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Characterization of the Slow Calcium Channel Binding Sites for [^{3}H]SR 33557 in Rat Heart Sarcolemmal Membranes

P. CHATELAIN, P. BEAUFORT, L. MEYSMANS, and M. CLINET
Sanofi-Labaz Research Centre, 1120 Brussels, Belgium
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SUMMARY

SR 33557 represents a new class of compounds (indolizine sulfone) that inhibit L-type Ca^{2+} channels. [^{3}H]SR 33557 has been shown to bind with high affinity ($K_d \simeq 0.36$ nM, calculated from saturation isotherms and association/dissociation kinetics) to a single class of sites in a purified preparation of rat cardiac sarcolemmal membranes. The binding was found to be saturable and reversible. The maximal binding capacity was in approximately 1:1 stoichiometry with that of other Ca^{2+} channel antagonists. Various divalent cations (Mg^{2+}, Mn^{2+}, Ca^{2+}, Ba^{2+}, and Cd^{2+}) were shown to inhibit specific [^{3}H]SR 33557 binding, with Cd^{2+} being the most potent. Among several receptor or channel ligands (including ω-conotoxin and Na^+ and K^+ channel modulators), only the L-type Ca^{2+} channel antagonists were found to displace [^{3}H]SR 33557. However, dihydropyridines, phenylalkylamines, benzothiazepines, and diphenylbutylpiperidines were found to inhibit [^{3}H]SR 33557 in a noncompetitive manner as demonstrated by displacement and saturation experiments in addition to dissociation kinetics. From these results, we suggest that SR 33557 binds with high affinity to a unique site on the L-type Ca^{2+} channel found in rat cardiac sarcolemmal membranes.

The fundamental role of Ca^{2+} as an intermediary in many regulatory and signaling processes in cellular activity is well established. In a variety of excitable cells, voltage-dependent Ca^{2+} channels allow the movement of Ca^{2+} across the cytoplasmic membrane (1). Cardiac sarcolemmal membranes have been shown to possess distinct high affinity binding sites for three different structural classes of Ca^{2+} entry blockers (also termed Ca^{2+} antagonists), dihydropyridines, phenylalkylamines, and benzothiazepines (1, 2). These sites are located on one of the proteins that form the Ca^{2+} channel complex that is functionally associated with the L-type Ca^{2+} channel (3). Each of these sites is coupled to the other sites by allosteric interactions.

Recently, several compounds, HOE 166 (4, 5), fluspirilene (6, 7), belfosdil (8), and SR 33557 (9–11), all representatives of chemical series structurally unrelated to the three previously described classes, have been shown to interact with the Ca^{2+} channel complex. SR 33557 (Fig. 1) is an indolizine sulfone derivative. It has a potent relaxant activity against Ca^{2+}-induced contractions in K^+-depolarized aorta, with the concentration relaxing the aorta by 50% being approximately 6 nM (9). Because SR 33557 is much less effective in inhibiting noradrenaline-induced contractions in the same tissue (9), it was concluded that the compound has Ca^{2+} channel antagonist properties. The Ca^{2+} antagonist activity was confirmed by electrophysiological studies (10), and studies of the binding profile of SR 33557 indicated a high specificity for the Ca^{2+} channel (9). In addition, we have recently shown (12) that SR 33557 possesses an interesting pharmacological profile in cardiac tissue, in that this compound displays a preferential negative chronotropic activity with regard to its negative inotropic actions.

Further characterization of the SR 33557 binding site using either [^{3}H]nitrendipine and [^{3}H]D888 in a guinea pig membrane preparation (9) or [^{3}H]PN200-110, [^{3}H]verapamil, [^{3}H]diltiazem, and [^{3}H]fluspirilene in skeletal muscle (10) provided evidence for a specific binding site of SR 33557. Photoaffinity labeling studies have confirmed that [^{3}H]SR 33557 associates with the same protein that binds to the classical Ca^{2+} channel antagonists (10). The aim of this study was to characterize the binding site of [^{3}H]SR 33557 in purified rat heart sarcolemmal membranes and to examine the interaction between the SR 33557 binding site and those previously identified for more classical Ca^{2+} channel antagonists.

Materials and Methods

Chemicals. [*methyl*-^{3}H]SR 33557 (1.8–3.2 TBq/mmol), (+)-[*methyl*-^{3}H]PN200-110 (2.6–3.2 TBq/mmol), (−)-[*N-methyl*-^{3}H]desmethoxyverapamil (2.2–3.1 TBq/mmol) were obtained from Amersham, England. (*cis*)-(+)-[*N-methyl*-^{3}H]Diltiazem was obtained from NEN (Dreieich, FRG). The ligands were stored, protected from light, at −18°. SR 33557 [2-isopropyl-1-((4-(3-(*N*-methyl-*N*-(3,4-dimethoxy-β-phenethyl)amino)propyloxy)benzenesulphonyl))indolizine], amioda-

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ABBREVIATIONS: DMSO, dimethyl sulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

rone, diclofurime, and BRL 34915 were synthetized in our Chemical Department.

The following drugs were generously donated by the companies listed: nifedipine, nitrendipine, nimodipine, nisoldipine, and BAY-K8644, Bayer (Wuppertal, FRG); (+)- and (−)-PN200-110 and (*S*)-(+)- and (*R*)-(−)-202-791, Sandoz (Basel, Switzerland); (*cis*)-(+)- and (*cis*)-(−)-diltiazem, Synthelabo (Paris, France); CGP 28392 and phentolamine, CIBA-GEIGY (Basel, Switzerland); tiapamil and diazepam, Hoffmann-La Roche (Basel, Switzerland); felodipine, Hässle A. B. (Mölndal, Sweden); flunarizine, lidoflazine, and fluspirilene, Janssen Pharmaceutica (Beerse, Belgium); and bepridil, CERM (Riom, France). The enzymes were from Boehringer and ω-conotoxin GVIA was from Peninsula Laboratories (Belmont, CA). All other chemicals were obtained from Sigma or Merck and were of the highest purity available. Stock solutions (5 mM) and all subsequent dilutions of unlabeled drugs were made in DMSO. Stock solutions of the radioligands were diluted in the appropriate buffers.

Purification of heart sarcolemma. Highly purified heart sarcolemma was prepared from adult rat heart, following the method previously described (13). In essence, the purification procedure consisted of collagenase digestion, treatment with KCl-pyrophosphate, differential centrifugation, and fractionation using a sucrose density gradient. The sarcolemma was obtained with a yield of 6–7 mg/10 g of heart. This was frozen in liquid nitrogen and stored at −80° in a 5 mM Tris·HCl buffer (pH 7.4) containing 250 mM sucrose and 0.1 mM dithioerythritol. Under these conditions, binding and enzymatic activities were stable for at least 3 months.

[^{3}H]SR 33557 binding assay. Binding of [^{3}H]SR 33557 was carried out in a final volume of 1 ml of Tris·HCl (50 mM) buffer, pH 7.4, containing 0.1% bovine serum albumin and 20 μg of sarcolemma. Incubations were carried out for 90 min at 25°, in the dark so as to avoid deterioration of the radioligand. The total radioactivity bound was measured after rapid filtration through Whatman GF/C filters, which has been pretreated with 0.3% polyethyleneimine, followed by five washings at 0° with Tris·HCl (5 mM) buffer, pH 8.0, containing 200 mM choline chloride. the nonspecific binding, defined as being the quantity of radioligand bound to the membranes in the presence of an excess of SR 33557 (1 μM), was subtracted from the total quantity bound. Duplicate assays were performed routinely for each experimental condition. The standard error of these results was typically less than 5%. As a general rule, all binding experiments with [^{3}H]SR 33557 were carried out in Minisorb polypropylene tubes and 0.1% (w/v) bovine serum albumin was added to all the media used, to reduce nonspecific fixation of the ligand to glass or plastic surfaces. (+)-[^{3}H]PN200-110, (−)-[^{3}H]D888, and (*cis*)-(+)-[^{3}H]diltiazem binding was carried at 25° under similar conditions as described elsewhere (11).

Antagonism between [^{3}H]SR 33557, unlabeled SR 33557, other drugs, and ions. Inhibition of [^{3}H]SR 33557 binding by different drugs and ions was measured under equilibrium conditions, as described above, in the presence of 0.5–0.8 nM [^{3}H]SR 33557. SR 33557 and the other organic products were added to the incubation medium dissolved in DMSO, so as to obtain a maximum final concentration in DMSO of 2% (v/v). It was established that the highest concentration of DMSO did not alter the density or affinity of [^{3}H]SR 33557 binding sites. For dissociation studies, membranes were incubated with the radioligand to establish equilibrium, and dissociation was induced by the addition of an excess of unlabeled ligand in the presence or absence of other drugs.

Analysis of data. Saturation isotherms were analyzed by a computer-assisted method of nonlinear regression, based on the Clark equation (14). The density of the sites (B_{max}, expressed in fmol/mg of protein) and the equilibrium dissociation constant (K_D, expressed in molarity) were calculated, taking into account the experimental values of B (specifically bound ligand) and of F (free ligand). The IC_{50} value (concentration inhibiting specific binding by 50%) and the Hill coefficient (n_H) were determined by a logit-log transformation of the inhibition curves. Correlation coefficients in both kinds of experiments were typically greater than 0.95. Where feasible, K_i values were obtained using the method of Cheng and Prusoff (15).

The rate of ligand dissociation (k_{-1}) was determined using the following expression:

$$\ln[LR_t]/[LR_{eq}] = -k_{-1}\cdot t$$

where $[LR_t]$ represents the concentration of the complex at time t and $[LR_{eq}]$, the concentration of the complex at equilibrium.

The rate of ligand association (k_{+1}) was determined using the following expression:

$$k_{+1} = k_{obs}\,[LR_{eq}]/[L][LR_{max}]$$

where $[L]$ is the concentration of ligand, $[LR_{max}]$ is the maximum number of receptors present, and k_{obs} is the slope of the pseudo-first-order plot of $\ln[LR_{eq}]/[LR_{eq}] - [LR_t]$ versus time. K_d was calculated from the ratio k_{-1}/k_{+1}.

Protein concentration measurements. The quantities of proteins were determined in accordance with the method described by Lowry *et al.* (16), using bovine serum albumin, fraction V, as standard.

Results

Characterization of [^{3}H]SR 33557 binding to cardiac sarcolemmal membranes. The binding of [^{3}H]SR 33557 to cardiac sarcolemmal membranes was shown to be saturable (Fig. 2), rapid, and reversible (Fig. 3). Analysis of the saturation curves by computer-assisted nonlinear regression (Fig. 2A) or by Scatchard analysis (17) (Fig. 2B) revealed only one high affinity (K_D = 0.37 ± 0.03 nM) class of sites (n_H = 0.96 ± 0.04) for radioligand concentrations ranging from 0.1 to 30 nM, with a maximal binding capacity of 3068 ± 180 fmol/mg of protein (11 experiments). In the presence of 1 mM $CaCl_2$, the maximal binding capacity was unchanged, but K_D was decreased by a factor of approximately 2 (K_D = 0.69 ± 0.06 nM six experiments). Specific binding of SR 33557 was shown to be linear with membrane protein concentration in the range of 5–75 μg of protein/ml. Specific binding represented about 85% of total binding at a concentration of [^{3}H]SR 33557 equivalent to its K_D and was independent of the concentration of nonradioactive SR 33557 between 10^{-6} and 10^{-5} M.

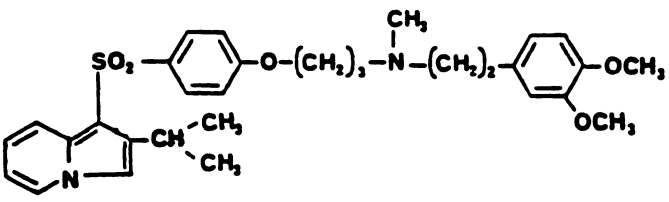

Fig. 1. Structure of SR 33557.

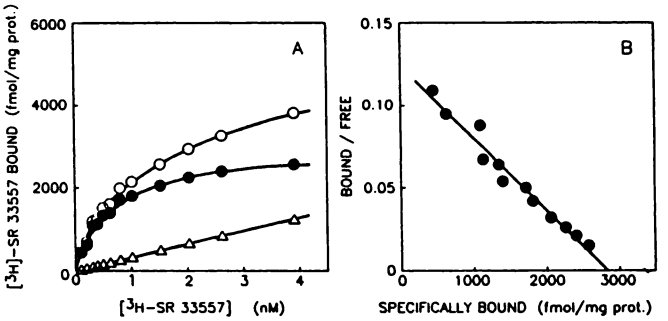

Fig. 2. Binding of [^{3}H]SR 33557 to cardiac sarcolemmal membranes. A, Equilibrium binding measured at 25°. Specific binding (●) was taken as the difference between binding in the absence (○) and in the presence (△) of 1 μM unlabeled SR 33557. B, Scatchard plot of the specific [^{3}H]SR 33557 binding component.

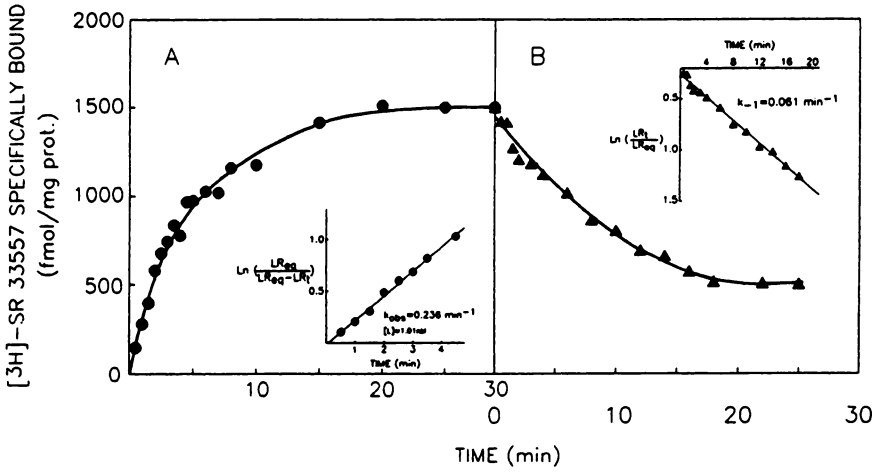

Fig. 3. Association and dissociation kinetics of [^{3}H] SR 33557 in cardiac sarcolemmal membranes. A, Association kinetics. Cardiac sarcolemmal membranes were incubated with 1.0 nM [^{3}H]SR 33557 at 25°. *Inset*, semilogarithmic representation of the pseudo-first-order association reaction. B, Dissociation kinetics. After equilibrium was reached, ligand dissociation was initiated by addition of 1 μM unlabeled SR 33557. *Inset*, semilogarithmic representation of the first-order dissociation reaction.

Saturation isotherms for cardiac sarcolemmal membranes were performed with (+)-[^{3}H]PN200-110, (−)-[^{3}H]D888, and (*cis*)-(+)-[^{3}H]diltiazem. The K_D values and the maximal binding capacities were, respectively, 3.1 ± 0.6 nM and 2418 ± 177 fmol/mg of protein (seven experiments) for (−)-[^{3}H]D888, 65 ± 7 nM and 2103 ± 99 fmol/mg of protein (four experiments) for (*cis*)-(+)-[^{3}H]diltiazem, and 0.042 ± 0.005 nM and 1564 ± 153 fmol/mg of protein (four experiments) for (+)-[^{3}H]PN200-110. In the case of (+)-[^{3}H] PN200-110, the K_D value and maximal binding capacity in the presence of 1 mM Ca^{2+} were 0.045 ± 0.007 nM and 2532 ± 150 fmol/mg of protein, respectively, (four experiments).

The binding constant for SR 33557 was also determined by two other means, namely binding kinetics (Fig. 3) and competition analysis (Fig. 4). The association of [^{3}H]SR 33557 to the sarcolemma was rapid, followed pseudo-first-order kinetics, and reached a plateau after 15 to 60 min, according to the concentration of the radioligand employed (0.1–2 nM). Binding was shown to remain stable for at least 2 hr. The value of the association constant (k_{+1}) derived from k_{obs} was 0.173 min^{-1} nM. The dissociation of [^{3}H]SR 33557, as measured by addition of an excess of nonradiolabeled ligand after equilibrium binding was attained, yielded first-order kinetics with a dissociation constant (k_{-1}) of 0.061 ± 0.007 min^{-1} (eight experiments). The calculated value of K_D was 0.35 nM and was identical to the value determined from the saturation isotherms (K_D = 0.37 ± 0.03 nM). The K_D of SR 33557 calculated by competition analysis [calculated by the Cheng-Prusoff relationship (15)] was 0.47 ± 0.04 nM (seven experiments) and was close to the values determined in the saturation and kinetics studies.

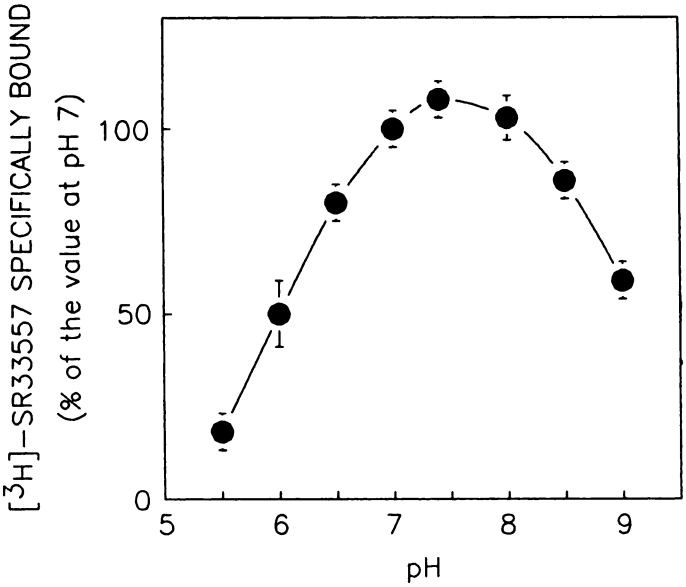

Fig. 4. Effect of pH on specific [^{3}H]SR 33557 binding to cardiac sarcolemmal membranes. The specific binding at pH 7.0 was taken as 100%. Each value represents the average ± standard error of three or four determinations.

Characteristics of [^{3}H]SR 33557 binding sites in cardiac sarcolemmal membranes. Preliminary studies performed by us showed that the specific binding of [^{3}H]SR 33557 increased when the pH value was raised from 5.5 to 7.0, reached a plateau between pH 7.0 and pH 8.0 (maximum at pH 7.5), and subsequently decreased when the pH exceeded 8.0 (Fig. 4). The ascending part of the bell-shaped curve suggests the presence of an ionizable group, with a pK_a value near 6, that is important for the association of [^{3}H]SR 33557 with its receptor. The descending part presumably corresponds to neutralization of SR 33557, because this compound has a pK_a value of 8.7.

In addition, the specific binding of [^{3}H]SR 33557 to sarcolemmal membranes was extremely heat sensitive (results not shown), being decreased by 80% when binding studies were performed at 50° for 10 min. Specific binding was also sensitive to proteolytic and phospholipid-degrading enzymes (Table 1).

TABLE 1

Enzymic degradation of [^{3}H]SR 33557 binding sites

Sarcolemma (50 μg/ml) was preincubated at 37° for 30 min, in the absence and in the presence of the enzymes tested at the indicated concentrations. The preparation was then washed and centrifuged before being placed in the normal incubation conditions at 25°, in the presence of 0.5 nM [^{3}H]SR 33557. Results are shown as the mean ± standard error of the number of determinations given in parentheses.

Enzymes	Concentration	[^{3}H]SR 33557 specifically bound
	mg/ml	%
None		100 ± 3 (4)
Trypsin	0.1	22 ± 3 (3)
Pronase	0.1	21 ± 3 (3)
Papain	1.5	31 ± 4 (3)
Phospholipase A_2 (bee venom)	0.01	54 ± 2 (3)
Phospholipase C (*Bacillus cereus*)	0.01	54 ± 1 (2)
Neuraminidase (*Clostridium perfringens*)	0.1	64 ± 6 (4)

These data indicated the proteinaceous nature of the binding sites; presumably, the integrity of these sites is maintained by the lipidic environment. Specific binding of [^{3}H]SR 33557 was also inhibited by preincubation (37° for 30 min) either with the reducing agent dithiothreitol (5 mM) (−55%) or with the alkylating agent *N*-ethylmaleimide (1 mM) (−60%). This indicates the importance of —SH groups and disulfide bridges to the structure of the binding site of the ligand.

Effect of inorganic cations on [^{3}H]SR 33557 binding to cardiac sarcolemmal membranes. The specific binding of [^{3}H]SR 33557 was shown to be particularly sensitive to the ionic composition of the medium. The cations inhibited the binding of [^{3}H]SR 33557 in the following order of activity (Table 2): $Cd^{2+} > La^{3+} > Mn^{2+} \simeq Ca^{2+} > Ni^{2+} \simeq Mg^{2+} > Ba^{2+} > Li^+ > K^+ > Na^+ > Rb^+$. The divalent cation Cd^{2+} was the most active, with an IC_{50} value of 0.04 mM. The trivalent cation La^{3+}, the classical antagonist of the slow calcium channel, was nearly 10 times less active than cadmium. Amongst the other divalent cations, Mn^{2+} and Ca^{2+} were the most potent antagonists, with respective IC_{50} values of 3.7 and 5 mM, i.e., 100 times less active than cadmium. Magnesium was 4 times less active than calcium, with an IC_{50} value of 19 mM. The monovalent cations were only slightly active. Sodium had no effect at 10 mM but inhibited binding by 30% at 100 mM. Fig. 5 shows the percentage of inhibition by various cations of the specific binding of [^{3}H]SR 33557. This is shown as a function of the ionic radius for the cations. A bell-shaped relationship between ionic radius and inhibition of binding is apparent for the divalent cations. It is also noteworthy that, for a given ionic radius (≃1 Å; Cd^{2+}, La^{3+}, Ca^{2+}, and Na^+), there is no relation with the charge of the ion (Fig. 5).

Studies undertaken by us revealed that EDTA and EGTA inhibited the binding of [^{3}H]SR 33557 at concentrations in excess of 1 μM. The maximal inhibition of 75% observed in the presence of EDTA was reached at an EDTA concentration of 30 μM and remained constant up to 10 mM. In the presence of EGTA, inhibition reached 40% at an EDTA concentration of 30 μM and remained more or less constant up to 1 mM, reaching 70% at an EDTA concentration of 10 mM. The inhibition profiles determined for EDTA, EGTA, and calcium were not altered when the temperature of the incubation medium was raised from 25° to 37°. Saturation isotherms were determined in order to further characterize the effects of calcium, magnesium, EGTA, and EDTA on the binding of [^{3}H]SR 33557.

TABLE 2

Effects of inorganic cations on the binding of [^{3}H]SR 33557 to cardiac sarcolemmal membranes

Results are the mean ± standard error of the number of determinations given in parentheses.

Cation	IC_{50}	n_H
	mM	
La^{3+}	0.3 ± 0.1 (3)	1.10 ± 0.01
Cd^{2+}	0.04 ± 0.02 (3)	1.02 ± 0.01
Mg^{2+}	19 ± 2 (3)	0.64 ± 0.12
Ni^{2+}	12 ± 2 (3)	0.89 ± 0.03
Mn^{2+}	3.7 ± 0.5 (3)	0.79 ± 0.10
Ca^{2+}	5.0 ± 0.6 (3)	0.60 ± 0.07
Ba^{2+}	>20 (2)	
Rb^+	>200 (2)	
Li^+	114 ± 15 (3)	1.02 ± 0.03
K^+	180 ± 11 (3)	1.04 ± 0.07
Na^+	218 ± 8 (3)	1.07 ± 0.09

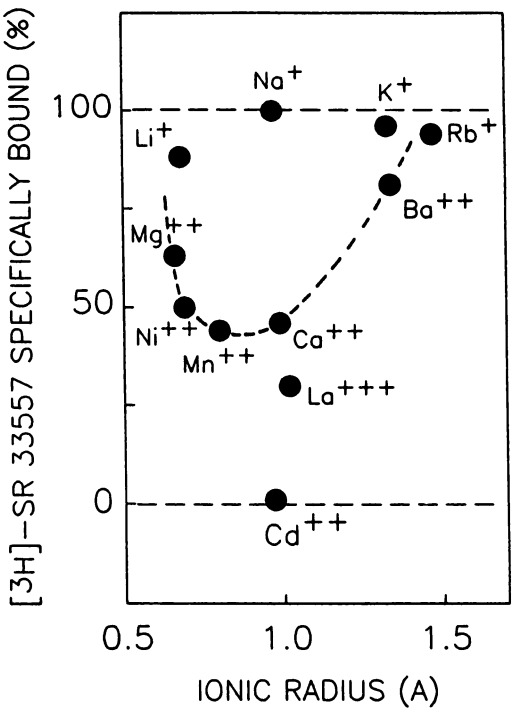

Fig. 5. Effects of inorganic cations on specific [^{3}H]SR 33557 binding to cardiac sarcolemmal membranes. The inhibitory effect of each cation (10 mM) is plotted as a function of ionic crystal radius.

TABLE 3

Pharmacological specificity of the binding of [^{3}H]SR 33557 to cardiac sarcolemmal membranes

Sarcolemma (20 μg) was incubated for 90 min at 25° in the presence of increasing or fixed (10^{-5} M) concentrations of product and 0.6 nM [^{3}H]SR 33557, a concentration sufficient to occupy about 50% of the sites. Results are the mean ± standard error of the number of determinations given in parentheses.

Agent	IC_{50}
	nM
ω-Conotoxin GVIA	>10.000 (3)
Fluspirilene	29 ± 6 (3)
Bepridil	678 ± 77 (3)
Flunarizine	1894 ± 148 (3)
Lidoflazine	643 ± 84 (3)
Calmidazolium	160 ± 11 (3)
Amiodarone	117 ± 2 (3)

These were determined for the ligand in the presence of 1 mM calcium, magnesium, EDTA, or EGTA. Calcium and magnesium increased the K_D of SR 33557 for its receptor without modifying the maximal binding capacity. The effect observed for calcium is identical at both 25° and 37°. The effects of EDTA and EGTA on the binding of [^{3}H]SR 33557 take the form of a marked reduction in the apparent number of sites without any change in K_D. These effects suggest that the binding of [^{3}H]SR 33557 requires the presence of calcium or magnesium in micromolar concentrations.

Pharmacological specificity of [^{3}H]SR 33557 binding. To determine the specificity of binding, several different classes of agents were examined as potential inhibitors of [^{3}H]SR 33557 binding (Tables 3 and 4). The representatives of the three main classes of calcium antagonists acting specifically on the L-type channel [nifedipine, verapamil, and (*cis*)-(+)-diltiazem] show themselves to be extremely potent with respect to the binding of [^{3}H]SR 33557 to the sarcolemma (Table 4). IC_{50}

values for these agents ranged from 6 to 600 nM. The other nonclassical calcium antagonists examined were less powerful, having IC_{50} values of 50 to 2000 nM, with fluspirilene showing the most activity. ω-Conotoxin GVIA, an antagonist that is specific for the N-type calcium channel, was inactive (Table 3). The agents examined that act on molecular targets other than those of the calcium channels were inactive at concentrations of 10^{-5} M and above. These agents included Na^+ and K^+ channel antagonists and ligands for β- and α-adrenergic, cholinergic, muscarinic, and histaminic receptors. Only calmidazolium and trifluoperazine, antagonists of calmodulin, and amiodarone produced a level of inhibition in excess of 50% at a concentration of 10^{-5} M (Table 3).

The results obtained from the systematic study of a range of Ca^{2+} channel modulators are shown in Fig. 6 and the IC_{50} values

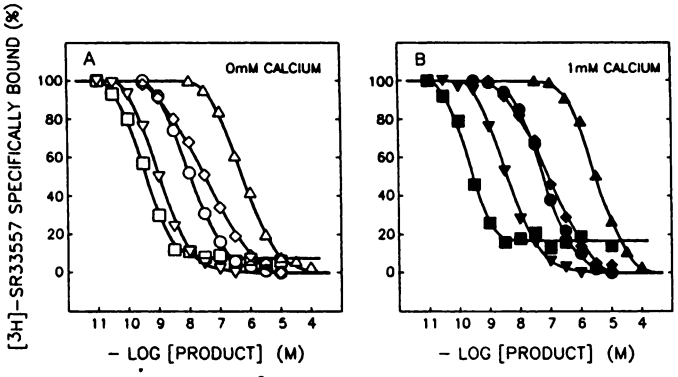

Fig. 6. Inhibition of specific [^{3}H]SR 33557 binding to cardiac sarcolemmal membranes by increasing concentrations of unlabeled SR 33557 (▽, ▼), (+)-PN200-110 (□, ■,), (−)-D888 (○, ●), (*cis*)-(+)-diltiazem (△, ▲), or fluspirilene (◇, ♦), in the absence of added $CaCl_2$ (A) or in the presence of 1 mM $CaCl_2$ (B). Each value represents the average of three to seven determinations.

are compiled in Table 4. At 25° in the absence of calcium (Fig. 6A), SR 33557 totally inhibited, in a competitive manner, the binding of [^{3}H]SR 33557, with an IC_{50} of 1.01 nM (K_i = 0.47 ± 0.04 nM (seven experiments). The presence of 1 mM calcium (Fig. 6B) caused the inhibition curves to shift towards higher concentrations with IC_{50} = 3.7 ± 0.3 nM (four experiments) and K_i = 1.4 ± 0.2 nM. An increase in temperature to 37° shifted the IC_{50} of SR 33557 towards higher concentrations (IC_{50} = 2.4 ± 0.2 nM, K_i = 1.2 ± 0.1 nM (five experiments). Thus, the effect of temperature on the IC_{50} values of SR 33557 seems to be less marked than the effect of calcium.

The 1,4-dihydropyridine derivatives, at 25° and in the absence of calcium, partially inhibited (≈92%) the binding of [^{3}H] SR 33557 to sarcolemma, with IC_{50} values ranging from 0.3 to 6 nM for the antagonist derivatives tested [(+)-PN200-110, felodipine, nitrendipine, nifedipine, nimodipine, nisoldipine, and (−)-R202-791] and from 50 to 1000 nM for the agonist derivatives [(+)-S202-791, BAY-K8644, and CGP 28392] (Table 4). The isomer (−)-PN200-110, an inactive antagonist, manifested a degree of activity that was 200 times lower than that of its isomer, (+)-PN200-110. The maximal degree of inhibition (93%) attained for (+)-PN200-110 in the presence of 0.5 nM [^{3}H]SR 33557 fell to 70% in the presence of 10 nM radioligand. This suggests an allosteric-type interaction between (+)-PN200-110 and the receptor site of SR 33557 (data not shown). Neither the presence of 1 mM calcium nor the increase in temperature (25° to 37°) modified the IC_{50} values observed for the 1,4-dihydropyridines. Furthermore, the maximal inhibition levels reached by the 1,4-dihydropyridines were not significantly different at 25° and at 37°. The phenylalkylamine and benzothiazepine derivatives at 25°, in the absence or presence of calcium, totally inhibited the binding of [^{3}H]SR 33557. The Hill coefficients were found to be close to 1. This

TABLE 4

Effects of SR 33557 and various calcium channel modulators on the specific binding of [^{3}H]SR 33557 to rat cardiac sarcolemmal membranes

Sarcolemma (20 μg) was incubated for 90 min at 25° in the presence of increasing quantities of product plus [^{3}H]SR 33557 at a concentration sufficient to occupy about 50% of the sites, namely 0.5 nM at 0 mM Ca^{2+} and 0.8 nM at 1 mM Ca^{2+}. Results are the mean ± standard error on the mean of the number of determinations given in parentheses.

Product	0 mM Calcium			1 mM Calcium		
	IC_{50}	K_i	n_H	IC_{50}	K_i	n_H
	nM	nM		nM	nM	
SR 33557	1.01 ± 0.06 (7)	0.47 ± 0.04	1.00 ± 0.02	3.7 ± 0.3 (4)	1.4 ± 0.2	0.92 ± 0.08
1,4-Dihydropyridines						
(+)-PN 200-110	0.33 ± 0.04 (4)	Partial inhibition 93%		0.21 ± 0.03 (4)	Partial inhibition 82%	
(−)-PN 200-110	6.5 ± 1.3 (4)	Partial inhibition 94%		4.0 ± 2.0 (2)	Partial inhibition 90%	
Felodipine	1.8 ± 0.4 (3)	Partial inhibition 93%		0.8 ± 0.3 (2)	Partial inhibition 96%	
Nitrendipine	2.2 ± 0.4 (3)	Partial inhibition 94%				
Nifedipine	5.6 ± 2.6 (3)	Partial inhibition 93%		1.5 ± 0.2 (3)	Partial inhibition 90%	
Nimodipine	1.8 ± 0.1 (3)	Partial inhibition 95%		1.0 ± 0.2 (2)	Partial inhibition 94%	
Nisoldipine	1.0 ± 0.3 (3)	Partial inhibition 97%		0.8 ± 0.2 (2)	Partial inhibition 93%	
(−)R202-791	5.9 ± 0.6 (3)	Partial inhibition 90%		5.6 ± 0.5 (3)	Partial inhibition 90%	
(+)-S202-791	863 ± 202 (3)	Partial inhibition 89%		849 ± 118 (3)	Partial inhibition 91%	
Bay K 8644	54 ± 8 (3)	Partial inhibition 91%		29 ± 5 (3)	Partial inhibition 94%	
CGP 28392	631 ± 178 (3)	Partial inhibition 86%		553 ± 107 (3)	Partial inhibition 89%	
Phenylalkylamines						
(−)-Desmethoxyverapamil [(-)-D888]	12.4 ± 2.8 (6)	5.7 ± 1.4	0.92 ± 0.04	62 ± 4 (3)	28 ± 2	0.97 ± 0.04
Verapamil	133 ± 11 (4)	61 ± 7	0.93 ± 0.05	374 ± 19 (2)	169 ± 4	0.80 ± 0.07
Tiapamil	378 ± 22 (3)	165 ± 13	0.96 ± 0.01	794 ± 204 (2)	362 ± 70	0.92 ± 0.10
Benzothiazepines						
(*cis*)-(+)-Diltiazem	592 ± 108 (6)	261 ± 49	0.94 ± 0.04	3,664 ± 820 (4)	1,648 ± 420	0.91 ± 0.10
(*cis*)-(−)-Diltiazem	11,400 ± 1,400 (4)	5,260 ± 770	1.01 ± 0.05	25,530 ± 1,300 (3)	11,420 ± 1,140	1.04 ± 0.04

suggests a competitive interaction between the phenylalkylamine or benzothiazepine derivatives and the binding site of SR 33557 (Table 4, Fig. 6). Interestingly, an increase in temperature did not seem to have any significant effect on the IC_{50} values. However, the presence of calcium shifted these values towards higher concentrations, whereas the Hill coefficient still remained close to 1. Fluspirilene totally inhibited the binding of [^{3}H]SR 33557 and the Hill coefficient was shown to be 0.64 ± 0.01. This suggests either multiple binding sites or allosteric interactions.

Characterization of the type of inhibition exerted by the classical calcium antagonists. The effects of (+)-PN200-110, (−)-D888, (*cis*)-(+)-diltiazem, and fluspirilene on the number and affinity of the binding sites for [^{3}H]SR 33557 were characterized by analysis, at 25°, of the saturation isotherms determined in the absence and in the presence of increasing concentrations of these ligands in the absence (Fig. 7) or in the presence of 1 mM Ca^{2+} (not shown). (+)-PN200-110 exerts its inhibitory effect by diminishing, at low concentration, the apparent affinity of [^{3}H]SR 33557 for its receptor site, as well as diminishing, at high concentration, the maximal binding capacity of this ligand. In the presence of 1 mM calcium, the inhibitory effect of (+)-PN200-110, at a concentration equivalent to the IC_{50}, was mainly shown as a change in apparent affinity (Fig. 7). In contrast, fluspirilene decreased both the apparent affinity of [^{3}H]SR 33557 and its maximal binding (Fig. 7).

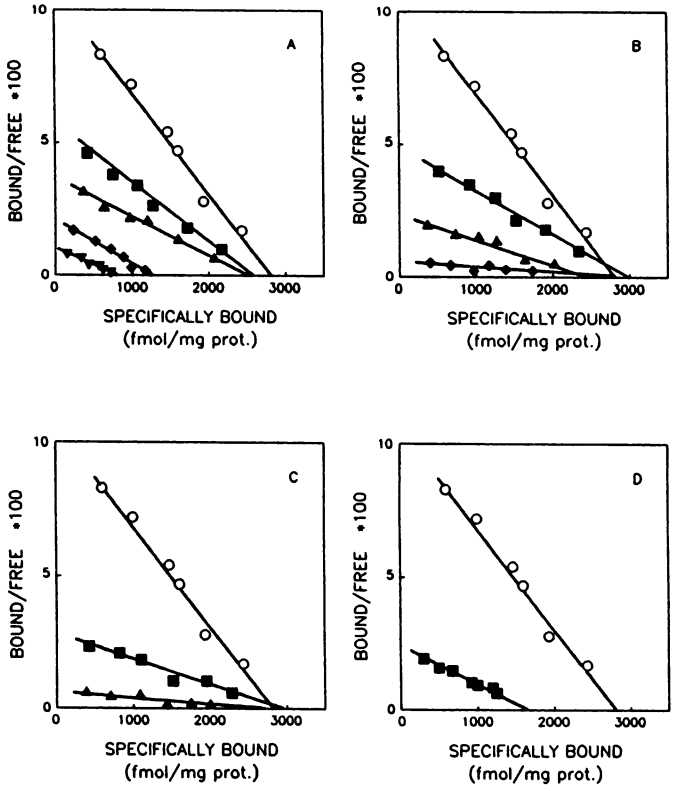

Fig. 7. Scatchard plot of equilibrium data for [^{3}H]SR 33557 binding in the absence (○) or presence of (+)-PN200-110 (A), (−)-D888 (B), (*cis*)-(+)-diltiazem (C), or fluspirilene (D). The concentrations of unlabeled calcium antagonists are, respectively, 0.2 nM (■), 0.5 nM (▲), 0.8 nM (♦), and 2.0 nM (▼) for (+)-PN200-110, 7 nM (■), 20 nM (▲), and 200 nM (♦) for (−)-D888, 0.8 μM (■) and 5 μM (▲) for (*cis*)-(+)-diltiazem, and 20 nM (■) for fluspirilene.

At all the concentrations tested, (−)-D888 and (*cis*)-(+)-diltiazem reduced the apparent affinity of [^{3}H]SR 33557 without affecting the maximal binding capacity (Fig. 7). In the absence of calcium, a linear relationship was observed between the dose ratio of the dissociation constants, K_D, measured in the absence and in the presence of (−)-D888 or (*cis*)-(+)-diltiazem, and the various concentrations of (−)-D888 or (*cis*)-(+)-diltiazem used (data not shown). Analysis of the Schild plot enables the affinity constants of (−)-D888 and (*cis*)-(+)-diltiazem to be determined. The values found, namely 4.7 nM and 200 nM, are in agreement with the values obtained by analysis of the inhibition curve of [^{3}H]SR 33557 binding (Table 4). The presence of 1mM calcium did not influence the observed inhibitory effects of (−)-D888 and (*cis*)-(+)-diltiazem on the apparent affinity of [^{3}H]SR 33557. At concentrations equivalent to the IC_{50} values, nifedipine, (−)-D888, and (*cis*)-(+)-diltiazem did not modify the constant of apparent association (k_{obs}) of [^{3}H]SR 33557 to sarcolemma. The saturation levels reached at equilibrium represent about 50% of the level obtained in the absence of product.

Fig. 8 shows that nifedipine, (−)-D888, (*cis*)-(+)-diltiazem, and fluspirilene accelerated the rate of dissociation of the [^{3}H]SR 33557/receptor complex. In the experimental conditions used in this study, the kinetic dissociation constants (k_{-1}) were doubled (Table 5), which suggests allosteric-type interactions between nifedipine, (−)-D888, or (*cis*)-(+)-diltiazem and the receptor site for [^{3}H]SR 33557.

Discussion

The results presented in this study strongly suggest that the newly synthetized agent SR 33557, selected from a novel chem-

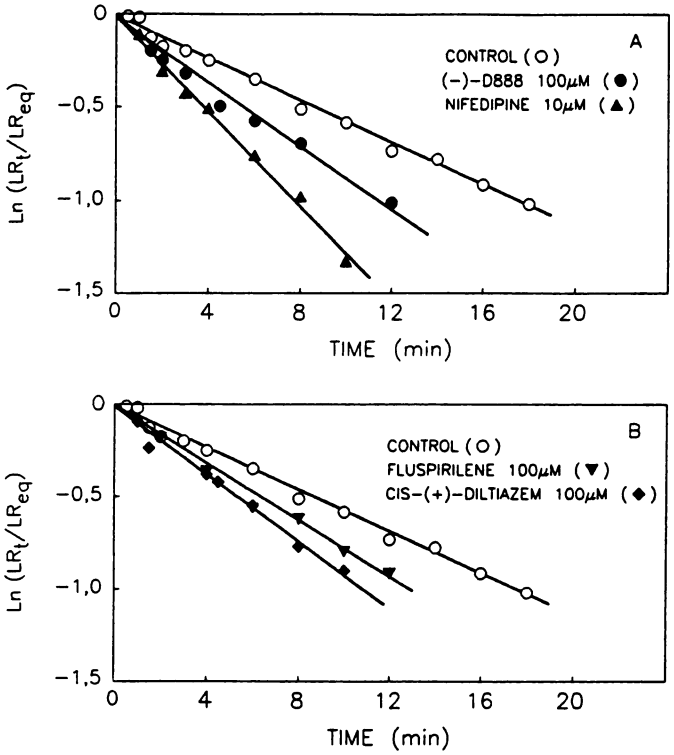

Fig. 8. Effects of calcium channel antagonists on [^{3}H]SR 33557 dissociation kinetics in cardiac sarcolemmal membranes. Cardiac sarcolemmal membranes were incubated at 25° with 0.6 nM [^{3}H]SR 33557. Ligand dissociation was initiated by addition of 1 μM unlabeled SR 33557 alone (○) or with 10 μM nifedipine (▲) or 100 μM (−)-D888 (●) (A) or 100 μM (*cis*)-(+)-diltiazem (♦) or 100 μM fluspirilene (▼) (B).

TABLE 5

Effects of nifedipine, (−)-D888, (*cis*)-(+)-diltiazem, and fluspirilene on the kinetics of dissociation of [^{3}H]SR 33557 from cardiac sarcolemmal membranes at 25°

Results are the mean ± standard error of three separate experiments.

	k_{-1}
	min^{-1}
Control	0.057 ± 0.001
+10 μM Nifedipine	0.123 ± 0.008
+100 μM (−)-D888	0.110 ± 0.010
+100 μM (*cis*)-(+)-Diltiazem	0.106 ± 0.004
+100 μM Fluspirilene	0.091 ± 0.012

ical series of Ca^{2+} antagonists (indolizine sulfone), binds to a protein that is part of the Ca^{2+} channel located in cardiac sarcolemmal membranes. The proteinaceous nature of the binding site is demonstrated by the effects of a range of different treatments. These included the effects of temperature changes, proteolytic enzymes, and reducing and alkylating agents. The effects of EDTA and EGTA on the binding of [^{3}H]SR 33557 indicate that the binding of the ligand requires the presence of calcium or magnesium in micromolar concentrations.

The identity of the binding site with the Ca^{2+} channel is based on its pharmacological specificity, which shows that only Ca^{2+} channel modulators interfere with the binding of [^{3}H]SR 33557. The pharmacological specificity is in agreement with the receptor binding profile previously demonstrated (9). Of particular interest is the lack of effect on channels other than the Ca^{2+} channel and even among the subtypes of Ca^{2+} channels; an interaction with the N-type is unlikely due to the lack of effect of ω-conotoxin in this study. In fact, our data confirm the absence of an interaction between ^{125}I-ω-conotoxin and SR 33557, suggested previously by Schmid *et al* (10).

The results of this study show that (a) [^{3}H]SR 33557 binds with high affinity to its receptor site and (b) only one class of binding sites for [^{3}H]SR 33557 is present in cardiac sarcolemmal membranes. The maximal binding capacity for [^{3}H]SR 33557 is of the same order as that found by us for (−)-[^{3}H]D888 and (*cis*)-(+)-[^{3}H]diltiazem. However, the maximal binding capacity of (+)-[^{3}H]PN200-110, measured under the same experimental conditions, was significantly lower. Addition of 1 mM Ca^{2+} doubled the maximal binding capacity of [^{3}H]PN200-110 without affecting its K_D, as already demonstrated with dihydropyridines (1). Thus, the binding sites of the four ligands, representative of different classes of Ca^{2+} channel effectors, are in 1:1:1:1 stoichiometry.

In guinea pig brain membranes, a K_i value of 0.19 ± 0.03 nM has been determined for SR 33557 (9); preliminary data obtained from saturation curves indicated a K_D value of 0.20 ± 0.3 nM, in close agreement with the K_i value. In transverse T tubule membranes from rabbit skeletal muscle, a K_D value of 0.08 nM has been reported (10). In cardiac sarcolemmal membranes, a K_D value of 0.37 ± 0.03 nM has been determined using various experimental protocols. In cardiac sarcolemmal membranes, [^{3}H]SR 33557 has a higher K_D than [^{3}H]PN200-110. In skeletal T tubule membranes, [^{3}H]SR 33557 has the lowest K_D yet found for the binding of Ca^{2+} channel effectors to the membrane (10). Thus, [^{3}H]SR 33557 binding displays a considerable degree of tissue heterogeneity. These observations performed with a chemically unrelated ligand are in agreement with similar results published for dihydropyridines (1, 18).

Because dihydropyridines were shown to be partial inhibitors of [^{3}H]SR 33557 binding in this study and their mode of action in equilibrium and kinetic binding studies did not display strictly competitive behavior, our results imply that [^{3}H]SR 33557 binds to a site quite distinct from that of the dihydropyridines. The inhibition curve of [^{3}H]SR 33557 binding by fluspirilene could correspond to either competitive inhibition on multiple binding sites or allosteric interactions. The effects of fluspirilene on the [^{3}H]SR 33557 saturation isotherm clearly indicate noncompetitive inhibition and suggest an allosteric interaction between the two compounds. In contrast, equilibrium studies performed by us indicated that (−)-D888 and (*cis*)-(+)-diltiazem reduce the apparent affinity of [^{3}H]SR 33557 without affecting the maximal binding capacity. This pattern could be due to either competitive or allosteric effects (3, 19). Kinetic studies indicate that the four ligands influence significantly the dissociation rate of SR 33557. This would suggest that [^{3}H]SR 33557 binds to a distinct site that is in positive or negative allosteric interaction with the other sites.

The bell-shaped curve for the effect of divalent ions on [^{3}H]SR 33557 binding is similar to that reported previously for the effects of the same ions on verapamil and (*cis*)-(+)-diltiazem binding to porcine heart sarcolemma (19). This relationship is the opposite of that observed for the effects of the same cations on dihydropyridine binding (20, 21). Calcium, which exerts the greatest inhibitory effect on the binding of [^{3}H]SR 33557, is the strongest activator with regard to the specific binding of [^{3}H]nitrendipine to heart sarcolemma (21). The effects of divalent ions on the binding of fluspirilene are completely at variance, with Cd^{2+} (and La^{3+}) stimulating binding at low concentrations followed by return to control levels, Ni^{2+}, Co^{2+}, and Mn^{2+} only stimulating, and Ca^{2+}, Ba^{2+}, and Sr^{2+} inhibiting binding (7). These characteristics indicate that fluspirilene and dihydropyridines bind to site(s) that are different from the binding site of SR 33557 in the cardiac Ca^{2+} channel complex. The difference in the effects of divalent ions on dihydropyridine and SR 33557 binding could be related to the membrane potential dependence of the blocking effect of SR 33557 being much smaller than that for the dihydropyridine (+)-PN200-110 (10).

In cardiac tissue, it has been shown that SR 33557 displays a preferential negative chronotropic activity with regard to its negative inotropic action (12). This pharmacological profile is not seen with nifedipine, verapamil, or diltiazem (12). SR 33557 has also been shown to possess a potent relaxant activity in K^+-depolarized aorta ($IC_{50} \simeq 6$ nM) (6, 12). Although the compound is 110 times more selective for vascular smooth muscle than cardiac muscle (12), it induces both an antihypertensive effect and bradycardia in spontaneously hypertensive rats (22). Whether this pharmacological profile is related to the binding of SR 33557 to a distinct site must await further studies.

Thus, in conclusion, the results of this study have shown that SR 33557 binds with high affinity to a single class of sites on the sarcolemmal Ca^{2+} channel. This binding is in a 1:1 stoichiometry with the binding of the classical Ca^{2+} channel antagonists (dihydropyridine, phenylalkylamine, and benzothiazepine). Extensive equilibrium and kinetics studies suggest that SR 33557 binds to a unique site distinct from that of the dihydropyridines, phenylalkylamines, benzothiazepines, and diphenylbutylpiperidines.

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Send reprint requests to: P. Chatelain, Sanofi-Labaz Research Centre, 1 Ave. de Béjar, 1120 Brussels, Belgium.