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Inhibition of P-Type and N-Type Calcium Channels by Dopamine Receptor Antagonists

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SUMMARY

P-type Ca^{2+} channels in cerebellar Purkinje neurons and N-type Ca^{2+} channels in sympathetic neurons were found to be inhibited by D_2 dopamine receptor antagonists with diverse structures, including phenothiazines (chlorpromazine and thioridazine), diphenylbutylpiperidines (fluspirilene and pimozide), butyrophenones (haloperidol and spiperone), and a piperazine (fluphenazine). Dopamine and quinpirole had no effect on P-type Ca^{2+} channels. In all cases, inhibition was characterized by slow onset and offset. The effects on P-type and N-type channels were very similar. Fluspirilene was the most potent of the drugs, with EC_{50} values of 6 μM for P-type current and 2 μM for N-type current. Block of P-type channels by fluspirilene was voltage dependent, being enhanced by depolarized holding potentials, and use dependent, being enhanced by higher stimulation frequencies. The effect of fluspirilene on the P-type Ca^{2+} channel current was not prevented by simultaneous exposure to the peptide toxin ω-agatoxin IVA, indicating that fluspirilene binds to a distinct site on the channel. The results suggest that N-type and P-type Ca^{2+} channels possess similar binding sites for dopamine receptor antagonists and that block of N-type and P-type channels is relatively weak, compared with that of some T-type and L-type Ca^{2+} channels.

Mammalian neurons contain multiple types of voltage-dependent calcium channels. Whole-cell and single-channel recordings from neurons have led to the identification of four families of channels, named T-type, L-type, N-type, and P-type, which have been distinguished based on differences in voltage dependence, kinetics, unitary current size, and pharmacology (1–6). Molecular biological studies have also identified multiple types of calcium channels in mammalian brain (3, 4, 7). Some of the cloned channel subunits have been clearly correlated with L-type calcium channels (8), and others have been identified as N-type channels (9, 10). Possible correspondence of the other cloned channels with T-type (11) or P-type (12) channels is less clear.

Calcium channels are known to be targets of many drugs. The pharmacology of L-type channels is extensive, largely because many calcium channel blockers were developed for their effects on the cardiovascular system, mediated by the L-type channels that predominate in cardiac muscle and vascular muscle. Potent L-type channel blockers include dihydropyridines like nifedipine and nimodipine, phenylalkylamines like verapamil, and benzothiazepines like diltiazem. A few drugs have been described as blockers of T-type calcium channels, including amiloride (13) and ethosuximide (14). In contrast, very little is known about block of N-type and P-type channels by small synthetic drugs. The pharmacological agents that have helped distinguish N-type and P-type channels from the other channel types are mainly large peptide toxins isolated from venoms, notably ω-conotoxin GVIA, identified as a selective N-type channel blocker (15–17), and ω-Aga-IVA, a selective blocker of P-type calcium channels (6).

The class of drugs known as neuroleptics, which are used to treat psychiatric disorders, are antagonists of D_{2-4} dopamine receptors (18–20). Interestingly, many of these drugs also display calcium channel-blocking activity. The selectivity of these drugs for different types of calcium channels is not yet clearly known. Diphenylbutylpiperidine drugs such as fluspirilene and pimozide have been shown to affect both L-type and T-type calcium channels at submicromolar concentrations (21–24). The effects of these and other neuroleptic drugs on identified N-type and P-type calcium channels have not yet been reported and may be of particular interest because of the likely involvement of these channels in synaptic transmission in the central nervous system (25–27).

Our goal was to characterize the effects of neuroleptic drugs on N-type and P-type calcium channels in rat neurons. Using cerebellar Purkinje neurons, where approximately 90% of the high-threshold current is carried by P-type channels (6), we found that a variety of dopamine antagonists are able to inhibit

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ABBREVIATIONS: ω-Aga-IVA, ω-agatoxin IVA; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethylsulfoxide; TEA, tetraethylammonium; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

P-type Ca^{2+} channels. In sympathetic neurons, where 65–95% of the high-threshold current is carried by N-type channels (6, 17, 28), the antagonists had very similar effects. Fluspirilene, the most potent of the drugs tested, interacted with P-type channels in a voltage- and use-dependent manner consistent with higher affinity binding to channels in open or inactivated states than to channels in the resting state. The potencies of the different drugs with P-type channels and N-type channels were very similar, and both of these channel types appear to be less sensitive than L-type and T-type channels to some of the neuroleptic drugs, especially those of the diphenylbutylpiperidine class.

Materials and Methods

Cell preparation. Purkinje neurons were enzymatically dissociated from the cerebellum of 6–14-day old Long-Evans rats as described previously (6, 29), with a few modifications. In brief, animals were anesthetized with chloral hydrate (0.1–0.2 ml of 25% chloral hydrate, injected intraperitoneally) and perfused (by injection into the left ventricle of the heart) with ice-cold Ca^{2+}-free Tyrode's solution (150 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.40 with NaOH). The perfusion rapidly cooled the brain and cleared it of blood. The cerebellum was then removed and placed in high-K^+ dissociation medium (81.4 mM Na_2SO_4, 30 mM K_2SO_4, 5.8 mM $MgCl_2$, 20.4 mM glucose, 10 mM HEPES, pH 7.40). Meninges were trimmed away, and the tissue was cut into 12–15 pieces. The enzymatic incubation was carried out with 3 mg/ml protease (type XXIII; Sigma Chemical Co., St. Louis, MO) in high-K^+ dissociation medium at 35° for 6 min, under oxygenation. Only one third of the tissue pieces were triturated immediately after the enzymatic incubation; the remaining tissue was kept under oxygenation, at room temperature, for 1–6 hr before trituration. Purkinje neurons were identified on the basis of their characteristic morphology, i.e., large cell somata and single dendritic stump. The accuracy of this identification procedure was confirmed previously by labeling of Purkinje neurons with propidium iodide (29).

Hippocampal pyramidal neurons were prepared from 9-day-old Long-Evans rats using the same enzymatic treatment as for Purkinje neurons. The hippocampus was cut into 400-μm slices with a tissue chopper. After the enzyme incubation, the slices remained under oxygenation until cells were needed. Then, a few slices were removed, and the CA3 regions were cut out for trituration.

Sympathetic neurons were enzymatically dissociated from the superior cervical ganglia of 7–16-day-old Long-Evans rats using the method of Bernheim *et al.* (30), with slight modifications (31). In brief, two to four ganglia were dissected into Leibovitz's L-15 medium, desheathed, and cut into two or three pieces each. The first enzyme incubation was carried out at 35° for 20 min, with 24 units/ml papain (Worthington, Freehold, NJ), 0.5 mM EDTA, and 2 mM L-cysteine in Ca^{2+}-free Tyrode's solution. For the second enzyme incubation, the ganglia were incubated at 35° for 45 min with 1.9 mg/ml collagenase (type I; Sigma) and 16 mg/ml dispase (grade II; Boehringer-Mannheim, Indianapolis, IN) in Ca^{2+}-free Tyrode's solution. During the second incubation, the ganglia were triturated approximately 15 times every 10 min, with a fire-polished Pasteur pipette. The dissociated cells were pelleted by centrifugation, resuspended in L-15 medium, and stored at room temperature.

Electrophysiology. Whole-cell recordings were obtained from freshly dissociated neurons. Pipettes (1–4 MΩ resistance) were pulled from Boralex glass (Dynalab, Rochester, NY), coated with Sylgard (Dow Corning, Midland, MI), fire-polished, and filled with internal solution containing 108 mM cesium methanesulfonate, 4 mM $MgCl_2$, 9 mM EGTA, 9 mM HEPES, 4 mM ATP, 14 mM creatine phosphate (Tris salt), and 0.3 mM GTP (Tris salt), pH 7.40 with CsOH. In some experiments, 10 mM BAPTA (tetracesium salt; Molecular Probes, Eugene, OR) replaced the internal EGTA. In other experiments, 0.3 mM GTPγS replaced the internal GTP. Whole-cell recordings were initially established in Tyrode's solution containing 2 mM $CaCl_2$ and 4 mM $BaCl_2$. Cells were then exposed to external solution (160 mM TEA-Cl, 5 mM $BaCl_2$, 10 mM HEPES, pH 7.40 with TEA-OH) containing 3 μM tetrodotoxin (Sigma). The control external solution and the solutions containing drugs flowed from an array of microcapillary tubes (internal diameter, 140 μm), driven by gravity. Drugs were applied by moving the cell from one stream to another, and solution exchange was complete in <1 sec. When drugs were diluted from a stock solution in DMSO, DMSO was added to the control external solution at a dilution equivalent to that in the external solution containing drug. In some experiments, 0.3–0.5 mM Cd^{2+} was applied at the end of the experiment to identify and subtract non-Ca^{2+} channel currents. In experiments with ω-Aga-IVA, 1 mg/ml cytochrome *c* was included in all external solutions.

Whole-cell currents were recorded using a Dagan 3900 patch-clamp amplifier and the BASIC-FASTLAB interface system (INDEC Systems, Capitola, CA). Currents were filtered at 10 kHz (four-pole Bessel low-pass filter) and digitized every 50 μsec. Series resistance compensation was used, typically for 75–90% of the series resistance measured from the uncompensated capacity transient (dividing the decay time constant by cell capacitance) or from the potentiometer used for nulling the capacity transient. Experiments were rejected if remaining voltage errors (calculated as current times uncompensated series resistance) were >5 mV. Reported potentials have been corrected for a liquid junction potential of −10 mV between the internal solution and the Tyrode's solution in which the pipette current was zeroed before sealing onto the cell. In all cases, except where noted, high-threshold Ca^{2+} channel currents were elicited every 5 sec by a 50-msec depolarizing step to a potential of −10 mV from a holding potential of −80 mV. Ca^{2+} channel currents were corrected for leak and capacitative currents by subtraction of an appropriately scaled current elicited by a hyperpolarization from −80 mV to −90 mV. High-threshold Ca^{2+} channel currents often ran up or down slowly for the first several minutes after establishment of the whole-cell configuration. As much as possible, currents were allowed to stabilize before drug applications. Because the modulatory effects of neuroleptic agents were characterized by slow onset (see Results), drugs were applied, in most cases, until steady state block was achieved. In a few cases where this was not feasible, drugs were applied for a minimum of 100 sec. All experiments were done at 21–25°. Statistics are given as mean ± standard error.

Drugs. Chlorpromazine, fluphenazine, fluspirilene, pimozide, spiperone, quinpirole, and thioridazine were obtained from Research Biochemicals (Natick, MA), ω-Aga-IVA from Peptides International (Louisville, KY), and all other drugs from Sigma. Stock solutions were 3 mM chlorpromazine, 3 mM thioridazine, 5 mM dopamine, 1 mM quinpirole, 3 mM fluphenazine in water, 3 mM fluspirilene, 10 mM haloperidol, 10 mM pimozide, 10 mM spiperone in DMSO, and 96 μM ω-Aga-IVA in external solution with 1 mg/ml cytochrome *c*. All drug stocks were stored frozen and diluted into external solution on the day of the experiment, except dopamine, which was made up fresh on the day of the experiment.

Results

Dopamine antagonist inhibition of P-type channels in Purkinje neurons. In Purkinje neurons, about 90% of the high-threshold Ca^{2+} channel current is carried by P-type channels that are sensitive to the spider toxin ω-Aga-IVA, with the small remaining current being carried by a mixture of L-type and N-type channels (6, 17, 29). We began by screening various dopamine receptor antagonists at a concentration of 30 μM for effects on the overall high-threshold current (Fig. 1).

Two phenothiazines, chlorpromazine (Fig. 1A) and thioridazine (Fig. 1B), reversibly inhibited the high-threshold Ca^{2+} channel current in Purkinje neurons. The potencies of the two drugs were similar, with 30 μM thioridazine blocking current by 70% and 30 μM chlorpromazine blocking current by 54%

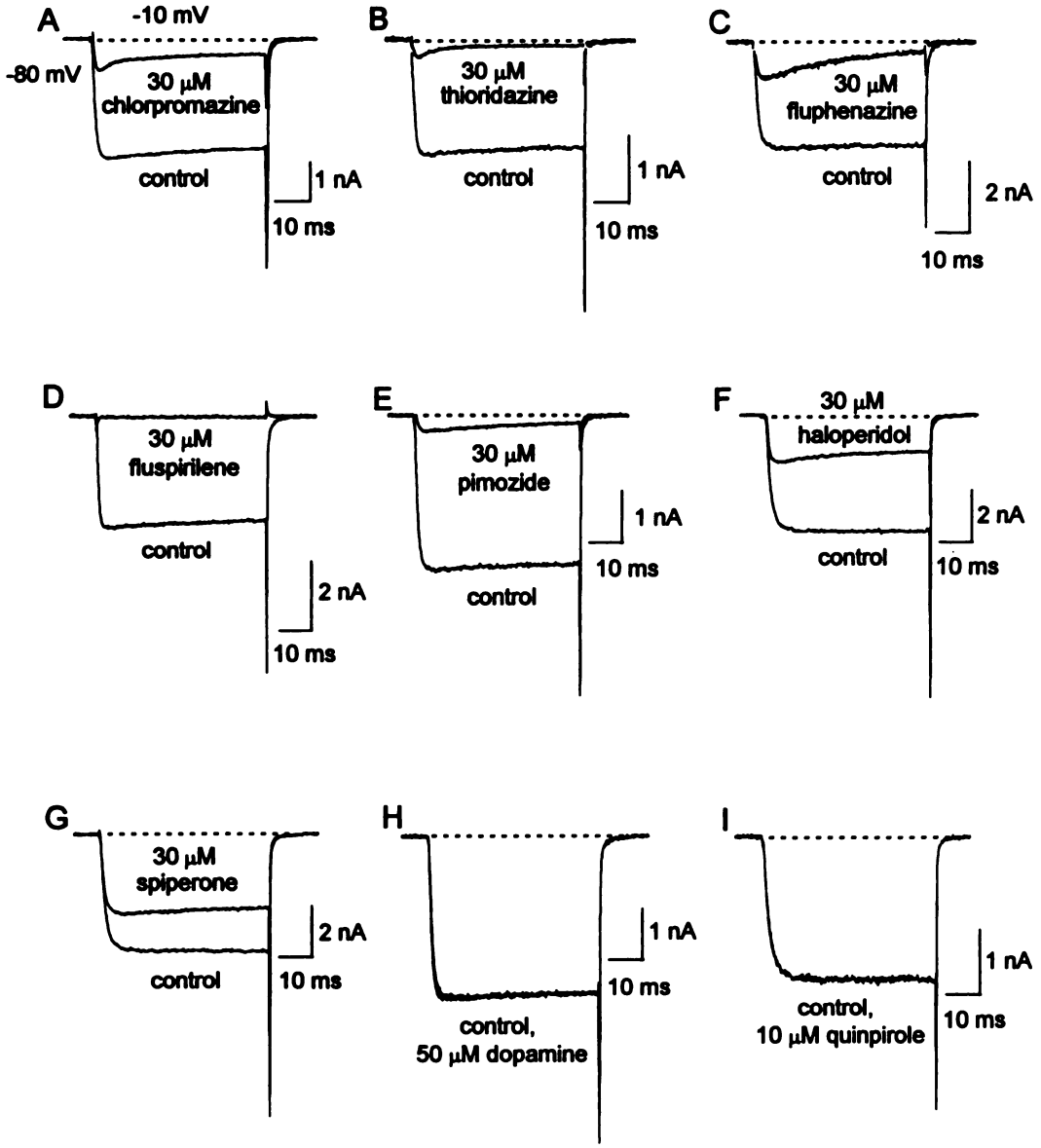

Fig. 1. Inhibition by dopamine receptor antagonists of high-threshold Ca^{2+} channel current in cerebellar Purkinje neurons. Currents were elicited by 50-msec depolarizations from −80 mV to −10 mV, delivered every 5 sec. Currents in the presence of drug represent block at steady state using this protocol.

(Table 1). The block by both drugs developed relatively slowly (Fig. 2, A and B), requiring several minutes for maximal effects (see Fig. 2, legend), and reversal was similarly slow. Fluphenazine, a piperazine neuroleptic agent, reversibly inhibited the high-threshold Ca^{2+} channel by 65% (Fig. 1C; Table 1) when current was measured early in the depolarization, when current in the presence of drug reached its peak. The diphenylbutylpiperidines fluspirilene and pimozide reversibly inhibited high-threshold Ca^{2+} channel current by 99% and 93%, respectively (Fig. 1, D and E; Table 1). The two butyrophenones studied, haloperidol (Fig. 1F) and spiperone (Fig. 1G), inhibited the high-threshold Ca^{2+} channel current by an average of 54% and 34%, respectively (Table 1). Besides producing less block, haloperidol and spiperone acted and reversed faster than any of the other neuroleptic agents tested, requiring about 30 sec for maximal effects to develop and about 1 min for reversal.

All of the neuroleptic drugs altered the kinetics of the current evoked by a 50-msec depolarization to −10 mV, inducing more rapid decay. This effect was most prominent with fluphenazine (Fig. 1C) but it was present to some degree with all of the drugs, including partially blocking concentrations of fluspirilene (e.g., Fig. 6A). This behavior is consistent either with extra open channel block or with acceleration of channel inactivation, which is normally minimal in 50 msec at −10 mV. In either case, the effect implies an interaction of the drugs with the voltage-dependent gating of the channel.

Because the neuroleptic drugs are dopamine receptor antagonists, they might act on the channels indirectly, by effects on dopamine receptors in the cells. This seems unlikely because effects on Ca^{2+} channels mediated by transmitter receptors usually vary greatly from cell to cell, whereas the effects of the neuroleptic drugs were highly reproducible from one cell to another. We tested directly for effects of dopamine receptors using dopamine (50 μM) and the D_2 agonist quinpirole (10 μM), neither of which had a significant effect (e.g., Figs. 1, H and I, and 2, H and I) in any cell tested ($n = 7$ for dopamine, $n = 8$ for quinpirole).

TABLE 1

Inhibition of high-threshold calcium channel current by dopamine receptor antagonists

The fraction of high-threshold current inhibited by neuroleptic drugs was measured in two types of neurons. All drugs were applied at 30 μM. Percentages represent block at steady state and are given as mean ± standard error, with the number of cells in parentheses.

	Inhibition	
	Purkinje	Sympathetic
	%	
Phenothiazines		
Chlorpromazine	54 ± 14 (5)	50 ± 9 (7)
Thioridazine	70 ± 14 (5)	81 ± 10 (4)
Piperazines		
Fluphenazine	63 ± 3 (5)	66 ± 14 (6)
Diphenylbutylpiperidines		
Fluspirilene	99 ± 1 (11)	93 ± 3 (7)
Pimozide	93 ± 3 (9)	88 ± 3 (8)
Butyrophenones		
Haloperidol	54 ± 4 (8)	68 ± 4 (6)
Spiperone	34 ± 3 (5)	50 ± 4 (6)

Dopamine antagonist inhibition of N-type channels in sympathetic neurons. The complete block of high-threshold current by fluspirilene in Purkinje neurons suggests that fluspirilene must block N- and L-type Ca^{2+} channel currents as well as P-type channels in these neurons, because ω-conotoxin- and dihydropyridine-sensitive currents each comprise approximately 5% of the total high-threshold current in these cells (3, 15, 27). To determine directly the effects of the neuroleptic drugs on N-type channels, we examined freshly dissociated sympathetic neurons from the superior cervical ganglion of young rats (postnatal days 7–16), where N-type current comprises an average of 85–95% of the high-threshold Ca^{2+} channel current (3, 15). All of the neuroleptic agents inhibited the high-threshold Ca^{2+} channel current in sympathetic neurons. The potency (Table 1) and kinetics of block were similar to those seen in Purkinje neurons. Thioridazine (blocking by an average of 81%) was somewhat more effective than chlorpromazine (blocking by 50%). Fluphenazine produced an average block of about two thirds, as in Purkinje neurons (Table 1). As in Purkinje cells, fluspirilene (producing 93 ± 3% block) was slightly more effective than pimozide (producing 88 ± 3% block). As in Purkinje neurons, haloperidol was somewhat more effective than spiperone in sympathetic neurons (Table 1); haloperidol inhibited the current by 68 ± 4% ($n = 6$), whereas spiperone inhibited the current by 50 ± 4% ($n = 6$). The onset of block by the butyrophenones (25–35 sec for complete block) was faster than for the other neuroleptic drugs (85–100 sec). Recovery was relatively slow for all of the drugs (complete recovery in 90–120 sec for chlorpormazine, haloperidol, and spiperone and in 200–325 sec for thioridazine and fluphenazine, with only partial recovery in >200–300 sec for fluspirilene and pimozide).

Concentration-dependent block by fluspirilene and thioridazine. We determined the concentration-response relations for block of current in Purkinje neurons and sympathetic neurons by fluspirilene and by thioridazine. In Purkinje neurons (Fig. 3A), the concentration-response relation for fluspirilene was fit by the logistic equation with an EC_{50} of 6 μM and a Hill coefficient of 2.4, and that for thioridazine was fit with an EC_{50} of 25 μM and a Hill coefficient of 2.5. The block at concentrations of <3 μM is likely underestimated because it is so slow; this would result in an overestimation of both the EC_{50} and the Hill coefficient.

In sympathetic neurons (Fig. 3B) the EC_{50} values for fluspirilene and thioridazine were 2 μM and 17 μM, respectively, with slope factors of 2.3 and 1.7. Interestingly, whereas the EC_{50} for fluspirilene was significantly lower for blocking the current in sympathetic neurons, compared with Purkinje neurons, there was more current left at 10 and 30 μM fluspirilene than predicted from the fitted dose-response curve, as if there is a small fraction of current that is less sensitive to fluspirilene. This may be connected to the greater variability and heterogeneity of contribution by different Ca^{2+} channel types to the current in sympathetic neurons.

Fluspirilene block of P-type channels in Purkinje neurons. In additional experiments, we focused on fluspirilene, because it was the most effective blocker of P-type and N-type channels among the dopamine receptor antagonists we studied. We studied block of high-threshold current in Purkinje neurons because this is likely more homogeneous than the high-threshold current in sympathetic neurons. Fig. 4 shows current-voltage relations for current in a Purkinje neuron before and after 30 μM fluspirilene, which eliminated the Ca^{2+} channel current at all test potentials from −40 mV to +60 mV.

Some of the neuroleptic agents have calmodulin antagonist properties (32). If fluspirilene were modulating P-type channels via calmodulin, then the effect would be dependent on internal Ca^{2+}. This seems unlikely, because the 10 mM EGTA in the internal solution would buffer intracellular Ca^{2+} to submicromolar levels, below that needed for activation of calmodulin. However, Ca^{2+} binding by EGTA is relatively slow, and it is possible to have Ca^{2+} transients in the presence of EGTA. We therefore conducted several experiments using 10 mM BAPTA (internally), a faster Ca^{2+} buffer, and saw no difference in Ca^{2+} channel currents or the effects of fluspirilene. With 10 mM BAPTA, fluspirilene inhibited the Ca^{2+} channel current completely (99 ± 0.5%; $n = 4$), just as with internal EGTA.

Like N-type channels, P-type channels can be inhibited by neurotransmitter activation of G proteins. Stimulation of γ-aminobutyric acid type B receptors in some Purkinje neurons produces partial inhibition of the P-type current, and the effects can be made irreversible by internally applied GTPγS, a poorly hydrolyzable GTP analog (33), implying mediation by G proteins. To test whether inhibition by fluspirilene involves G proteins, we included GTPγS in the internal solution. There was no effect on the ability of fluspirilene to inhibit the current (92 ± 1% block by 30 μM fluspirilene; $n = 3$), and the inhibition reversed normally.

Cd^{2+} and other inorganic divalent and trivalent ions can inhibit Ca^{2+} channels, most likely by binding tightly to sites within the channel pore that are intimately involved in permeation by Ca^{2+} and Ba^{2+}. Recovery from Cd^{2+} block is much more rapid (Fig. 5A) than recovery from fluspirilene (Fig. 2D). If fluspirilene bound to the same sites as Cd^{2+}, a saturating concentration of Cd^{2+} might prevent fluspirilene binding. In that case, after cumulative addition of the two blockers, the reversal of block should be fast, as is characteristic of recovery from Cd^{2+}. However, this was not the case ($n = 2$), as shown in Fig. 5A. An intial 100-sec application of 0.3 mM Cd^{2+} alone produced a rapid complete block of high-threshold Ca^{2+} channel current, which reversed readily (complete recovery in 1 min). When fluspirilene was added during the middle 90 sec of a 110-sec Cd^{2+} application, the block reversed slowly (17% recovery in 1 min), similar to recovery from fluspirilene alone. This result shows that Cd^{2+} block does not prevent binding of

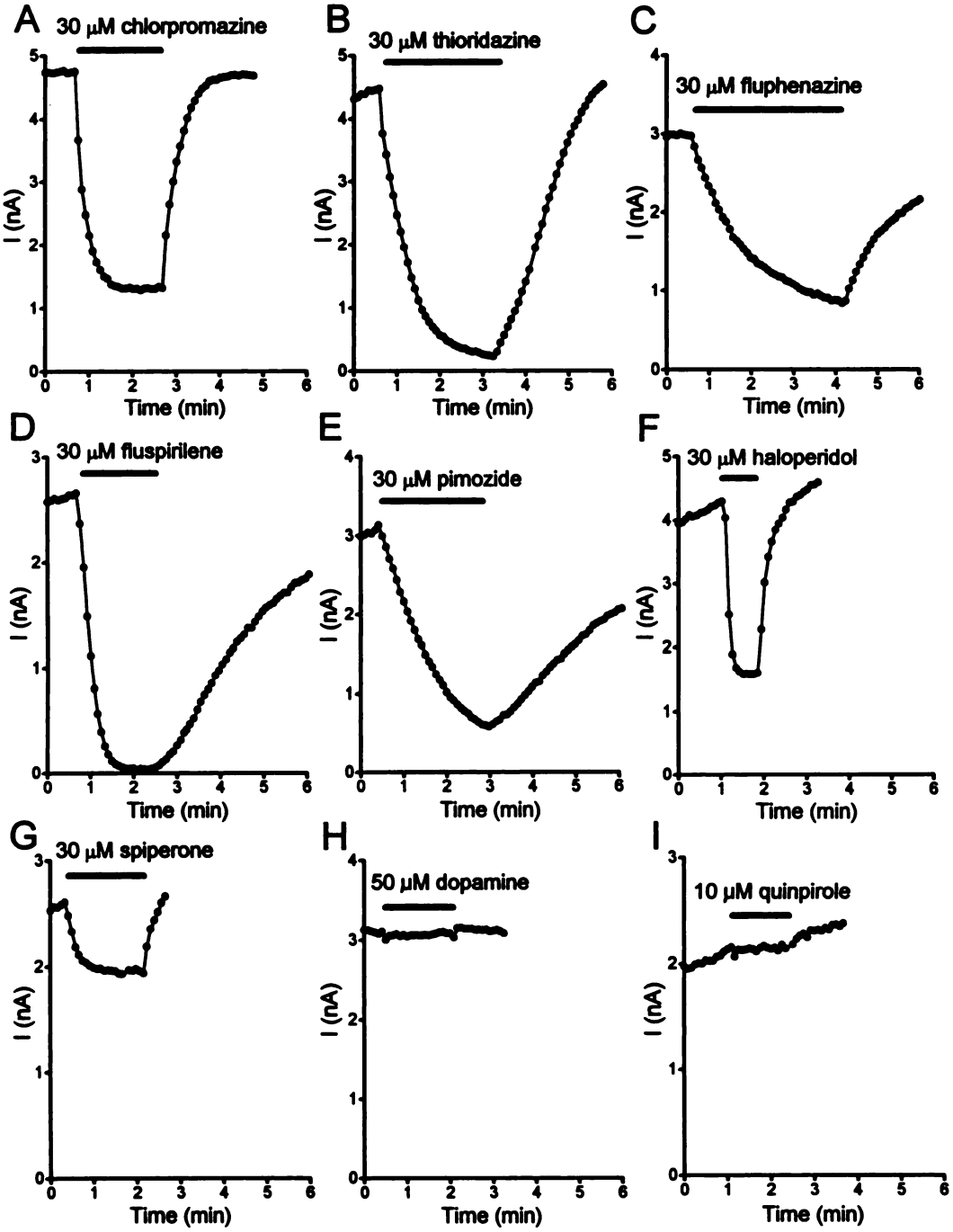

Fig. 2. Time course of inhibition and recovery with dopamine receptor antagonists applied to cerebellar Purkinje neurons. Solution changes were complete in <1 sec. Currents were elicited by 50-msec depolarizations from −80 mV to −10 mV, delivered every 5 sec. Currents were measured at the time of peak current in the presence of drug. Typical examples are shown. Collected results for the time (in sec) required for development (at 30 μM) are as follows: chlorpromazine, 66 ± 16 (n = 5); thioridazine, 119 ± 25 (n = 4); fluphenazine, 134 ± 36 (n = 4); fluspirilene, 75 ± 6 (n = 6); pimozide, 106 ± 11 (n = 9); haloperidol, 27 ± 3 (n = 8); spiperone, 38 ± 4 (n = 5). Time (in sec) required for reversal was as follows: chlorpromazine, 104 ± 21 (n = 4); thioridazine, 178 (n = 2); fluphenazine, 100 ± 35 (n = 3); fluspirilene, 390 (n = 2) for ~90% recovery; pimozide, 300 (n = 2) for ~75% recovery; haloperidol, 75 (n = 2); spiperone, 55 (n = 1).

fluspirilene, and it suggests that the two blockers bind to different sites on the P-type Ca^{2+} channel.

We tested similarly for an interaction between fluspirilene and the spider toxin ω-Aga-IVA for blocking P-type channels. ω-Aga-IVA blocks P-type Ca^{2+} channels specifically and potently, acting from the external side of the channel (6). Block by ω-Aga-IVA can be relieved within seconds by application of a series of large depolarizations. If ω-Aga-IVA and fluspirilene bind to the same site on the P-type Ca^{2+} channel, then bound ω-Aga-IVA would prevent the subsequent binding of fluspirilene. Applying a series of depolarizing steps would result in a rapid alleviation of block, as is characteristic of the recovery from ω-Aga-IVA inhibition. Fig. 5B illustrates the results of this experiment. An initial 200-sec application of 200 nM ω-Aga-IVA produced an 87% inhibition of the high-threshold Ca^{2+} channel current. This block was relieved by 66% by a series of 60 depolarizations (60 msec each) to +70 mV at 1 Hz. When fluspirilene was added during the last 100 sec of a 200-

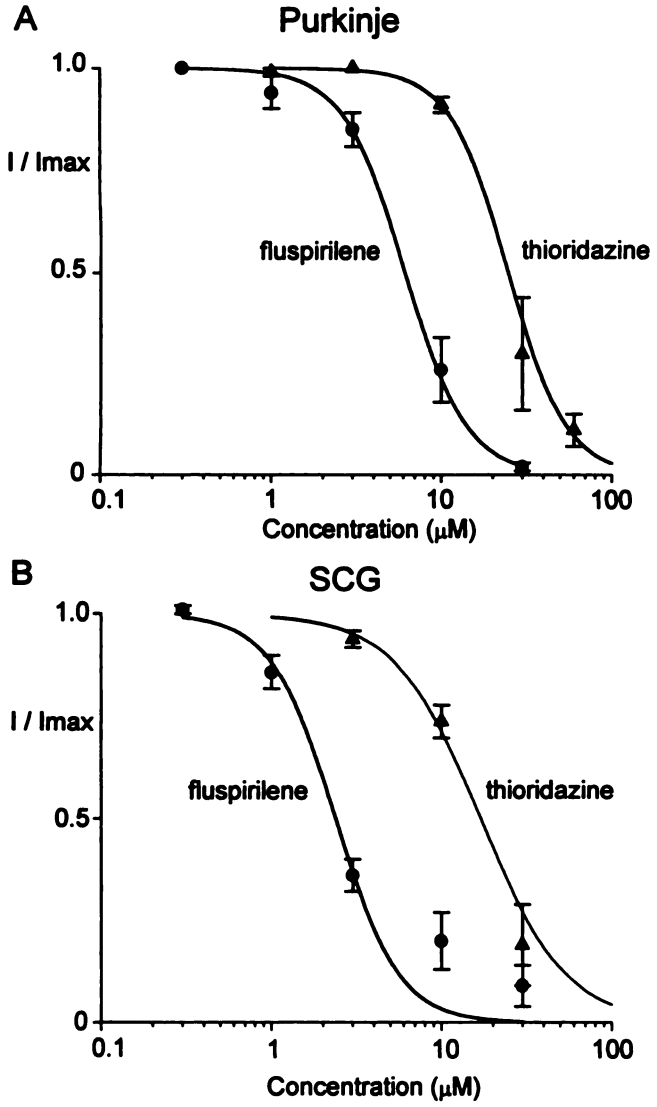

Fig. 3. Dose-dependent inhibition of high-threshold Ca^{2+} channel current in cerebellar Purkinje and sympathetic neurons by fluspirilene and thioridazine. Fifty-millisecond depolarizations from −80 mV to −10 mV were delivered every 5 sec. Currents were measured after about 100 sec of drug application. Each *point* represents the mean ± standard error for 3–11 cells. *Smooth curves*, best fits to the equation $1/(1 + ([drug]/EC_{50})^n)$. A, Purkinje neurons. For fluspirilene, EC_{50} = 6.1 μM and n = 2.4. For thioridazine, EC_{50} = 25 μM and n = 2.5. B, Superior cervical ganglion (*SCG*) neurons. For fluspirilene, EC_{50} = 2.4 μM and n = 2.3. For thioridazine, EC_{50} = 17 μM and n = 1.7.

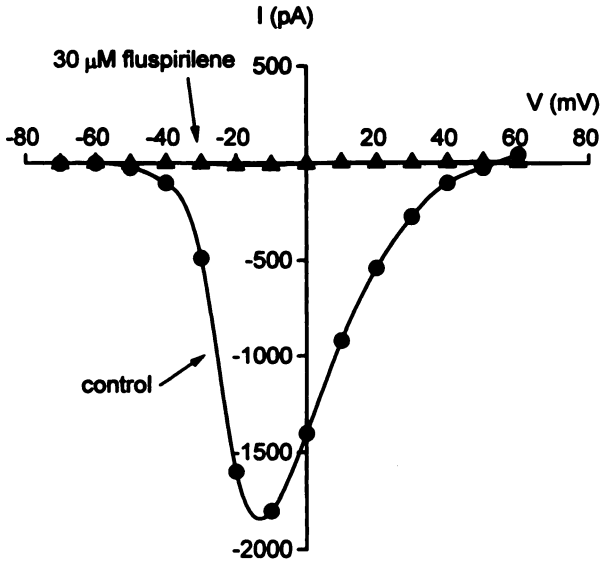

Fig. 4. Current-voltage relationship for block of high-threshold Ca^{2+} channel current by fluspirilene in cerebellar Purkinje neurons. Currents were elicited by depolarizations from a holding potential of −80 mV in control solution, with 30 μM fluspirilene (after an 80-sec exposure), and with 500 μM Cd^{2+}; the small Cd^{2+}-resistant currents were subtracted. Block by fluspirilene was complete at all test potentials between −40 and +60 mV.

sec exposure to ω-Aga-IVA, the depolarizing train no longer produced a relief of block. In three experiments, there was <5% recovery as a result of 60–90 depolarizations to +70 mV after addition of fluspirilene during the application of ω-Aga-IVA. This result suggests that ω-Aga-IVA and fluspirilene bind to distinct sites on the P-type Ca^{2+} channel.

Fluspirilene block of P-type current was modulated by the voltage protocol used to elicit current. Block was enhanced by depolarized holding potentials, as shown in Fig. 6. In this experiment, 7 μM fluspirilene was applied while P-type current was monitored with short infrequent test pulses (3-msec depolarizations to −10 mV every 20 sec). This minimized block by fluspirilene. When the inhibition reached steady state, current was elicited by a longer, 50-msec depolarization to −10 mV (Fig. 6A), and the holding potential was then changed to −40 mV for 10 sec, followed by another 50-msec test pulse to −10 mV. Block by fluspirilene was potentiated at the holding potential of −40 mV, compared with that at −80 mV. In controls, the holding potential of −40 mV reduced current by 21 ± 1% (n = 3). Fluspirilene at 7 μM reduced current elicited from −80 mV by only 7 ± 9% and reduced current elicited from −40 mV by 31 ± 8%.

Fluspirilene block was also potentiated by faster stimulation rates. Fig. 7 shows the effect of the rate of stimulation. In controls, the current elicited by 50-msec steps to −10 mV showed little dependence on the rate of stimulation in the range of 0.03–0.33 Hz. However, changing the stimulation rate from 0.03 Hz to 0.33 Hz dramatically enhanced inhibition by fluspirilene.

Inhibition of high-threshold Ca^{2+} channels in hippocampal neurons. The results so far show that fluspirilene is an effective blocker of both P-type and N-type Ca^{2+} channels, and previous studies have shown that it can block both T-type and L-type Ca^{2+} channels as well (21–24). Some central neurons, including hippocampal CA3 neurons, spinal cord interneurons, and cerebral cortical neurons, have components of high-threshold Ca^{2+} channel current that are resistant to blockers of L-type, N-type, and P-type channels and are still unclassified (6, 33). We tested the effect of fluspirilene on such current in hippocampal CA3 neurons, as shown in Fig. 8. The current in a CA3 neuron was blocked by the cumulative addition of ω-conotoxin GVIA, nimodipine, ω-Aga-IVA, and fluspirilene. ω-Contoxin GVIA at 3 μM inhibited about 20% of the high-threshold current, 3 μM nimodipine inhibited another 20% of the current, and 200 nM ω-Aga-IVA had no effect, as was previously found in about half of the CA3 neurons tested (6). The current remaining in these blockers was then completely inhibited by 30 μM fluspirilene. In three CA3 neurons, the combined application of 3 μM ω-conotoxin GVIA, 3 μM nimodipine, and 200 nM ω-Aga-IVA inhibited 53 ± 7% of the overall high-threshold current, and 30 μM fluspirilene completely inhibited the remaining inward current.

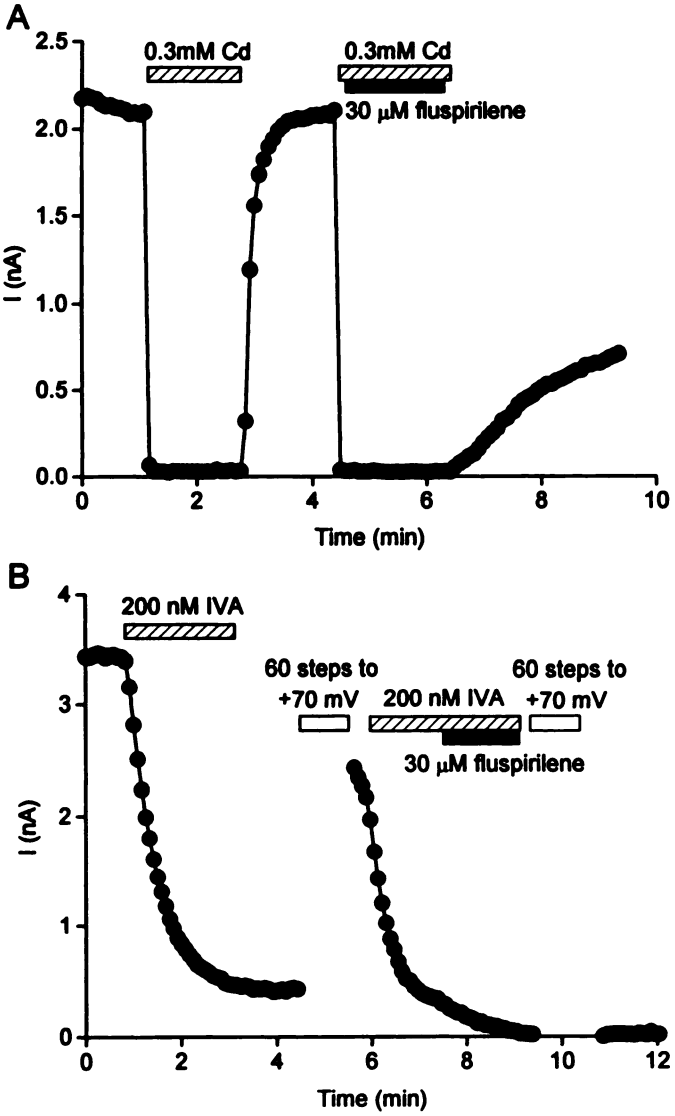

Fig. 5. Lack of effect of Cd^{2+} and ω-Aga-IVA on recovery from block of high-threshold Ca^{2+} channel current by fluspirilene in cerebellar Purkinje neurons. Currents were elicited by 50-msec depolarizations from −80 mV to −10 mV, delivered every 5 sec. A, Recovery of current after application of Cd^{2+} (0.3 mM) alone is rapid, being complete within 1 min. Binding of fluspirilene (30 μM) was not prevented by the previous and simultaneous presence of Cd^{2+}, as evidenced by the slow recovery from block. B, Recovery of current after application of ω-Aga-IVA (*IVA*) (200 nM) is rapid if a burst of 60-msec steps from −80 mV to +70 mV at 1 Hz is applied. In this cell, about two thirds of the block was relieved after such a burst. Binding of fluspirilene (30 μM) was not prevented by the previous and simultaneous presence of ω-Aga-IVA, as evidenced by the lack of recovery from block after a burst of depolarizations.

Discussion

These results show that both P-type Ca^{2+} channels and N-type Ca^{2+} channels can be inhibited by a variety of neuroleptic drugs, including phenothiazines, piperazines, diphenylbutylpiperidines, and butyrophenones. Previous studies have shown block by these agents of identified T-type channels (23, 24), identified L-type channels in skeletal (21) and cardiac (22) muscle, and unresolved mixtures of high-threshold currents in cell lines of neural origin (24). We made use of the predominance of P-type channels in freshly isolated Purkinje neurons and of N-type channels in freshly isolated sympathetic neurons to study the effects of the drugs on relatively homogeneous populations of these channels. In Purkinje cells, 85–100% of the current is inhibited by the P-type channel toxin ω-Aga-IVA (6). Thus, block of overall high-threshold current in Purkinje neurons can be interpreted as reflecting the actions of the drugs on P-type channels. The small currents remaining in the presence of ω-Aga-IVA were also blocked by 30 μM fluspirilene (Fig. 5B), consistent with the other evidence for its ability to block the small N-type and L-type currents also present in Purkinje cells. Although some Purkinje neurons possess T-type current (29), it was not evident in the cells under the conditions in which we studied them. In the freshly isolated superior cervical ganglion neurons we used, 65–95% of the current is carried by ω-contoxin GVIA-sensitive N-type channels (6, 17, 28), with variable contributions by L-type current and an unidentified current resistant to dihydropyridines, ω-conotoxin GVIA, and ω-Aga IVA.[2] There was no T-type current in the cells we used. The effects of the drugs on the overall high-threshold current in the sympathetic neurons clearly reflect mainly effects on N-type current, but other channels may contribute significantly in individual cells. The fraction of current with a lower sensitivity to fluspirilene (Fig. 3B) might come from other channel types.

Previous studies have identified the diphenylbutylpiperidines as unique among the neuroleptic drugs in producing block of both T-type and L-type Ca^{2+} channels (21–24) at submicromolar levels, and there are high affinity binding sites for diphenylbutylpiperidines on L-type channels (34, 35). Our results with P-type and N-type channels also identify the diphenylbutylpiperidines as the most effective Ca^{2+} channel blockers among the neuroleptic drugs. Fluspirilene was the most potent of the drugs we tested, with EC_{50} values (at a negative holding potential, with a low stimulation rate) of 6 μM for P-type channels and 2 μM for N-type channels.

Fluspirilene block of P-type channels was enhanced by depolarized holding potentials (that produced partial inactivation under control conditions) and by more frequent depolarizing pulses. Enyeart *et al.* (22–24) previously found similar effects for diphenylbutylpiperidine block of both T-type channels and L-type channels. These effects suggest that, for diphenylbutylpiperidine block of all of these channel types, drug binding is tighter to the open or inactivated states of the channel than to the resting state. It is hard to distinguish whether it is drug binding to open channels or to inactivated channels that is more important, because it is difficult or impossible to put channels purely in the open state or the inactivated state.

Block of N-type and P-type Ca^{2+} channels by diphenylbutylpiperidines seems somewhat weaker than block of L-type and T-type channels. Enyeart *et al.* (24) found that fluspirilene at 1 μM inhibited T-type current in the thyroid C cell line by >90%, implying significantly greater potency than the EC_{50} values of 2 μM and 6 μM that we estimate for N-type and P-type channels, respectively. However, in the GH_3 anterior pituitary cell line, T-type channels were about one-half blocked by 2 μM fluspirilene (23), similar to its potency on N-type channels. Pimozide at 200 nM blocked cardiac L-type current by about one half when applied from a holding potential of −80 mV (22), suggesting a much greater potency than for N-type or P-type currents tested under similar voltage protocols. Even more potent effects of diphenylbutylpiperidines on skeletal

[2] L. M. Boland, I. M. Mintz, J. Morrill, and B. P. Bean, unpublished observations.

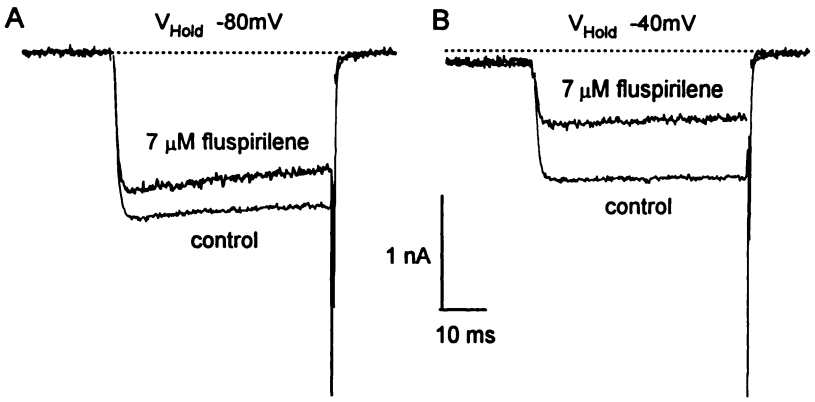

Fig. 6. Voltage dependence of fluspirilene block of high-threshold Ca^{2+} channel current in cerebellar Purkinje neurons. Current was elicited by a 50-msec depolarization to −10 mV delivered from a holding potential of −80 mV (A) or after the holding potential was changed to −40 mV for 10 sec (B). Fluspirilene (7 μM) was applied at a holding potential of −80 mV, whereas current was elicited every 20 sec with short (3-msec) depolarizations to −10 mV; after 2 min, the effects of fluspirilene reached steady state and the records shown were obtained. Tail currents were truncated to save space.

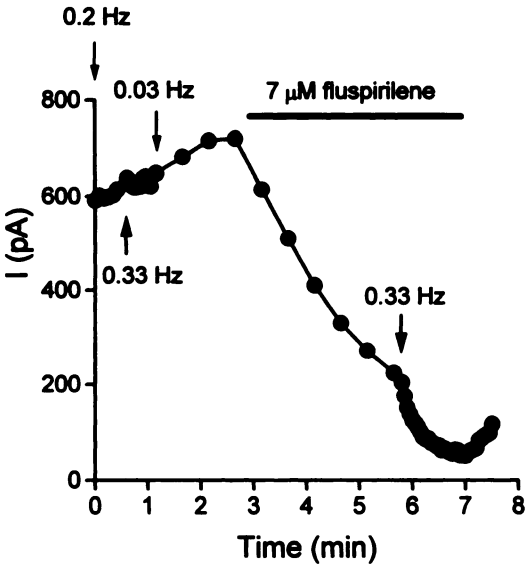

Fig. 7. Frequency dependence of inhibition of high-threshold Ca^{2+} channel current by fluspirilene in cerebellar Purkinje neurons. Current was elicited every 3, 5, or 30 sec with 50-msec depolarizations to −10 mV from a holding potential of −80 mV. In control solution, depolarizing steps were initially applied every 5 sec. Increasing the frequency to 0.33 Hz produced no significant run-down in current. The stimulation frequency was then reduced to 0.03 Hz, and 7 μM fluspirilene was applied to the cell. This resulted in a steady state inhibition of 74%. When the stimulation rate was increased to 0.33 Hz in the presence of fluspirilene, there was additional block; steady state inhibition of Ca^{2+} channel current was 93%.

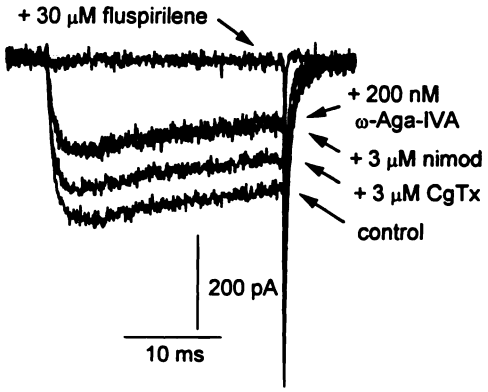

Fig. 8. Fluspirilene block of current resistant to ω-Aga-IVA, ω-conotoxin, and nimodipine in hippocampal CA3 neurons. Currents were elicited by 50-msec depolarizations from −90 mV to −10 mV, delivered every 5 sec. Drugs were applied cumulatively in the following order: 3 μM ω-conotoxin (*CgTx*), 3 μM nimodipine (*nimod*), 200 nM ω-Aga-IVA, and 30 μM fluspirilene.

muscle L-type current have been reported (21). The high potency block of L-type currents by diphenylbutylpiperidines is consistent with their ability to bind to L-type channels with nanomolar affinity in biochemical experiments (34, 35).

In comparison with the diphenylbutylpiperidines, the other classes of neuroleptic drugs show both less potent block of calcium channels and less selectivity among the different types of calcium channels. Phenothiazines have previously been shown to interact with L-type Ca^{2+} channels (36, 37). In our experiments, chlorpromazine inhibited P-type and N-type calcium channels with similar potencies (one-half block by 30 μM), as previously found by Ogata and Narahashi (38) for both low-threshold (EC_{50} ~ 15 μM) and high-threshold (EC_{50} ~ 15 μM) Ca^{2+} channels in N1E-115 neuroblastoma cells. Chlorpromazine inhibition of all of these Ca^{2+} channel types is somewhat weaker than that of voltage-dependent Na^+ channels (EC_{50} ~ 2.5 μM) (39) and comparable to that of delayed rectifier K^+ channels in epithelial cells (EC_{50} ~ 30 μM) (40). Apparently, chlorpromazine interacts with all types of voltage-activated channels. Interaction with the gating machinery of the channels is suggested by the voltage-dependent and use-dependent block seen for Na^+ channels (39) and low-threshold Ca^{2+} channels (38) and by the speeding of the decay seen for high-threshold Ca^{2+} current in neuroblastoma cells (38), delayed rectifier K^+ channels (40), and P-type Ca^{2+} channels (Fig. 1). The dependence on gating state makes it hard to define the blocking affinity of chlorpromazine for each type of channel; it is interesting that block is most potent for the channels with the most pronounced inactivation (Na^+ channels and T-type Ca^{2+} channels) when studied at holding potentials that partially inactivate the channels. Similar conclusions apply to the butyrophenone haloperidol. The 50–70% block of P-type and N-type channels with 30 μM haloperidol is comparable to the EC_{50} of 36 μM estimated for overall Ca^{2+} current in neuroblastoma cells (41), and Na^+ and K^+ channels are blocked with somewhat higher potency (EC_{50} values of 5 and 14 μM, respectively) (41). The potency for haloperidol block of T-type Ca^{2+} channels in a neural crest-derived cell line is also in this range (24).

It has previously been suggested that diphenylbutylpiperidine binding to L-type and T-type channels could occur at clinically relevant concentrations (24, 34), whereas our results suggest that considerably higher concentrations are needed to block N-type or P-type Ca^{2+} channels. The functions of L-type and T-type channels in brain neurons are not yet clearly understood. Other potent L-type channel blockers, including the dihydropyridines, have remarkably little effect on the normal function

of the nervous system. The consequences of inhibiting various subtypes of T-type channels in the brain are also not well understood (see Refs. 14 and 42). Whatever the effects of clinical concentrations of diphenylbutylpiperidines on L-type and T-type channels in the brain, the relative weakness of block of P-type and N-type channels is probably significant for the clinical utility of these agents. N-type and P-type Ca^{2+} channels may well be involved in normal synaptic transmission in many brain regions (e.g., Refs. 25–27), so the weak effects of the neuroleptic drugs may help ensure that normal brain function is maintained while higher order processes are selectively affected.

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References

1. Bean, B. P. Classes of calcium channels in vertebrate cells. *Annu. Rev. Physiol.* **51**:367–384 (1989).
2. Hess, P. Calcium channels in vertebrate cells. *Annu. Rev. Neurosci.* **13**:337–356 (1990).
3. Tsien, R. W., P. T. Ellinor, and W. A. Horne. Molecular diversity of voltage-dependent Ca^{2+} channels. *Trends Pharmacol. Sci.* **12**:349–354 (1991).
4. Miller, R. J. Voltage sensitive Ca^{2+} channels. *J. Biol. Chem.* **267**:1403–1406 (1992).
5. Usowicz, M. M., M. Sugimori, B. Cherskey, and R. Llinas. P-type calcium channels in the somata and dendrites of adult cerebellar Purkinje cells. *Neuron* **8**:1185–1199 (1992).
6. Mintz, I. M., M. E. Adams, and B. P. Bean. P-type calcium channels in central and peripheral neurons. *Neuron* **9**:1–20 (1992).
7. Snutch, T. P., and P. B. Reiner. Ca^{2+} channels: diversity of form and function. *Curr. Opin. Neurobiol.* **2**:247–253 (1992).
8. Williams, M., D. H. Feldman, A. F. McCue, R. Brenner, G. Velicelebi, S. B. Ellis, and M. M. Harpold. Structure and function of $\alpha 1$, $\alpha 2$, and β subunits of a novel human neuronal calcium channel subtype. *Neuron* **8**:71–84 (1992).
9. Williams, M. E., P. F. Brust, D. H. Feldman, P. Saraswathi, S. Simerson, A. Maoufi, A. F. McCue, G. Velicelebi, S. B. Ellis, and M. M. Harpold. Structure and functional expression of an ω-conotoxin-sensitive human N-type calcium channel. *Science (Washington D. C.)* **257**:389–395 (1992).
10. Fujita, Y., M. Mynlieff, R. T. Dirksen, M.-S. Kim, T. Niidome, J. Nakai, T. Friedrich, N. Iwabe, T. Miyata, T. Furuichi, D. Furutama, K. Mikoshiba, Y. Mori, and K. G. Beam. Primary structure and functional expression of the ω-conotoxin-sensitive N-type calcium channel from rat brain. *Neuron* **10**:585–598 (1993).
11. Soong, T. W., A. Stea, C. D. Hodson, S. J. Dubel, S. R. Vincent, and T. P. Snutch. Structure and functional expression of a member of the low voltage-activated calcium channel family. *Science (Washington D. C.)* **260**:1133–1136 (1993).
12. Mori, Y., T. Friedrich, M. S. Kim, A. Mikami, J. Nakai, P. Ruth, E. Bosse, F. Hofmann, V. Flockerzi, T. Furuichi, K. Mikoshiba, K. Imoto, T. Tanabe, and S. Numa. Primary structure and functional expression from complementary DNA of a brain calcium channel. *Nature (Lond.)* **350**:398–402 (1991).
13. Tang, C. M., F. Presser, and M. Morad. Amiloride selectively blocks the low threshold (T) calcium channel. *Science (Washington D. C.)* **240**:213–215 (1988).
14. Coulter, D. A., J. R. Huguenard, and D. A. Prince. Characterization of ethosuximide reduction of low-threshold calcium current in thalamic relay neurons. *Ann. Neurol.* **25**:582–593 (1989).
15. Aosaki, T., and H. Kasai. Characterization of two kinds of high-voltage-activated Ca-channel currents in chick sensory neurons: differential sensitivity to dihydropyridines and ω-conotoxin GVIA. *Pfluegers Arch.* **414**:150–156 (1989).
16. Plummer, M. R., D. E. Logothetis, and P. Hess. Elementary properties and pharmacological sensitivities of calcium channels in mammalian peripheral neurons. *Neuron* **2**:1453–1463 (1989).
17. Regan, L. J., D. W. Sah, and B. P. Bean. Ca^{2+} channels in rat central and peripheral neurons: high-threshold current resistant to dihydropyridine blockers and ω-conotoxin. *Neuron* **6**:269–280 (1991).
18. Seeman, P., T. Lee, M. Chau-Wong, and K. Wong. Antipsychotic drug doses and neuroleptic/dopamine receptors. *Nature (Lond.)* **261**:717–719 (1976).
19. Sokoloff, P., B. Giros, M.-P. Martres, M.-L. Bouthenet, and J.-C. Schwartz. Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. *Nature (Lond.)* **347**:146–151 (1990).
20. Van Tol, H. H., J. R. Bunzow, H.-C. Guan, R. K. Sunahara, P. Seeman, H. B. Niznik, and O. Civelli. Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine. *Nature (Lond.)* **350**:610–614 (1991).
21. Galizzi, J.-P., M. Fosset, G. Romey, P. Laduron, and M. Lazdunski. Neuroleptics of the diphenylbutylpiperidine series are potent calcium channel inhibitors. *Proc. Natl. Acad. Sci. USA* **83**:7513–7517 (1986).
22. Enyeart, J. J., R. T. Dirksen, V. K. Sharma, D. J. Williford, and S.-S. Sheu. Antipsychotic pimozide is a potent calcium channel blocker in heart. *Mol. Pharmacol.* **37**:752–757 (1990).
23. Enyeart, J. J., B. A. Biagi, R. N. Day, S.-S. Sheu, and R. A. Maurer. Blockade of low and high threshold calcium channels by diphenylbutylpiperidine antipsychotics linked to inhibition of prolactin gene expression. *J. Biol. Chem.* **265**:16373–16379 (1990).
24. Enyeart, J. J., B. A. Biagi, and B. Mlinar. Preferential block of T-type calcium channels by neuroleptics in neural crest-derived rat and human C cell lines. *Mol. Pharmacol.* **42**:364–372 (1992).
25. Kamiya, H., S. Sawada, and C. Yamamoto. Synthetic ω-conotoxin blocks synaptic transmission in the hippocampus *in vitro*. *Neurosci. Lett.* **91**:84–88 (1988).
26. Kuan, Y. F., and C. N. Scholfield. Ca-channel blockers and the electrophysiology of synaptic transmission of the guinea-pig olfactory cortex. *Eur. J. Pharmacol.* **130**:273–278 (1986).
27. Turner, T. J., M. E. Adams, and K. Dunlap. Ca^{2+} channels coupled to glutamate release identified by ω-Aga-IVA. *Science (Washington D. C.)* **258**:310–313 (1992).
28. Ikeda, S. R. Double-pulse calcium channel current facilitation in adult rat sympathetic neurons. *J. Physiol. (Lond.)* **439**:181–214 (1991).
29. Regan, L. J. Voltage-dependent calcium currents in Purkinje cells from rat cerebellar vermis. *J. Neurosci.* **11**:2259–2269 (1991).
30. Bernheim, L., D. J. Beech, and B. Hille. A diffusible second messenger mediates one of the pathways coupling receptors to calcium channels in rat sympathetic neurons. *Neuron* **6**:859–867 (1991).
31. Swartz, K. J. Modulation of Ca^{2+} channels by protein kinase C in rat central and peripheral neurons: disruption of G protein-mediated inhibition. *Neuron* **11**:1–20 (1993).
32. Prozialeck, W. C., and B. Weiss. Inhibition of calmodulin by phenothiazines and related drugs: structure-activity relationships. *J. Pharmacol. Exp. Ther.* **222**:509–516 (1982).
33. Mintz, I. M., and B. P. Bean. $GABA_B$ receptor inhibition of P-type Ca^{2+} channels in central neurons. *Neuron* **10**:889–898 (1993).
34. Gould, R. J., K. M. M. Murphy, I. J. Reynolds, and S. H. Snyder. Antischizophrenic drugs of the diphenylbutylpiperidine type act as calcium channel antagonists. *Proc. Natl. Acad. Sci. USA* **80**:5122–5125 (1983).
35. King, V. F., M. L. Garcia, J. L. Shevell, R. S. Slaughter, and G. J. Kaczorowski. Substituted diphenylbutylpiperidines bind to a unique high affinity site on the L-type calcium channel. *J. Biol. Chem.* **264**:5633–5641 (1989).
36. Luchowski, E. M., F. Yousif, D. J. Triggle, S. C. Maurer, J. G. Sarmiento, and R. A. Janis. Effects of metal cations and calmodulin antagonists on [^{3}H] nitrendipine binding in smooth and cardiac muscle. *J. Pharmacol. Exp. Ther.* **230**:607–613 (1984).
37. Nakazawa, K., K. Higo, K. Abe, Y. Tanaka, H. Saito, and N. Matsuki. Blockade by calmodulin inhibitors of Ca^{2+} channels in smooth muscle from rat vas deferens. *Br. J. Pharmacol.* **109**:137–141 (1993).
38. Ogata, N., and T. Narahashi. Potent blocking action of chlorpromazine on two types of calcium channels in cultured neuroblastoma cells. *J. Pharmacol. Exp. Ther.* **252**:1142–1149 (1990).
39. Ogata, N., M. Yoshii, and T. Narahashi. Differential block of sodium and calcium channels by chlorpromazine in mouse neuroblastoma cells. *J. Physiol. (Lond.)* **420**:165–183 (1990).
40. Jacobs, E. R., and T. E. DeCoursey. Mechanisms of potassium channel block in rat alveolar epithelial cells. *J. Pharmacol. Exp. Ther.* **255**:459–472 (1990).
41. Ogata, N., M. Yoshii, and T. Narahashi. Psychotropic drugs block voltage-gated ion channels in neuroblastoma cells. *Brain Res.* **476**:140–144 (1989).
42. Huguenard, J. R., and D. A. Prince. A novel T-type current with slowed inactivation contributes to prolonged Ca^{2+}-dependent burst firing in GABAergic neurons of rat thalamic reticuluar nucleus. *J. Neurosci.* **12**:3804–3817 (1992).

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