Differential Inhibition of T-Type Calcium Channels by Neuroleptics

Celia M. Santi,[1] Francisco S. Cayabyab,[2] Kathy G. Sutton,[1] John E. McRory,[1] Janette Mezeyova,[2] Kevin S. Hamming,[1] David Parker,[2] Anthony Stea,[3] and Terrance P. Snutch[1]

[1]Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, Canada, V6T 1Z3, [2]NeuroMed Technologies, Vancouver, British Columbia, Canada, V6T 1Z4, and [3]University-College of the Fraser Valley, Abbotsford, British Columbia, Canada, V2S 7M8

T-type calcium channels play critical roles in cellular excitability and have been implicated in the pathogenesis of a variety of neurological disorders including epilepsy. Although there have been reports that certain neuroleptics that primarily target D_2 dopamine receptors and are used to treat psychoses may also interact with T-type Ca channels, there has been no systematic examination of this phenomenon. In the present paper we provide a detailed analysis of the effects of several widely used neuroleptic agents on a family of exogenously expressed neuronal T-type Ca channels (α_{1G}, $\alpha_{1H,}$ and α_{1I} subtypes). Among the neuroleptics tested, the diphenylbutylpiperidines pimozide and penfluridol were the most potent T-type channel blockers with K_d values (~30–50 nM and ~70–100 nM, respectively), in the range of their antagonism of the D_2 dopamine receptor. In contrast, the butyrophenone haloperidol was ~12- to 20-fold less potent at blocking the various T-type Ca channels. The diphenyldiperazine flunarizine was also less potent compared with the diphenylbutylpiperadines and preferentially blocked α_{1G} and α_{1I} T-type channels compared with α_{1H}. The various neuroleptics did not significantly affect T-type channel activation or kinetic properties, although they shifted steady-state inactivation profiles to more negative values, indicating that these agents preferentially bind to channel inactivated states. Overall, our findings indicate that T-type Ca channels are potently blocked by a subset of neuroleptic agents and suggest that the action of these drugs on T-type Ca channels may significantly contribute to their therapeutic efficacy.

Key words: T-type; neuroleptics; cDNA; calcium channels; schizophrenia

The rapid entry of calcium into cells through voltage-dependent Ca channels triggers a variety of intracellular events such as muscle contraction, hormone secretion, synaptic transmission, and gene expression. Ca channels have been traditionally classified into high voltage-activated (HVA) and low voltage-activated (LVA) subtypes (Tsien et al., 1988; Bean, 1989). HVA Ca channels first activate at relatively depolarized potentials and comprise L-, P/Q-, N-, and R-types. These channels exhibit a high single-channel conductance and varied patterns of inactivation and deactivation. LVA Ca channels, also known as T-type, show a more negative range of activation and inactivation, rapid inactivation, slow deactivation, and smaller single-channel conductances. T-type Ca channels are present in a variety of cell types where they appear to mediate low-threshold spikes and rebound burst firing patterns (for review, see Huguenard, 1996), pacemaker activity (Hagiwara et al., 1988), hormone secretion (Rossier et al., 1996), cell growth and proliferation (Xu and Best, 1990), and are also involved in fertilization (Arnoult et al., 1996; Santi et al., 1996). Abnormal activity of T-type Ca channels has been implicated in the pathophysiology of epilepsy (Huguenard and Prince, 1994; Tsakiridou et al., 1995; Huguenard, 1996), hypertension (Self et al., 1994), and cardiac hypertrophy (Nuss and Houser, 1993). Furthermore, the activity of T-type channels may play a role in enlarging the area of damage produced by a transient ischemic insult (Ito et al., 1994).

Three different T-type Ca channels have been cloned and expressed from mammals: α_{1G}, $\alpha_{1H,}$ and α_{1I} (Cribbs et al., 1998; Perez-Reyes et al., 1998; Klugbauer et al., 1999; Lee et al., 1999; McRory et al., 2001). Although HVA Ca channels have a well defined pharmacology, it has proven difficult to identify specific high-affinity blockers of LVA Ca channels. Ni^{2+} and amiloride are often described as effective blockers of T-type channels in many native cells, although the effects of these agents is quite variable in different cell types (Huguenard, 1996; Todorovic and Lingle, 1998).

Neuroleptic agents comprise several chemically distinct classes of compounds (diphenylbutylpiperidines, butyrophenones, and phenothiazine) that act as antagonists of the D_2 dopamine receptors and are widely prescribed to treat a variety of psychiatric disorders (Seeman et al., 1976). Interestingly, many of these drugs also display Ca channel blocking activity, although their selectivity for the different Ca channel subtypes has not been systematically examined. Although some neuroleptics inhibit to some extent P-, N-, and L-type Ca channels, they appear to block T-type channels with relatively higher affinity than HVA Ca channels (Enyeart et al., 1990, 1993; Takahashi and Akaike, 1991; Sah and Bean, 1993). The diphenylbutylpiperidines (DPBPs) pimozide and penfluridol have been shown to block T-type currents in various cell types: heart (Enyeart et al., 1990), adrenal fasciculata (Enyeart et al., 1993), mouse spermatogenic (Arnoult et al., 1998), neural crest cells, and human C cell lines (Enyeart et al., 1992). Moreover, the neuroleptic-like agent flunarizine has

Received May 30, 2001; revised Oct. 3, 2001; accepted Oct. 12, 2001.

This work was supported by a grant from Canadian Institutes for Health Research (CIHR) (T.P.S.), fellowship support from the Human Frontiers Science Program (C.M.S.), from the CIHR (J.E.M.), and from the Natural Sciences and Engineering Research Council of Canada and Killam Foundation (K.S.C.H.), and a CIHR Senior Scientist Award (T.P.S.).

Correspondence should be addressed to Dr. Terrance P. Snutch, Biotechnology Laboratory, Room 237–6174, University Boulevard, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3. E-mail: snutch@zoology.ubc.ca.

 0270-6474/02/220396-08$15.00/0

also been reported to act both as a D_2 dopamine receptor antagonist and as blocker of Ca currents in the CNS where it acts as a potent organic blocker of the T-type currents from dissociated hypothalamic neurons (Akaike et al., 1989) as well as hypothalamic T-type currents expressed in *Xenopus* oocytes (Dzura et al., 1996).

In the present paper we explored the inhibitory effects of the neuroleptic agents pimozide, penfluridol, haloperidol, and flunarizine on three exogenously expressed neuronal T-type Ca channels. The results demonstrate that the DPBP class of neuroleptics are high-affinity T-type Ca channel blockers and suggest that Ca channel block may represent a significant contribution to the clinical efficacy of these agents.

MATERIALS AND METHODS

Cell culture. Stable cell lines expressing $\alpha_{1G,}$ $\alpha_{1H,}$ or α_{1I} were generated by transfecting either $\alpha_{1G,}$ $\alpha_{1H,}$ or α_{1I} cDNAs (all in pcDNA3.1 vector) into human embryonic kidney (HEK) tsa 201 cells using standard Ca-phosphate precipitation, and clones were selected with zeocin. In some cases standard Ca-phosphate precipitation was also used for transient cotransfection in HEK tsa 201 cells of α_{1I} (3 μg in pcDNA3.1 vector), CD8 (2 μg) marker plasmid, and 15 μg of pBluescript SK carrier DNA for a total of 20 μg of cDNA mix. Transiently transfected cells were selected for expression of CD8 by adherence of Dynabeads (Dynal, Great Neck, NY). The cells were grown in standard DMEM (10% fetal bovine serum and 50 U/ml penicillin–streptomycin), maintained at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2. Stably expressing cell lines were enzymatically dissociated with trypsin–EDTA and plated on 35 mm Petri dishes 12 hr before recordings. Functional transient expression of α_{1I} was evaluated 24 hr after transfection.

Electrophysiological recordings. Macroscopic currents were recorded using the whole-cell patch-clamp technique (Hamill et al., 1981). The external recording solution contained in mM: 2 $CaCl_2$ or 2 $BaCl_2$, 1 $MgCl_2$, 10 HEPES, 40 TEA Cl, 92 CsCl, and 10 glucose, pH 7.2. The internal pipette solution contained in mM: 105 CsCl, 25 TEA Cl, 1 $CaCl_2$, 11 EGTA, and 10 HEPES, pH 7.2. α_{1G} and α_{1I} currents were recorded in the presence of 2 mM external Ca. Because α_{1H} Ca currents are significantly smaller than Ba currents (McRory et al., 2001), we used Ba as a charge carrier for α_{1H} (Ca was also used where indicated). Whole-cell currents were recorded using an Axopatch 200B or 200A amplifier (Axon Instruments, Foster City, CA), controlled and monitored with a personal computer running pClamp software version 6.03 (Axon Instruments). Patch pipettes (borosilicate glass BF150-86-10; Sutter Instruments, Novato, CA), were pulled using a Sutter P-87 puller and fire-polished using a Narishige (Tokyo, Japan) microforge, with typical resistances of 2.5–4 MΩ (filled with internal solution). Series resistance was electronically compensated by at least 60%. Whole-cell currents that exceeded 2 nA were not examined, minimizing voltage error (<2–3 mV). Only cells exhibiting adequate voltage control [judged by smoothly rising current–voltage (I–V) relationship and monoexponential decay of capacitive currents] were included in the analysis. The bath was connected to ground via a 3 M KCl Agar bridge. All recordings were performed at room temperature (20–24°C).

Drugs effects were investigated using 150 msec steps to peak potentials every 15–30 sec from a holding potential of −100 mV. Current–voltage relations were measured by a series of 150-msec-long depolarizing pulses applied from a holding potential of −100 mV to membrane potential between −90 and +20 mV every 15 sec.

Data were low-pass filtered at 2 kHz using the built-in Bessel filter of the amplifier, and in most cases, subtraction of capacitance and leakage current was performed on-line using P/4 protocol. Recordings were analyzed using Clampfit 6.03 (Axon Instruments), and figures were generated using the software program Microcal Origin (version 3.78). Data from drug concentration–response studies were fitted with the equation $y = [(A_1 - A_2)/\{1 + (x/x_o)^P\}] + A_2$, where A_1 is initial (= 0) and A_2 final value, x_o is IC_{50} (concentration causing 50% inhibition of currents), and p gives a measure of steepness of curve.

Time courses of channel blockade were well described by single exponential curves from which τ_{on} and the fraction of unblocked channels at equilibrium ($a = I_{drug}/I_{control}$) were obtained. K_d values were estimated using the following equation:

$$K_d = a[\text{drug}]/(1-a).$$

The steady-state inactivation curves were constructed by plotting the normalized current during the test pulse as a function of the conditioning potential. The data were fitted with a Boltzmann equation: $I/I_{max} = \{1 + \exp[V - V_{0.5i}/k_i]\}^{-1}$, where I is the peak current, I_{max} is the peak current when the conditioning pulse was −120 mV, V and $V_{0.5i}$ are the conditioning potential and the half-inactivation potential respectively, and k_i is the inactivation slope factor.

Current–voltage relationships were fitted with the Boltzmann equation: $I = \{1/1 + \exp(V_m - V_{0.5a})/k_a)\}\{(V - E_{rev})G_{max}\}$, where V_m is the test potential, $V_{0.5a}$ is the half-activation potential, E_{rev} is the extrapolated reversal potential, G_{max} is the maximum slope conductance, and k_a reflects the slope of the activation curve.

Statistical significance was determined by Student's t test or one-way ANOVA followed by Bonferroni multiple comparison post-test, as appropriate, and significant values were set as indicated in the text and figure legends.

Solutions and drugs. Penfluridol was from Research Diagnostics Inc. (Flanders, NJ). All other drugs were purchased from Sigma (St. Louis, MO), unless otherwise specified. Test solutions containing drugs were prepared fresh for each experiment from concentrated stock solutions and added to the recording solution. Concentrated stock solutions were as follows: pimozide (1 mM in DMSO); penfluridol (500 μM in ethanol), haloperidol (1 mM in DMSO), and flunarizine (1 mM in DMSO). The highest concentration of ethanol and DMSO in the recording solution did not exceed 0.1%, a concentration that did not detectably affect calcium channel properties. The perfusion system consisted of a custom-made multiple solution perfusion manifold consisting of three input and three output capillary tubes (custom microfil, 28 gauge, 250 μm inner diameter and 350 μm outer diameter; World Precision Instruments, Sarasota, FL)

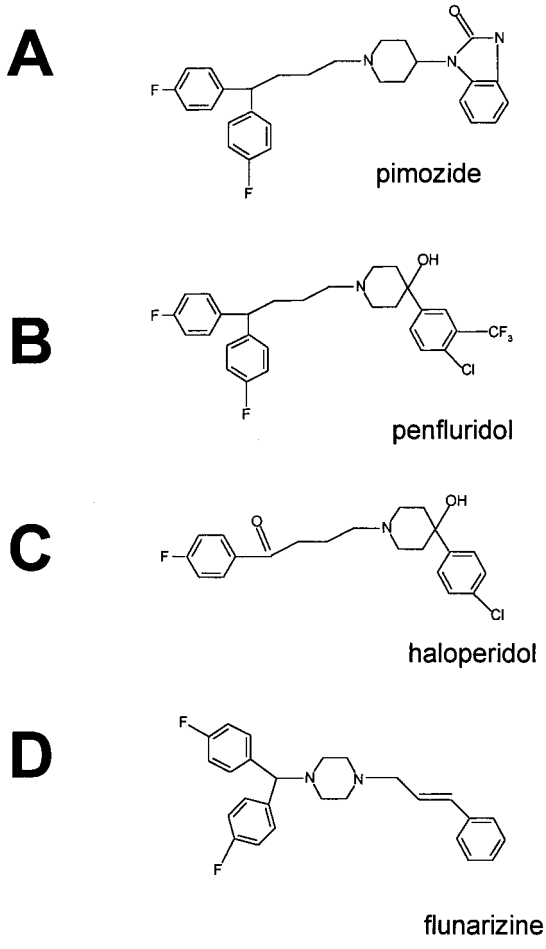

Figure 1. Chemical structures of the three different structural classes of neuroleptic agents used in this study: the DPBPs pimozide (*A*) and penfluridol (*B*). An example of a butyrophenone (*C*, haloperidol) and a diphenyldiperazine, flunarizine (*D*).

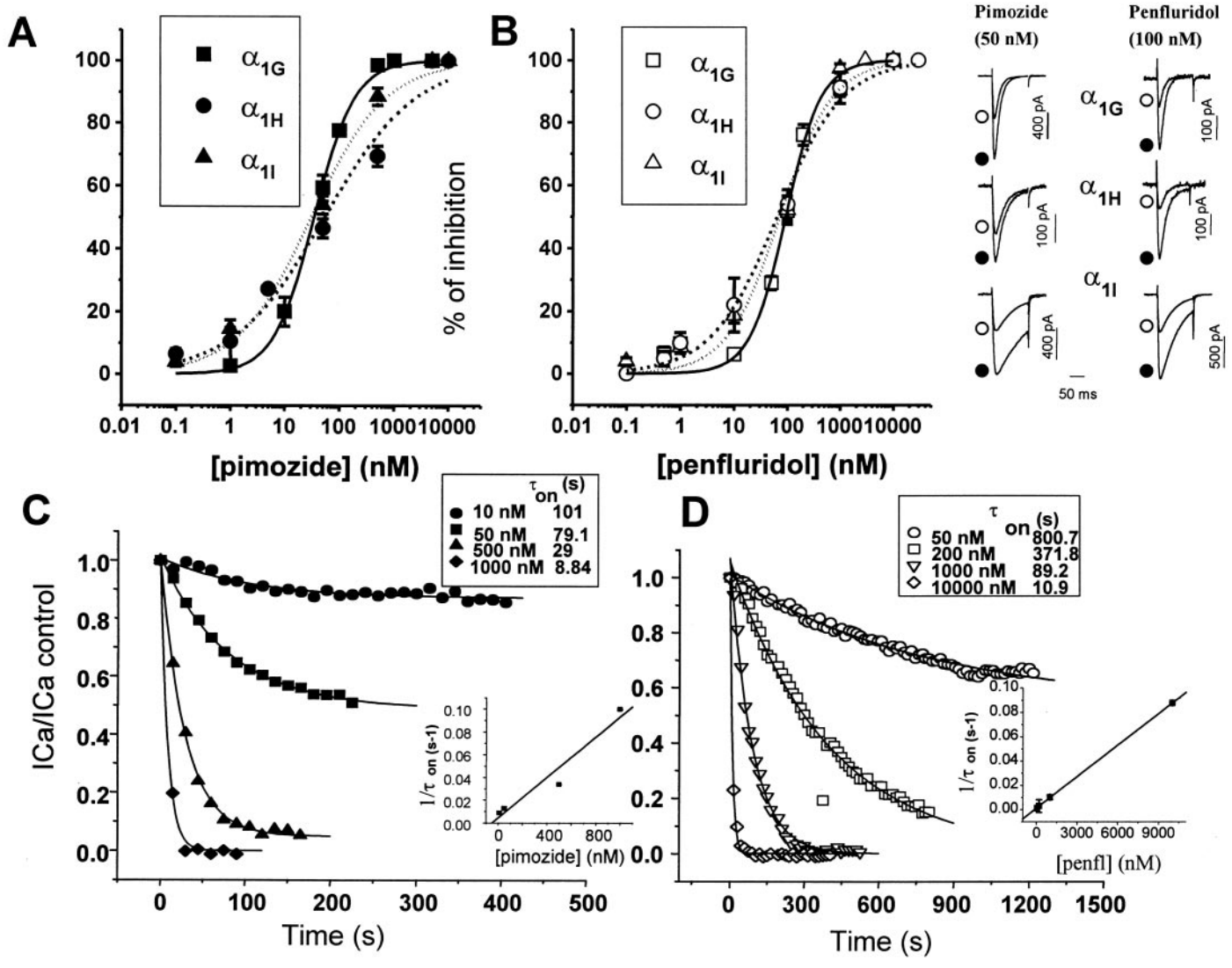

Figure 2. Summary of the effect of DPBPs on T-type calcium channels. *A, B,* Concentration–response curves for pimozide and penfluridol of α_{1G}, $\alpha_{1H,}$ and α_{1I} T-type channels. Data points reflect mean ± SE of two to six determinations. Half-maximal inhibition (IC_{50}) were determined from fitting the data as described in the methods. IC_{50} values for pimozide were 34.6, 53.5, and 30.4 nM, for α_{1G}, $\alpha_{1H,}$ and $\alpha_{1I,}$ respectively. IC_{50} values for penfluridol were 93.1, 64.1, and 71.6 nM, for α_{1G}, $\alpha_{1H,}$ and $\alpha_{1I,}$ respectively. *Inset*, Representative current traces in control (*filled circles*) and 5 min after addition of drugs (*open circles*). *C, D,* Time course of block of α_{1G} currents in eight different cells exposed to 10, 50, 500, and 1000 nM pimozide and 50, 200, 1000, and 10,000 nM penfluridol. Ca currents were evoked every 15 sec with 150 msec step depolarizations from −100 to −40 mV. *Solid lines,* Fits of single exponential decays to a plateau. *Insets,* Dependence of $1/\tau_{on}$ on [drug]. Values are mean and SE of three to five determinations. Solid line fit was made with the following equation: $1/\tau_{on} = k_{on}[\text{drug}] + k_{off}$, where $k_{on} = 0.00432$/sec, $k_{off} = 0.000088$/sec, and $K_d = 48.4$ nM for pimozide, and $k_{on} = 0.00117$/sec, $k_{off} = 0.0000087$/sec, and $K_d = 135.1$ nM for penfluridol.

ensheathed in a glass pipette. The lengths of lines from valve to the manifold were kept to a minimum, and the outputs of the manifold were placed within five cell lengths, resulting in cells being bathed with new solutions with minimal delay (within 1 sec) and minimal dead space volume.

RESULTS

Diphenylbutylpiperidines are high-affinity antagonists of T-type Ca channels

The DPBPs (e.g., pimozide, penfluridol) (Fig. 1*A,B*) are a class of neuroleptic drugs clinically used to relieve the symptoms of schizophrenia (Pinder et al., 1976). The DPBPs were initially identified as potential Ca channel antagonists when it was discovered that these agents inhibited dihydropyridine (DHP) binding to specific brain receptors and also inhibited depolarization-dependent Ca uptake and contraction in muscle (Gould et al., 1983). A number of reports have shown that DPBPs are relatively potent blockers of L-type Ca channels in endocrine (e.g., pituitary) and heart muscle cells (Enyeart et al., 1990). In some of these same preparations it has been observed that low concentrations of DPBPs also block T-type currents (Enyeart et al., 1990, 1992). While some DPBPs (e.g., pimozide) have been used to probe the physiological functions of T-type channels (Arnoult et al., 1998), it has not been established whether these agents are selective for particular subtypes of T-type channel. We tested the effect of two DPBPs, penfluridol and pimozide (Fig. 1*A,B*), on exogenously expressed α_{1G}, $\alpha_{1H,}$ and α_{1I} T-type Ca channels from rat brain (Fig. 2). Penfluridol and pimozide were not subtype-selective because both of these agents blocked the three neuronal T-type Ca channels to similar extents (Fig. 2, Table 1). A detailed analysis of the concentration–response relations of

Table 1. Differential affinity of three classes of neuroleptic agents for three cloned T-type channels

	K_d values (nM)		
	α1G	α1H	α1I
Penfluridol	109.8 ± 14.8 (n = 3)	72.1 ± 7.2 (n = 4)	95.1 ± 18.2 (n = 6)
Pimozide	43.5 ± 6.5 (n = 3)	57.7 ± 6.7 (n = 4)	39.2 ± 3.1 (n = 4)
Haloperidol	1163.3 ± 128.7 (n = 4)	1387.1 ± 70.8 (n = 4)	1259.9 ± 102 (n = 4)
Flunarizine	530.2 ± 107.9 (n = 3)	3551.7 ± 119 (n = 3)*	837.2 ± 59.03 (n = 3)

K_d values were obtained as described in Materials and Methods. Statistical significance was determined by one-way ANOVA followed by Bonferroni multiple comparison post-test. The K_d values were not significantly different except for flunarizine where $^*p < 0.01$ both for α_{1H} versus α_{1G} and α_{1H} versus α_{1I}.

pimozide and penfluridol with the three T-type Ca channels revealed a similar range of IC_{50} values in the case of pimozide (34.6, 53.5, and 30.4 nM for α_{1G}, $\alpha_{1H,}$ and α_{1I}, respectively) and IC_{50} values equal to 93.1, 64.1, and 71.6 nM for α_{1G}, $\alpha_{1H,}$ and α_{1I}, respectively, for penfluridol (Fig. 2*A,B*)

Examination of current–voltage relations and kinetic parameters showed that the DPBPs did not significantly affect these properties of T-type Ca channels (data not shown).

The blocking rate of penfluridol was ~10 times slower than pimozide with τ_{on} values at 1 μM of 98.5 ± 17.2 sec (n = 5) for penfluridol and 10.01 ± 1.8 sec (n = 3) for pimozide (Fig. 2*C,D,* see for the α_{1G} data). The blocking effect of both drugs was faster at higher concentrations. The time course of onset for pimozide and penfluridol block for α_{1G} could be fitted well with a single exponential (Fig. 2*C,D*), and the plot of $1/\tau_{on}$ exhibited a linear concentration dependence as would be expected for a 1:1 interaction (Fig. 2*C,D, insets*). The values of the binding (k_{on}) and unbinding (k_{off}) rate constants were obtained from the slope and y-intercept, respectively, of the linear equation with K_d values of 48.8 and 135.1 nM for pimozide and penfluridol, respectively, which are very close to the IC_{50} values obtained from the dose–response curves. Washout of pimozide was faster and more complete than for penfluridol (see Fig. 5*A,B*), and washout of penfluridol was largely incomplete. The extent of recovery could be increased only slightly by holding at more negative potentials (data not shown). Complete recovery of DPBP block has been seen for T-type currents in some native cells (Enyeart et al., 1992), whereas in other cell types DPBP block is reported to be more persistent (Arnoult et al., 1998).

The butyrophenone haloperidol blocks T-type channels with lower affinity than DPBPs

Haloperidol (Fig. 1*C*) is a potent butyrophenone antipsychotic commonly used to treat disorders such as schizophrenia (DiMascio, 1972). Cardiac arrhythmias have been associated with haloperidol therapy (Hunt and Stern, 1995), although the mechanism for this effect is unclear. It has been proposed that haloperidol could mediate cardiac effects by the block of repolarizing potassium currents, in particular HERG channels (Hunt and Stern, 1995). However, because T-type Ca channels are present in pacemaker heart cells, they may also be involved in the pathogenesis of arrhythmias. Figure 3 summarizes the effects of application of 1 μM haloperidol on α_{1G}, α_{1H}, and α_{1I} T-type Ca channels. The estimated K_d values for haloperidol interaction were similar among all three T-type channel subtypes (range, 1.2–1.4 μM) (Table 1). However, haloperidol is 12-to 20-fold less potent on the T-type channels compared with the DPBP neuroleptics pimozide and penfluridol (Table 1). The block of haloper-

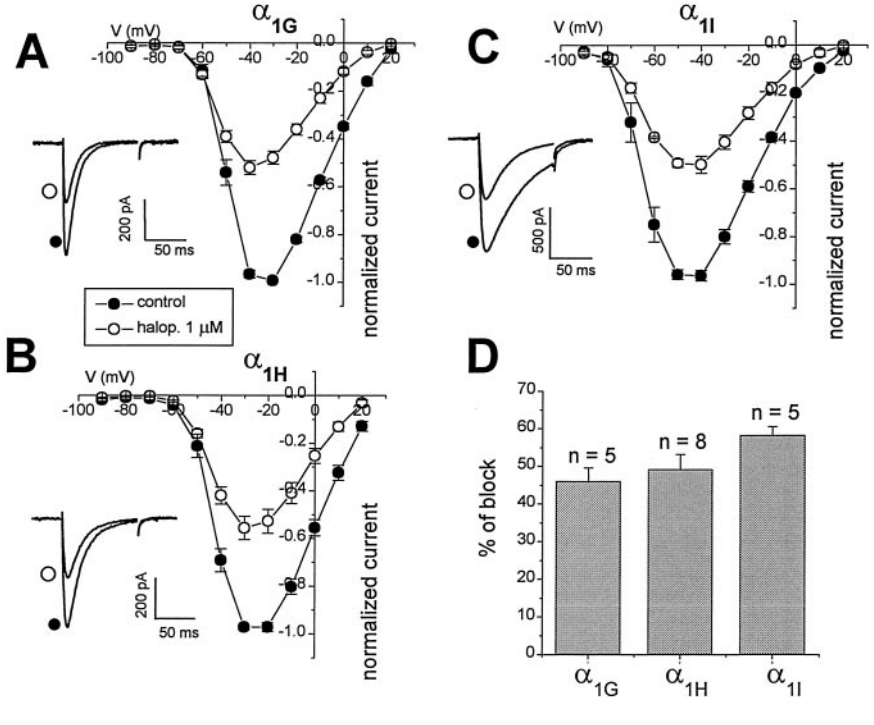

Figure 3. Summary of the effect of the butyrophenone haloperidol, on α_{1G}, α_{1H}, and α_{1I} T-type Ca channels. Mean *I–V* relationships of T-type currents in the absence (*filled circles*) or presence (*open circles*) of haloperidol (1 μM) on currents through α_{1G} (*A*), α_{1H} (*B*), and α_{1I} (*C*). Normalized mean *I–V* data represent the mean ± SE from three to eight cells. *Insets* show representative current traces in absence (control, *filled circles*) and presence (*open circles*) of 1 μM haloperidol, elicited with test pulses to −40 mV (α_{1G}, α_{1I}) or −30 mV (α_{1H}). *D,* All currents were inhibited to similar levels (≈50% inhibition) by 1 μM haloperidol, as summarized in this bar chart (n = 5 for α_{1G}; n = 8 for α_{1H}; n = 5 for α_{1I}).

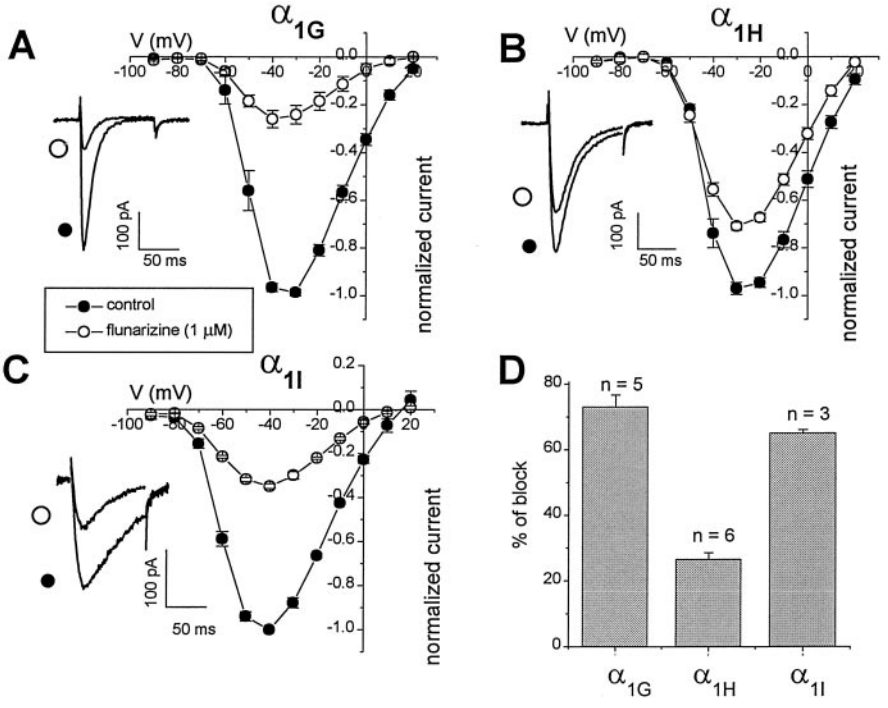

Figure 4. Differential blockade of T-type Ca channels by the diphenyldiperazine flunarizine. Mean *I–V* relationships of T-type currents in the absence (*filled circles*) or presence (*open circles*) of flunarizine (1 μM), were obtained from normalized currents (mean ± SE from 3–6 cells) through α_{1G} (*A*), α_{1H} (*B*), and α_{1I} (*C*). Normalized mean *I–V* data represent the mean ± SE from three to six cells. *Insets* show representative current traces in absence (control, *filled circles*) and presence (*open circles*) of 1 μM flunarizine, elicited with test pulses to −40 mV (α_{1G}, α_{1I}) or −30 mV (α_{1H}). *D,* Flunarizine (1 μM) differentially inhibited the cloned T-type currents, inhibiting α_{1G} and α_{1I} more potently (~70%) than α_{1H} (~30%).

idol developed faster than the other neuroleptic drugs with τ_{on} values ranging from 13 to 20 sec, and drug washout was similarly faster and more complete (see Fig. 5*C*).

Flunarizine exhibits subtype-specific blocking activity for T-type calcium channels

Flunarizine is a diphenyldiperazine derivative (Fig. 1*D*) that has been used clinically to treat a number of diseases such as vertigo (Olesen, 1988), migraine (Spierings, 1988), and some heart disorders (Koch et al., 1990). Flunarizine is also a potent anticonvulsant (Greenberg, 1987) and displays some neuroprotective properties (Desphande and Wieloch, 1986; Rich and Hollowell, 1990; Kaminski Schierle et al., 1999). Flunarizine has been shown to block native L-, N-, and T-type Ca channels (Tytgat et al., 1988, 1991, 1996), and some neurons possess T-type currents characterized by their high sensitivity to this neuroleptic compound (Kaneda and Akaike, 1989; Panchenko et al., 1993; Takahashi and Akaike, 1991). In certain preparations, flunarizine has been described as the most potent organic blocker of hypothalamic T-type Ca channels (Akaike et al., 1989; Dzura et al., 1996).

Figure 4 summarizes the effects of 1 μM flunarizine on α_{1G}, $\alpha_{1H,}$ and α_{1I} T-type Ca channels. Unlike the other neuroleptics studied, flunarizine displayed a preferential block of α_{1G} and α_{1I} (K_d = 0.53 and 0.84 μM, respectively) (Table 1) compared with α_{1H} (K_d = 3.6 μM) (Table 1). As with pimozide, penfluridol, and haloperidol, flunarizine did not significantly alter the current–voltage relations or current kinetics of the three T-type channels (Fig. 4). The onset of blockade by flunarizine was the slowest (Fig. 5*D*) of the neuroleptics tested with τ_{on} values at 1 μM ranging from 72 to 197 sec for α_{1G} and α_{1I}. Washout of flunarizine was likewise slow and essentially complete.

Neuroleptics affect T-type Ca channel steady-state inactivation properties

To further characterize the biophysical effects of neuroleptics on the three types of neuronal T-type Ca channels, a detailed analysis of voltage-dependent inactivation of the α_{1G} Ca channel was performed. A conditioning pulse of 15 sec duration at various potentials between −120 and −50 mV was applied and followed by a test pulse to −30 mV of 150 msec duration. Figure 6 shows that addition of flunarizine, haloperidol, pimozide, and penfluridol caused a significant hyperpolarizing shift (from 7 to 10 mV) of $V_{0.5inact}$ compared with the steady-state inactivation profiles of the α_{1G} currents under control conditions. Similar voltage-dependent block of α_{1H} and α_{1I} channels by penfluridol, haloperidol, or flunarizine is suggested by comparing data at more depolarized potentials (i.e., −85 vs −100 mV; data not shown).

Whereas inactivation-gating processes were profoundly affected by all neuroleptic drugs tested, the voltage dependence of activation was not significantly altered. The $V_{0.5}$ of activation obtained from *I–V* curves were not significantly shifted (<5 mV) in the presence of the drugs (data not shown).

DISCUSSION

Neuroleptics represent chemically diverse classes of compounds that share the ability to alleviate the symptoms of CNS disorders such as schizophrenia and some mood disorders. Several previous studies have shown that these agents can inhibit native T-type Ca channels in a number of cell types (Enyeart et al., 1990, 1992; Arnoult et al., 1998). In a systematic analysis of the cloned α_{1G}, $\alpha_{1H,}$ and α_{1I} T-type Ca channel subtypes, we show here that distinct classes of neuroleptics: the DPBPs (pimozide and penfluridol), butyrophenones (haloperidol), and the diphenyldiperazine derivative flunarizine, differentially affect neuronal T-type Ca channels.

Diphenybutylpiperidines are potent blockers of T-type Ca channels

The DPBPs are a class of neuroleptic agents used in the treatment of schizophrenia and related psychoses such as delusional disorder. Pimozide is also used in the treatment of Tourette's syndrome, which is a familial neurobehavioral disorder characterized by fluctuating involuntary motor and/or vocal tics (Cohen et

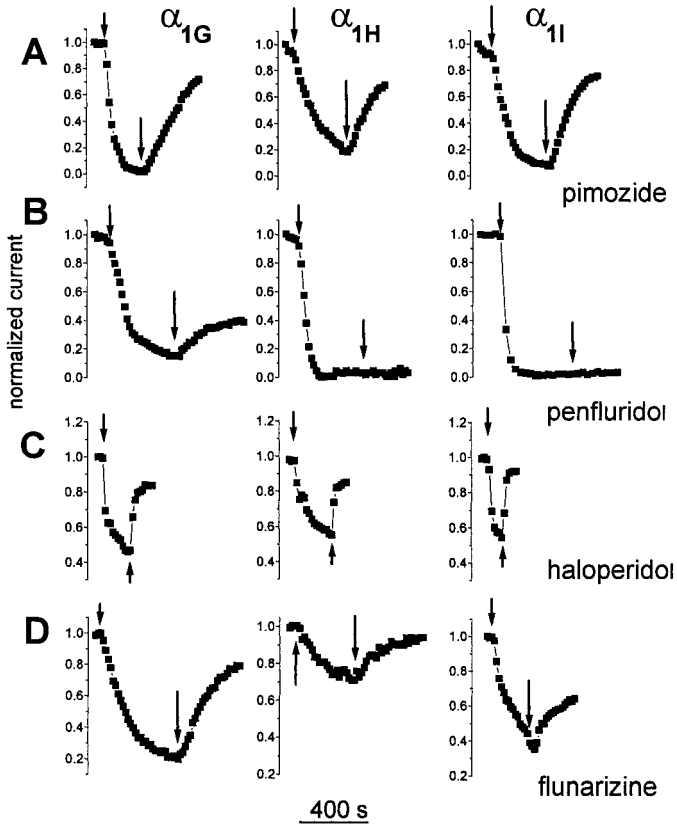

Figure 5. Time course of neuroleptic blockade of the three T-type Ca channels. Shown are representative time courses of the inhibitory effects of 500 nM pimozide (*A*), 1 μM penfluridol (*B*), 1 μM haloperidol (*C*), and 1 μM flunarizine (*D*) on cloned rat α_{1G}, α_{1H}, and α_{1I} channels. Drug application (*first arrow*) followed by washout of drugs (*second arrow*) appear as indicated. The DPBPs penfluridol (1 μM) and pimozide (500 nM), showed τ_{on} of inhibition ranging from 60.3 ± 16 to 109.9 ± 29 and from 29.5 ± 0.4 to 84.2 ± 12.4 sec (n = 3–6) for penfluridol and pimozide, respectively. In contrast, the butyrophenone haloperidol showed τ_{on} values from 13.3 ± 0.9 to 20 ± 2.6 sec (n = 4). Flunarizine exhibits the slowest on-rate (from 39.2 ± 8.8 to 197.3 ± 23.8 sec; n = 3). The T-type Ca currents were elicited by test pulses to −40 mV (α_{1G}, α_{1I}) or −30 mV (α_{1H}) from −100 mV holding potential (every 15 sec before and during drug application).

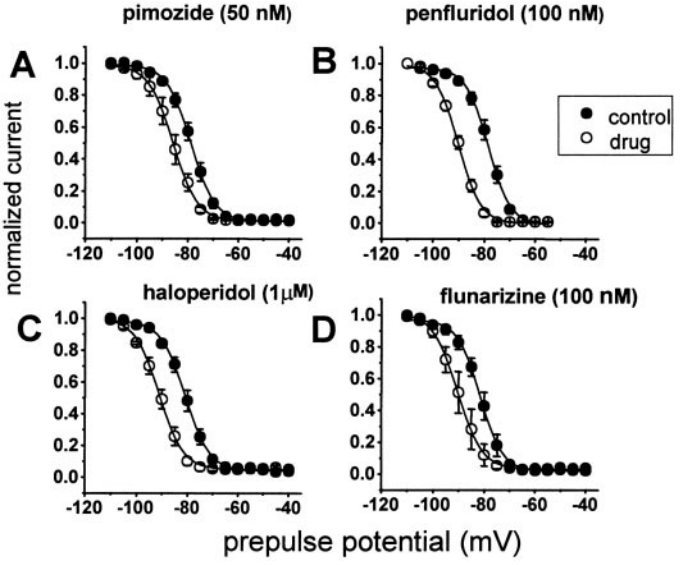

Figure 6. Neuroleptic agents induce a negative shift in the voltage dependence of steady-state inactivation of α_{1G} T-type Ca channels. The $V_{0.5i}$ values of rat α_{1G} channel inactivation were shifted to hyperpolarized potentials as follows: *A,* 50 nM pimozide (control $V_{0.5i}$ = −78.6 ± 0.2 mV vs treated $V_{0.5i}$ = −85.8 ± 0.2 mV; $p < 0.01$; n = 4). *B,* 100 nM penfluridol (control $V_{0.5i}$ = −78.5 ± 0.3 mV vs treated $V_{0.5i}$ = −90.2 ± 0.3 mV; $p < 0.01$; n = 4). *C,* 1 μM haloperidol (control $V_{0.5i}$ = −80.8 ± 0.2 mV vs treated $V_{0.5i}$ = −91.1 ± 0.3 mV; $p < 0.01$; n = 3). *D,* 100 nM flunarizine (control $V_{0.5i}$ = −81.6 ± 0.3 mV vs treated $V_{0.5i}$ = −90.4 ± 0.2 mV; $p < 0.01$; n = 4). Steady-state inactivation curves were generated as described in Materials and Methods.

al., 1992). It is thought that DPBPs owe much of their antipsychotic effectiveness to their antagonism of dopamine receptors (Seeman et al., 1976). However, several studies have shown they also interact with other nervous system targets, including Ca channels (Enyeart et al., 1992; Sah and Bean, 1993). Our results have identified the DPBPs penfluridol and pimozide as two of the most effective T-type Ca channel antagonists among the available organic blockers. We find that the K_d values for pimozide (39–60 nM) are very similar to published K_d values for dopamine D_2 receptors (29 nM; Richelson and Souder, 2000). Studies on neural crest-derived cell lines and adrenal zona fasciculata (AZF) cells have shown that penfluridol potently blocks T-type channels with IC_{50} values of 224 nM (Enyeart et al., 1992) and 300 nM (Enyeart et al., 1993), respectively, whereas pimozide inhibited T-type currents in the AZF cells and spermatogenic cells with IC_{50} of 500 and 460 nM, respectively. Because the concentration of permeant ion has been shown to affect piperidine blocking affinity (Zamponi et al., 1996), the discrepancy between the blocking potency observed in native T-type channels and that which we report in the cloned channels may be explained by the different experimental conditions used (10 mM Ca was used in native channels vs 2 mM Ca/Ba used in our experiments). To explore this possibility, experiments were performed in 10 mM external Ca for comparison. These experiments showed that 1 μM penfluridol blocked α_{1G} 71.6 ± 5.6% (n = 3) in 10 mM Ca, whereas the same concentration blocked T-type currents 90.9 ± 2.8 (n = 7) α_{1G} in 2 mM Ca (data not shown). Thus, the difference in Ca concentrations probably accounts for only a small portion of the difference.

The potency of DPBPs as T-type Ca channel antagonists parallels their potency as D_2 receptor antagonists (cf. Gould et al., 1983; this study). In contrast, butyrophenone and phenothiazine are much weaker Ca channel antagonists compared with D_2 dopamine receptor blockers (haloperidol K_d values: D_2 receptor, 2.6 nM, Richelson and Souder, 2000; T-type channels, ~1200 nM; this study). Thus, it is possible that at therapeutic doses DPBPs occupy receptor sites on both T-type Ca channels and D_2 dopamine receptors, whereas butyrophenones like haloperidol bind almost exclusively to D_2 dopamine receptors. Clinically, DPBPs offer an advantage over other drugs (e.g., butyrophenones) because they are able to relieve the negative symptoms of schizophrenia such as emotional withdrawal and poverty of speech that are resistant to treatment with other classical neuroleptic drugs. The only known pharmacological action that distinguishes DPBPs from other neuroleptics such as the butyrophenones (e.g., haloperidol) is their high-potency T-type Ca-antagonist activity. This raises the possibility that the selective clinical improvement in negative symptoms observed for DPBPs is attributable to their ability to block T-type calcium channels (Gould et al., 1983; Snyder and Reynolds, 1985).

Neuroleptic agents preferentially bind to the inactivated state

We find that the effects of all the neuroleptic drugs examined are potentiated at more depolarized holding potentials. This suggests that these drugs bind with higher affinity to the inactivated state of the channels. Similar results in native cells showed that penfluridol (300 nM) and pimozide (500 nM) shifted the steady-state inactivation of T-type currents by −8.2 and −10 mV, respectively (Enyeart et al., 1993). In addition T-type currents from spermatogenic cells have been shown to be blocked by pimozide in a voltage-dependent manner (Arnoult et al., 1998). Flunarizine has also been reported to have voltage-dependent inhibitory effects on many native T-type currents; for example, 5 μM flunarizine shifted the steady-state inactivation of T-type currents in rat Purkinje neurons by −10 mV (Panchenko et al., 1993) and similarly in mouse neuroblastoma cells (Wang et al., 1990) and guinea pig ventricular myocytes (Tytgat et al., 1996). This voltage dependence of block is particularly relevant in cells that fire action potentials because more depolarized potentials would be predicted to enhance the potency of blockade.

Flunarizine preferentially blocks α_{1G} and α_{1I} T-type channels

Flunarizine is a diphenyldiperazine derivative described as a Ca channel antagonist (Tytgat et al., 1988; Akaike et al., 1989; Wang et al., 1990), although it also displays moderate dopamine receptor antagonist activity with reported K_d values of 100–500 nM (Ambrosio and Stefanini, 1991; Brucke et al., 1995). Interestingly, these values are similar to those found in our study for α_{1G} and α_{1I} T-type Ca channel blockade (~530 and 840 nM, respectively) (Table 1). Clinically, flunarizine has been mainly prescribed to treat vertigo (Olesen, 1988), migraine (Spierings, 1988), and epilepsy (Greenberg, 1987). The mechanisms underlying the clinical efficacy of flunarizine are considered to be related to modulation of the activity of neuronal Ca channels (Moron et al., 1989). Flunarizine has been identified as a moderately potent T-type Ca channel blocker in native cells such as hypothalamic neurons (IC_{50} = 0.7 μM; Akaike et al., 1989). Similar effects of flunarizine on T-type Ca currents have been observed in rat amygdaloid neurons (Kaneda and Akaike, 1989), hippocampal CA1 pyramidal neurons (Takahashi and Akaike, 1991), N1E-115 neuroblastoma cells (Wang et al., 1990), and in Purkinje cells (Panchenko et al., 1993). In contrast, T-type channels from guinea pig ventricular myocytes and rat calcitonin secreting C-cells display lower affinity for this compound (K_d, ~10 μM and IC_{50} ~10 μM, respectively) (Enyeart et al., 1992; Tytgat et al., 1996). The different blocking potencies of flunarizine on native T-type currents could be explained by the recently described diversity in the molecular composition of the T-type channels. Of particular relevance, we report here that among the neuroleptic drugs tested, flunarizine displayed preferential blockade of the α_{1G} and α_{1I} subtypes with K_d values approximately five times higher for the α_{1H} channels (Table 1). A similar difference in K_d was observed when Ca was used as a charge carrier for α_{1H} (K_d, 3.04 μM).

The blockade of T-type Ca channels by flunarizine is consistent with its depressive action on repetitive neuronal firing and may contribute to its efficacy in suppressing seizure discharge (Bingmann and Speckmann, 1989). The α_{1G} T-type channel is highly expressed in the thalamus (McRory et al., 2001) and may represent the molecular target for flunarizine action.

In summary, we have characterized the effects of clinically important neuroleptic agents on the α_{1G}, α_{1H}, and α_{1I} T-type Ca channels expressed in the mammalian CNS. The results show that the DPBPs antagonize T-type Ca channels with a potency similar to their affinity for D_2 dopamine receptors, and thus Ca channel block may represent a significant contribution to the clinical efficacy of these agents. Flunarizine exhibited preferential block of α_{1G} and α_{1I} compared with the α_{1H} T-type Ca channel that may contribute to the differential therapeutic efficacy of this agent compared with DPBPs. Overall, the inhibitory effects of the DPBP neuroleptics on T-type Ca channels may underlie some of the clinical efficacy (and side effects) of antipsychotic treatments.

REFERENCES

Akaike N, Kostyuk PG, Osipchuck YV (1989) Dihydropyridine-sensitive low-threshold calcium channels in isolated rat hypothalamic neurons J Physiol (Lond) 412:181–195.

Ambrosio C, Stefanini E (1991) Interaction of flunarizine with dopamine D_2 and D_1 receptors. Eur J Pharmacol 197:221–223.

Arnoult C, Cardullo RA, Lemos JR, Florman HM (1996) Activation of mouse sperm T-type Ca^{2+} channels by adhesion to the egg zona pellucida. Proc Natl Acad Sci USA 12:93:13004–13009.

Arnoult C, Villaz M, Florman HM (1998) Pharmacological properties of the T-type Ca^{2+} current of mouse spermatogenic cells. Mol Pharmacol 53:1104–1111.

Bean BP (1989) Classes of calcium channels invertebrate cells. Annu Rev Physiol 51:367–384.

Bingmann D, Speckmann EJ (1989) Specific suppression of pentylenetetrazol-induced epileptiform discharges in CA3 neuron (hippocampal slice, guinea pig) by the organic calcium antagonists flunarizine and verapamil. Exp Brain Res 74:239–248.

Brucke T, Wober C, Podreka I, Wober-Bingol C, Asenbaum S, Aull S, Wenger S, Ilieva D, Harasko-van der Meer C, Wessely P (1995) D_2 receptor blockade by flunarizine and cinnerizine explains extrapyramidal side effects. A SPECT study. J Cerb Blood Flow Metab 15:513–518.

Cohen DJ, Riddle MA, Leckman JF (1992) Pharmacotherapy of Tourette's syndrome and associated disorders. Psychiatr Clin North Am 15:109–129.

Cribbs LL, Lee J-H, Yang J, Satin J, Zhang Y, Daud A, Barclay A, Williamson MP, Fox M, Rees M, Perez-Reyes E (1998) Cloning and characterization of α_{1H} from human heart, a member of the T-type calcium channel gene family. Circ Res 83:103–109.

Desphande JK, Wieloch T (1986) Flunarizine, a calcium entry blocker, ameliorates ischemic brain damage in the rat. Anesthesiology 64:215–224.

DiMascio A (1972) The butyrophenones. In: Butyrophenones in Psychiatry (DiMascio A, Shade RI, eds), p 117. New York: Raven.

Dzura IO, Naidenov VG, Lyubanova OP, Kostyuk PG, Shuba YM (1996) Characterization of hypothalamic low-voltage activated Ca channels based on their functional expression in *Xenopus* oocytes. Neuroscience 70:729–738.

Enyeart JJ, Dirksen RT, Sharma VK, Williford DJ, Sheu SS (1990) Antipsychotic pimozide is a potent Ca^{2+} channel blocker in heart. Mol Pharmacol 37:752–757.

Enyeart JJ, Biagi BA, Mlinar B (1992) Preferential block of T-type calcium channels by neuroleptics in neural crest-derived rat and human C cell lines. Mol Pharmacol 42:364–372.

Enyeart JJ, Mlinar B, Enyeart JA (1993) T-type channels are required for adrenocorticotropin-stimulated cortisol production by bovine adrenal zona fasciculata cells. Mol Endrocrinol 7: 1031–1040.

Gould RJ, Murphy KMM, Reynolds IJ, Snyder SH (1983) Antischizophrenic drugs of the dyphenybutylpiperidine type act as calcium channel antagonists. Proc Natl Acad Sci USA 80:5122–5125.

Greenberg DA (1987) Calcium channels and calcium channel antagonist. Ann Neurol 21:317–330.

Hagiwara N, Irisawa H, Kemeyama M (1988) Contribution of two types of calcium currents to the pacemaker potentials of rabbit sino-atrial cells. J Physiol (Lond) 395:233–253.

Hamill OP, Marty E, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. Pflügers Arch 381:85–100.

Huguenard JR (1996) Low threshold calcium currents in central nervous system neurons. Annu Rev Physiol 58:329–348.

Huguenard JR, Prince DA (1994) Intrathalamic rhythmicity studied in vitro: nominal T-current modulation causes robust antioscillatory effects. J Neurosci 14:5485–5502.

Hunt N, Stern TA (1995) The association between intravenous haloperidol and tordsades de pointes. Psychosomatics 36:541–549.

Ito C, Im WB, Takagi H, Takahashi M, Tsuzuki S-YL, Kunihara M (1994) U-92032, a T-type Ca^{2+} channel blocker and antioxidant, reduces neuronal ischemic injuries. Eur J Pharmacol 257:203–210.

Kaminski Schierle GS, Hansson O, Brundin P (1999) Flunarizine im-

proves the survival of grafted dopaminergic neurons. Neuroscience 94:17–20.
Kaneda M, Akaike N (1989) The low-threshold Ca current in isolated amygdaloid neurons in the rat. Brain Res 497:187–190.
Klugbauer N, Marais E, Lacinová L, Hofmann F (1999) A T-type calcium channel from mouse brain. Plügers Arch 437:710–715.
Koch P, Wilffert B, Peters T (1990) R56865: a new anti-ischemic principle. Cardio Drug Rev 8:238–254.
Lee, J-H, Daud AN, Cribbs LLLacerda, AE, Pereverzev, A, Klockner, U, Schneider, T, Perez-Reyes E (1999) Cloning and expression of a novel member of the low voltage-activated T-type calcium channel family. J Neurosci 19:1912–1921.
McRory JE, Santi CM, Hamming KS, Mezeyova J, Sutton KG, Baillie DL, Stea A, Snutch TP (2001) Molecular and functional characterization of a family of rat brain T-type calcium channels. J Biol Chem 276:3999–3401.
Moron MA, Stevens CW, Yaksh TL (1989) Diltiazem enhances and flunarizine inhibits nimodipine's antiseizure effects. Eur J Pharmacol 163:299–307.
Nuss HB, Houser SR (1993) T-type Ca^{2+} current is expressed in hypertrophied adult feline left ventricular myocytes. Circ Res 73:777–782.
Olesen J (1988) Calcium entry blockers in the treatment of vertigo Ann. NY Acad Sci 522:690–697.
Panchenko VA, Kristhal OA, Tegtmeier F, Tsyndrenko A (1993) R56865 as a Ca^{2+}-channel blocker in Purkinje neurons of rat: comparison with flunarizine and nimodipine. Neuroscience 54:587–594.
Perez-Reyes E, Cribbs LL, Daud A, Lacerda AE, Barclay J, Williamson MP, Fox M, Rees M, Lee J-H (1998) Molecular characterization of a neuronal T-type calcium channel. Nature 391:896–900.
Pinder RM, Brogden RN, Sawyer PR, Speight TM, Spencer R, Avery GS (1976) Pimozide: a review of its pharmacological properties and therapeutic uses in psychiatry. Drugs 12:1–40.
Rich KM, Hollowell JP (1990) Flunarizine protects neurons from death after axotomy or NGF deprivation. Science 248:1419–1421.
Richelson E, Souder T (2000) Binding of antipsychotic drugs to human brain receptors. Focus on newer generation compounds. Life Sci 68:29–39.
Rossier MF, Burnay MM, Valloton MB, Capponi AM (1996) Distinct functions of T- and L-type calcium channels during activation of bovine adrenal glomerulosa cells. Endocrinology 137:4817–4826.
Sah DWY, Bean BP (1993) Inhibition of P-type and N-type calcium channels by dopamine receptor antagonists. Mol Pharmacol 45:84–92.
Santi CM, Darszon A, Hernandez-Cruz A (1996) A dihydropyridine-sensitive T-type Ca^{2+} current is the main Ca^{2+} current carrier in mouse primary spermatocytes. Am J Physiol 271:C1583–C1593.
Seeman P, Lee T, Chau-Wong M, Wong K (1976) Antipsychotic drug doses and neuroleptic/dopamine receptors. Nature 261:717–719.
Self DA, Bian K, Mishra SK, Hermsmeyer K (1994) Stroke-prone SHR vascular muscle Ca^{2+} current amplitudes correlate with lethal increases in blood pressure, 1994. J Vasc Res 31:359–366.
Snyder SH, Reynolds IJ (1985) N Engl J Med 313:995–1002.
Spierings EL (1988) Clinical and experimental evidence for a role of calcium entry blockers in the treatment of migraine. Ann NY Acad Sci 522:676–689.
Takahashi K, Akaike N (1991) Calcium antagonist effects on low-threshold (T-type) calcium current in rat isolated hippocampal CA1 pyramidal neurons. J Pharmacol Exp Ther 256:169–175.
Todorovic SM, Lingle CJ (1998) Pharmacological properties of T-type Ca^{2+} current in adult rat sensory neurons: effects of anticonvulsant and anesthetic agents. J Neurophysiol 79:240–252.
Tsakiridou E, Bertollini L, de Curtis M, Avanzini G, Pape HC (1995) Selective increase in T-type calcium conductance of reticular thalamic neurons in a rat model of absence epilepsy. J Neurosci 15:3110–3117.
Tsien RW, Lipscombe D, Madison DV, Bley KR, Fox AP (1988) Multiple types of neuronal calcium channels and their selective modulation. Trends Neurosci 11:431–438.
Tytgat J, Vereeke J, Carmeliet E (1988) Differential effects of verapamil and flunarizine on cardiac L-type and T-type Ca channels. Naunyn Schmiedergs Arch Pharmacol 337:690–692.
Tytgat J, Pauwels PJ, Vereeke J, Carmeliet E (1991) Flunarizine inhibits a high-threshold inactivating calcium channel (N-type) in isolated hippocampal neurons. Brain Res 549:112–117.
Tytgat J, Vereecke J, Carmeliet (1996) Mechanism of L- and T-type Ca^{2+} channel blockade by flunarizine in ventricular myocytes of the guinea-pig. Eur J Pharmacol 296:189–197.
Wang R, Karpinski E, Wu L, Pag P (1990) Flunarizine selectively blocks transient calcium channel currents in NIE-115 cells. J Pharmacol Exp Ther 254:1006–10011.
Xu X, Best PM (1990) Increase in T-type calcium current in atrial myocytes from adult rats with growth hormone-secreting tumours. Proc Natl Acad Sci USA 87:4655–4659.
Zamponi GW, Soong TW, Bourinet E, Snutch TP (1996) β subunit coexpression and the α_1 subunit domain I-II linker affect piperidine binding to neuronal calcium channels. J Neurosci 16:2430–2443.