Identification of a novel calcium antagonist binding site in rat brain by SR 33557

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1 In K⁺-depolarized taenia preparations from guinea-pig caecum SR 33557 was a potent antagonist of Ca²⁺-induced contractions and antagonized the effect of the calcium channel activator Bay K 8644.

2 SR 33557 displayed high affinity (pK_i 9.54 ± 0.04, *n*H 1.01) for the [³H]-(±)-PN 200-110 binding site in rat cerebral cortex membranes. In the presence of 5 mM Ca²⁺ this affinity was reduced (pK_i 8.82 ± 0.01, *n*H 1.05) whilst the affinity of nitrendipine was unaffected by this concentration of Ca²⁺.

3 Saturation binding experiments in rat cerebral cortex carried out in the absence and presence of SR 33557 (0.1–1.0 nM) indicated an apparently competitive interaction at the dihydropyridine site, in that SR 33557 reduced the K_D of [³H]-(±)-PN 200-110 binding without any effect on B_{max}. In kinetic experiments, the rate of dissociation of [³H]-(±)-PN 200-110 from rat cerebral cortex was unchanged in the presence of SR 33557 (5 nM).

4 D-cis-diltiazem fully reversed the inhibition [³H]-nitrendipine binding to rat cerebral cortex produced by SR 33557 indicating the site of action of SR 33557 to be distinct from the dihydropyridine (DHP) binding site.

5 Saturation analysis indicated that [³H]-SR 33557 (0.01–0.8 nM) labelled a single class of binding sites in rat cerebral cortex membranes with high affinity (K_D 0.12 ± 0.01, B_{max} 222 ± 20 fmol mg⁻¹ protein), although kinetic data indicated the existence of negative cooperativity between the binding sites.

6 In competition studies, a variety of different calcium antagonists displayed similar affinity for [³H]-SR 33557 and [³H]-(±)-PN 200-110 sites. The [³H]-SR 33557 site was sensitive to the inhibitory effect of divalent cations. The affinity of Cd²⁺ was 0.026 ± 0.015 mM and the rank order of affinity was Cd²⁺ > Ca²⁺ > Mn²⁺ > Mg²⁺ > Na⁺.

7 We propose that SR 33557 labels a distinct site in rat cerebral cortex. The coupling between the SR 33557 and DHP site appears to be very close, resulting in apparently competitive interactions in some experimental protocols but can be revealed as negatively allosteric in other circumstances.

Keywords: SR 33557; ligand binding; dihydropyridines; L-type calcium channels

Introduction

It is now well established that at least three distinct sites exist on the L-type voltage sensitive calcium channel (Glossmann *et al.*, 1982; Glossmann & Ferry, 1985; Mir & Spedding, 1987) for the diverse group of agents classified as calcium antagonists (Fleckenstein, 1983). Distinct sites for dihydropyridines (DHPs), phenylalkylamines and benzothiazepines (chemically represented by the prototypical drugs nitrendipine, verapamil and diltiazem respectively) have been demonstrated in brain, skeletal muscle and cardiac tissue (Glossmann & Ferry, 1985; Qar *et al.*, 1987; Lazdunski *et al.*, 1988). Galizzi *et al.* (1986) first demonstrated a putative fourth binding site for fluspirilene, a diphenylbutylpiperidine molecule, in skeletal muscle T-tubule membranes, at a site distinct from the sites of action of other calcium antagonists. Similar high affinity binding sites for fluspirilene have also been demonstrated in cardiac sarcolemmal membranes with an interaction consistent with a distinct but allosterically coupled binding site (King *et al.*, 1989), although several studies have failed to identify this site in brain membranes (Qar *et al.*, 1987; Kenny *et al.*, 1990). Indeed, in skeletal muscle and cardiac membrane preparations at least five separate, mutually coupled binding sites for different chemical classes of calcium antagonist have now been demonstrated (for a review see Janis & Triggle, 1991). Although heterogeneous in nature, this group of compounds have all been shown to

interact at distinct, high affinity, channel linked domains, each being reciprocally and allosterically coupled and residing on a 165–200 kDa polypeptide or α_1 subunit of the L channel (Striessnig *et al.*, 1987; Schmid *et al.*, 1989; Glossmann *et al.*, 1989). However, in brain membranes, only three definitive sites remain clearly established for DHPs, phenylalkylamines and benzothiazepines.

SR 33557, a novel indolizine sulfone calcium antagonist, also possesses high affinity for a site in skeletal muscle associated with the L-channel α_1 polypeptide (Schmid *et al.*, 1989). The compound is a potent antagonist of calcium-induced contractions in K⁺-depolarized rat aorta and displays high affinity for DHP and phenylalkylamine sites in rat brain membranes (Nokin *et al.*, 1989). Recently, a novel high affinity site for [³H]-SR 33557 has been demonstrated in rat heart sarcolemmal membranes (Chatelain *et al.*, 1991) with properties similar to those described in skeletal muscle (Schmid *et al.*, 1989; Sol-Rolland *et al.*, 1991). In this paper, we have fully characterized the interaction of SR 33557 with [³H]-DHP sites in rat cerebral cortex and identified a site for [³H]-SR 33557 which appears to be distinct but allosterically coupled to the DHP site. A preliminary account of some of these findings has been presented to the British Pharmacological Society (Kenny & Spedding, 1992).

Methods

K⁺-depolarized smooth muscle

Strips of taenia (1–2 mm diameter, 1.5–2.0 cm relaxed length) from the caecum of female guinea-pigs (200–350 g)

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were set up in 30 ml isolated organ baths containing K⁺-Tyrode solution which was gassed with 95% O₂:5% CO₂ and maintained at 35°C. The K⁺-Tyrode solution had the following composition (mM): NaCl 97, KCl 40, NaHCO₃ 11.9, NaH₂PO₄ 0.4, glucose 5.5. The preparations were washed in the Ca²⁺-free, 40 mM K⁺-Tyrode solution for 30 min, by which time they were fully relaxed. Contractile responses were measured under isotonic conditions (1 g load) with Harvard or Bioscience isotonic transducers connected to BBC Goetz SE460 potentiometric recorders. Cumulative concentration-response curves to Ca²⁺ (10–3000 μ M) were obtained by increasing the Ca²⁺ concentration at 3–5 min intervals in logarithmic increments (Van Rossum, 1963). When concentration-response curves were repeated the curves were obtained at 30 min intervals. Antagonists were preincubated for 30 min. Concentration-ratios were calculated as the ratios of the concentrations of Ca²⁺ which produced a 50% maximal response (EC₅₀) in the presence and in the absence of the antagonists. In the experiments where Bay K 8644 was used the preparations were partially contracted with Ca²⁺ and Bay K 8644 added in a cumulative fashion. The increase in contraction caused by Bay K 8644 was calculated as a % increase over the tone established by the prior addition of Ca²⁺ (normally 25–45% of Ca²⁺ maximum).

[³H]-dihydropyridine binding assays

Membranes were prepared from the cerebral cortex of male Sprague-Dawley rats by homogenization in ice-cold 50 mM Tris-HCl (pH 7.4 at 4°C) with a polytron (Kinematica PT 10) tissue disruptor at setting 8, by us of two 10 s bursts followed by centrifugation at 48,000 *g*_{av} at 4°C for 15 min. The resultant pellet was washed twice by resuspension and centrifugation and the final pellet was resuspended in 50 mM Tris-HCl (pH 7.4 at 4°C) at a protein concentration of 4–5 mg ml⁻¹. Membranes were stored in liquid nitrogen until required for assay. Aliquots of membrane homogenate (80–100 μ g protein per assay) were incubated in 50 mM Tris-HCl (pH 7.4 at 25°C) with [³H]-(-)-PN 200-110 in a total volume of 2.0 ml for 120 min at 25°C under subdued light. Non-specific binding was determined in the presence of 1 μ M nitrendipine. Reactions were terminated by the addition of 5 ml ice-cold Tris-HCl and rapid filtration over Whatman GF/B filters, followed by two washes with ice cold buffer by use of a Brandel Cell harvester under a constant vacuum of 22 mmHg. Bound radioactivity was determined by liquid scintillation counting. In experiments with [³H]-nitrendipine, incubations were carried out for 90 min and non-specific binding was determined in the presence of 1.0 μ M nifedipine.

[³H]-SR 33557 binding assay

Aliquots of Tris-washed membranes (prepared as above) were incubated with [³H]-SR 33557 in buffer (50 mM Tris-HCl, pH 7.4 at 25°C, containing 0.1% bovine serum albumin) in a total volume of 1.0 ml. Non-specific binding was determined in the presence of 1 μ M SR 33557. Incubations were carried out for 100 min under subdued light before filtration over Whatman GF/B filters as described for [³H]-DHP binding. For [³H]-SR 33557 experiments, filters were pretreated with 0.05% polyethyleneimine for at least 45 min. Failure to include bovine serum albumin in the assay media or pretreatment of the filters with polyethyleneimine resulted in low levels of specific binding.

Data analysis

Competition curves were generated by the use of at least ten concentrations of competing drug. Assay points were determined in duplicate and the resultant displacement isotherms were analysed by a non linear least square fitting program by a microcomputer as described by Michel & Whiting (1988). The *K_i*, where appropriate, was obtained from the equation

of Cheng & Prusoff (1973). Equilibrium saturation parameters (*K_D* and *B_{max}*) of assay points carried out in triplicate were determined by the non linear least square fitting program LIGAND (Munson & Rodbard, 1980). Protein concentration was determined by use of a Pierce BSA protein assay reagent kit with bovine serum albumin as standard.

In kinetic experiments the association rate constant (*K_i*) and dissociation constant (*K_{-i}*) were determined according to Weiland & Molinoff (1981).

Drugs

The following drugs were used: [³H]-SR 33557 (2-isopropyl-1-((4-(3-N-methyl-N-(3,4-dimethoxy-B-phenethyl) amino) propoxy) benzenesulphonyl))-inolazine) was a gift from Sanofi (Bejar, Belgium), specific activity 83 Ci mmol⁻¹ (98% purity) and was stored at -18°C and protected from light. [³H]-nitrendipine (86 Ci mmol⁻¹) and [³H]-(+)-PN 200-110 (82 Ci mmol⁻¹) were obtained from DuPont. SR 33557 was a kind gift from Dr P. Chatelain, Sanofi (Bejar, Belgium). Lidoflazine, fluspirilene and flunarizine were obtained from Janssen (Beerse, Belgium), D-cis-diltiazem from Synthelabo (Paris, France), nifedipine, nitrendipine and Bay K 8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethyl-phenyl)-pyridine-5-carboxylate) from Bayer AG (Leverkusen). Verapamil and D-600 (methoxyverapamil) were obtained from Sigma (U.K.). All other drugs and chemicals were obtained from Sigma (U.K.) and were of the highest purity available. All drugs were dissolved in ethanol or distilled water at a stock concentration of 1 mM and subsequent dilutions made in distilled water or assay buffer, ensuring that the concentration of assay ethanol never exceeded 0.1% vol. All inorganic ions were used as their chloride salts.

Results

Functional effects of SR 33557 in smooth muscle

SR 33557 (1–1000 nM) caused a concentration-dependent inhibition of the contractions induced by the addition of Ca²⁺ in K⁺-depolarized taenia preparations from the guinea-pig caecum, with threshold antagonism of 1 nM (Figure 1a). Functional antagonism of the effects of Bay K 8644 has been shown to be a simple method for differentiating between subtypes of calcium antagonists (Spedding & Berg, 1984; Spedding, 1985b). Concentration-response curves to Bay K 8644 were constructed under constant depolarizing conditions (40 mM K⁺) in preparations which are partly (25–45% of the maximal response) contracted to Ca²⁺ (0.1 mM). Under these conditions, SR 33557 1.0 nM (*n* = 4) caused a rightward shift and slight depression of the maximum response to Bay K 8644 (Figure 1b). Similarly, at concentrations of 10 and 100 nM (*n* = 4; experiments where the tissues were initially contracted with 0.3 and 3.0 mM Ca²⁺) SR 33557 caused a rightward shift of the Bay K 8644 curve without depression of the maximum response, indicating a high affinity interaction of SR 33557 at the DHP site in K⁺-depolarized smooth muscle.

Effects of SR 33557 on [³H]-DHP binding parameters

The binding of [³H]-(+)-PN 200-110 to rat cortical membranes at 25°C was saturable, reversible, and of high affinity. Iterative non linear analysis of the binding isotherms indicated a single class of high affinity sites with a *K_D* of 0.046 ± 0.006 nM and a *B_{max}* of 186 ± 5 fmol mg⁻¹ protein. In competition experiments (Table 1) SR 33557 displayed high affinity for the [³H]-(+)-PN 200-110 site (p*K_i* 9.54 ± 0.04, *n*H 0.97) and the Hill slope of unity was consistent with simple competitive interaction. In the presence of 5 mM Ca²⁺ the affinity of SR 33557 for the DHP site was reduced more than five fold (p*K_i* 8.82 ± 0.01, *n*H 1.05). The potency of nitren-

dipine was not affected by this concentration of Ca^{2+} (pK_i values of 9.48 and 9.53 respectively, $n = 2$).

Saturation binding experiments carried out with [^3H]-(+)-

Figure 1 (a) Antagonism of the Ca^{2+} -induced contractions in K^{+} -depolarized taenia preparations from the guinea-pig caecum by SR 33557. Control (●) and in the presence of 1 nM (○), 10 nM (■), 100 nM (□) and 1 μM SR 33557 (▲). (b) Cumulative concentration-response curves to Bay K 8644 in K^{+} -depolarized taenia preparations from the guinea-pig caecum which were contracted to 24–45% of maximum with Ca^{2+} (0.1–3.0 mM). Control (●), and in the presence of SR 33557, 1 nM (○), 10 nM (■) and 1000 nM (□).

PN 200-110 in the presence and absence of SR 33557 (Figure 2) indicated that the compound caused a decrease in binding affinity (K_D) without any effect on binding capacity (B_{max}). Similar findings were obtained with the unlabelled DHP nifedipine (control values K_D 0.029 ± 0.008 nM, B_{max} 221 ± 20 fmol mg^{-1} protein, and in the presence of 5.0 nM nifedipine K_D 0.17 ± 0.02 nM, B_{max} 196 ± 6 fmol mg^{-1} protein). In both cases these results were indicative of a competitive interaction at the [^3H]-DHP site.

In order to characterize the interaction of SR 33557 further, the effect of the compound on [^3H]-(+)-PN 200-110 dissociation kinetics was examined. The concentration of SR 33557 employed in these experiments was more than 25

Figure 2 Scatchard transformation of [^3H]-(+)-PN 200-110 binding to rat cerebral cortex in the presence and absence of SR 33557. Control (●), $n = 4$, K_D 0.04 ± 0.006 nM, B_{max} 186 ± 5 fmol mg^{-1} protein; 0.1 nM SR 33557 (■), $n = 4$, K_D 0.09 ± 0.004 nM, B_{max} 192 ± 11 fmol mg^{-1} protein; 0.5 nM SR 33557 (□), $n = 4$, K_D 0.15 ± 0.02 nM, B_{max} 193 ± 16 fmol mg^{-1} protein, 1.0 nM SR 33557 (▲), $n = 4$, K_D 0.24 ± 0.03 nM, B_{max} 188 ± 12 fmol mg^{-1} protein.

Table 1 Comparative affinity values at the [^3H]-dihydropyridine and [^3H]-SR 33557 sites in rat cerebral cortex for calcium channel antagonists, activators and cations

A				
Compound	[^3H] SR 33557		[^3H] PN 200-110	
	pK_i	nH	pK_i or pIC_{50}	nH
SR 33557	9.60 ± 0.10	0.82	9.54 ± 0.04	1.00
Nitrendipine	9.48 ± 0.14	0.90	9.67 ± 0.15	0.94
Bay K 8644	8.23 ± 0.17	0.87	8.43 ± 0.20	0.96
Fluspirilene	8.13 ± 0.17	0.87	7.69 ± 0.02	0.66*
D-600	8.09 ± 0.02	0.92	Not determined	
Verapamil	7.97 ± 0.03	0.98	7.69 ± 0.09	0.92 (*42)
D-cis-diltiazem	7.47 ± 0.09	0.93	7.58 ± 0.13	1.00 (*37)
Flunarizine	7.15 ± 0.14	1.06 (*92)	6.10 ± 0.20	1.28 (*92)
Lidoflazine	7.14 ± 0.11	0.82	6.44 ± 0.06	0.84 (*90)
B				
Ion	IC_{50} (mM)			
Cd^{2+}	0.026 ± 0.015			
Ca^{2+}	2.09 ± 0.48			
Mn^{2+}	5.01 ± 0.7			
Mg^{2+}	11.06 ± 1.1			
Na^{2+}	> 100			

Values represent mean $\pm$ s.e.mean of 3–5 separate determinations. (A) Competition curves were generated by use of 12 concentrations of competing drug in duplicate. Experiments were performed as described in Methods with 0.03 nM [^3H]-(+)-PN 200-110 and 0.1 nM [^3H]-SR 33557. Values represent pK_i or pIC_{50} where inhibition of binding is incomplete or the Hill slope (nH) was < 1.0 () maximal inhibition at 10 μM .

(B) Affinity values (IC_{50}) for inorganic cations at the [^3H]-SR 33557 site. Ions were present as their chloride salts and values represent mean $\pm$ s.e.mean of 3 separate experiments.

fold greater than its K_i value for this site. Figure 3 illustrates that the rate of dissociation of [3 H]-(+)-PN 200-110 from the DHP binding site at equilibrium was unaffected in the presence of SR 33557. This was further evidence for an apparently competitive interaction with SR 33557.

Comparative reversal of calcium antagonism by D-cis-diltiazem

When [3 H]-nitrendipine was employed as a radioligand in rat cerebral cortex at 25°C, D-cis-diltiazem caused a concentration-dependent enhancement of specific binding through a positive allosteric mechanism (Figure 4). In this series of experiments we examined the ability of D-cis-diltiazem to reverse the inhibition of [3 H]-nitrendipine binding produced by nifedipine, SR 33557 and flunarizine. Figure 4 illustrates that in the presence of 0.1–2.0 nM SR 33557 ($n = 4$), D-cis-diltiazem fully reversed the inhibition of [3 H]-nitrendipine binding. This effect was dependent on the concentration of SR 33557, with diltiazem reversal curves shifted to the right with increasing concentrations of SR 33557. The observation that SR 33557 did not block the positive allosteric interaction of D-cis-diltiazem indicated that the interaction of SR 33557 was not mediated through the DHP site. Thus, D-cis-diltiazem was unable to exert a positive allosteric effect in the presence of the competitive dihydropyridine antagonist nifedipine (2 nM, $n = 3$) with this experimental protocol. In contrast to the full reversal of SR 33557, D-cis-diltiazem only partially reversed the inhibitory effects of flunarizine (Figure 4; 0.1–2.0 μ M, $n = 3$). Furthermore, the extent of this reversal was similar at all flunarizine concentrations, irrespective of the amount of [3 H]-nitrendipine initially displaced.

[3 H]-SR 33557 binding to rat cerebral cortex membranes

Saturation experiments carried out with [3 H]-SR 33557 over the concentration range 0.01–0.8 nM indicated that the compound labelled a single population of binding sites in rat cerebral cortex with high affinity (B_{max} 222 $\pm$ 20 fmol mg $^{-1}$ protein, K_D 0.12 $\pm$ 0.01 nM). Specific binding was saturable and reversible, and at a concentration equivalent to the K_D represented 65–70% of the total binding. However, kinetic studies of [3 H]-SR 33557 binding to cerebral cortical membranes illustrated that when dissociation was initiated from equilibrium by the addition of excess SR 33557 the resulting dissociation rate was rapid (K_{-1} , 0.052 $\pm$ 0.014 min $^{-1}$, $n = 4$) in comparison to the observed pseudo first order association rate (K_{obs} 0.013 $\pm$ 0.001 min $^{-1}$, $n = 4$) thus making a kinetic derivation of K_D impossible under this experimental protocol since

$$K_D = \frac{K_{-1}}{K_1}$$

where $K_1 = (K_{obs} - K_{-1})/[L]$, when $[L]$ represents the concentration of radioligand employed. These data indicated the presence of complex binding phenomena for [3 H]-SR 33557, since on the basis of the data obtained above, specific binding would not be observed if true $K_{-1} \gg K_{obs}$. However, when the dissociation rate of [3 H]-SR 33557 was monitored under conditions where dissociation was initiated from equilibrium by infinite dilution, a slow rate of dissociation was observed (0.005 $\pm$ 0.001 min $^{-1}$, $n = 3$; Figure 5). Furthermore, when dissociation was initiated by infinite dilution in the presence of excess unlabelled SR 33557, a fast curvilinear dissociation rate was observed. These results suggested the existence of negative co-operativity between the sites, since occupation of the sites with unlabelled ligand would reduce the overall affinity of the binding site population, thus resulting in an increased dissociation rate.

Pharmacological specificity of [3 H]-SR 33557 binding

Competition studies carried out with a range of calcium antagonists against [3 H]-(+)-PN 200-110 and [3 H]-SR 33557

Figure 3 The effect of SR 33557 on the rate of [3 H]-(+)-PN 200-110 dissociation from rat cerebral cortex at 25°C initiated by 1 μ M nitrendipine alone (control, $\bullet$) or in the presence of 5 nM SR 33557. Mean data ($\pm$ s.e.mean) of 3 separate experiments performed in duplicate: Control, 0.007 $\pm$ 0.0009 min $^{-1}$, and in the presence of 5 nM SR 33557, 0.006 $\pm$ 0.001 min $^{-1}$.

Figure 4 Comparative effect of D-cis-diltiazem on the inhibition of [3 H]-nitrendipine by (a) flunarizine and (b) SR 33557. Rat cerebral cortex membranes were incubated with 0.2 nM [3 H]-nitrendipine with D-cis-diltiazem alone ($\bullet$), control and in the presence of (a) flunarizine, ($\circ$) 0.1 μ M, ($\blacktriangle$) 0.5 μ M, ($\blacksquare$) 1.0 μ M and ($\square$) 2.0 μ M or (b) SR 33557 ($\circ$) 0.1 nM, ($\blacktriangle$) 0.5 nM and ($\blacksquare$) 2.0 nM. Representative experiments of 3 others are shown.

Figure 5 Dissociation of [^3H]-(+)-PN 200-110 from rat cerebral cortex membranes at 25°C by infinite dilution (●). Dissociation was achieved by dilution of pre-equilibrated 30 ml total and non-specific assay mixtures (containing 0.1 nM [^3H]-SR 33557) into 3 litres of an identical assay buffer and the specific binding of 50 ml aliquots determined at the indicated time points. A parallel experiment was performed with the dilution buffer containing 1 μM unlabelled SR 33557 (■).

binding in rat cerebral cortex are shown in Table 1a. SR 33557 displayed high and similar affinity for both sites, although the lower Hill slope at the [^3H]-SR 33557 site may be a reflection of the negative cooperativity confirmed in kinetic experiments. Both dihydropyridine agonists and antagonists showed high affinity for the [^3H]-SR 33557 site and the rank order of potency and affinity of other compounds including benzothiazepines, phenylalkylamines, and diphenylbutylpiperidines was essentially the same for both sites, although several agents such as fluspirilene, flunarizine and lidoflazine displayed higher affinity for the [^3H]-SR 33557 site. The allosteric interactions whereby D-*cis*-diltiazem and verapamil inhibited [^3H]-(+)-PN 200-110 binding incompletely was not seen at the [^3H]-SR 33557 site. A positive allosteric effect of diltiazem (comparative to its effects against [^3H]-nitrendipine) at the [^3H]-SR 33557 binding site was not observed, and in all cases (with the exception of flunarizine) competing agents displaced specific binding maximally.

Effect of inorganic cations

In contrast to the lack of effect on [^3H]-DHP binding (the presence of low concentrations of divalent cations in washed membranes being a requisite for high affinity binding), all the divalent cations tested appeared to inhibit [^3H]-SR 33557 binding in a concentration-dependent manner. The rank order of potency was $\text{Cd}^{2+} > \text{Ca}^{2+} > \text{Mn}^{2+} > \text{Mg}^{2+}$ (Table 1b). The monovalent cation Na^+ was inactive at concentrations up to 100 mM.

Discussion

In this paper we have demonstrated a high affinity interaction of SR 33557 to a novel site in rat cerebral cortex which appears to be distinct from, but tightly coupled to the DHP site. In functional experiments, SR 33557 was a highly potent calcium antagonist in K^+ -depolarized smooth muscle and the interaction resembled those of the dihydropyridines in that Ca^{2+} -concentration curves were displaced to the right

without depression of the maximum response until the solubility of the Ca^{2+} in the K^+ -Tyrode solution was exceeded (10–30 mM, Spedding, 1982). Furthermore, SR 33557 was a potent antagonist of the effects of Bay K 8644. DHPs have been shown to antagonize competitively the effects of Bay K 8644, whereas verapamil and diltiazem non-competitively antagonize contractile effects and diphenylpiperidines are highly potent antagonists which depress the maximum response (Spedding & Berg, 1984; Spedding, 1985a,b). SR 33557 appeared to interact with the DHP site in functional tests and the effects did not appear to resemble the effects of verapamil, diltiazem or diphenylalkylamines. A full analysis of the interaction of SR 33557 with the DHP site was therefore performed by use of radioligand binding techniques.

Schmid *et al.* (1989) first demonstrated specific binding of [^3H]-SR 33557 to skeletal muscle membranes and a variety of studies in cardiac and skeletal muscle membranes have confirmed a distinct binding site associated with the α_1 subunit of the channel which appears to be allosterically coupled to other calcium antagonist sites (Chatelain *et al.*, 1991; Sol-Rolland *et al.*, 1991). On the basis of the interaction at the [^3H]-(+)-PN 200-110 binding site in rat cortex, the site for SR 33557 and DHPs appeared to be very tightly coupled, to the extent that the interaction of SR 33557 at the [^3H]-(+)-PN 200-110 site appeared to be competitive under several experimental approaches. This is consistent with a previous report in which the only suggestive evidence for an allosteric interaction was obtained with SR 33557 when the compound was found to increase the rate of [^3H]-DHP dissociation at 10 μM (Nokin *et al.*, 1989), a concentration inappropriate relative to its nM K_i for the DHP site. Furthermore, in competition experiments the rank order and affinity for a range of different calcium antagonists was similar to the affinities of calcium antagonists for the DHP site. The affinities obtained in the present work are also similar to those found at the [^3H]-SR 33557 site in skeletal muscle (Schmid *et al.*, 1989; Sol-Rolland *et al.*, 1991) and cardiac sarcolemmal membranes (Chatelain *et al.*, 1991). In cardiac and skeletal muscle membranes SR 33557 has affinities at [^3H]-verapamil, [^3H]-diltiazem and [^3H]-fluspirilene sites comparable to the homologous compounds indicating mutually high affinity for other calcium antagonist sites (Schmid *et al.*, 1989). In brain tissue, however, the affinity displayed by verapamil and diltiazem was similar for both [^3H]-DHP and [^3H]-SR 33557 sites indicating that the site which [^3H]-SR 33557 labelled with low nM affinity did not appear to correspond to the phenylalkylamine or benzothiazepine site. The maximal inhibition of binding observed with both diltiazem and verapamil indicated a possible difference in the interaction of these compounds at the [^3H]-SR 33557 site in comparison to their positive and negative allosteric regulation of [^3H]-DHP binding. However, due to the negative cooperativity amongst [^3H]-SR 33557 binding sites, it was not possible to examine the interaction of other classes of calcium antagonist at this site in kinetic experiments. Indeed, we also detected the appearance of non linear Scatchard transformations of [^3H]-SR 33557 saturation binding when the ligand was employed over a wide (0.002–2.0 nM) concentration range. This also precluded an examination of competing calcium antagonists in saturation shift experiments.

Nevertheless, the observation that the inhibition of [^3H]-nitrendipine binding by SR 33557 could be fully reversed by D-*cis*-diltiazem was evidence for a distinct site of action for SR 33557. This allosteric interaction has been used to differentiate the site of action of several calcium antagonists such as prenylamine (Murphy *et al.*, 1983), amiodarone (Nokin *et al.*, 1986) and fluspirilene (Qar *et al.*, 1987). However, in these previous studies where diltiazem has been used to reverse the inhibition of [^3H]-DHP binding, reversal curves have been generated only in the presence of one concentration of calcium antagonist. In this respect, whilst we found the inhibitory effect of flunarizine on [^3H]-nitrendipine binding to be partially reversed, the efficacy of diltiazem was

different in comparison to the reversal with SR 33557 when both drugs were employed at several different concentrations. In the case of flunarizine, the degree of reversal produced by diltiazem was similar to all concentrations, irrespective of the amount of [3 H]-nitrendipine binding initially displaced. In contrast, the inhibition of [3 H]-nitrendipine binding by SR 33557 was reversed by diltiazem in a surmountable fashion, producing a rightward shift in the diltiazem reversal curves. This latter effect was an accurate reflection of the allosteric interaction of diltiazem, as the extent of the reversal is likely to be dependent on the degree of allosteric control from other mutually coupled sites. In the case of flunarizine, the relatively weak effect of diltiazem may reflect membrane interactions unrelated to specific interactions for [3 H]-nitrendipine and other allosterically coupled sites. This would not be surprising given the relative weakness of flunarizine at [3 H]-DHP sites, its poor selectivity for L-type channel (Spedding, 1985a; Tytgat *et al.*, 1988), and extremely high concentrations present in the membrane due to its high partition coefficient (Mason *et al.*, 1991). The membrane concentration arising from aqueous concentrations around 1 μ M (likely to be nearer 1 mM in the membrane) would give rise to many non-selective effects.

The site labelled in rat cerebral cortex by [3 H]-SR 33557 was found to be susceptible to the inhibitory effects of divalent cations. In this respect, the rank order and affinity of a variety of divalent cations closely resembled that found at other [3 H]-SR 33557 binding sites (Chatelain *et al.*, 1991), with Cd^{2+} displaying the highest affinity. Similar inhibitory properties of divalent cations have been demonstrated at

verapamil and diltiazem sites (Galizzi *et al.*, 1985; Balwierczak & Schwartz, 1985) in contrast to their requirement for high affinity DHP binding. Thus, the decreased affinity of SR 33557 at the [3 H]-(+)-PN 200-110 site in rat cortical membranes probably reflects an ionic effect at the SR 33557 recognition site. Interestingly, the inhibitory effect of Cd^{2+} at the SR 33557 site is in contrast to the marked enhancement of [3 H]-fluspirilene binding which occurs in the presence of this ion (Garcia *et al.*, 1990) providing further evidence of ligand specific variation in the ionic regulation of calcium antagonist binding interactions.

Several lines of evidence now indicate the distribution and coupling between the various sites for calcium antagonists to exist in a 1:1 stoichiometrical arrangement (King *et al.*, 1989). Whilst in the present experiments we found the number of [3 H]-SR 33557 and DHP sites to be similar in rat cortical membranes, we have previously suggested that specific sites for other ligands such as fluspirilene appear to be absent in this membrane preparation. Indeed, the present paper represents the first pharmacological characterization of a specific, high affinity binding site in rat cortical membranes for a calcium antagonist other than a prototypical DHP, phenylalkylamine or benzothiazepine. However, the coupling with the DHP site appears to be very tight resulting in apparently competitive interactions under several experimental approaches, although these can be revealed as being allosteric under certain defined conditions. Nevertheless, these data do support the proposal that SR 33557 binds to a distinct site associated with the L-type calcium channel.

References

- BALWIERCZAK, J.L. & SCHWARTZ, A. (1985). Specific binding of [3 H]-d-cis-diltiazem to cardiac sarcolemma and its inhibition by calcium. *Eur. J. Pharmacol.*, **116**, 193–194.
- CHATELAIN, P., BEAUFORT, P., MEYSMANS, L. & CLINET, M. (1991). Characterisation of the slow calcium channel binding sites for [3 H] SR 33557 in rat sarcolemmal membranes. *Mol. Pharmacol.*, **39**, 64–71.
- CHENG, Y.C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099–3108.
- FLECKENSTEIN, A. (1983). *Calcium Antagonism in Heart and Smooth Muscle*. New York: John Wiley.
- GALIZZI, J.P., FOSSET, M. & LAZDUNSKI, M. (1985). Characterisation of the Ca^{2+} coordination site regulating binding of Ca^{2+} channel inhibitors d-cis-diltiazem, ($\pm$) flunarizine and (–) desmethoxyverapamil to their receptor site in skeletal muscle transverse tubule membranes. *Biochem. Biophys. Res. Commun.*, **132**, 49–55.
- GALIZZI, J.P., FOSSET, M., ROMEY, G., LADURON, P. & LAZDUNSKI, M.C. (1986). Neuroleptics of the diphenylbutylpiperidine series are potent calcium channel inhibitors. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 7513–7517.
- GARCIA, M.L., KING, F.V., SHEVELL, J.L., SLAUGHTER, R.S., SUAREZ-KURTZ, G., WINQUIST, R.J. & KACZOROWSKI, G.J. (1990). Amiloride analogues inhibit L-type calcium channels and display calcium antagonist blocker activity. *J. Biol. Chem.*, **265**, 3763–3771.
- GLOSSMANN, H. & FERRY, D.R. (1985). Assay for calcium channels. *Methods Enzymol.*, **109**, 513–550.
- GLOSSMANN, H., FERRY, D.R., LUBBECKE, F., MEWES, R. & HOFMANN, F. (1982). Calcium channels: direct identification in radioligand binding studies. *Trends Pharmacol. Sci.*, **3**, 431–437.
- GLOSSMANN, H., STREISSNIG, J., KNAUS, H.G., MULLER, J., GRASSEGGER, F., HOLTJE, H.D., MARRER, S., HYMEL, L. & SCHINDLER, H. (1989). Structure of calcium channels. *Ann. NY. Acad. Sci.*, **560**, 150–161.
- JANIS, R.A. & TRIGGLE, D. (1990). Drugs acting on calcium channels. In *The Calcium Channel: Its Properties, Function and Clinical Relevance*. ed. Hurwitz, L., Partridge, L.D. & Leach, J.K., Telford Press, N.J., U.S.A.
- KENNY, B.A., FRASER, S., KILPATRICK, A.T. & SPEDDING, M. (1990). Selective antagonism of calcium channel activators by fluspirilene. *Br. J. Pharmacol.*, **100**, 211–216.
- KENNY, B.A. & SPEDDING, M. (1992). The novel calcium antagonist SR 33557 displays high affinity for a distinct binding site in rat brain. *Br. J. Pharmacol.*, **106**, 38P.
- KING, F.V., GARCIA, M.L., SHEVELL, J.L., SLAUGHTER, R.S. & KACZOROWSKI, G.J. (1989). Substituted diphenylbutylpiperidines bind to a unique high affinity site on the L-type calcium channel. *J. Biol. Chem.*, **264**, 5633–5641.
- LAZDUNSKI, M., BARHANIN, J., BORSETTO, M., COGNARD, C., COOPER, C., COPPOLA, T., FOSSET, M., GALIZZI, J.P., HOSEY, M.M., MOURRE, C., RENAUD, J., ROMEY, G., SCHMID, A. & VANDAELE, S. (1988). Molecular properties of structure and regulation of calcium channels. *Ann. NY. Acad. Sci.*, **522**, 134–149.
- MASON, P.R., RHODES, D. & HERBETTE, L.G. (1991). Reevaluating equilibrium and kinetic binding parameters for lipophilic drugs based on a structural model for drug interaction with biological membranes. *J. Med. Chem.*, **34**, 869–877.
- MICHEL, A.D. & WHITING, R.L. (1985). Methoctramine, a polymethylene tetraamine, differentiates three subtypes of muscarinic receptors in direct binding studies. *Eur. J. Pharmacol.*, **145**, 61–66.
- MIR, A.K. & SPEDDING, M. (1987). Calcium antagonist properties of diclofurime isomers. II. Molecular aspects: allosteric interactions with dihydropyridine recognition sites. *J. Cardiovasc. Pharmacol.*, **9**, 469–477.
- MUNSON, P.J. & RODBARD, S. (1980). Ligand: a versatile computerized approach for the characterization of ligand binding systems. *Anal. Biochem.*, **107**, 220–239.
- MURPHY, K.M.M., GOULD, R.J., LARGENT, B.L. & SNYDER, S.H. (1983). A unitary mechanism of calcium antagonist drug action. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 860–864.
- NOKIN, P., CLINET, M., SWILLENS, S., DELISEE, C., MEYSMANS, L. & CHATELAIN, P. (1986). Allosteric modulation of [3 H] nitrendipine binding to cardiac and cerebral membranes by amiodarone. *J. Cardiovasc. Pharmacol.*, **8**, 1051–1057.

- NOKIN, P., CLINET, M., POLSTER, P., BEAUFORT, P., MEYSMANS, L., GOUGAT, J. & CHATELAIN, P. (1989). SR 33557, a novel calcium-antagonist: interaction with [^3H](+)-nitredipine and [^3H](−)-desmethoxyverapamil binding sites in cerebral membranes. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **339**, 31–36.
- QAR, J., GALIZZI, J.-P., FOSSET, M. & LAZDUNSKI, M. (1987). Receptors for diphenylbutylpiperidine neuroleptics in brain, cardiac, and smooth muscle membranes. Relationship with receptors for 1,4-dihydropyridines and phenylalkylamines and with Ca^{2+} channel blockade. *Eur. J. Pharmacol.*, **141**, 261–268.
- SCHMID, A., ROMÉY, G., BARHANIN, J. & LAZDUNSKI, M. (1989). SR33557, an indolizinsulfone blocker of Ca^{2+} channels: identification of receptor sites and analysis of its mode of action. *Mol. Pharmacol.*, **35**, 766–773.
- SOL-ROLLAND, J., JOSEPH, M. & RINALDI-CARMONA, M. (1991). Interaction of SR 33557 with skeletal muscle calcium channel blocker receptors in the baboon: characterization of its binding sites. *J. Pharmacol. Exp. Ther.*, **257**, 595–601.
- SPEEDING, M. (1982). Assessment of Ca^{2+} antagonist effects of drugs in K^+ -depolarised smooth muscle: differentiation of antagonist subgroups. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **318**, 234–240.
- SPEEDING, M. (1985a). Activators and inactivators of Ca^{++} channels: new perspectives. *J. Pharmacol. (Paris)*, **16**, 319–343.
- SPEEDING, M. (1985b). Calcium antagonist subgroups. *Trends Pharmacol. Sci.*, **6**, 109–114.
- SPEEDING, M. & BERG, C. (1984). Interactions between a "calcium agonist" Bay K 8644 and calcium antagonists differentiate calcium antagonist subgroups. *Naunyn Schmiedeberg's Arch. Pharmacol.*, **328**, 69–75.
- SPEEDING, M. & KENNY, B.A. (1991). Voltage-dependent calcium channels: structure and drug binding sites. *Biochem. Soc. Trans.*, **20**, 147–153.
- STRIESSNIG, J., KNAUS, H.G., GRABNER, M., MOOSBURGER, K., SEITZ, W., LIETZ, H. & GLOSSMANN, H. (1987). Photoaffinity labelling of the phenylalkylamine receptor of the skeletal muscle transverse tubule calcium channel. *FEBS Lett.*, **212**, 247–253.
- TYTGAT, J., VEREECKE, J. & CARMELIET, E. (1988). Differential effects of verapamil and flunarizine on cardiac L-type and T-type Ca^{2+} channels. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **337**, 690–692.
- VAN ROSSUM, J.M. (1963). Cumulative dose-response curves. II. Technique for the making of dose-response curves in isolated organs and the evaluation of drug parameters. *Arch. Int. Pharmacodyn.*, **143**, 299–330.
- WEILAND, G.A. & MOLINOFF, P.B. (1981). Quantitative analysis of drug-receptor interactions. 1. Determination of kinetic and equilibrium properties. *Life Sci.*, **29**, 313–330.

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