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Neuroleptics of the diphenylbutylpiperidine series are potent calcium channel inhibitors

(fluspirilene/desmethoxyverapamil/binding/voltage-clamp/skeletal muscle)

JEAN-PIERRE GALIZZI, MICHEL FOSSET, GEORGES ROMEY, PIERRE LADURON*, AND MICHEL LAZDUNSKI

Centre de Biochimie du Centre National de la Recherche Scientifique, Parc Valrose, 06034 Nice Cedex, France

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ABSTRACT **[^{3}H]Fluspirilene, a neuroleptic molecule of the diphenylbutylpiperidine series, binds to skeletal muscle transverse tubule membranes with a high affinity corresponding to a K_d of 0.11 ± 0.04 nM. A 1:1 stoichiometry was found between [^{3}H]fluspirilene binding and the binding of (−)-[^{3}H]desmethoxyverapamil {(−)[^{3}H]D888}, one of the most potent Ca^{2+} channel inhibitors. Ca^{2+} channel inhibitors such as D888, verapamil, gallopamil, bepridil, or diltiazem antagonize [^{3}H]fluspirilene binding besides antagonizing (−)[^{3}H]-D888 binding. Neuroleptics, especially those of the diphenylbutylpiperidine family, also antagonize both (−)[^{3}H]D888 binding and [^{3}H]fluspirilene binding. There is an excellent correlation between affinities found from [^{3}H]fluspirilene binding experiments and those found from (−)[^{3}H]D888 binding experiments. Analysis of the properties of these cross-inhibitions indicates that [^{3}H]fluspirilene binds to a site that is not identical to that for phenylalkylamine derivatives (gallopamil, verapamil, diltiazem, and bepridil). Voltage-clamp experiments have shown that fluspirilene is an efficient inhibitor of the voltage dependent Ca^{2+} channel, achieving a half-maximal effect near 0.1–0.2 nM and nearly complete blockade at 1 nM. Fluspirilene blockade has little voltage dependence.**

The different types of voltage-dependent Ca^{2+} channels in neurons and in cardiac and skeletal muscle cells vary in biophysical and pharmacological properties (1–4). The type of voltage-dependent Ca^{2+} channels that has been most studied is that inhibited by 1,4-dihydropyridines such as nitrendipine and also by phenylalkylamines [such as desmethoxyverapamil (D888) and verapamil], diltiazem, and bepridil (5–7). The transverse-tubular (T-tubule) membrane of skeletal muscle (8, 9), is an excellent source for the biochemical isolation of Ca^{2+} channels, containing large amounts of 1,4-dihydropyridine and phenylalkylamine receptors (5); Ca^{2+} channel protein has now been purified from skeletal muscle T-tubule membranes, and its subunit structure has been determined (10, 11).

Neuroleptic drugs [phenothiazines, butyrophenones, diphenylbutylpiperidines (DPBPs), etc.] are a group of chemically diverse molecules that have the ability to relieve schizophrenic symptoms. The DPBP class includes molecules such as fluspirilene, penfluridol, and pimozide. Fluspirilene and penfluridol, for example, are effective long-acting neuroleptics particularly useful in the maintenance therapy of schizophrenic patients (12). Recent work (13, 14) has shown that this class of molecules inhibits [^{3}H]nitrendipine binding to rat brain and heart microsomes and has suggested that these antipsychotic drugs may have inhibitory properties at the Ca^{2+} channel (13).

We have studied the properties of the DPBP receptor in skeletal muscle T-tubule membranes with the ligand [^{3}H]fluspirilene and directly demonstrate that molecules in the DPBP series are among the most potent inhibitors of the skeletal muscle Ca^{2+} channel.

MATERIALS AND METHODS

Materials. (−)[^{3}H]D888 (82 Ci/mmol; 1 Ci = 37 GBq) and the 1,4-dihydropyridine (+)[^{3}H]PN 200-110 (80 Ci/mmol) were obtained from Amersham; (−)D888, verapamil, and gallopamil were from Knoll AG (Ludwigshafen, F.R.G.); bepridil and the bepridil analogs 4205 {1-[(1-propynyl)-cyclohexyloxy]-2-(1-pyrrolidinyl)-3-[(2-methyl)propoxy]propane chlorhydrate} and 11787 [*N*-(4-chloro)phenylmethyl-*N*-phenyl-(*β*-propoxymethyl)-1-morpholineethaneamine hydrochloride] were from CERM (Riom, France); *d-cis-* and *l-cis*-diltiazem were from Synthelabo (Paris). [^{3}H]Fluspirilene (8.9 Ci/mmol), fluspirilene, and the fluspirilene analogs 6728 {8-[5,5-bis(*p*-fluorophenyl)pentyl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one}, 6798 {8-[4,4-bis(*p*-methylphenyl)-butyl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one}, 6655 {8-[4-(phenyl),4-(*p*-methoxyphenyl)butyl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one}, and 5573 {8-[4,4-bis(phenyl)-propyl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one} as well as penfluridol, pimozide, clopimozide, and domperidone, were gifts from Janssen Pharmaceutica (Beerse, Belgium).

Preparation of Membranes from Rabbit White Skeletal Muscle. T-tubule membranes were prepared from rabbit white skeletal muscle as previously described (8, 15). Protein concentrations were assayed as described (9).

Standard [^{3}H]Fluspirilene Equilibrium Binding Assays. Samples of membranes (0.7 μg/ml) were incubated at 25°C in 7 ml of a solution containing 20 mM Hepes/NaOH buffer at pH 7.5, 0.01% bovine serum albumin, and the various [^{3}H]fluspirilene concentrations. In all binding experiments [^{3}H]fluspirilene was added last. In standard equilibrium binding experiments, incubation lasted 45–60 min. Incubations were stopped by rapid filtration of 4.5-ml samples as described (16). Experiments were done in duplicate. Two-milliliter samples of the incubation mixture were measured for [^{3}H]fluspirilene concentration. Stock solutions were diluted in the presence of 0.1% bovine serum albumin.

(−)[^{3}H]D888, [^{3}H]nitrendipine, and [^{3}H]PN 200-110 Binding Assays. Assays were done using previously described techniques (refs. 9, 8, and 17, respectively).

Cell Cultures. Primary cultures of thigh muscle of newborn rats were prepared as previously described (18). Myoballs were obtained by adding colchicine (10 nM) to the culture medium when cells had reached the early myotubular stage of development (18).

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Abbreviations: DPBP, diphenylbutylpiperidine; D888, desmethoxyverapamil; T-tubules, transverse tubules.

*Permanent address: Janssen Pharmaceutica, Beerse, Belgium.

Electrophysiological Measurements. Voltage-clamp experiments were done at room temperature (20–22°C) on rat myoballs in the whole-cell configuration as previously described (19, 20).

RESULTS

Equilibrium Binding of [³H]Fluspirilene to T-Tubule Membranes. Fig. 1 shows a typical equilibrium binding experiment of [³H]fluspirilene to skeletal muscle T-tubule membranes in the absence (total binding) and in the presence of 0.1 μM unlabeled fluspirilene (nonspecific binding). A Scatchard plot for the specific binding component (Fig. 1, *Inset A*) shows that [³H]fluspirilene specifically binds to a single type of site. The corresponding K_d is 0.11 ± 0.04 nM, and the maximal binding capacity (B_{max}) is 80 ± 15 pmol/mg of protein. Specific [³H]fluspirilene binding to T-tubule membranes was inhibited by increasing concentrations of unlabeled fluspirilene (Fig. 1 *Inset B*). The concentration (0.15 nM) found for half-maximal inhibition, $K_{0.5}$, is equivalent to the true K_d.

Effect of Unlabeled Neuroleptics and Calcium Channel Inhibitors on [³H]Fluspirilene Binding to T-Tubule Membranes. Relative inhibitory effects of neuroleptic molecules on the specific binding of [³H]fluspirilene to T-tubule membranes are illustrated in Fig. 2*A*. K_d values for penfluridol, clopimozide, and pimozide were in the range of 0.25–0.9 nM (Fig. 2*A* and Table 1). Neuroleptics that do not belong to the DPBP class, such as trifluoperazine, chlorpromazine, haloperidol, and the dopamine receptor antagonist domperidone have a higher K_d value, around 350–900 nM. Fig. 2*B* shows the inhibition of specific [³H]fluspirilene binding by Ca^{2+} channel inhibitors such as (−)D888, verapamil, bepridil, *d-cis-* and *l-cis*-diltiazem. These molecules inhibit [³H]fluspirilene binding by >95%. Concentrations for $K_{0.5}$ are between 6 and 1500 nM (Table 1). The rank order of potency was as follows: (−)D888 ($K_{0.5}$ = 6 nM) > verapamil ($K_{0.5}$ = 50 nM) > bepridil ($K_{0.5}$ = 60 nM) > *d-cis*-diltiazem ($K_{0.5}$ = 200 nM) > *l-cis*-diltiazem ($K_{0.5}$ = 1500 nM).

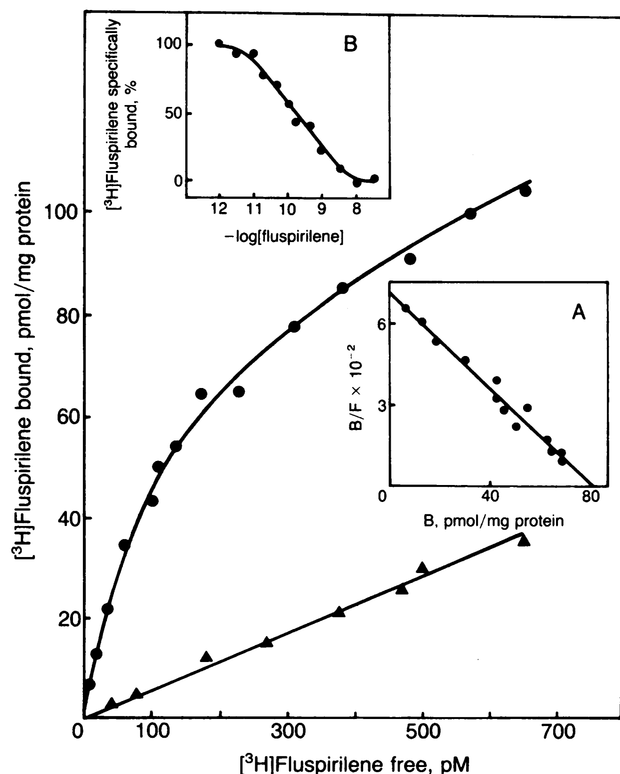

FIG. 1. Equilibrium binding of [³H]fluspirilene to T-tubule membranes. Equilibrium binding was measured using increasing concentrations of [³H]fluspirilene and membrane protein at 0.7 μg/ml (pH 7.5, 25°C). Binding of [³H]fluspirilene to T-tubule membranes in the absence (●) or in the presence (▲) of 0.1 μM fluspirilene. (*Inset A*) Scatchard plot for the specific [³H]fluspirilene binding component. (*Inset B*) Inhibition of specific [³H]fluspirilene (0.06 nM) binding by increasing concentrations of unlabeled fluspirilene. *B*, bound, and *F*, free, in pM.

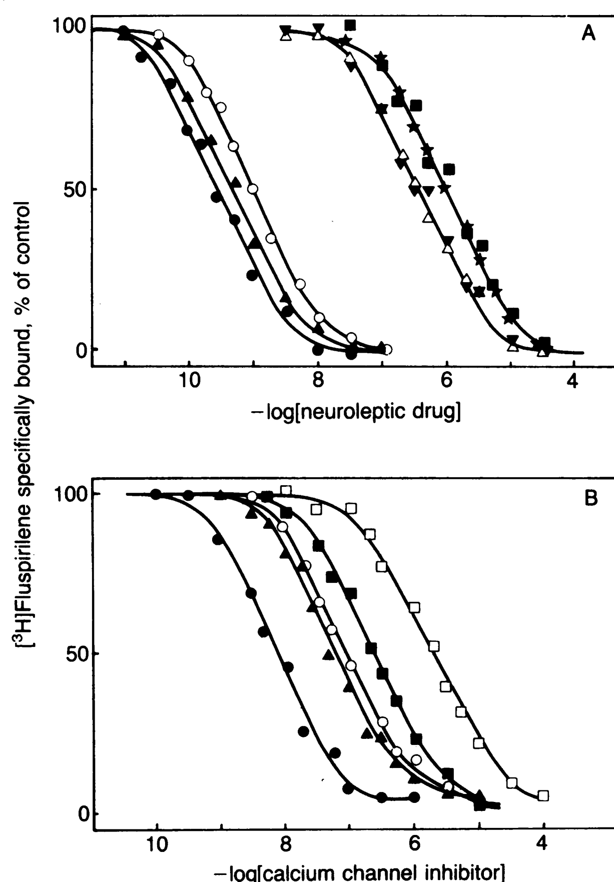

FIG. 2. Effects of neuroleptics and of calcium channel inhibitors on [³H]fluspirilene binding to T-tubule membranes at equilibrium. Binding of [³H]fluspirilene to T-tubule membranes was done under equilibrium conditions as described in the legend of Fig. 1*B*. (*A*) Specific binding of [³H]fluspirilene (0.06 nM) measured in the presence of increasing concentrations of penfluridol (●), clopimozide (▲), pimozide (○), trifluoperazine (△), chlorpromazine (▼), haloperidol (★), and domperidone (■). (*B*) Specific binding of [³H]fluspirilene measured in the presence of increasing concentrations of calcium channel inhibitors: (−)D888 (●), (±)-verapamil (▲), (±)-bepridil (○), *d-cis*-diltiazem (■), *l-cis*-diltiazem (□). Nonspecific binding represents 15% of total binding (not shown).

Effect of Unlabeled Calcium Channel Inhibitors and of Molecules of the DPBP Family on (−)[³H]D888 Binding to T-Tubule Membranes at Equilibrium. Scatchard plots for specific (−)[³H]D888 binding (Fig. 3, *Inset*) are consistent with the existence of a single class of sites with a K_d of 2 nM and a B_{max} of 83 ± 10 pmol/mg of protein. Molecules belonging to the DPBP class inhibited (−)[³H]D888 binding with $K_{0.5}$ values between 0.4 and 2 nM (Fig. 3 and Table 1). The order of potency of these molecules for the inhibition of (−)[³H]D888 binding ($K_{0.5}$) is in close correlation with their order of potency in the inhibition of [³H]fluspirilene binding (K_d values) (Table 1 and Fig. 4*A*): fluspirilene (K_d = 0.15 nM, $K_{0.5}$ = 0.4 nM) > penfluridol (K_d = 0.25 nM, $K_{0.5}$ = 0.9 nM) > clopimozide (K_d = 0.35 nM, $K_{0.5}$ = 0.9 nM) > pimozide (K_d = 0.9 nM, $K_{0.5}$ = 2 nM). (−)[³H]D888 binding was also inhibited by the dopamine receptor antagonist domperidone ($K_{0.5}$ = 1000 nM) and by neuroleptics such as haloperidol

Table 1. Inhibitors of (−)[^{3}H]D888 and [^{3}H]fluspirilene binding to T-tubule membranes

Compound	(−)[^{3}H]D888 $K_{0.5}$, nM	(−)[^{3}H]D888 K_d,* nM	[^{3}H]Fluspirilene $K_{0.5}$, nM	[^{3}H]Fluspirilene K_d, nM
Phenylalkylamines				
Verapamil		20	50	
(−)D888		2	6	
Gallopamil		40	100	
Bepridils				
Bepridil		20	60	
4205		100	250	
11787		2800	3500	
Benzothiazepines				
d-cis-diltiazem		60	200	
l-cis-diltiazem		900	1500	
DPBPs				
Fluspirilene	0.4			0.15
6728	4			1
6798	10			3
6655	5			5.5
5573	50			40
Penfluridol	0.9			0.25
Clopimozide	0.9			0.35
Pimozide	2			0.9
Butyrophenones				
Haloperidol	1000			900
Phenothiazines				
Trifluoperazine	630			350
Chlorpromazine	400			350
Domperidone	1000			900

K_d, the equilibrium dissociation constant, was obtained for neuroleptic compounds by inhibition of [^{3}H]fluspirilene binding and for calcium channel inhibitors by inhibition of (−)[^{3}H]D888 binding. $K_{0.5}$, the half-maximal inhibition concentration, was obtained for neuroleptic drugs by inhibition of (−)[^{3}H]D888 binding and for calcium channel inhibitors by inhibition of [^{3}H]fluspirilene binding. *Values for the inhibition of (−)[^{3}H]D888 are obtained under the same equilibrium conditions described for Fig. 3.

($K_{0.5}$ = 1000 nM), trifluoperazine ($K_{0.5}$ = 630 nM) and chlorpromazine ($K_{0.5}$ = 400 nM) (Fig. 3). Fluspirilene induces a decrease of the B_{max} value for (−)[^{3}H]D888 binding with no significant change in the K_d (2.0 ± 0.5 nM) (Fig. 3 *Inset*).

Verapamil, gallopamil, bepridil, and diltiazem are known to be competitive inhibitors of (−)[^{3}H]D888 binding to T-tubule membranes (9, 16). The order of potency of these calcium channel inhibitors to inhibit (−)[^{3}H]D888 binding (K_d) is also in good correlation with their order of potency (Table 1) in the inhibition of [^{3}H]fluspirilene binding ($K_{0.5}$) (Fig. 4*B*).

Comparative Electrophysiological Analysis of the Effects of Fluspirilene and (−)D888 on Rat Skeletal Muscle Cells (Myoballs) in Culture. Voltage-clamp experiments on rat skeletal myoballs have revealed two pharmacologically distinct types of Ca^{2+} channels (4, 20). This study was restricted to Ca^{2+} channels that are inhibited by 1,4-dihydropyridines. The blocking effects of (−)D888 and fluspirilene were investigated with two experimental protocols. In the first protocol (I) depolarizing test pulses of increasing amplitude were applied from a holding potential of −80 mV before and after the external application of the compounds. In the second protocol (II) the possible voltage dependence of the drug effect was investigated by exposing the myoball to a fixed concentration of (−)D888 or fluspirilene at a holding potential of −80 mV until a steady-state effect on the current was reached. Next, the membrane potential was held for 3 min at −40 mV in order to expose the inactivated state of the Ca^{2+} channel to the drug, and then the membrane potential was returned to −80 mV for 2 min to return the channel to its resting state before the test pulse. Fig. 5*A* shows that 1 μM (−)D888 blocked about 70% of the Ca^{2+} current under protocol I and almost 100% of the current with protocol II. Under the two protocols, fluspirilene was a more potent blocker of the Ca^{2+} channel than (−)D888, since it blocked about 40% of the Ca^{2+} current at 0.1 nM (Fig. 5 *B* and *D*) and over 80% at 1 nM (Fig. 5 *C* and *D*). Comparative current-voltage relationships for the peak Ca^{2+} current before and after application of 0.1 nM and 1 nM fluspirilene are presented in Fig. 5*D*. The degree of Ca^{2+} current block is almost independent of the test pulse amplitude.

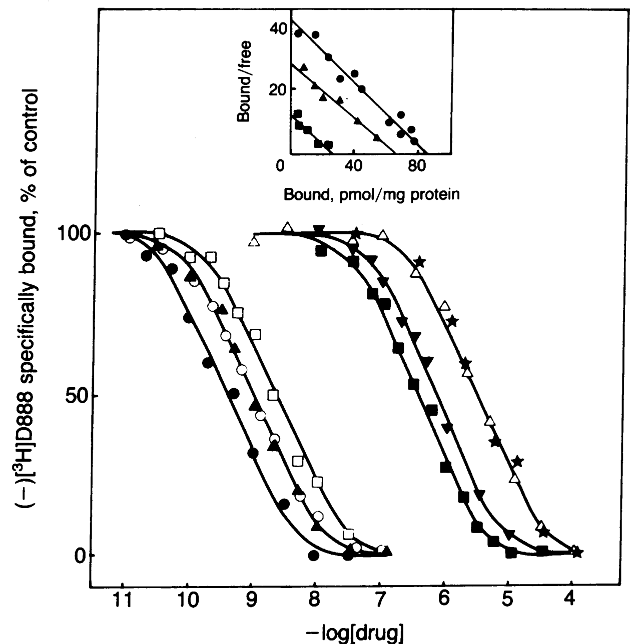

FIG. 3. Inhibitory effects of neuroleptics and related compounds on (−)[^{3}H]D888 binding to T-tubule membranes at equilibrium. Specific equilibrium binding of (−)[^{3}H]D888 (0.3 nM) to T-tubule membranes (8 μg of protein per ml) (pH 7.5, 25°C) with increasing concentrations of neuroleptic drugs: fluspirilene (●), penfluridol (▲), clopimozide (○), pimozide (□), chlorpromazine (■), trifluoperazine (▼), haloperidol (△), and domperidone (★). Nonspecific binding represents 10% of total binding (not shown). (*Inset*) Scatchard plots of equilibrium data for specific (−)[^{3}H]D888 binding in the absence (●) or presence of 0.12 nM (▲) or 1.2 nM (■) fluspirilene.

DISCUSSION

This report describes the identification of T-tubule membrane receptors for DPBP neuroleptics by using [^{3}H]fluspirilene. The two salient properties of [^{3}H]fluspirilene binding to T-tubule membranes are (*i*) a high affinity for DPBP receptors indicated by a K_d value of 0.11 ± 0.04 nM, and (*ii*) a large number of receptors, corresponding to a B_{max} value of 80 ± 15 pmol/mg of protein. Two kinds of results immediately associate the DPBP receptor with the voltage-dependent Ca^{2+} channel: (*i*) fluspirilene prevents the binding of (−)[^{3}H]D888, and (−)D888 prevents [^{3}H]fluspirilene binding to the DPBP receptor; (*ii*) binding stoichiometries of [^{3}H]fluspirilene and (−)[^{3}H]D888 are nearly identical.

A more detailed analysis of the inhibitory effect of fluspirilene on (−)[^{3}H]D888 binding revealed that the inhibition was noncompetitive and that consequently the phenylalkylamine receptor for (−)D888 and the DPBP receptors are probably two distinct types of receptors. In skeletal muscle T-tubules, DPBP molecules inhibit the 1,4-dihydropyridines [^{3}H]nitrendipine and (+)[^{3}H]PN 200-110 binding in a non-

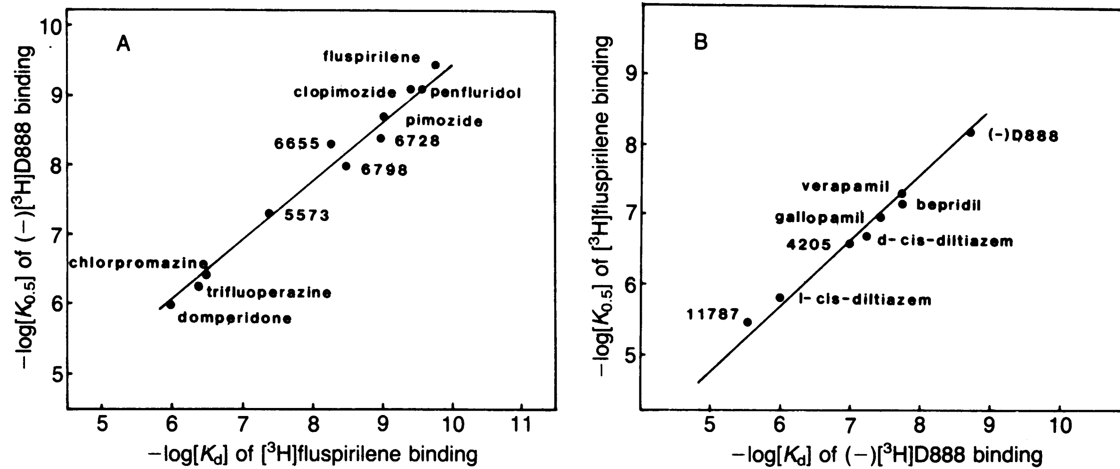

FIG. 4. Correlation between (−)[^{3}H]D888 and [^{3}H]fluspirilene binding inhibition by neuroleptic or calcium antagonist drugs. Half-maximal inhibition concentrations ($K_{0.5}$) or K_d were reported from Table 1. (*A*) −log[$K_{0.5}$] (for inhibition of (−)[^{3}H]D888 binding by neuroleptic drugs) was plotted versus −log[K_d] (inhibition of [^{3}H]fluspirilene binding). The plot has a slope of 0.87 and a correlation coefficient r = 0.99. (*B*) −log[$K_{0.5}$] for the inhibition of [^{3}H]fluspirilene binding by calcium channel inhibitors was plotted versus −log[K_d] (inhibition of (−)[^{3}H]D888 binding). The plot has a slope of 0.93 and a correlation coefficient r = 1.0.

competitive way ($K_{0.5}$ = 300 nM) (not shown); this noncompetitive interaction has been previously reported for rat brain membranes (13). At concentrations that saturate the DPBP receptor (1–10 nM) fluspirilene only inhibited (+)[^{3}H]PN 200-110 and [^{3}H]nitrendipine binding by <20%. Maximal binding capacities for both [^{3}H]fluspirilene and the 1,4-dihydropyridine (+)[^{3}H]PN 200-110 (17) were 85 ± 15 pmol/mg of protein. All these results strongly suggest that there are three different types of receptors on the Ca^{2+} channel protein: one for 1,4-dihydropyridine, one for phenylalkylamines, and one for DPBP molecules. The stoichiometry of the three receptors is 1:1:1.

In another group of experiments a series of neuroleptics has been tested for capacity to prevent the binding of (−)[^{3}H]D888 and [^{3}H]fluspirilene at the respective receptor sites. The correlation (r = 0.99) between binding constants in the two assays was very high, showing that compounds that are less potent in inhibiting (−)[^{3}H]D888 binding are also less potent in inhibiting [^{3}H]fluspirilene binding (Fig. 4*A*). Neuroleptics of the phenothiazine and butyrophenone series proved to be much less active inhibitors of (−)[^{3}H]D888 and [^{3}H]fluspirilene binding ($K_{0.5}$ and K_d values of 350–900 nM) (Fig. 2*A*, Fig. 3, and Table 1) than DPBP neuroleptics. These affinities are lower by a factor of 300 to 1000 than those found

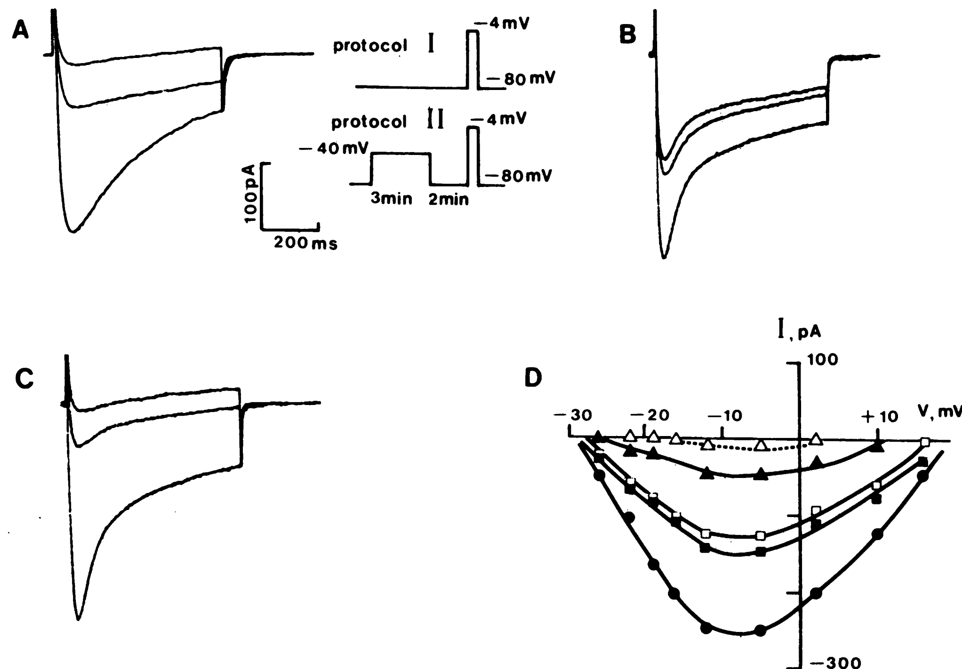

FIG. 5. Blocking effects of (−)D888 and fluspirilene on Ca^{2+} channels of rat myoballs. The external solution contained 2.5 mM Ba^{2+}. (*A–C*) Ca^{2+} currents associated with a step depolarization to −4 mV from a holding potential V_H = −80 mV. Lower traces: control current. Middle traces: steady-state effect of 1 μM D888 (*A*), 0.1 nM fluspirilene (*B*) and 1 nM fluspirilene (*C*) (protocol I). Upper traces: after the membrane potential was maintained to −40 mV for 3 min and returned to −80 mV for 20 min (protocol II). (*D*) Peak Ca^{2+} current–membrane potential relationships: control current (●), steady-state effects of fluspirilene (■, ▲) after a 3-min depolarization to −40 mV (□, △). Fluspirilene concentrations are 0.1 nM (■, □) and 1 nM (▲, △).

for the same compounds for the D_2-dopamine receptor in brain membranes (K_d = 1–10 nM) (13, 21).

A series of well-established Ca^{2+} channel inhibitors have also been tested for their inhibitory effect on [^{3}H]fluspirilene and (−)[^{3}H]D888 binding. Again the Ca^{2+} channel inhibitors that are less potent in inhibiting (−)[^{3}H]D888 binding are also less potent in inhibiting [^{3}H]fluspirilene binding (r = 1.0).

Electrophysiological experiments using the whole cell patch-clamp technique have definitively shown that fluspirilene, which is the most active DPBP molecule in binding assays, is a very effective Ca^{2+} channel blocker. Nearly complete block was obtained at 1 nM, and half-maximal inhibition was seen near 0.1–0.2 nM (Fig. 5 *B* and *C*), a concentration that is very close to the K_d found for [^{3}H]fluspirilene binding to its receptor site. (−)D888 is a much less potent Ca^{2+} channel inhibitor; concentrations higher than 1 μM are necessary to block Ca^{2+} channel activity. Ca^{2+} channel blockade by fluspirilene was little affected by variations of the membrane potential. Conversely, blockade of the Ca^{2+} channel by the most potent 1,4-dihydropyridine (+)PN 200-110 has been shown to be extremely voltage dependent (20). The K_d of the (+)PN 200-110–Ca^{2+} channel complex when the Ca^{2+} channel is in its resting state is 13 nM (20). Clearly, fluspirilene is also more active than (+)PN 200-110 on muscle Ca^{2+} channels in the resting state.

The K_d values that have been found for fluspirilene in nerve, cardiac, and smooth muscle membranes are 35, 50, and 50 nM respectively, as compared with 0.11 nM for skeletal muscle membranes (unpublished data). This observation substantiates the hypothesis that different types of voltage-dependent Ca^{2+} channels exist in different tissues.

Fluspirilene and pimozide have a higher affinity for Ca^{2+} channels in skeletal muscle than for D_2-dopamine receptors (IC_{50} is 6.5–22 nM and 4–8 nM, respectively) in brain membranes (13, 22). One could easily envision that specific regions exist in the central nervous system containing Ca^{2+} channels that interact as efficiently with pimozide and fluspirilene as do Ca^{2+} channels in skeletal muscle. The relationship between Ca^{2+} channel blockade and the antischizophrenic activity of DPBP molecules is still unclear, because other neuroleptics not belonging to the DPBP family of drugs such as haloperidol, the archetype of neuroleptic drugs, appear decidedly less active on Ca^{2+} channels (Table 1, Fig. 2). The Ca^{2+} channel-blocking activity of DPBP molecules may be related to the activating and anti-anxiety properties of these drugs in psychovegetative disorders (23, 24). The efficacy of fluspirilene in blocking Ca^{2+} channels at very low concentrations can be paralleled with the very low doses of the molecule (1.5 mg per week) known to influence mental condition in the direction of anxiolysis, relaxation, and increased self-confidence (23).

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