHP in our model allowed us to calculate the interaction energies of the DHPs with the hypothetical receptor. The interaction energies for (−)- and (+)-sadopine were −24.7 and −37.3 kJ/mol, corresponding to calculated dissociation con-

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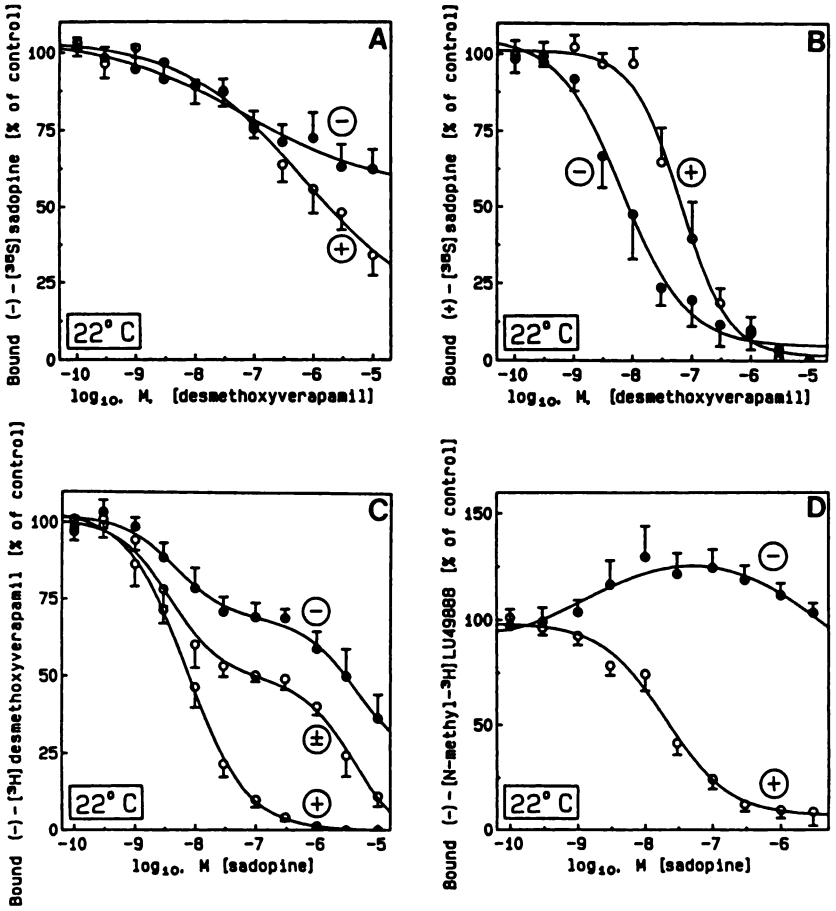

Fig. 3. Allosteric interaction of the sadopine diastereomers with the phenylalkylamine binding domain. A, Skeletal muscle L-type Ca^{2+} channels (0.021–0.028 mg/ml membrane protein) were labeled with (−)-[^{35}S]sadopine in the absence or presence of increasing concentrations of (−)- and (+)-desmethoxyverapamil. For (−)-[^{35}S]sadopine, the following binding parameters were determined: ●, IC_{50} = 69.19 ± 14.2 nM; n_H = 0.36 ± 0.16; maximal inhibition = 45.1%; ○, IC_{50} = 794.3 ± 103.2 nM; n_H = 0.41 ± 0.11; maximal inhibition = 88.4%. Specifically bound radioligand (control = 100%) was 6.8 pM. B, Same as above, with (+)-[^{35}S]sadopine as radioligand. Bound ligand in the absence of desmethoxyverapamil was 7.3 pM (0.021–0.028 mg of protein/ml was employed). ●, IC_{50} = 6.60 ± 1.31 nM; n_H = 0.80 ± 0.12; ○, IC_{50} = 64.56 ± 7.80 nM; n_H = 1.07 ± 0.15. C, Membrane-bound skeletal muscle L-type Ca^{2+} channels [0.026–0.031 mg/ml membrane protein were labeled with (−)-[^{3}H]desmethoxyverapamil in the absence or presence of increasing concentrations of (−)-sadopine (IC_{50}[I] = 4.78 ± 1.21 nM; IC_{50}[II] = 4.46 ± 0.73 μM), (±)-sadopine (IC_{50}[I] = 3.89 ± 0.67 nM; IC_{50}[II] = 4.16 ± 0.45 μM), or (+)-sadopine (IC_{50} = 7.14 ± 1.56 nM; n_H = 0.86 ± 0.21)]. IC_{50}[I] and IC_{50}[II] refer to the apparent high and low affinity components of the (−)- and (±)-sadopine inhibition curve. Specifically bound ligand at equilibrium in the absence of sadopine was 61.3–69.4 pM. D, Purified L-type Ca^{2+} channels (0.0024–0.0036 mg of protein/ml) were labeled with [*N-methyl*-^{3}H]LU49888 in the presence of increasing concentrations of (+)-sadopine (○) and (−)-sadopine (●). The following binding parameters were obtained: ●, EC_{50} = 1.94 ± 0.76 nM; ○, IC_{50} = 20.41 ± 4.56 nM, n_H 0.91 ± 0.17. Specifically bound ligand at equilibrium in the absence of sadopine was 79 pM.

stants of 0.23 and 1.32 nM, respectively, values very close to the calculated interaction energy of the equipotent DHP (+)-PN200-110 (Table 2). These results show that our model correctly predicted not only the antagonistic properties of the sadopine diastereomers but also their binding affinities, in reasonable agreement with the experimentally observed data.

Discussion

Sadopine is the first ^{35}S-labeled, DHP receptor-selective Ca^{2+} channel probe. Its high specific activity (800–1400 Ci/mmol) and high affinity for neuronal as well as muscle L-type Ca^{2+} channels, in combination with its low nonspecific labeling (Fig. 1), make it a unique probe for high resolution autoradiography of L-type Ca^{2+} channels. As shown for guinea pig brain, the distribution of [^{35}S]sadopine-labeled receptors is identical to that of (+)-[^{35}S]PN200-110- or [^{3}H]nimodipine-labeled Ca^{2+} channels. This confirms that [^{35}S]sadopine is selective for L-type Ca^{2+} channel-associated DHP receptors and does not label DHP binding sites unrelated to L-type Ca^{2+} channels (23, 24). Racemic sadopine and its two labeled and unlabeled diastereomers can be synthesized with good yields by coupling commercially available labeled and unlabeled *t*-Boc-L-methionine-*N*-hydroxysuccinimidylester to the respective DHP amino precursor.

It was not expected that the two sadopine diastereomers recognize the DHP receptor with equal dissociation constants. This is in contrast to other optically active DHPs studied so far, which display eudismic ratios of 10–300-fold (11, 24). Due to the low affinity of the distomers, the characterization of both labeled enantiomers [e.g., (*R*)- and (*S*)-[^{3}H]202-791 or (−)- and (+)-[^{3}H]azidopine (22, 25)] by direct labeling has so far been precluded. To our knowledge, our studies with (+)- and (−)-[^{35}S]sadopine are, therefore, the first example in the literature in which the equilibrium binding, allosteric, and kinetic properties of two labeled optical antipodes of a DHP Ca^{2+} antagonist

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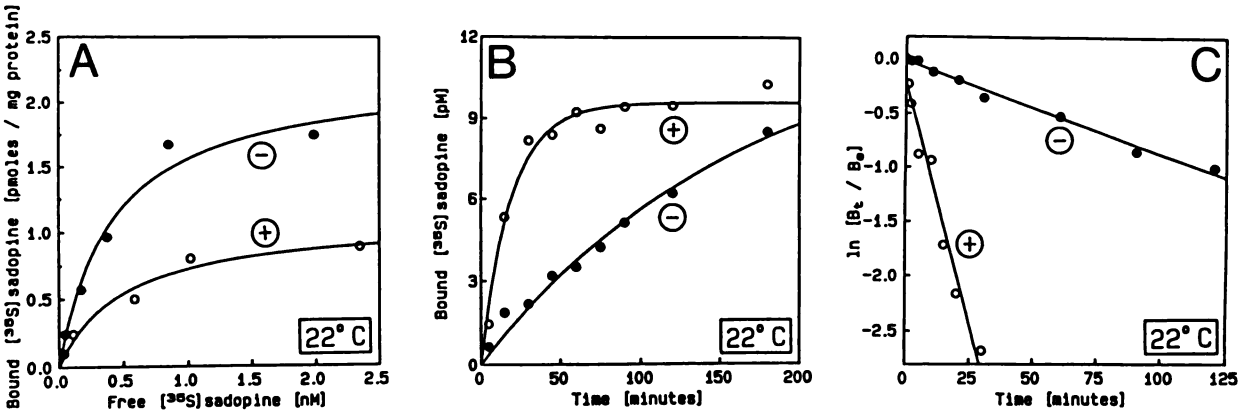

Fig. 4. Binding properties of (+)-[^{35}S]sadopine and (−)-[^{35}S]sadopine to membrane-bound skeletal muscle L-type Ca^{2+} channels. A, Equilibrium saturation analysis (0.125-ml assay volume, 22°). The following binding parameters were obtained: (+)-[^{35}S]sadopine (○) (0.040 mg/ml membrane protein), K_D = 0.511 ± 0.19 nM, B_{max} = 1.08 ± 0.14 pmol/mg of protein; (−)-[^{35}S]sadopine (●) (0.027 mg/ml membrane protein), K_D = 0.441 ± 0.11 nM, B_{max} = 2.24 ± 0.21 pmol/mg of protein. B, Association kinetics. Membrane protein at 0.066 mg/ml was incubated at 22° with 48.1 pM (+)-[^{35}S] sadopine or 57.05 pM (−)-[^{35}S]sadopine for the indicated times, and specifically bound radioligand was determined at the indicated times. ○, k_{obs} = 0.051 ± 0.012 min^{-1}; ●, k_{obs} = 0.0056 ± 0.002 min^{-1}. The corresponding means of the K_{+1}, the association rate constant, are given in Table 1. C, Dissociation kinetics. Membrane protein at 0.069 mg/ml was incubated with 42.5 pM (+)-[^{35}S]sadopine for 75 min or 34.3 pM (−)-[^{35}S]sadopine for 180 min. Ligand dissociation was initiated with 1 μM (±)-PN200-110. A semilogarithmic representation of the first-order dissociation reaction is given. ○, k_{-1} = 0.089 ± 0.007 min^{-1}; ●, k_{-1} = 0.0086 ± 0.0004 min^{-1}.

TABLE 1

Kinetic constants of (+)- and (−)-[^{35}S]sadopine at different temperatures

To determine association kinetics, skeletal muscle L-type Ca^{2+} channels (0.055–0.069 mg/ml membrane protein) were labeled with 34.6–57.0 pM (+)- or (−)-[^{35}S] sadopine. The data were fitted by linear methods to the second-order rate equation as recently described in detail (13). To determine dissociation kinetics, skeletal muscle L-type Ca^{2+} channels (0.046–0.069 mg/ml membrane protein) were labeled with 32.6–57.0 pM (+)- or (−)-[^{35}S]sadopine. After equilibrium was reached, dissociation was induced by the addition of 1 μM (+)-PN200-110. Semilogarithmic transformation of the dissociation data (as in Fig. 4C) revealed monophasic dissociation (regression coefficient in all cases was >0.96).

	K_{+1}	K_{-1}	K_D[a]
	$nM^{-1}\ min^{-1}$	min^{-1}	nM
(+)-[^{35}S]Sadopine			
37°	0.2201	0.815	3.70
22°	0.0878	0.0945	1.07
2°	0.00197	0.0052	2.64
(−)-[^{35}S]Sadopine			
37°	0.0636	0.0821	1.29
22°	0.00501	0.0095	1.89
2°	0.00182	0.00106	0.582

[a] Calculated from kinetic rate constants.

are directly compared. We discuss the significant differences between the two structures in context with our current knowledge of the properties of the DHP receptor of L-type Ca^{2+} channels.

Confirmation of a previously developed computer model of the DHP receptor using (+)- and (−)-sadopine. We previously developed a hypothetical computer model of the DHP receptor to predict whether a DHP acts as an Ca^{2+} channel agonist or antagonist (10, 20), as well as its affinity for the receptor. This model assumes interaction of the DHP with three sites within the receptor binding domain, represented by a tryptophan (MEP sensor), a positively charged residue (lysine), and another aromatic amino acid (phenylalanine). When this model was tested with the sadopine diastereomers, both behaved like antagonists. The calculated interaction energies were close to the K_D values obtained experimentally. This finding provides further support for the validity of our model. It will be interesting to apply the structural assumptions of our model to the amino acid sequences of the α_1 subunit found to be involved in the formation of the DHP binding domain.

Allosteric model of the DHP receptor. One of the most interesting findings in our study is the opposite allosteric effects of the sadopine diastereomers on phenylalkylamine and benzothiazepine receptor domains. As shown elsewhere (11, 13), stimulation and inhibition of Ca^{2+} channel labeling can be explained by differential effects of the allosteric modulators on the rate constants. Clearly, the different ester substituents in positions 3 and 5 of the pyridine ring are a crucial determinant of the channel conformation induced by the binding of these DHPs. Because the DHP- and phenylalkylamine-binding domains are believed to be accessible from opposite sides of the channel pore (26, 27), these conformational changes, underlying the modulation, must affect one or more of the hydrophobic, membrane-spanning helices (28) of the α_1 subunit. These changes induce or stabilize transitions between different functional states of the channel protein (e.g., between open and closed states) (29, 30). Earlier observations with the enantiomers of 202-791 and BAY K 8644 and other DHP agonists and antagonists suggested to us that opposite channel conformations (reflected in opposite allosteric coupling of the DHP receptor to the phenylalkylamine and benzothiazepine receptors) correspond to conformations of the "open" and "closed" channel, respectively. However, our experiments with sadopine disprove this hypothesis and suggest that the opposite allosteric states of the DHP receptor observed after binding of (−)- or (+)-sadopine must both be associated with a closed conformation of the channel. Recent studies with stimulators of DHP binding suggest that the positive heterotropic allosteric effect of these drugs on the DHP receptor is always associated with an increased affinity of Ca^{2+} ions for the channel (11, 13). These experiments are based on the fact that high affinity DHP binding to brain and muscle L-type channels requires occupation of high affinity divalent cation binding sites by Ca^{2+}. Therefore, DHPs may exert their channel-blocking effect by increasing the affinity of Ca^{2+} ions for the channel (7, 13). According to this model, our results with (+)- and (−)-sadopine

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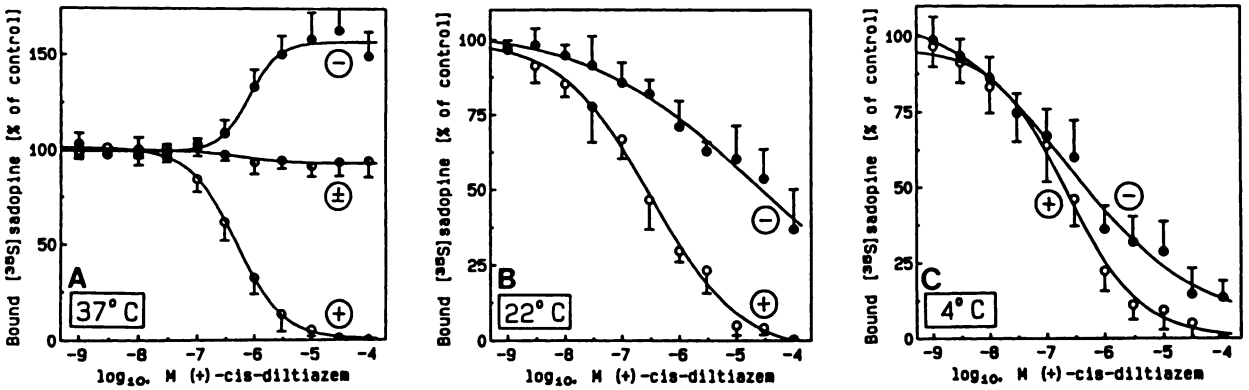

Fig. 5. Temperature-dependent regulation of the (+)- and (−)-[^{35}S]sadopine-labeled DHP receptor by (+)-*cis*-diltiazem. Membrane-bound skeletal muscle L-type Ca^{2+} channels (0.026–0.044 mg/ml membrane protein) were labeled with (−)-[^{35}S]sadopine (●), (±)-[^{35}S]sadopine (◐), or (+)-[^{35}S]sadopine (○), in the absence or presence of increasing concentrations of (+)-*cis*-diltiazem, at 37°, 22°, or 2°. The following binding parameters were obtained: 37°, ●, EC_{50} = 83.1 ± 4.64 nM, n_H = 1.68 ± 0.23; ◐, 570 ± 58 nM; ○, IC_{50} = 478 ± 21.3 nM, n_H = 1.05 ± 0.18; 22°, ○, IC_{50} 20.89 ± 1.34 μM, n_H = 0.29 ± 0.11; ●, IC_{50} = 316 ± 34.3 nM, n_H = 0.55 ± 0.24; 2°, ●, IC_{50} = 257 ± 21.4 nM, n_H = 0.40 ± 0.09; ○, IC_{50} = 229 ± 41.9 nM, n_H = 0.69 ± 0.21.

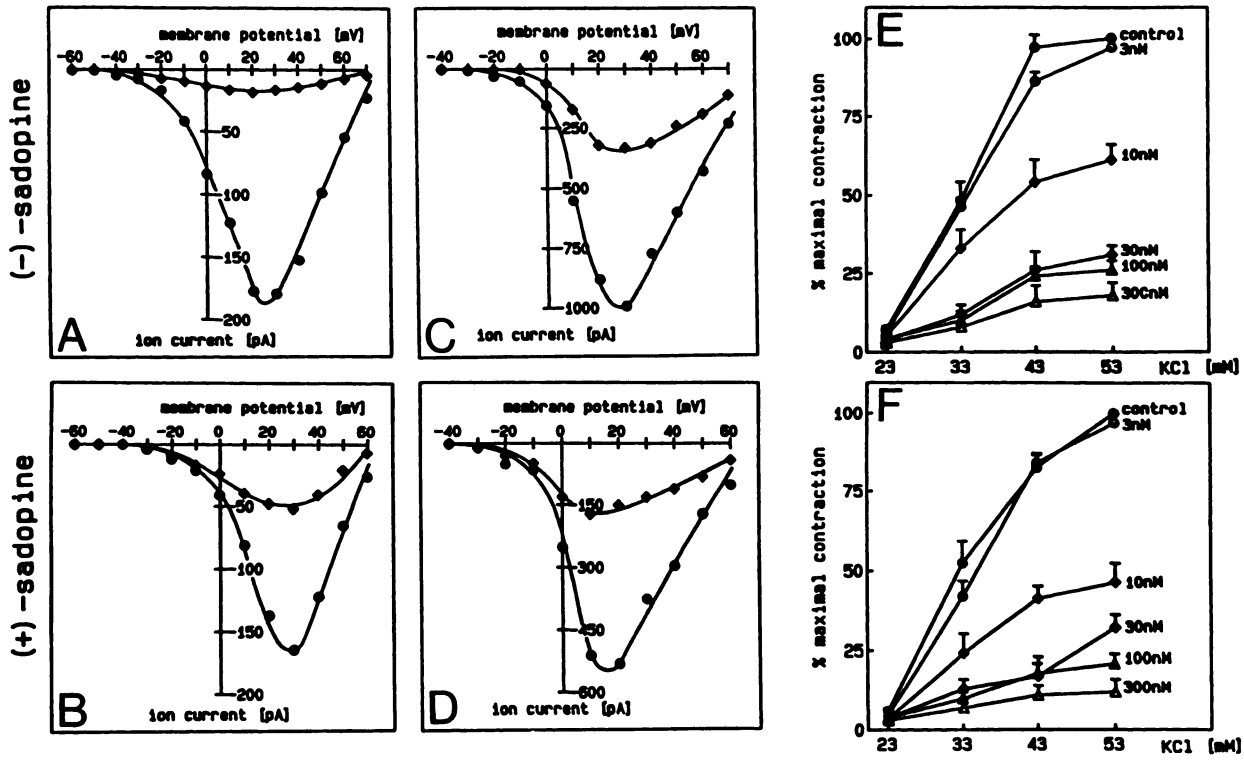

Fig. 6. Effect of (−)- and (+)-sadopine on L-type Ca^{2+} channel currents and on smooth muscle contraction. Current-voltage relationships of I_{Ba} in a rabbit ear artery smooth muscle cell (A and B) or a guinea pig cardiomyocyte (C and D), under control conditions (●) and after 3-min superfusion (◆) with 1 μM (−)- or (+)-sadopine as indicated. E and F, Inhibition of K^+-induced smooth muscle contraction of rabbit aortic rings. Data were normalized to the maximal contraction obtained with 53 mM K^+ before drug addition. IC_{50} values were calculated after construction of concentration-response curves plotting the percentage of maximal contraction (53 mM K^+) against drug concentration: (−)-sadopine, IC_{50} = 17.6 ± 4.5 nM; (+)-sadopine, IC_{50} = 6.41 ± 3.2 nM.

suggest that at least two different DHP-induced channel conformations exist with high affinity for Ca^{2+}, whereas only one [i.e., the (−)-sadopine-occupied state] favors the simultaneous binding of non-DHP Ca^{2+} channel blockers, such as (−)-[^{3}H]desmethoxyverapamil or (+)-*cis*-[^{3}H]diltiazem.

L-type Ca^{2+} channel DHP receptor. The labeled sadopine diastereomers are the first example in which the kinetic constants could be determined for two optical antipodes of a DHP. An unexpected finding was the significant difference in the rate constants for the two diastereomers, despite equal binding affinity. (−)-[^{35}S]Sadopine associated and dissociated slowly, with rate constants comparable to those of the benzoxadiazole DHP (+)-[^{3}H]PN200-110 (compare with Ref. 11). The association and dissociation rate constants for (+)-[^{35}S]sadopine were considerably faster, at least 10 times, at 22°, than those for the (−)-diastereomer. Our data clearly show that, in the case of the sadopine diastereomers, receptor affinities are not determined only by dissociation but also by association rate constants. Another example where association was the crucial determinant of binding affinity is the interaction of a ring-substituted *p*-

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TABLE 2

Interaction of DHP antagonists with a hypothetical DHP receptor model

Calculated MEPs for the isolated molecules (column 1) and the corresponding tryptophan complexes (column 2) and the effective potential changes (column 3). Column 4 presents the interaction energies of antagonistic DHPs with the proposed binding site model, column 5 the biological activities (IC_{50} values) for inhibition of K^+-induced contraction of strips of rabbit aorta (IC_{50} values for the reference DHPs are taken from Ref. 39). Column 6 shows our measured IC_{50} values and column 7 the n_H values for displacement of specific (+)-[^{3}H]PN200-110 binding of guinea pig hippocampus membranes. These data were determined as described (11) and in the legend to Fig. 1. Column 8 are the calculated predicted K_i values for the hypothetical DHP receptor model.

DHP	MEP		Potential change	Interaction energy	IC_{50}	IC_{50}	n_H	K_i
	Isolated molecule	Tryptophan complex						
	KJ		KJ	KJ/mol	nM	nM		nM
(+)-Nimodipine	171.6	401.1	140.3	−55.8	12.6	12.4 ± 1.64	0.87 ± 0.12	7.94
(−)-Nimodipine	126.9	376.7	115.9	−52.3	2.6	1.96 ± 0.26	1.12 ± 0.14	1.58
(+)-Nitrendipine	125.9	375.4	114.6	−59.8	19.9	8.58 ± 0.34	1.03 ± 0.23	50.1
(−)-Nitrendipine	109.5	357.9	97.1	−52.5	2.0	2.40 ± 0.65	0.95 ± 0.09	1.99
Nifedipine	57.9	318.9	58.1	−55.8	11.2	8.26 ± 2.25	0.71 ± 0.11	7.94
(+)-PN200-110	57.5	332.2	44.5	−34.1	1.4	0.273 ± 0.06	1.14 ± 0.15	0.12
(+)-Sadopine	47.6	324.5	36.6	−37.3	6.41	0.85 ± 0.24	0.95 ± 0.15	1.32
(−)-Sadopine	45.3	324.5	36.8	−24.7	17.6	0.91 ± 0.18	0.91 ± 0.28	0.23

carboxy-benzenesulfonamide and its uncharged nitro congener with carbonic anhydrase (35).

Kinetic models and X-ray diffraction studies postulate a "membrane bilayer pathway" to be involved in the receptor binding of DHPs (31, 32). This first requires drug partitioning into the membrane, followed by lateral diffusion to the binding domain. The latter was found to be localized close to the external mouth of the channel pore (27). The two sadopine diastereomers are expected to possess equal membrane partition coefficients. Hence, their different kinetics more likely reflect differences in the interaction with the channel protein itself, rather than with the lipid bilayer. The association rate constants for both diastereomers are below the diffusion-limited rate for a membrane approach (33). This suggests that additional time-limiting processes have to be involved in the binding reaction. Such processes could be the isomerization of the receptor protein between different states (33), orientational or conformational constraints of the DHP after penetration into the bilayer (32), or a "flip-flop" of the ligand between the monolayers of the bilayer (34), thereby affecting drug-receptor interaction. Although these mechanisms could account for the different association behavior, they cannot explain the different dissociation rates. We, therefore, suggest that the overall accessibility of the DHP receptor site within the α_1 subunit is different for the two diastereomers and must be a function of their stereochemical properties. A localization of the receptor so that it is easily accessible from the surrounding membrane bilayer on the lipid-oriented surface of the channel is, therefore, very unlikely.

Photoaffinity labeling studies with different DHPs (36–38) identified regions within the α_1 subunit that contribute to the formation of the DHP receptor domain. The major site of DHP binding is located very close to or within transmembrane helix S6 in domain III (IIIS6). In addition, portions of the connecting loop between IIIS5 and IIIS6 and a region close to IVS6 interact preferentially with the long ester substituent in position 3 of azidopine, which is structurally related to sadopine (37). These regions must, therefore, be located very close to each other in the folded structure of the α1 subunit, forming a single high affinity DHP receptor domain. Taking together structural data (36, 37), functional data (27), and data presented here, we favor the idea that the DHP receptor domain is formed between trans- or intramembrane helices of the α_1 structure close to the extracellular surface of the channel, with different accessibility for the two sadopine diastereomers.

In summary, the DHP Ca^{2+} antagonist [^{35}S]sadopine is a novel, ^{35}S-labeled radioligand that is highly suitable for autoradiographic studies of L-type Ca^{2+} channels. Its labeled and unlabeled diastereomers will be useful probes to further characterize the molecular basis of DHP-α_1 subunit interaction and the pharmacological and physiological significance of the different allosteric conformations of the channel induced by Ca^{2+} channel-active drugs.

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