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SR33557, an Indolizinsulfone Blocker of Ca^{2+} Channels: Identification of Receptor Sites and Analysis of Its Mode of Action

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SUMMARY

SR33557 belongs to a new class of molecules (indolizinsulfones) that act on the same receptor complex that has been characterized for other classical calcium channel effectors. The main binding properties of SR33557 to rabbit skeletal muscle are as follows. (i) Unlabeled SR33557 completely inhibits the specific binding of all classes of calcium channel antagonists such as dihydropyridines [(+)-[^{3}H]PN200-110], phenylalkylamines ([^{3}H]verapamil), benzothiazepines (*d*-(*cis*)-[^{3}H]diltiazem), and diphenybutylpiperidines ([^{3}H]fluspirilene). In all these cases inhibition of binding is of a noncompetitive nature. (ii) [^{3}H]SR33557 binds with high affinity to T tubule membranes (K_D = 0.08 nM) and the maximum binding capacity (B_{max} = 78 pmol/mg of protein) is the same as that found for other classes of Ca^{2+} channel antagonists. Photoaffinity labeling confirms that [^{3}H]SR33557 associates with the same protein of M_r 165,000 that binds the classical calcium channel inhibitors. $^{45}Ca^{2+}$ uptake experiments performed with the rat aortic cell line A7r5, the insulin-secreting cell line RINm5F, and the pheochromocytoma cell line PC12 demonstrate that SR33557 fully inhibits the 1,4-dihydropyridine-sensitive $^{45}Ca^{2+}$ uptake elicited by depolarization. A very good correlation was found between inhibition of $^{45}Ca^{2+}$ uptake and of [^{3}H]dopamine release in PC12 cells and between inhibition of $^{45}Ca^{2+}$ uptake and of L-type Ca^{2+} current in A7r5 cells under whole-cell patch-clamp conditions.

Voltage-dependent Ca^{2+} channels are the major entry pathway for Ca^{2+} in many cell types. In excitatory cells such as muscle and nerve cells, they are postulated to play a role in excitation-contraction coupling and in neurotransmitter release (1–4).

They are also found in nonexcitable cells such as fibroblasts (5), osteoblasts (6), and probably even plant cells (7), where regulation of Ca^{2+} entry appears to be important for cell function. Different types of voltage-dependent Ca^{2+} channels exist, which are characterized by differences in their voltage sensitivities, kinetic properties, and pharmacological patterns (8–12).

The group of compounds known as Ca^{2+} channel effectors (13), which form an important class of therapeutic agents (14), have been particularly useful in the study of the physiological, biophysical, and biochemical properties of the L-type Ca^{2+} channels (15). Ca^{2+} channel effectors form a chemically heterogeneous series of compounds. Three families of molecules have received particular attention. They are the DHP family, which includes nitrendipine, PN200-110, and Bay K8644, the phenylalkylamine family, with compounds such as verapamil, D_{600}, and D_{888}, and the benzothiazepine family, typified by diltiazem. Members of the DHP family bind with high affinity to a specific receptor site that does not bind phenylalkylamines and benzothiazepines (16–18). These two other classes of agents bind to one or two (16, 17, 19) other distinct binding sites. Affinity labeling studies (17, 20, 21) have demonstrated that the receptor sites for DHPs, phenylalkylamines, and benzothiazepines are contained within the same protein of M_r 165,000. These receptor sites are allosterically linked via negative or positive heterotropic interaction, so that occupation of one site affects ligand binding at other sites. Other Ca^{2+} channel effectors are known; some of them act at one of the previously described binding sites, such as bepridil at the phenylalkylamine binding site (17) and tetrandine at the benzothiazepine binding site (22). Other Ca^{2+} channel blockers have their own binding sites. This is the case for neuroleptic molecules in the diphenylbutylpiperidine series (23, 24) or for benzolactams, a novel family of blockers that has been recently described (25).

SR33557 (Fig. 1) is a structurally new molecule of the indolizinsulfone series, chemically unrelated to structures of previously described Ca^{2+} channel effectors. This paper shows that SR33557 interacts with receptors of classical L-type Ca^{2+} chan-

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ABBREVIATIONS: DHP, 1,4-dihydropyridine; MOPS, 3-morpholinopropanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethylether)-*N*,*N*,*N*′*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SR33557, (2-isopropyl-1-((4-(3-*N*-methyl-*N*-(3,4-dimethoxy-β-phenethyl)amino)propyloxy)benzenesulfonyl))indolizine.

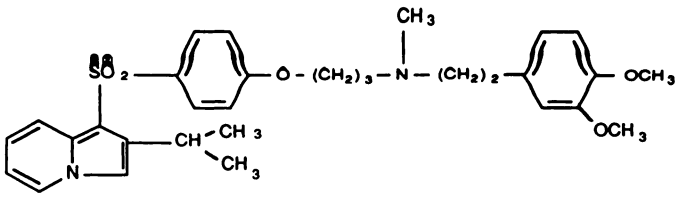

Fig. 1. Structure of SR33557.

nel blockers in muscle transverse tubule (T tubule) membranes, which are the best source available for these receptors (26). Tritiated SR33557 was also used to directly study the interactions of the new molecule with its own binding site and to label the protein on which it is located. Moreover, SR33557 was shown to block DHP-sensitive $^{45}Ca^{2+}$ uptake in aortic, insulin-secreting, and neurotransmitter-secreting cell lines. SR33557 action was also analyzed with the whole-cell patch-clamp technique.

Materials and Methods

Chemicals. Unlabeled SR33557 and [^{3}H]SR33557 (1.89 TBq/mmol) were from Sanofi-Labaz (Brussels, Belgium). Unlabeled (+)-PN200-110 was from Sandoz (Basel, Switzerland), (±)-verapamil was from Knoll AG (FRG), *d*-(*cis*)-diltiazem was from Synthelabo (Paris, France), and fluspirilene was from Janssen Pharmaceutica (Beerse, Belgium). Tritiated (+)-PN200-110 (3.11 TBq/mmol) and *d*-(*cis*)-diltiazem (6.06 TBq/mmol) were from Amersham. Tritiated (±)-verapamil (2.5 TBq/mmol) and fluspirilene (2.48 TBq/mmol) were from DuPont de Nemours.

Cell culture. The A7r5 embryonic aortic smooth muscle cell line was obtained from the American Type Culture Collection, (Rockville, MD). Cells were plated at a density of 10^5 cells/well (Falcon 24-well tissue culture plates) and were grown in Dulbecco's modified Eagle's medium that was supplemented with 10% fetal calf serum (GIBCO, Grand Island, NY). RINm5F is an insulin-secreting cell line derived from a rat islet cell tumor and was a gift from Dr. Y. Le Marchand. Cells were plated at a density of 2×10^5 cells/well and were grown in RPMI 1640 medium that was supplemented with 10% fetal calf serum (GIBCO). PC12 rat pheochromocytoma cells were kindly provided by Dr. L. A. Green. Cells were plated at a density of 10^5 cells/well (Falcon 24-well tissue culture plates) or 5×10^5 cells/well (Falcon 12-well tissue culture plates) and were grown in Dulbecco's modified Eagle medium that was supplemented with 10% heat inactivated horse serum (GIBCO) and 5% fetal calf serum (GIBCO).

Preparation of membranes. Transverse T tubule membranes from rabbit skeletal muscle were prepared according to the method of Fosset *et al.* (26), in the presence of 0.1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, and 10 mM EDTA. Cell membranes were prepared as described (24), from 5×10^8 to 10^9 cells grown in 100-mm diameter Falcon tissue culture dishes.

[^{3}H]Verapamil, (+)-[^{3}H]PN200-110, *d*-(*cis*)-[^{3}H]diltiazem, and [^{3}H]fluspirilene binding assays. Assays for the inhibition of the binding of different labeled ligands to T tubule and cell membranes by unlabeled SR33557 were performed in a 1-ml solution of 20 mM MOPS-NaOH, pH 7.5, 0.1 mM EDTA, and 0.01% bovine serum albumin for [^{3}H]verapamil, [^{3}H]fluspirilene, and *d*-(*cis*)-[^{3}H]diltiazem or 0.1 M Tris-HCl, pH 7.5, 2 mM $CaCl_2$, and 0.01% bovine serum albumin for (+)-[^{3}H]PN200-110. Concentrations of membrane proteins and of [^{3}H]-ligands are indicated in the legends to the figures. Dilutions of unlabeled SR33557 were performed in 20 mM MOPS-NaOH, pH 7.5, and 0.01% bovine serum albumin. After an incubation time of 45 min for (+)-[^{3}H]PN200-110 (27), 60 min for [^{3}H]fluspirilene (23), 90 min for [^{3}H]verapamil (28), and 120 min for *d*-(*cis*)-[^{3}H]diltiazem (17), two 400-μl aliquots were filtered on GF/C glass fiber filters that were soaked in 0.05% polyethylenimine, 100 mM Tris-HCl, pH 7.5.

Dissociation kinetics. After equilibrium had been reached, the rate of dissociation of the [^{3}H]-ligand-receptor complex was monitored, following a 20-fold dilution of the incubation medium with 20 mM MOPS-NaOH, pH 7.4, 0.1 mM EDTA, and 0.01% bovine serum albumin. At time *t*, two 10-ml aliquots were filtered through Whatman GF/C filters.

Standard [^{3}H]SR33557 equilibrium binding assays. Membranes were incubated at 25° in 5 ml of a solution containing 20 mM MOPS-NaOH, pH 7.4, 0.01% bovine serum albumin, 0.1 mM EDTA, and the required [^{3}H]SR33557 concentrations. Incubations lasted 30 to 45 min and were stopped by rapid filtration of 4.5 ml of the incubation medium through polyethylenimine (0.05%)-treated Whatman GF/C filters. Experimental points were systematically determined in duplicate. For measurement of nonspecific binding, unlabeled SR33557 was present in the medium at a final concentration of 0.1 μM. Samples of 500 μl of the incubation mixture were counted to determine the total [^{3}H]SR33557 concentrations.

$^{45}Ca^{2+}$ uptake experiments. Ca^{2+} uptake studies on A7r5, RINm5F, and PC12 cells were carried out in 24-well culture plates at 37°. Cells were washed in buffer containing 20 mM HEPES-NaOH, pH 7.4, 135 mM NaCl, 5 mM KCl, 0.1 mM EGTA, and 0.01% bovine serum albumin and were preincubated for 15 min with 200 μl of the same buffer without EGTA, in the presence of different concentrations of unlabeled SR33557 or (+)-PN200-110. $^{45}Ca^{2+}$ uptake was started by addition to the preincubation mixture of 20 μl of medium containing 550 mM KCl, 1 mM $CaCl_2$, and 6 μCi/ml $^{45}CaCl_2$. $^{45}Ca^{2+}$ uptake measurements were made after 3 min of incubation.

[^{3}H]Dopamine release from PC12 cells. [^{3}H]Dopamine release from PC12 cells was carried out in 12-well culture plates at 37° as described for [^{3}H]norepinephrine (29). PC12 cells were preloaded for 45 min with 30 nM [^{3}H]dopamine (5 Ci/mmol), in buffer containing 135 mM NaCl, 5 mM KCl, 25.9 mM $NaHCO_3$, 10 mM glucose, 1.3 mM $MgSO_4$, 1.5 mM $CaCl_2$, and 25 mM HEPES-NaOH, pH 7.4 (5K buffer), in the presence of 0.1 mM pargyline and 20 μM dithiothreitol. Cells were washed for 10 min in 5K buffer and preincubated 15 min in 5K buffer in the presence of the drug to be assayed (SR33557 or PN200-110). [^{3}H]Dopamine release was elicited in a 70K buffer (same as 5K buffer but containing 70 mM KCl and 70 mM NaCl) during 15 min.

Photoaffinity labeling. T tubule membranes were incubated (0.3 mg of protein/ml) in 20 mM MOPS-NaOH, pH 7.4, 0.1 mM EDTA, in the presence of 0.1 mM phenylmethylsulfonylfluoride, 1 mM iodoacetamide, 10 μg/ml leupeptin, and 1 μM pepstatin, at 20° for 35 min with 20 nM [^{3}H]SR33557. Nonspecific binding was determined in the presence of 20 μM unlabeled SR33557. Samples were irradiated with high intensity UV light with a 2000-W mercury lamp (Philips HP 2000), at 4° for 30 sec at a distance of 20 cm from the lamp. After UV irradiation, samples were centrifuged for 60 min at 100,000 × *g*. The pellets were dissolved in 2% sodium dodecyl sulfate, 9% glycerol, 75 mM Tris-HCl, pH 6.8, and 2.5% β-mercaptoethanol (disulfide-reducing conditions) or 8 mM iodoacetamide (nonreducing conditions) and were electrophoresed on sodium dodecyl sulfate gels that contained a gradient of 4–14% polyacrylamide (30). After being stained with Coomassie blue, the gels were treated for fluorography with 1 M sodium salicylate (31) for 45 min, dried, and exposed at −70°, using X-Omat XAR-5 film, for 5 days.

Electrophysiology. Voltage-clamp experiments were done at 30 ± 2° on aortic A7r5 cells by using the whole-cell configuration of the patch-clamp method (32). Ca^{2+} channel activity was measured with Ba^{2+} as charge carrier. The external solution contained 130 mM NaCl, 10 mM $BaCl_2$, and 10 mM tetraethylammonium. This solution was buffered at pH 7.4 with 10 mM HEPES-KOH. The pipette solution contained 140 mM CsCl, 5 mM EGTA, 4 mM $MgCl_2$, and 3 mM ATP and was buffered at pH 7.2 with 10 mM HEPES-CsOH. Patch pipettes (2–6 MΩ) were connected to the head stage of the recording apparatus (RK 300; Bio-Logic, Grenoble, France).

Results

Effects of SR33557 on binding of classical Ca^{2+} channel effectors. Binding sites for different Ca^{2+} channel effec-

tors are best characterized in purified T tubule membranes from skeletal muscle (18). By monitoring the binding of representative tritiated Ca^{2+} channel effectors to these membranes in the presence of unlabeled SR33557, it is possible to determine whether SR33557 interacts at any of the receptor sites present in the Ca^{2+} channel protein complex. Protection experiments with increasing concentrations of SR33557 and a fixed low concentration of either (+)-[^{3}H]PN200-110 (DHP) (Fig. 2A), [^{3}H]verapamil (phenylalkylamine) (Fig. 3A), *d*-(*cis*)-[^{3}H]diltiazem (benzothiazepine) (Fig. 3C), or [^{3}H]fluspirilene (diphenylbutylpiperidine) (Fig. 3E) demonstrate that SR33557 completely inhibits the binding of all these labeled molecules in a concentration-dependent manner. Concentrations of SR33557 that inhibit 50% of each type of binding ($K_{0.5}$ values) are listed in Table 1 and are compared with $K_{0.5}$ values found under the same conditions for the homologous nonlabeled compound. It appears that, except for [^{3}H]fluspirilene binding, SR33557 is more potent than each of the homologous unlabeled compounds tested. $K_{0.5}$ values found in these experiments performed at 10° were not significantly different at 25° or 37°.

To investigate in more detail the mechanisms by which SR33557 inhibits binding of the other Ca^{2+} channel blockers, dissociation kinetics of the complex formed with (+)-PN200-110 (data not shown), (+)-verapamil (Fig. 3B), *d*-(*cis*)-diltiazem (Fig. 3D), and fluspirilene (Fig. 3F) were measured in the

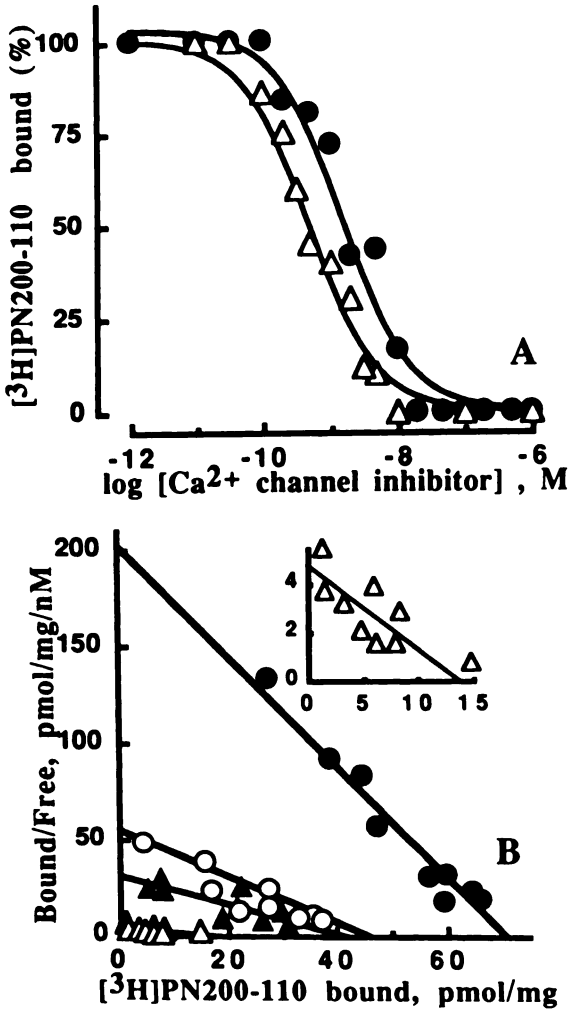

Fig. 2. Inhibition of (+)-[^{3}H]PN200-110 binding to T tubule membranes by unlabeled SR33557. A, Specific binding of (+)-[^{3}H]PN200-110 (0.2 nM) was measured at 25° with T tubule membranes (2 μg of protein/ml) in the presence of increasing concentrations of unlabeled SR33557 (△) and (+)-PN200-110 (●). B, Scatchard plot of equilibrium data for (+)-[^{3}H]PN200-110 binding to T tubule membranes (4 μg of protein/ml) in the absence (●) or presence of 1 nM (○), 3 nM (▲) or 10 nM (△) of unlabeled SR33557. *Inset*, an expansion of the Scatchard plot for the specific binding of (+)-[^{3}H]PN200-110 to T tubule membranes in the presence of 10 nM SR33557.

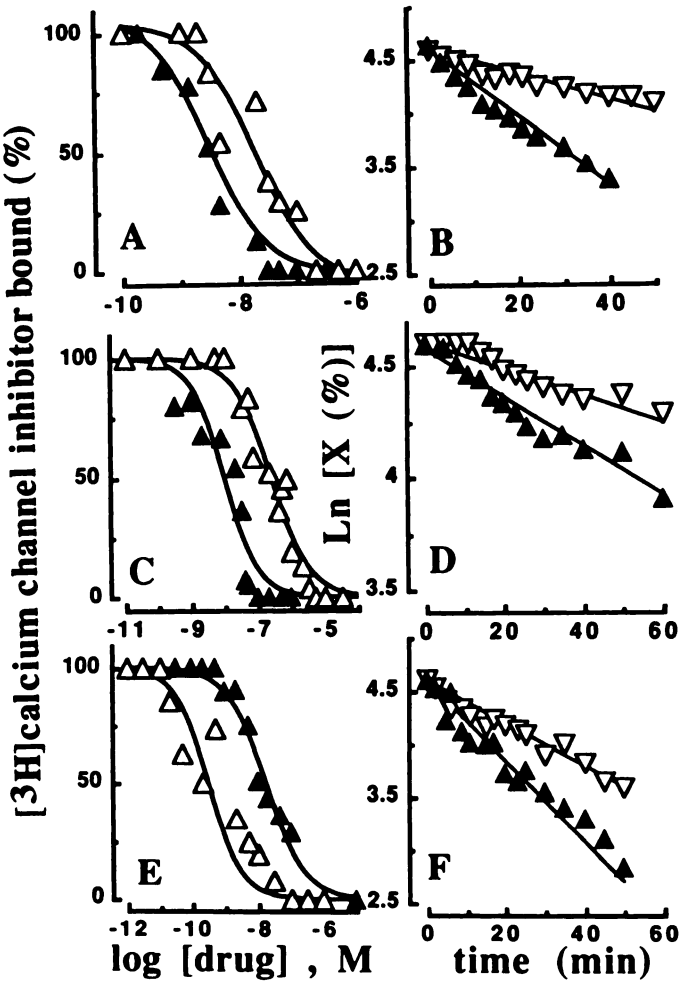

Fig. 3. Effect of unlabeled SR33557 on [^{3}H]verapamil (A and B), *d*-(*cis*)-[^{3}H]diltiazem (C and D), and [^{3}H]fluspirilene (E and F) binding and on dissociation kinetics of [^{3}H]-labeled calcium channel inhibitors bound to T tubule membranes. A, C, and E, Specific binding of [^{3}H]-labeled calcium channel inhibitors was measured under equilibrium conditions with T tubule membranes, in the presence of increasing concentrations of the homologous unlabeled drug (△) or SR33557 (▲). Equilibrium binding assays were carried out at 10° in the presence of 1 nM [^{3}H]verapamil (70 μg of protein/ml), 2 nM *d*-(*cis*)-[^{3}H]diltiazem (100 μg of protein/ml), and 0.4 nM [^{3}H]fluspirilene (4.3 μg of protein/ml). B, D, and F, [^{3}H] Verapamil (B), *d*-(*cis*)-[^{3}H]diltiazem (D), and [^{3}H]fluspirilene (F) were first associated to T tubule membranes under the equilibrium conditions used above. The amount of ^{3}H-ligand specifically bound before dilution was 0.28 nM for [^{3}H]verapamil, 0.16 nM for *d*-(*cis*)-[^{3}H]diltiazem, and 0.056 nM for [^{3}H]fluspirilene; the amount of nonspecific binding was 2, 7, and 10% of total binding, respectively. The dissociation of the ^{3}H-ligand-receptor complex was followed after a 20-fold dilution of the incubation mixture in the absence (▽) or presence (▲) of 5 nM (B), 10 nM (D), or 20 nM (F) unlabeled SR33557. All the experiments were performed at 10° except for fluspirilene experiments, which were performed at 25°. *X*, Percentage of specifically bound ligand at time *t*. Error bars on the inhibition and dissociation curves are not shown because their sizes do not exceed the size of the symbols.

TABLE 1

Effects of SR33557 on Ca^{2+} channel effectors binding to skeletal muscle T tubule membranes

Values of $K_{0.5}$ are from experiments illustrated in Fig. 1A and Fig. 2, A, C, and E and of k_{-1} from Fig. 2, B, D, and F. Standard deviations are calculated from results of three independent experiments.

[^{3}H]ligand	$K_{0.5}$		k_{-1}	
	Homologous compound	SR33557	−SR33557	+SR33557
	nM		min^{-1}	
(+)PN200-110	0.5 ± 0.1	0.2 ± 0.05	0.039 ± 0.005	0.040 ± 0.008
(−)-Verapamil	17 ± 2	3 ± 0.5	0.012 ± 0.002	0.032 ± 0.008
d-(*cis*)-Diltiazem	260 ± 20	10 ± 2	0.006 ± 0.001	0.011 ± 0.003
Fluspirilene	0.5 ± 0.1	20 ± 2	0.020 ± 0.005	0.038 ± 0.006

absence and in the presence of SR33557. For each ligand, the SR33557 concentration used was the one corresponding to the $K_{0.5}$ value. The dissociation rate constant (k_{-1}) was not modified by SR3357 in the case of (+)-PN200-110 (Table 1) but was approximately doubled for all the other ligands (Table 1). The accelerated dissociation found for (±)-verapamil, *d*-(*cis*)-diltiazem, and fluspirilene indicates that allosteric interactions take place between the receptors for these agents and the SR33557 receptor.

Because of the lack of SR33557 effect on (+)-[^{3}H]PN200-110 off-rate, saturation binding experiments with (+)-[^{3}H]PN200-110 were carried out in the presence of different concentrations of unlabeled SR33557. Scatchard plot analyses are shown in Fig. 2B. One observes a decrease both of the maximal binding capacity (B_{max}) and of the apparent equilibrium dissociation constant (K_d) when the SR33557 concentration is increased from 0 (B_{max} = 71 pmol/mg of protein; K_D = 0.35 nM) to 1 nM ($B_{max_{app}}$ = 45 pmol/mg of protein; $K_{D_{app}}$ = 0.83 nM), 3 nM ($B_{max_{app}}$ = 41 pmol/mg of protein; $K_{D_{app}}$ = 1.52 nM), and 10 nM ($B_{max_{app}}$ = 14 pmol/mg of protein; $K_{D_{app}}$ = 2.9 nM). Again, this result is consistent with allosteric interactions and not with competitive binding inhibition.

Specific binding of [^{3}H]SR33557 to rabbit skeletal muscle T tubule membranes. The availability of SR3357 in a tritiated form (1.887 GBq/mol) allowed us to analyze its interaction with the receptor complex for Ca^{2+} channel effectors in skeletal muscle. Fig. 4 shows a typical equilibrium binding experiment of [^{3}H]SR33557 to T tubule membranes. The nonspecific binding is very low compared with the total binding. The Scatchard plot is linear (Fig. 4B), consistent with a single type of binding site with a K_D value of 0.08 nM and a B_{max} of 97 pmol/mg of protein. The B_{max} value in this experiment is slightly higher than the one measured with (+)-[^{3}H]PN200-110 in the same membrane preparation. However, data from 10 similar independent experiments indicate that the (+)-[^{3}H]PN200-110 and [^{3}H]SR33557 binding sites are in a 1:1 stoichiometry (B_{max} = 82 ± 5 pmol/mg of protein for (+)-PN200-110 and SR33557). Binding parameters of [^{3}H]SR33557 to T tubule membranes are independent of the presence of free Ca^{2+} ions in the incubation mixture, from 0 to 10 mM. Dissociation of the [^{3}H]SR33557-receptor complex is a relatively slow process at 25°. It displays first-order kinetics, as shown by the data in Fig. 4A, *inset*. The dissociation rate constant is 0.010 ± 0.001 min^{-1} (six experiments) corresponding to a $t_{1/2}$ of 69 min.

Unlabeled SR33557 inhibits binding of [^{3}H]SR33557 to T tubule membranes in a dose-dependent manner. The $K_{0.5}$ value found in the experiment illustrated in Fig. 5 is 1 nM, which

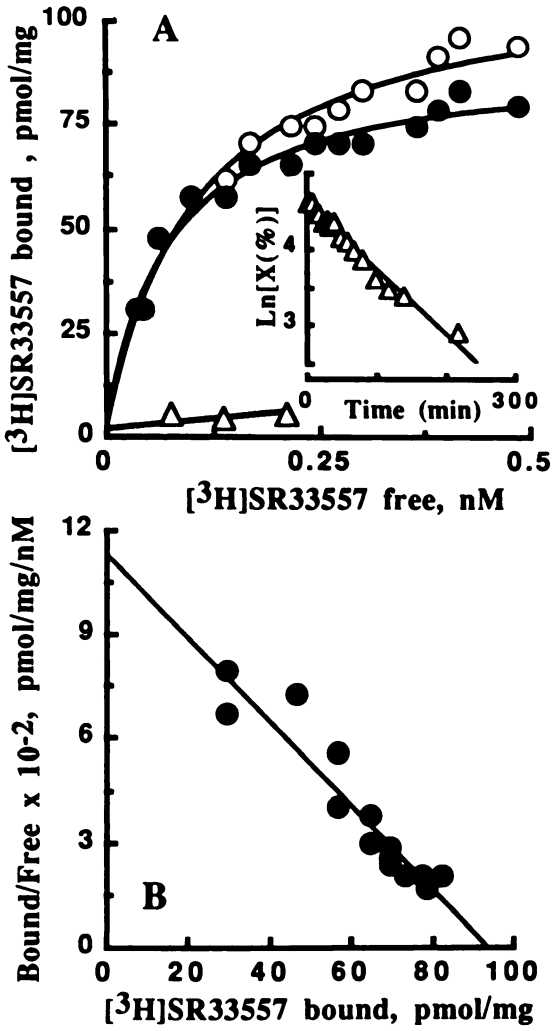

Fig. 4. Equilibrium binding of [^{3}H]SR33557 to T tubule membranes. A, Equilibrium binding was measured, at 25°, using 0.2 μg/ml T tubule membranes and increasing concentrations of [^{3}H]SR33557. Specific binding (●) of [^{3}H]SR33557, is the difference between the binding in the absence (○) and the presence (△) of 0.1 μM unlabeled SR33557. *Inset*, semilogarithmic representation of the [^{3}H]SR33557 dissociation kinetics on T tubule membranes at 25°. [^{3}H]SR33557 (0.3 nM) was first associated to T tubule membranes (3.5 μg of protein/ml) before the dissociation of the [^{3}H]SR33557-receptor complex was initiated by a 20-fold dilution of the incubation mixture. The amount of [^{3}H]SR33557 specifically bound (90% of total binding) before dilution was 0.2 nM. *X*, Percentage of specifically bound ligand at time *t*. *B*, Scatchard plot for the specific [^{3}H]SR33557 binding component.

corresponds to a true K_D value of 0.14 nM after correction for the experimental conditions. This is in good agreement with the value of 0.08 nM found in direct binding experiments. Other structurally unrelated Ca^{2+} channel effectors also inhibit [^{3}H]SR33557 binding, with $K_{0.5}$ values ranging from 1 to 3000 nM. The rank order of potencies is as follows: fluspirilene ($K_{0.5}$ = 1 nM > (+)-PN200-110 ($K_{0.5}$ = 8 nM) > verapamil ($K_{0.5}$ = 1000 nM > *d*-(*cis*)-diltiazem ($K_{0.5}$ = 3000 nM). True K_D values cannot be calculated from these data because of the allosteric nature of the observed interactions.

Photoaffinity labeling of the [^{3}H]SR33557 binding protein in T tubule membranes. SR33557 is a fluorescent compound that can form covalent bonds with its binding site upon irradiation with high intensity UV light. Fig. 6 shows fluorogram patterns of photoaffinity labeling experiments to

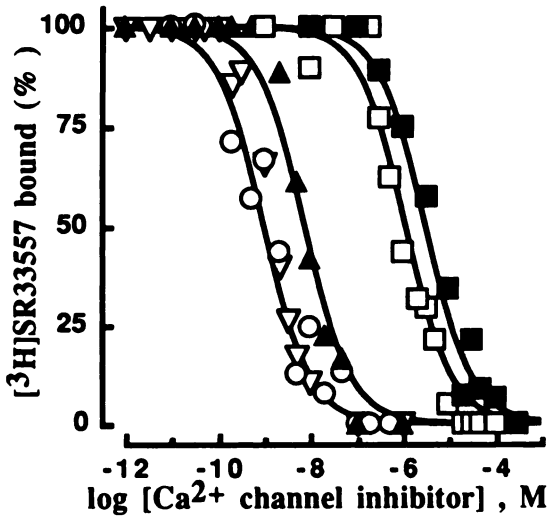

Fig. 5. Inhibition of specific [^{3}H]SR33557 binding by increasing concentrations of unlabeled SR33557 (▽), fluspirilene (○), (+)-PN200-110 (▲), (−)-verapamil (□), and *d*-(*cis*)-diltiazem (■). Experimental conditions were 0.5 nM [^{3}H]SR33557 and 1.25 μg/ml T tubules. Sizes of error bars do not exceed the size of symbols.

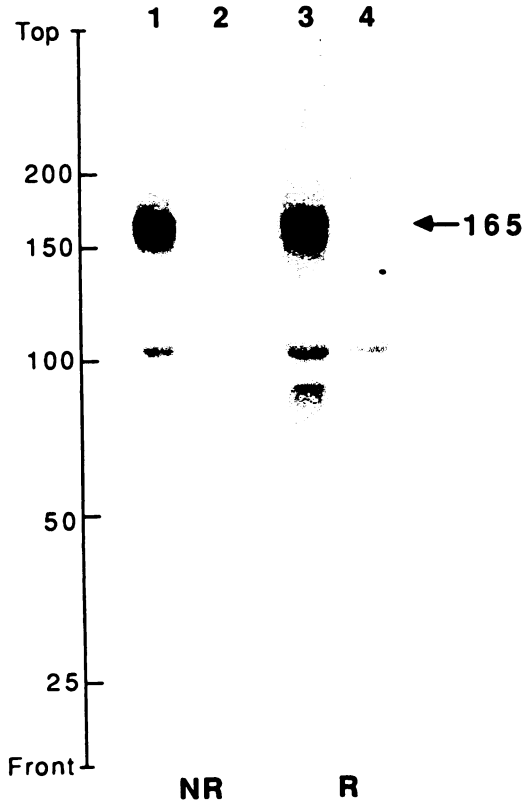

Fig. 6. Fluorogram pattern of [^{3}H]SR33557 photoaffinity-labeled T tubule membranes. Membranes were incubated with [^{3}H]SR33557 and irradiated, as described in Materials and Methods, in the absence (*lanes 1* and *3*) or in the presence (*lanes 2* and *4*) of 20 μM unlabeled SR33557. Polyacrylamide gels were run under nonreducing (*NR*) or reducing conditions (*R*). *Numbers on right* and *left* represent $M_r \times 10^{-3}$.

the T tubule [^{3}H]SR33557 receptor. A band of M_r 165,000 is the major specifically labeled product both in reducing and nonreducing conditions. This M_r corresponds perfectly to the M_r of the protein labeled with other Ca^{2+} channel ligands (17, 21, 33–35). A minor band of M_r 85,000 is also specifically labeled and probably corresponds to a degradation product of the M_r 165,000 band, which is known to be very labile (33).

Effect of SR33557 on DHP-sensitive $^{45}Ca^{2+}$ uptake in A7r5, RINm5F, and PC12 cells in culture. Effects of SR33557 on the activity of voltage-dependent Ca^{2+} channels were investigated by Ca^{2+} flux experiments performed on three different cell lines, the aortic cell line A7r5, the insulin-secreting cell line RINm5F, and the pheochromocytoma cell line PC12.

In polarized conditions (5 mM external KCl), SR33557 was without effect on the basal $^{45}Ca^{2+}$ uptake in all cell types. Conversely, the $^{45}Ca^{2+}$ uptake component due to Ca channel activity elicited by depolarization (55 mM external KCl) was completely inhibited by SR33557. Concentration dependencies for inhibition by SR33557 and by PN200-110 are presented in Fig. 7A for A7r5 cells, in Fig. 7B for RINm5F cells, and in Fig. 7C for PC12 cells. Concentrations of SR33557 and PN200-110 giving half maximal effects (EC_{50} values) on Ca^{2+} uptake in these cell lines are 25 ± 2 nM and 0.3 ± 0.05 nM in A7r5 cells, 50 ± 2 nM and 0.5 ± 0.1 nM in RINm5F cells, and 50 ± 3 nM and 0.4 ± 0.07 nM in PC12 cells for SR33557 and PN200-110, respectively (three experiments).

Although SR33557 binds with a higher affinity than (+)-PN200-110 to T tubule membranes, it is by far less potent in inhibiting $^{45}Ca^{2+}$ uptake in the cell lines examined. Direct binding experiments of [^{3}H]SR33557 to A7r5 membranes indicate a B_{max} of 90 fmol/mg of protein [as compared with 87 fmol/mg for (+)-[^{3}H]PN200-110 in the same membrane prep-

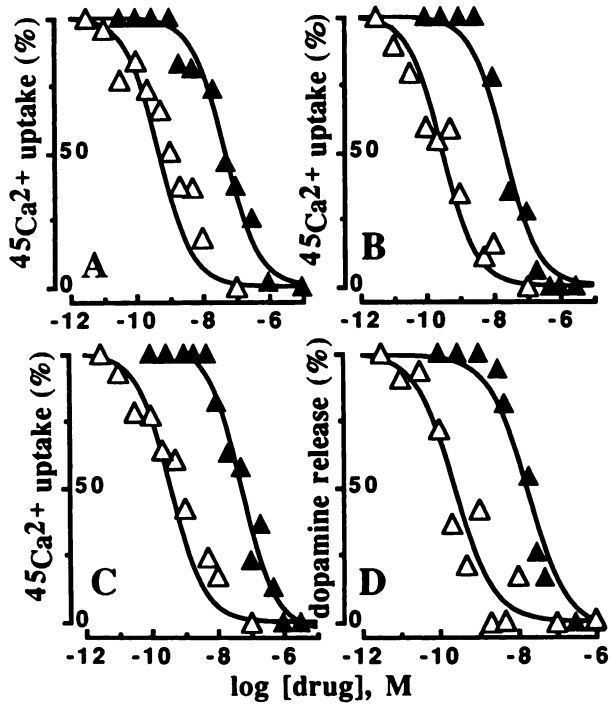

Fig. 7. Effect of SR33557 on K^+-induced $^{45}Ca^{2+}$ uptake into A7r5, RINm5F, and PC12 cells and on evoked [^{3}H]dopamine release from PC12 cells. A, B, and C, Inhibition of K^+-induced $^{45}Ca^{2+}$ uptake by increasing concentrations of SR33557 (▲) or (+)-PN200-110 (△) was measured on A7r5 (A), RINm5F (B), and PC12 (C) cells. Times of uptake were 3 min. D, Evoked [^{3}H]dopamine release was measured in preloaded PC12 cells in the presence of increasing concentrations of SR33557 (▲) or (+)-PN200-100 (△) under depolarization conditions, with 70 mM K^+ buffer.

aration] and a K_D value of 0.16 nM, close to the value found on T tubule membranes (Fig. 8A). Reliable identification of a specific [^{3}H]SR33557 binding component to PC12 and RINm5F cell membranes was not possible because of the high value of the nonspecific binding component. Curves describing the inhibition of specific (+)-[^{3}H]PN200-110 binding by SR33557 have provided $K_{0.5}$ values of 2, 1.8, and 4 nM for A7r, PC12, and RINm5F membranes, respectively (Fig. 8B). These values are similar to the corresponding value of 1 nM found with T tubule membranes.

Effects of SR33557 on [^{3}H]dopamine release from PC12 cells. PC12 cells preloaded with [^{3}H]dopamine rapidly release the neurotransmitter upon depolarization with 70 mM KCl (29). It has been demonstrated that the evoked release of [^{3}H]dopamine from undifferentiated cells is greatly enhanced by the voltage-sensitive Ca^{2+} channel agonist Bay K8644 and is blocked by voltage-sensitive Ca^{2+} channel antagonists. SR33557 also totally blocked release of [^{3}H]dopamine from PC12 cells. The dose-response curve for this inhibition is presented in Fig. 7D, together with the dose-response curve for (+)-PN200-110. The EC_{50} values are 20 and 0.2 nM for SR33557 and (+)-PN200-110, respectively. Hence, a very good correlation was found between the IC_{50} values for inhibition of $^{45}Ca^{2+}$ uptake and those for [^{3}H]dopamine release. For the latter effect, (+)-PN200-110 is again 100 times more potent than SR33557.

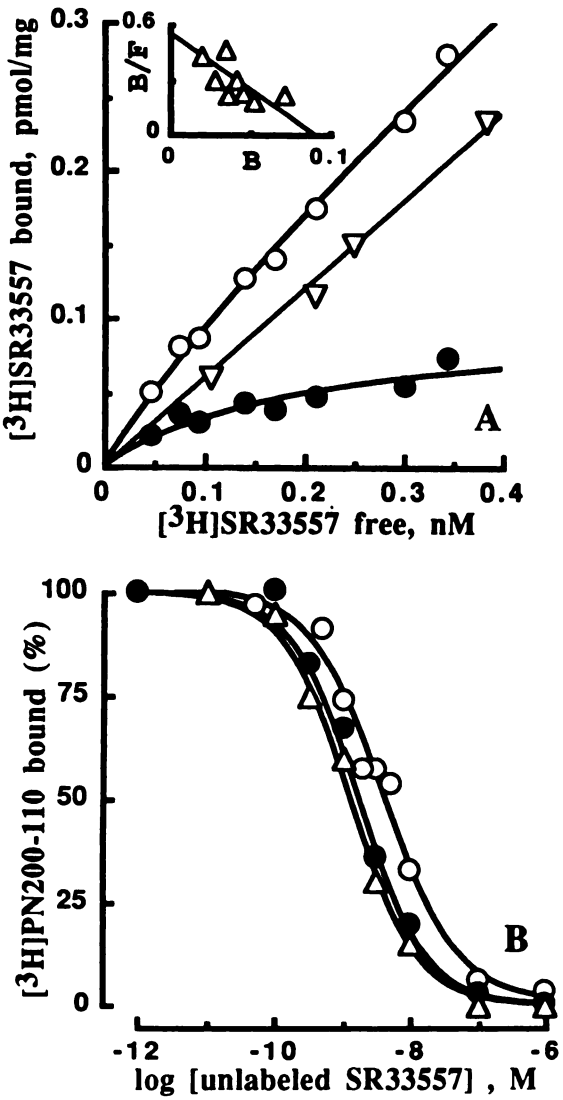

Fig. 8. A, equilibrium binding of [^{3}H]SR33557 to A7r5 cell membranes. Equilibrium binding was measured at 25°, using 200 μg of protein/ml and increasing concentrations of [^{3}H]SR33557. Specific binding (●) of [^{3}H]SR33557 is the difference between the binding in the absence (○) and in the presence (△) of 0.1 μM unlabeled SR33557. *Inset*, Scatchard plot for the specific [^{3}H]SR33557 binding component. (*B*, bound, in pmol/mg of protein; *F*, free, in nM). B, Inhibition of specific (+)-[^{3}H]PN200-110 binding to PC12 (△), A7r5 (●), and RINm5F (○) cell membranes by increasing concentrations of unlabeled SR33557. Experimental conditions are 0.2 nM [^{3}H]PN200-110 and 1 mg/ml membrane preparation.

Voltage-clamp analysis of SR33557 action on A7r5 cells. Two experimental protocols have been used to investigate the blocking effect of SR33557 on the Ca^{2+} current of A7r5 cells. In protocol 1, the effect of the drug was tested under polarized conditions, i.e., at a holding potential $V_H = -80$ mV. The voltage dependence of the drug effect was investigated by first exposing the cell to a fixed concentration of the drug under polarized conditions (protocol 1) until a steady state effect on the Ca^{2+} current was reached. Next, V_H was held for 3 min at −10 mV in order to set the Ca^{2+} channel in the inactivated state (36). V_H was then returned to −80 mV for 1 min before the test pulse (protocol 2). The comparative blocking effects of SR33557 and (+)-PN200-110 on Ca^{2+} channel activity are presented in Fig. 9, A and B. SR33557 at 10 nM blocked 30% of the peak Ca^{2+} current under polarized conditions (protocol 1), whereas the same concentration of (+)-PN200-110 blocked 40% of the Ca^{2+} current. An exposure to depolarized conditions (protocol 2) increased blockade by SR33557 from 30 to 43%, whereas blocking by (+)-PN200-110 was increased from 40 to 80%, indicating a higher voltage dependence for the blocking effect of the DHP. Current-voltage relationships for the peak Ca^{2+} current before and after application of 10 nM SR33557 (protocol 2) are presented in Fig. 9D. The degree of Ca^{2+} current blocking was almost independent of the test pulse amplitude.

Discussion

Although the study of Ca^{2+} channel pharmacology began more than 20 years ago, it is still a very dynamic topic in biomedical and fundamental research on Ca^{2+} channel physiology. Recent developments in these fields include the use of well known Ca^{2+} channel effectors (for review see Ref. 37) in new therapeutic applications and further investigations into the properties and functions of Ca^{2+} channels. Another active area of investigation is the search for novel effectors, either obtained from natural sources (22) or chemically synthesized (25).

This report presents strong evidence showing that the newly synthesized molecule SR33557, from the indolizinsulfone series, acts on L-type Ca^{2+} channels. Binding data on T tubule membranes clearly demonstrate that SR33557 binds at a specific site that is distinct from all other binding sites previously characterized and that interacts in an allosteric manner with them. Unlabeled SR33557 completely prevents the binding of all known classes of Ca^{2+} channel effectors studied, including (+)-[^{3}H]PN200-110, [^{3}H]verapamil, (*d*)-(*cis*)-[^{3}H]diltiazem, and [^{3}H]fluspirilene, and accelerates the rate of dissociation of all ligands except (+)-[^{3}H]PN200-110 from their respective binding sites. However, Scatchard analysis reveals that SR33557 influences both the affinity of (+)-PN200-110 binding and the maximal density (B_{max}) of the DHP binding sites. This

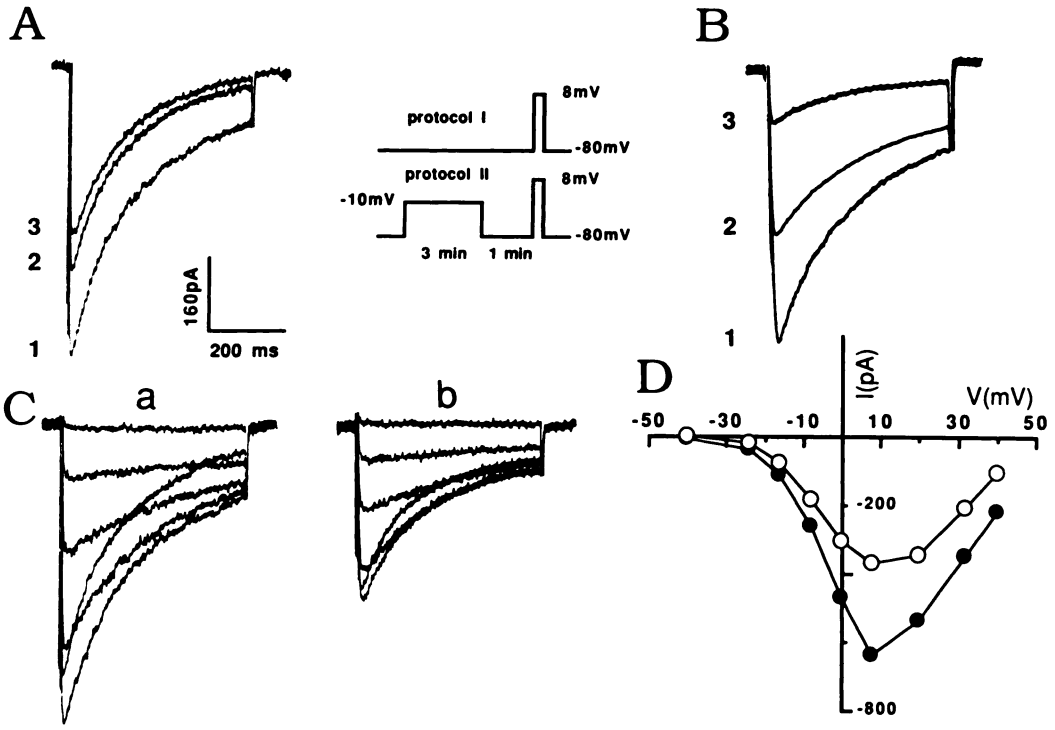

Fig. 9. Blocking effects of SR33557 and (+)-PN200-110 on Ca^{2+} channels of A7r5 cells. Ca^{2+} channel activities were measured with Ba^{2+} (10 mM) as charge carrier. Ca^{2+} blockers were applied only in stable control experiments, i.e., after having checked that Ca^{2+} currents remained constant following protocols 1 and 2 for at least a period of 15 min. A and B, Ca^{2+} currents associated with a step depolarization to +8 mV from a holding potential of −80 mV. A, *1*, control (protocol 1); *2*, steady state effect of 10 nM SR33557 (protocol 1); *3*, additional blockage of the Ca^{2+} current (protocol 2). B, *1*, control (protocol 1); *2*, steady state effect of 10 nM (+)-PN200-110; *3*, additional blockage following protocol 2. C and D, Superimposed Ca^{2+} current traces associated with depolarizing steps to −40, −16, −8, 0, +8, and +20 mV from a holding potential of −80 mV. C, *a*, Control current (protocol 2); *b*, effect of 10 nM SR33557 (protocol 2). D, peak Ca^{2+} current-membrane potential relationship for the experiment illustrated in C; ●, *a*; ○, *b*. *Traces* in A and C were low pass-filtered at 2 KHz; those in B were low pass-filtered at 500 Hz.

noncompetitive type of inhibition indicates again that distinct sites for (+)-PN200-110 and SR33557 exist, in agreement with data from an independent binding study in guinea pig cerebral membranes (38).

[^{3}H]SR33557 binds with a very high affinity to its receptor site. The K_D value of 0.08 nM is the lowest one yet found for the binding of a Ca^{2+} channel effector to skeletal muscle T tubules. Only one class of binding sites is present and the maximal binding capacity for [^{3}H]SR33557 is the same as that found for the other ligands. As expected, all other Ca^{2+} channel effectors tested inhibit [^{3}H]SR33557 binding, with the exception of ω-conotoxin, which has no binding sites on muscle (39). Attempts to inhibit ^{125}I-ω-conotoxin binding to rat brain membranes indicate the total absence of interaction between ω-conotoxin and SR33557 binding sites (data not shown). The same situation has been found with the other classic Ca^{2+} channel effectors (39). Finally, photoaffinity labeling confirms that [^{3}H]SR33557 binds to the same protein that binds the other effectors used in this study (17, 20, 21, 33). The structure of SR33557 is particularly suitable for photolabeling experiments and a large amount of radioactivity was covalently incorporated into the M_r 165,000 protein.

Although binding and photoaffinity studies leave no doubt that SR33557 binds to the Ca^{2+} channel complex, they do not provide information on the effect of the new molecule on Ca^{2+} channel function. Measurements of channel activity by $^{45}Ca^{2+}$ uptake experiments or by electrophysiology are necessary to answer this question. Three very different cell lines have been used in this work, (i) a vascular smooth muscle model with the aortic cell line A7r5, (ii) an endocrine cell model with the insulin-secreting cell line RINm5F, and (iii) a nerve-like cell model with the pheochromocytoma cell line PC12. With this latter model, SR33557-induced inhibition of $^{45}Ca^{2+}$ uptake and neurotransmitter release have been correlated. In flux as well as in electrophysiology experiments using the aortic cell line, half-maximal effects are obtained for relatively high SR33557 concentrations (25 to 50 nM), in comparison with the very high affinity found in binding to membrane preparations (K_D of 0.08 nM for T tubule and 0.16 nM for the A7r membranes). This discrepancy could be due to the fact that properties of binding to membranes are not directly comparable to properties of binding to living cells, as has been very often observed for other drugs such as DHPs (27, 40–42), ω-conotoxin (12, 39), or apamin (43). Another possibility could be that SR33557 recognizes two different types of binding sites. One type could have a high affinity and its occupation would not block Ca^{2+} currents. The other type, with a lower affinity, would be responsible for blocking L-type Ca^{2+} currents. Because of its lower affinity, the second binding site could not have been detected in direct binding experiments with [^{3}H]SR33557. Clearly, more work is needed to answer this question. One particularly interesting property of the DHP blocking of L-type Ca^{2+} channels is the voltage dependence of the drug effect, which is due to the fact that the Ca^{2+} channel blocker has a higher affinity for the inactivated form of the Ca^{2+} channel (27, 40–42). The effect of SR33557 on Ca^{2+} channel activity in the aortic cell is also increased by a transient exposure to the drug at a more depolarized potential (Fig. 9). However, the membrane potential dependence of this effect is much smaller than it is for the DHP (+)-PN200-110. It is interesting to note that,

even for some of the DHPs, large discrepancies exist between drug concentrations needed for the half-maximal electrophysiological effects and the corresponding K_D values from binding studies that cannot be fully explained by the voltage dependence of the binding (42). Possible explanations for these differences have been proposed (42) that may also hold for SR33557.

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