Selective antagonism of calcium channel activators by fluspirilene

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1 Fluspirilene has been claimed to bind to a high affinity site in the calcium channel in skeletal muscle. We have investigated its calcium-antagonistic effects in smooth muscle and affinity for the channel in radioligand binding assays.

2 Fluspirilene was weakly active as an antagonist of Ca^{2+} -induced contractions in K^{+} -depolarized taenia preparations from the guinea-pig caecum, with threshold antagonism starting from concentrations of 30 nM. Nitrendipine, nicardipine and nimodipine were very potent antagonists in this model (threshold antagonism, > 1 nM).

3 In contrast, fluspirilene (10–1000 nM) was a potent non-competitive antagonist of the effects of Bay K 8644 (1–3000 nM) on Ca^{2+} -induced contractions and, at 10 nM, selectively antagonised the effects of Bay K 8644, abolished the Ca^{2+} -channel activator effects of CGP 28392, without changing the calcium antagonist effects of nitrendipine, or modifying the sensitivity of the tissues to Ca^{2+} . In contrast, the dihydropyridines were more effective as antagonists of Ca^{2+} than of Bay K 8644. Fluspirilene therefore selectively antagonised the effects of dihydropyridine Ca^{2+} channel activators without affecting the antagonist potency.

4 In radioligand binding experiments, fluspirilene was a potent displacer of [^3H]-PN-200-110 binding to rat cerebral cortical membranes (EC_{50} 30 nM), albeit with a low Hill slope (0.66), and was more potent than other lipophilic diphenylalkylamines such as flunarizine and lidoflazine. Fluspirilene interacted non-competitively with [^3H]-PN-200-110 and increased dissociation of the radioligand.

Introduction

It is now clear that there are three distinct binding sites for selective calcium-antagonists on the α_1 protein of the L-type calcium channel: a dihydropyridine site, a site for phenylalkylamines such as verapamil and a site of benzothiazepines such as diltiazem (Glossmann *et al.*, 1985; Mir & Spedding, 1987). However, although the lipophilic diphenylalkylamines, fendiline, prenylamine, flunarizine, cinnarizine, lidoflazine and pimozide (class III calcium antagonists, Spedding, 1985a,b) have been claimed to be calcium antagonists since the initiation of the concept of this class of drugs (e.g. Godfraind & Kaba, 1969; Fleckenstein, 1983), their mode of action has been much disputed, on the basis that the drugs have little selectivity for Ca^{2+} channels compared with Na^{+} channels (Grima *et al.*, 1986), may inhibit the contractile proteins directly (Spedding, 1983) and may interfere with calmodulin-dependent processes (see Spedding, 1985b for a review). Thus it is not clear if these drugs act directly at the calcium channel, or at other sites. In this respect, the lipophilic nature of these agents (Spedding, 1985a) and their low affinity for the Ca^{2+} channel has precluded their being used in the tritiated form to define their site of action.

We were therefore interested by the finding that fluspirilene, a lipophilic diphenylalkylamine used as a neuroleptic, had very high affinity for the Ca^{2+} channels in skeletal muscle (Galizzi *et al.*, 1986; Qar *et al.*, 1987), so that [^3H]-fluspirilene could be used as a radioligand. We have tested fluspirilene in a well characterized model for studying the functional effects of calcium antagonists in K^{+} -depolarized smooth muscle: the taenia from the guinea-pig caecum. Interactions with Ca^{2+} channel activators such as Bay K 8644 (Schramm *et al.*, 1983) may be used to define the site of action of calcium antagonists. Dihydropyridine activators and antagonists interact competitively (Spedding & Berg, 1984; Su *et al.*, 1984; Spedding, 1985b), verapamil and diltiazem interact non-competitively with Bay K 8644 (Su *et al.*, 1984; Spedding & Berg, 1984; Spedding, 1985a,b), whereas the effects of the lipophilic diphenylalkylamines are not reversed by Bay K 8644

(Spedding & Berg, 1984; Spedding, 1985a; Boddeke *et al.*, 1988; Patmore & Duncan, 1988). These functional interactions reflect the allosteric interactions of the drugs at their respective binding sites (Glossmann *et al.*, 1985; Janis *et al.*, 1987) and are considered to be criteria for the classification of calcium antagonists (Vanhoutte & Paoletti, 1987). Using these criteria to define the site of action of fluspirilene, we found that fluspirilene selectively antagonised Bay K 8644 without modifying the effects of dihydropyridine calcium antagonists.

The potency of fluspirilene in displacing tritiated ligands from the Ca^{2+} channels in rat cerebral cortex membranes was also investigated; binding of dihydropyridines to cortical membranes is similar to that in smooth muscle membranes (Janis *et al.*, 1987). Our data confirm and extend previous results indicating high affinity for an undefined site in the Ca^{2+} channel in skeletal muscle, with slightly lower affinity in brain tissue (Quirion *et al.*, 1985; Galizzi *et al.*, 1986; Qar *et al.*, 1987). Also, they show that fluspirilene interacts non-competitively with the dihydropyridine site, but do not fully explain the selectivity against the calcium channel activators which we find in functional experiments.

Methods

Strips of taenia (1–2 mm diameter, 1.5–2 cm relaxed length) from the caecum of female guinea-pigs (200–350 g) were set up in 30 ml isolated organ baths containing K^{+} -Tyrode solution which was gassed with 95% O_2 : 5% CO_2 and maintained at 35°C (Spedding, 1982). The K^{+} -Tyrode solution had the following composition (mmol l^{-1}): NaCl 97, KCl 40, NaHCO_3 11.9, NaH_2PO_4 0.4, glucose 5.5. The preparations were washed in the Ca^{2+} -free, 40 mmol l^{-1} 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 Ca^{2+} (10–3000 $\mu\text{mol l}^{-1}$) were obtained by increasing the Ca^{2+} 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

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preincubated for 30 min. Concentration-ratios were calculated as the ratios of the concentrations of Ca^{2+} which produced a 50% maximal response (EC_{50}) 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 Ca^{2+} and Bay K 8644 added in a cumulative fashion, so that the increase in tone was taken as a percentage of the increase in contraction, as a fraction of the total increase possible (taken from the maximum response to Ca^{2+}).

Student's *t* test was used for comparisons of mean values and the Mann-Whitney U test for comparison of % changes. Concentration-ratios were calculated, and tested for statistical differences, in logarithmic units and the antilogarithms are quoted in the test. Standard errors of the mean are illustrated on percentage changes for representational purposes only. All concentrations refer to final bath concentrations of drugs.

$[^3\text{H}]\text{-}(+)\text{-PN 200-110}$ binding to rat cerebral cortex

Membranes were prepared from the cerebral cortex of male Sprague-Dawley rats by homogenization of the tissue in 30 volumes of ice-cold 50 mM Tris-HCl (pH 7.4 at 25°C) with a polytron tissue disruptor (setting 8, two 10 s bursts) followed by centrifugation at 48,000 g_{av} at 4°C for 15 min. The resulting pellet was washed twice by resuspension and centrifugation under the conditions described. The final pellet was resuspended in buffer and aliquots were stored under liquid nitrogen until required for assay.

Membrane aliquots (0.1–0.15 mg ml^{-1}) were incubated in 50 mM Tris-HCl (pH 7.4 at 25°C) with $[^3\text{H}]\text{-}(+)\text{-PN 200-110}$ (30 pM for competition studies, 0.005–1.0 nM for saturation studies) in a total volume of 2.0 ml. Incubations were carried out for 100 min at 25°C, after which the reaction was terminated by the addition of 5.0 ml ice-cold 50 mM Tris-HCl and rapid filtration over Whatman GF B filters by use of a Brandel cell harvester. The filters were washed twice with ice-cold buffer and the bound radioactivity determined by liquid scintillation counting. Non-specific binding was determined in the presence of 1.0 μM nitrendipine. For competition experiments, assays were carried out with at least 10 concentrations of competing drug and the resulting displacement curves were analysed by a non-linear least square parametric curve fitting program. Equilibrium binding parameters (K_d and B_{max}) were

obtained by the non-linear least square fitting programme 'ligand' (Munson & Rodbard, 1980).

Drugs

The following drugs were used: Bay K 8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate), nifedipine, nisoldipine (Bayer AG, Leverkusen, FRG), nicardipine hydrochloride (Syntex), fluspirilene, lidoflazine and flunarizine tartrate (Jannssen), PN 200-110 (isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-methoxy-carbonylpyridine-3-carboxylate) and CGP 28392 (4-[2-(difluoromethoxy)-phenyl]1,4,5,7-tetrahydro-2-methyl-5-oxofluoro[3,4-b]pyridine-3-carboxylic acid ethylester).

Dihydropyridines were dissolved in ethanol (1 or 30 mmol l^{-1}), before being diluted in distilled water. Stock solutions were kept at 0°C for up to 12 h and aqueous dilutions prepared immediately before use. All solutions were shielded from light. The other drugs were dissolved in distilled water, with the exception of fluspirilene which has very low aqueous solubility (10 $\mu\text{g ml}^{-1}$) and was consequently dissolved in ethanol. The maximum concentration of ethanol in the bathing medium was <0.03%; this concentration did not affect responses to Bay K 8644.

Results

K^+ -depolarised smooth muscle

Nitrendipine, nimodipine and nicardipine caused concentration-dependent inhibition of the contractions induced by cumulative addition of Ca^{2+} in K^+ -depolarized taenia preparations from the guinea-pig caecum, with threshold antagonism occurring at concentrations of 1 nM (Figure 1). The antagonism by the dihydropyridines was non-competitive in these experiments. Fluspirilene was considerably less potent, in that threshold antagonism occurred at 100 nM (Figure 1d). An apparent pA_2 could be calculated for fluspirilene (7.5 ± 0.1 , slope 1.1, $n = 5$), but not for the dihydropyridines.

Addition of Bay K 8644 (1–3000 nM) to an established sub-maximal contraction induced by Ca^{2+} (0.1 mM; 25–45% of the maximal contraction to Ca^{2+}) caused a concentration-

Figure 1 Antagonism of Ca^{2+} -induced contractions (●, controls) in K^+ -depolarized taenia preparations from the guinea-pig caecum by the dihydropyridines (○, 0.1 nM; ■, 1 nM; □, 10 nM; ▲, 100 nM) nicardipine (a), nimodipine (b), and nitrendipine (c) and fluspirilene (□, 10 nM; ▲, 100 nM; △, 1000 nM; d). Vertical bars represent s.e.mean, $n = 4-6$.

Figure 2 Cumulative concentration-response curves to Bay K 8644 in K^+ -depolarised taenia preparations from the guinea-pig caecum ($\bullet$, controls) which were contracted to 25–45% of maximum with Ca^{2+} (0.1–0.3 mM in control and fluspirilene experiments; 0.3–10 mM in dihydropyridine experiments) in the presence of nicardipine ($\blacklozenge$, 0.1 nM; $\blacksquare$, 1 nM; $\square$, 10 nM; a), nimodipine ($\circ$, 0.1 nM; $\blacksquare$, 1 nM; $\square$, 10 nM; b), nitrendipine ($\circ$, 0.1 nM; $\blacksquare$, 1 nM; $\square$, 10 nM; c) or fluspirilene ($\blacksquare$, 1 nM; $\square$, 10 nM; $\blacktriangle$, 100 nM; $\triangle$, 1000 nM; d). Vertical bars represent s.e.mean, $n = 4-8$. The responses are expressed as % augmentation of the Ca^{2+} -induced contraction.

dependent augmentation of the contraction. Fluspirilene (1–1000 nM) antagonised the responses to Bay K 8644 in a concentration-dependent, but non-competitive, manner, reducing the maximum response, with a pD_2 of 8.3 (Figure 2d). In contrast, nicardipine (0.1, 1 nM) did not significantly affect the response to Bay K 8644 and at higher concentrations (10 nM) shifted the curve to the right, without depressing the maximum response (Figure 2). Similarly, nitrendipine and nimodipine did not depress the maximum response to Bay K 8644 and antagonism was seen only at low concentrations of Bay K 8644; indeed some augmentation of the response was apparent with high concentrations (Figure 2). In these experiments, progressively higher concentrations of Ca^{2+} (0.3, 1, 3, 10 mM) were used to recontract the tissues to 25–45% of the maximum contraction before the addition of Bay K 8644 when the dihydropyridines were used, but this was not necessary in the fluspirilene experiments as the sensitivity to Ca^{2+} was hardly impaired. This means that the apparently increased responses to Bay K 8644 seen after exposure to nimodipine and nitrendipine could be secondary to an increase in the driving force for Ca^{2+} entry, so that channels which were unblocked by Bay K 8644 would pass more Ca^{2+} than under control conditions.

In order to control for this difference, fluspirilene (100 nM) was added in the presence or absence of nimodipine (10 nM) and the effects of Bay K 8644 tested (Figure 3). Fluspirilene (100 nM for 25 min) did not antagonise the responses to Ca^{2+} in that 0.3 mM Ca^{2+} caused a large contraction ($47 \pm 5\%$ max), but the activator effects of Bay K 8644 were almost abolished. Nimodipine (10 nM for 25 min) in these experiments abolished the contraction to 3 mM Ca^{2+} , yet this inhibition was reversed fully by Bay K 8644. A combination of fluspirilene (100 nM) and nimodipine (10 nM) also abolished the effects of 3 mM Ca^{2+} , but the sensitivity to Bay K 8644 was reduced 30 fold by fluspirilene. The slightly greater contraction induced by Bay K 8644 in the presence of fluspirilene and nimodipine, compared with that seen with fluspirilene alone, can probably be attributed to the increased Ca^{2+} concentration (3 mM compared with 0.3 mM) increasing Ca^{2+} flux through such channels as were activated by Bay K 8644.

In order to assess whether fluspirilene affected the potency of dihydropyridine calcium antagonists a series of experiments was performed in parallel (Figure 4a). Fluspirilene (10 nM) did not change the sensitivity of the taenia to Ca^{2+} , nor did it change the antagonistic effects of nitrendipine (1 nM; control mean Ca^{2+} concentration-ratio 4.4, $n = 10$; in the presence of fluspirilene 5.5, $n = 10$, $P > 0.5$). However, this concentration of fluspirilene markedly antagonised the effects of Bay K 8644 and completely antagonised the calcium channel activator effects of CGP 28392, revealing weak antagonistic effects (Figure 4b).

Fluspirilene is a potent dopamine antagonist, but this property does not relate directly to the antagonism of the effects of Bay K 8644 because sulpiride (10 μ M) did not antagonise the effects of Bay K 8644 in four experiments (data not shown).

Figure 3 Effects of fluspirilene ($\bullet$, 100 nM), nimodipine ($\circ$, 10 nM) or a combination of fluspirilene (100 nM) and nimodipine (10 nM) ($\blacksquare$) on the sensitivity of taenia preparations to Bay K 8644. Ca^{2+} (0.3 mM) was used to contract the tissues in the presence of fluspirilene alone, otherwise 3 mM was added. The responses are expressed as the maximum response of the tissues to Ca^{2+} (3 mM). Vertical bars represent s.e.mean, $n = 5$.

Figure 4 (a) Effects of fluspirilene (○, 10 nM) and nitrendipine (□, 1 nM) on cumulative concentration-response curves to Ca^{2+} (●, controls) in K^{+} -depolarised taenia preparations from the guinea-pig caecum. The effects of a combination of fluspirilene (10 nM) and nitrendipine (1 nM) are also shown (□). (b) Cumulative concentration-response curves to Bay K 8644 (●, ○) or CGP 28392 (■, □) in K^{+} -depolarised taenia preparations from the guinea-pig caecum which were contracted to 25–45% of maximum with Ca^{2+} in the presence of fluspirilene (○, □ 10 nM; ●, ■, controls). In (a) and (b) vertical bars represent s.e.mean, $n = 5$ (a) or 8 (b). (b) Note that fluspirilene abolishes the augmentation of the Ca^{2+} -induced contraction caused by CGP 28392.

Characteristics of [^3H]-PN 200-110 binding to rat cerebral cortex

Binding of [^3H]-(+)-PN 200-110 to rat cerebral cortex was saturable, reversible and of high affinity. Iterative non-linear

analysis of the binding isotherms demonstrated a single class of high affinity sites with a K_d of 0.029 ± 0.008 nM and a B_{max} of 221 ± 20 fmol mg^{-1} protein. The affinity of [^3H]-PN 200-110 derived from transformations of saturation isotherms was similar to the value derived kinetically (0.026 ± 0.003 nM). Specific binding was linear between 20 and 270 $\mu\text{g ml}^{-1}$ protein, and at a ligand concentration equivalent to K_d , represented 90% of the total binding ($n = 4$).

Modulation of [^3H]-PN 200-110 binding by calcium antagonists

Structurally-related dihydropyridines displaced [^3H]-PN 200-110 with high affinity and gave rise to 100% inhibition of specific binding (Table 1). Hill slopes were not significantly different from unity. Of the class III calcium antagonists (Spedding, 1985b), fluspirilene and pimozone displayed the highest affinity, although the former compound possessed a low Hill slope. This finding was also confirmed in competition experiments with another dihydropyridine ligand, [^3H]-nitrendipine (results not shown). Other class III agents displayed lower affinity.

The class II agents D-*cis*-diltiazem and verapamil inhibited binding of [^3H]-PN 200-110 but only displaced between 35 and 41% of the specific binding, respectively.

The effect of fluspirilene on [^3H]-PN 200-110 binding parameters and kinetics

Saturation isotherms of [^3H]-PN 200-110 binding to rat cerebral cortical membranes in the presence of fluspirilene indicated a non-competitive allosteric interaction, producing a decrease in K_d and B_{max} from control (K_d 0.03 ± 0.007 nM, B_{max} 224 ± 21 fmol mg^{-1} to 0.13 ± 0.03 nM, 138 ± 21 fmol mg^{-1} in the presence of 10^{-7} M fluspirilene, and 0.063 ± 0.01 nM, 211 ± 16 fmol mg^{-1} in the presence of 10^{-8} M fluspirilene, $n = 4$). In contrast, Scatchard transformations in the presence of nifedipine, a dihydropyridine antagonist, indicated a competitive interaction in that K_d was lowered without a significant change in the number of binding sites (control values: K_d 0.029 ± 0.008 nM, B_{max} 221 ± 20 fmol mg^{-1} ; 0.17 ± 0.2 nM, 196 ± 6 fmol mg^{-1} in the presence of 0.5 nM nifedipine, $n = 4$).

The effect of fluspirilene on the dissociation rate of the PN 200-110: Ca channel complex is shown in Figure 5, which is a representative experiment from four similar experiments. Dissociation was induced at steady-state by the addition of 1 μM nitrendipine. The dissociation rate was increased from

Table 1 Binding affinities of calcium channel antagonists and activators at the [^3H]-PN 200-110 binding site in rat cerebral cortex at 25°C

Drug	pK_i	% maximal inhibition of specific binding	nH
Nifedipine	8.97 ± 0.25	100	0.88 ± 0.1
Nitrendipine	9.67 ± 0.15	100	0.94 ± 0.04
(+)-PN 200-110	10.1 ± 0.04	100	1.06 ± 0.1
(-)-PN 200-110	7.92 ± 0.02	110	1.14 ± 0.12
Bay K 8644	7.39 ± 0.12	100	0.96 ± 0.04
CGP 28392	6.65 ± 0.03	100	1.03 ± 0.11
Pimozone	7.72 ± 0.13	100	0.92 ± 0.02
IC_{50} (μM)			
Fluspirilene	0.03 ± 0.001	100	0.66 ± 0.09
Lidoflazine	0.54 ± 0.14	90	0.84 ± 0.02
Flunarizine	1.18 ± 0.24	92	1.28 ± 0.08
Verapamil	0.03 ± 0.009	41	0.92 ± 0.06
D- <i>cis</i> -Diltiazem	0.04 ± 0.013	35	1.00 ± 0.04
L- <i>cis</i> -Diltiazem		Not effective	

Values represent the means of 2–4 experiments performed in duplicate at a ligand concentration of 0.03–0.05 nM.

pK_i values (where appropriate) were derived from the equation $K_i = IC_{50}/[1 + (L/K_d)]$ where L represents the free concentration of [^3H]-PN 200-110, and K_d represents the equilibrium dissociation constant. nH —Hill slope.

Figure 5 The effect of calcium antagonists on the dissociation rate of the $[^3\text{H}]\text{-PN 200-110-Ca}^{2+}$ -channel complex. Data are shown as linear transformation, where B_0 is specific $[^3\text{H}]\text{-PN 200-110}$ bound at equilibrium, and B_t is that at time t . Dissociation was initiated at steady-state by the addition of $1\text{ }\mu\text{M}$ nitrendipine in the absence, control ($\bullet$), and presence of $1\text{ }\mu\text{M}$ fluspirilene ($\blacktriangle$), $10\text{ }\mu\text{M}$ verapamil ($\circ$), $10\text{ }\mu\text{M}$ lidoflazine (∇) or 10 nM nifedipine ($\blacksquare$).

$0.078 \pm 0.0009\text{ min}^{-1}$ in the absence of fluspirilene to $0.014 \pm 0.001\text{ min}^{-1}$ in the presence of $1\text{ }\mu\text{M}$ fluspirilene.

An increase in the dissociation rate was also observed with verapamil $10\text{ }\mu\text{M}$ ($0.017 \pm 0.002\text{ min}^{-1}$), lidoflazine $10\text{ }\mu\text{M}$ ($0.016 \pm 0.003\text{ min}^{-1}$) and flunarizine $10\text{ }\mu\text{M}$ (data not shown). No significant change was observed in the presence of nifedipine 10 nM (0.0086 min^{-1}). These findings indicate that fluspirilene, by an allosteric interaction with the Ca^{2+} channel, increases the rate of dissociation of the dihydropyridine ligand.

Discussion

In this paper we have shown that fluspirilene has weak antagonistic effects on Ca^{2+} -induced contractions in K^{+} -depolarized smooth muscle, but only at much higher concentrations than are required to interact with binding sites in the putative Ca^{2+} channels in skeletal muscle (Galizzi *et al.*, 1986; Qar *et al.*, 1987). However, fluspirilene was much more potent (threshold 1 nM) in antagonising the effects of Bay K 8644 and at 10 nM abolished the augmentation of Ca^{2+} -induced contractions seen with CGP 28392. CGP 28392 is a less powerful activator of calcium channels than Bay K 8644 and these weak effects were more readily antagonised by fluspirilene. At concentrations which abolished the effects of CGP 28392, fluspirilene did not significantly affect the contractile responses to Ca^{2+} or the antagonistic effects of nitrendipine, indicating that the drug selectively antagonised the activator, but not the antagonistic effects of dihydropyridines.

How could this effect come about? A simple explanation would be that fluspirilene prevents the binding of Bay K 8644 but not of other dihydropyridines. However, it would appear likely that activator and antagonist dihydropyridines bind to a single site and can stabilize different states of the Ca^{2+} channel, with evidence from electrophysiological studies (e.g. Sanguinetti *et al.*, 1986) and from conformational energy calculations (Mahmoudian & Richards, 1986; Holtje & Marrer, 1987; see Janis *et al.*, 1987). The high affinity binding of $[^3\text{H}]\text{-Bay K 8644}$ to cell membranes that has been obtained by several groups (Bellemann, 1984; Lee *et al.*, 1987; Maan & Hosey, 1987; Sarmiento *et al.*, 1987) appears to correlate with 'antagonist' binding to a state of the channel corresponding to the inactivated state, i.e. there is competition for a single site between all the dihydropyridines tested. However, Maan & Hosey (1987) considered that activator binding was enthalpy driven and antagonist binding was entropy driven. Unfortunately, as yet, there is no clear method of investigating acti-

vated states of the channel in radioligand binding experiments and we do not consider that Bay K 8644 binding labels the activated form of the channel. We used $[^3\text{H}]\text{-PN-200-110}$ to label the channel, as it has high affinity and labels a large number of channels (high B_{max}) due to its high affinity for the inactivated state (Glossmann *et al.*, 1985). Saturation studies indicated a non-competitive interaction of fluspirilene with the $[^3\text{H}]\text{-PN-200-110}$ binding site. Fluspirilene increased the dissociation of the radioligand from its site, compatible with an allosteric interaction between the site for fluspirilene and the dihydropyridine binding site. Further studies indicated similar interactions if $[^3\text{H}]\text{-nitrendipine}$ was used as the radioligand (data not shown). Fluspirilene may therefore resemble other diphenylalkylamines such as flunarizine and lidoflazine, but not verapamil on the grounds that verapamil does not fully displace dihydropyridine binding (Glossmann *et al.*, 1985; Janis *et al.*, 1987) and the effects of verapamil are readily reversed by Bay K 8644 (Spedding & Berg, 1984; Su *et al.*, 1984; Spedding, 1985a). Thus, fluspirilene has affinity for a distinct site on the calcium channel, as assessed by dihydropyridine binding, but we have no evidence for a selective inhibition of 'activator' binding by the drug. Indeed the weak inhibitory effects of CGP 28392 in the presence of fluspirilene may point to binding being unaffected, but the activator effects being selectively antagonised.

The conclusion from these results is that the site for fluspirilene is only weakly linked to the dihydropyridine site, but that binding of fluspirilene changes the channel such that the ability of activators to be effective is suppressed, whereas the effects of antagonists are less affected.

The low Hill slope observed in the $[^3\text{H}]\text{-PN-200-110}$ displacement studies indicates that the EC_{50} for fluspirilene can only be used as an empirical estimate of potency, even though fluspirilene was considerably more potent than the other diphenylalkylamines tested. In this respect, it may be considered as a prototypical agent for this class of drugs. Thus, the selective antagonism of Bay K 8644 does indicate that the site of these drugs is, if not at the dihydropyridine site, at least tightly coupled to the calcium channel. Flunarizine and lidoflazine have also been shown to be effective antagonists of the effects of Bay K 8644 (Spedding & Berg, 1984; Boddeke *et al.*, 1988; Patmore & Duncan, 1988), but the present study is the first to show that certain concentrations may selectively antagonise the effects of calcium channel activators.

However, despite the high affinity of fluspirilene for a site in the Ca^{2+} channel, in preliminary experiments we did not observe specific binding of $[^3\text{H}]\text{-fluspirilene}$ to rat cerebral cortex membranes under a range of experimental conditions,

presumably because of high non-specific binding as demonstrated by Galizzi *et al.* (1986). These authors only found specific binding in skeletal muscle, where the high density of sites increases the signal to noise ratio. Further progress in this field with radioligand binding techniques will require conditions that allow stabilization of the activated state of the channels and demonstrable high affinity binding by [³H]-fluspirilene in all relevant tissues.

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