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Preferential Block of T-type Calcium Channels by Neuroleptics in Neural Crest-Derived Rat and Human C Cell Lines

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SUMMARY

We have used the whole-cell version of the patch-clamp technique to analyze the inhibition of Ca^{2+} currents by antipsychotic agents in neural crest-derived rat and human thyroid C cell lines. Diphenylbutylpiperidine (DPBP) antipsychotics, including penfluridol and fluspirilene, potently and preferentially block T-type Ca^{2+} current in the rat medullary thyroid carcinoma 6-23 (clone 6) cell line. When step depolarizations were applied at 0.1 Hz from a holding potential of −80 mV, with 10 mM Ca^{2+} as the charge carrier, the DPBP penfluridol inhibited T-type current with an IC_{50} of 224 nM. High voltage-activated L and N currents were less potently blocked. At a concentration of 500 nM, penfluridol inhibited 78.0 ± 2.3% (n = 29) of inactivating T-type Ca^{2+} current, whereas the sustained high voltage-activated current was reduced by 25.6 ± 3.5% (n = 28). Block of T-type current by penfluridol was enhanced by depolarizing test pulses applied at frequencies above 0.03 Hz. The use-dependent component of block was largely reversed by pulse-free periods at −80 mV. T-type Ca^{2+} channels in the human TT C cell line were blocked by penfluridol, and the potency was enhanced by reduction of extracellular Ca^{2+}. Non-DPBP antipsychotics, including haloperidol, clozapine, and thioridazine, also blocked T-type channels, but these were 20-100 times less potent than the DPBPs. These results identify the DPBPs as a new class of organic Ca^{2+} channel antagonists, which are distinctive in their ability to preferentially block T-type channels. These agents will be useful in defining the function of T channels in various excitable cells. Their potent block of T-type Ca^{2+} channels, which would be enhanced in rapidly firing cells, suggests that this action may be relevant to the therapeutic or toxic effects of these drugs when used in clinical pharmacology.

At least four different types of voltage-gated Ca^{2+} channels have been identified in voltage-clamp studies on excitable cells. These can be distinguished by their voltage dependence, kinetics, unitary conductance, ionic selectivity, modulation, and pharmacology (1-4). Three types of high voltage-activated Ca^{2+} channels have been identified. L-type Ca^{2+} channels are the predominant channels in many cells and are blocked by the major classes of organic Ca^{2+} antagonists, particularly the dihydropyridines. N-type channels may be specific to neurons and neural crest-derived cells and are present in nerve terminals (1-4). These channels are less sensitive to organic Ca^{2+} antagonists but are specifically blocked by the peptide neurotoxin ω-conotoxin (fraction GV1A) from the venom of *Conus geographicus*. A distinct class of P-type Ca^{2+} channel, blocked by funnel web spider venom, has been proposed to exist in cerebellar Purkinje neurons (3). Ca^{2+} channels that may correspond to these P channels have recently been cloned from rat brain (5).

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A fourth type of Ca^{2+} channel, the T-type, has been identified in many types of excitable cells, including neurons, various myocytes, and endocrine cells. These low voltage-activated, rapidly inactivating channels regulate the firing pattern of spontaneously active cells, including thalamic neurons and cardiac pacemaker cells (6-8). They may serve a similar function in other cells, including hormone-secreting cells (9). T-type Ca^{2+} channels may be involved in certain pathophysiological states involving disorders of impulse generation, such as absence seizures of epilepsy (10).

Low voltage-activated Ca^{2+} channels are relatively insensitive to the major classes of organic Ca^{2+} antagonists, which block primarily L-type Ca^{2+} channels. Several drugs, including phenytoin and amiloride, have been reported to block T-type Ca^{2+} channels selectively, but only at relatively high concentrations (11, 12). Because of the importance of T-type channels in physiological and possibly pathological processes, it would be desirable to identify pharmacological agents that selectively and potently block these channels in excitable cells.

A class of neuroleptic compounds, the DPBPs, have been

ABBREVIATIONS: DPBP, diphenylbutylpiperidine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; G protein, guanine nucleotide-binding protein; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; GDPβS, guanosine-5′-*O*-(2-thio)diphosphate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

shown to inhibit L-type Ca^{2+} channels in a variety of excitable cells, including muscle cells and endocrine cells (13–16). Recently, we discovered that these agents block both T- and L-type Ca^{2+} channels in an endocrine pituitary cell line (17). The interaction of DPBP antipsychotics with neuronal Ca^{2+} channel subtypes, their selectivity, and their mechanism have not been explored. Importantly, the ability of these agents to block neuron-specific Ca^{2+} channels and their relative potency as antagonists of T-, L-, and N-type channels have not been established. We demonstrated recently that a neural crest-derived rat thyroid C cell line possessed T-, L-, and N-type Ca^{2+} channels (18), whereas a human C cell line is unusual in expressing only T-type Ca^{2+} channels (19). In the present study, we have used these two cell lines to investigate the selective block of Ca^{2+} channel subtypes by the DPBPs and several other classes of antipsychotic agents. Some of these results have been published in an abstract (20).

Experimental Procedures

Materials. Tissue culture media, horse serum, and fetal calf serum were obtained from GIBCO (Grand Island, NY). Culture dishes were purchased from Corning (Corning, NY). Tetrodotoxin, GTP, MgATP, poly-D-lysine, thioridazine, flunarizine, and haloperidol were obtained from Sigma Chemical Co. (St. Louis, MO). The dihydropyridines (−)-Bay K8644 and nimodipine were kindly donated by Dr. Alexander Scriabine, Miles Institute of Preclinical Pharmacology (West Haven, CT). ω-Conotoxin was obtained from Peninsula (Belmont, CA). Penfluridol and fluspirilene were purchased from Janssen Pharmaceuticals (Beerse, Belgium). Clozapine was kindly donated by Dr. William Houlihan, Sandoz Research Institute (East Hanover, NJ).

Cell culture. The rat medullary thyroid carcinoma 6–23 (clone 6) cell line was purchased from the American Type Culture Collection and was grown in 35-mm dishes on polylysine-coated coverslips, in Dulbecco's modified Eagle's medium supplemented with 10% horse serum, at 37° in a humidified atmosphere of 95% air and 5% CO_2. The human medullary thyroid carcinoma TT cell line (21), kindly provided by Andrée de Bustros (Johns Hopkins University), was grown on coverslips as described above, in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Solutions for whole-cell patch clamp. Solutions for whole-cell patch-clamp experiments were designed to eliminate Na^+ and K^+ currents and to minimize Ca^{2+} channel washout. The standard intracellular solution was 120 mM CsCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 11 mM BAPTA, 10 mM HEPES, and 1 mM MgATP, with pH titrated to 7.2 using CsOH. The external solution consisted of 117 mM tetraethylammonium chloride, 5 mM CsCl, 10 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM HEPES, and 5 mM glucose, with pH adjusted to 7.4 using tetraethylammonium hydroxide. Deviations from these solutions are noted in the text. In some early experiments, 2 μM tetrodotoxin was included in the external saline, to block sodium channels. This addition had no apparent effect on membrane currents. All solutions were filtered through 0.22-μm cellulose acetate filters.

Recording conditions. Rat and human C cells were used in patch-clamp experiments 1–4 days after plating. Many C cells developed processes over periods of days in culture. Recordings from these cells frequently included prolonged irregular tail currents, suggesting that adequate voltage control was not possible, even with low-resistance patch electrodes. Consequently, only spherical cells 20–35 μm in diameter, without processes, were selected for recordings. Capacitances of these cells ranged from 15 to 35 pf. Results reported here were obtained in recordings from >250 cells.

Coverslips were transferred from 35-mm culture dishes to the recording chamber (volume, 1.5 ml), which was continuously perfused by gravity at a rate of 5–6 ml/min. Patch electrodes with resistances of 1.5–2.5 MΩ were fabricated from RC-6 glass (Garner Glass Co., Claremont, CA). Whole-cell currents were recorded at room temperature (22–24°), following the procedure of Hamill *et al.* (22), using a List EPC-7 or Axopatch 1D patch-clamp amplifier.

Pulse generation and data acquisition were done using an IBM-AT computer and pCLAMP software, with an Axolab interface (Axon Instruments, Inc., Burlingame, CA). Currents were digitized at 1–50 kHz after filtering with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA). Linear leak and capacity currents were subtracted from current records by using scaled hyperpolarizing steps of ½ to ¼ amplitude. Data were analyzed and plotted using pCLAMP (CLAMPAN and CLAMPFIT) and GRAPHpad.

Drugs were applied by continuous bath perfusion, controlled manually by a six-way rotary valve. Bath exchanges were made by flushing at least 15 ml through the 1.5-ml recording chamber, at rates of 2–5 ml/min. Dihydropyridines penfluridol, haloperidol, and fluspirilene were dissolved in ethanol and diluted into test solutions. Fluspirilene was dissolved in dimethylsulfoxide. The final ethanol or dimethylsulfoxide concentration never exceeded 0.05%, which by itself did not affect currents.

Results

Preferential block of T current by penfluridol. In whole-cell patch-clamp experiments, three separate components of Ca^{2+} current could be detected in most rat medullary thyroid carcinoma 6–23 (clone 6) cells. Based on biophysical and pharmacological properties, we have previously identified these as T-, L-, and N-type currents (18). One of these, the low voltage-activated, rapidly inactivating, T-type current was prominent (>200 pA) in approximately 25% of the cells. Cells with large T currents were chosen for pharmacological studies. This current was potently and preferentially blocked by the DPBP penfluridol. The results illustrated in Fig. 1 are from an experiment in which Ca^{2+} currents were activated by depolarizing steps of increasing size, in 5-mV increments, from a holding potential of −80 mV, before and after superfusion of the cell with saline containing 500 nM penfluridol. Weak depolarizations to test potentials between −30 and −10 mV activated primarily rapidly inactivating T-type current. Stronger depolarizations activated additional decaying as well as maintained current (Fig. 1A). At a concentration of 500 nM, penfluridol blocked approximately 87% of low voltage-activated current, whereas the maximum maintained current (measured at the end of the 300-msec test pulse) was reduced by only 27% (Fig. 1B). Current-voltage plots of the peak (Fig. 1C), maintained (Fig. 1E), and inactivating (peak minus maintained) (Fig. 1D) currents before and after exposure to penfluridol clearly illustrate the preferential block of the rapidly inactivating, low threshold current component. The results shown in Fig. 1 were typical of many other experiments. In a total of 29 cells, 500 nM penfluridol inhibited 78.0 ± 2.3% T-type current, whereas the maintained high threshold component of Ca^{2+} current was less potently blocked (25.6 ± 3.5%).

In addition to its voltage dependence of activation and rapid inactivation, the current inhibited by penfluridol was clearly identified as T-type current based on its pharmacology and deactivation kinetics (Fig. 2, A–C). The low threshold current potently blocked by penfluridol was insensitive to the N channel-specific toxin ω-conotoxin (Fig. 2C). A distinguishing feature of T-type Ca^{2+} current in all types of excitable cells is their slow rate of closing with repolarization, observable as prolonged "tail" currents in whole-cell recordings. Fig. 2B shows decaying tail currents recorded at a potential of −80 mV after a 10-msec

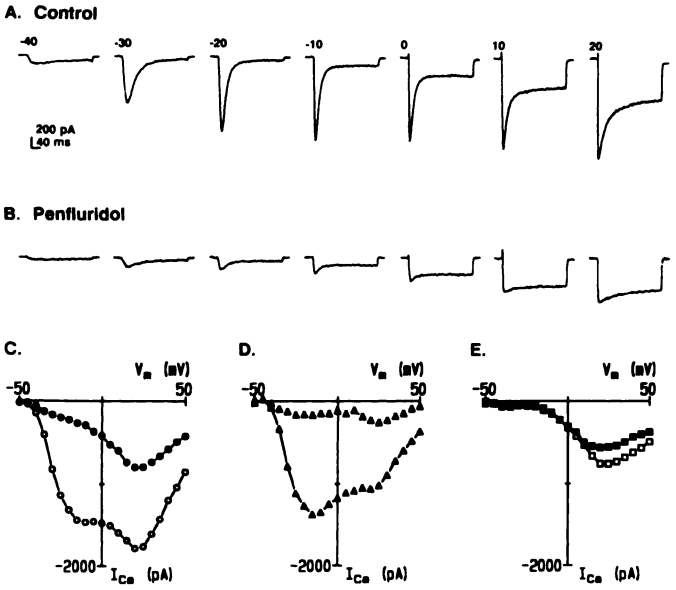

Fig. 1. Inhibition of calcium currents by penfluridol in rat C cells. Ca^{2+} currents were activated by 300-msec depolarizing steps of increasing size, from a holding potential of −80 mV, before and after superfusion of the cell with 500 nM penfluridol. A, Control current records at the indicated test potential. B, Current records after steady state block was reached with 500 nM penfluridol, while test pulses to −20 mV were applied at 0.1 Hz. C, Peak current-voltage curves before (○) and after (●) exposure to penfluridol. Peak current is plotted against test voltage. D, Inactivating current-voltage curves. The inactivating component of current (peak − maintained) is plotted against test potential before (△) and after (▲) exposure to penfluridol. E, Current-voltage curves of maintained current. Current measured at the end of each 300-msec test pulse is plotted against test potential before (□) and after (■) penfluridol. All test pulses were applied at 0.1 Hz in 5-mV increments.

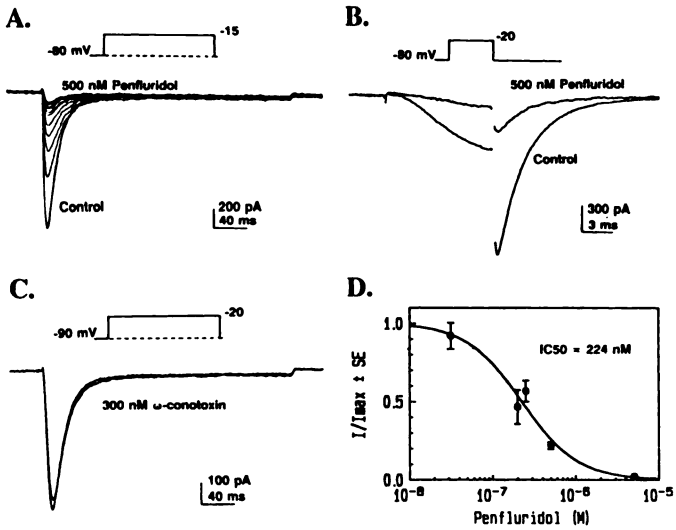

Fig. 2. Inhibition of T type Ca^{2+} current by penfluridol. A, Low voltage-activated Ca^{2+} currents activated by test pulses to −15 mV, applied at 0.1 Hz, from a holding potential of −80 mV. After recording of control currents, the cell was superfused with 500 nM penfluridol. Current records show progression of block over 90 sec. B, Tail currents recorded at −80 mV after 10-msec test pulses to −20 mV, before and after penfluridol, as indicated. C, Low voltage-activated Ca^{2+} currents before and 5 min after superfusion of the cell with ω-conotoxin. At this time, block of high threshold N current had reached a steady state value. D, Inhibition curve for penfluridol block of T current. Data were fit to the equation: $y = 1/1 + (C/x)^p$, where C is the IC_{50} and D is the Hill coefficient.

depolarizing step to −20 mV. The tail current could be fit with a single exponential, with a time constant of 2.5 msec. Penfluridol (500 nM) reduced this tail current and the current activated during the test pulse identically, by 80%. An inhibition curve derived from experiments in which T-type Ca^{2+} currents were blocked by penfluridol at concentrations from 30 nM to 5 μM gave a calculated IC_{50} value of 224 nM, with 10 mM Ca^{2+} as the charge carrier (Fig. 2D).

High voltage-activated current. The high voltage-activated current, which was less sensitive to penfluridol, included N- and L-type components. Fig. 3 shows results of an experiment in which test pulses of varying size were applied before

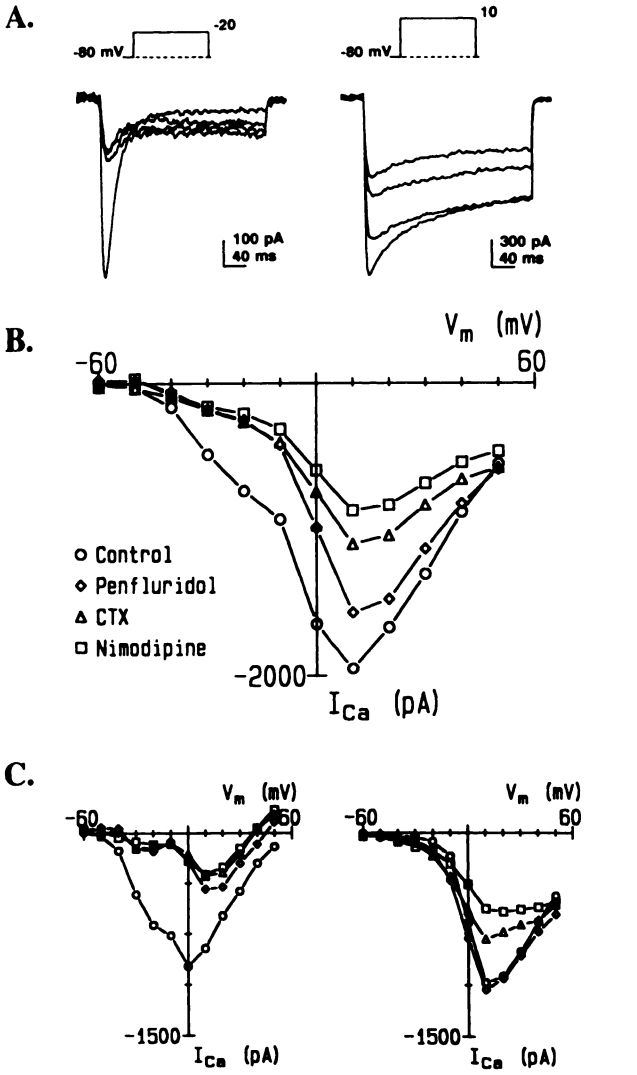

Fig. 3. Block of low and high threshold Ca^{2+} current by multiple antagonists. Individual current traces and current-voltage relationships were obtained from a single cell in control saline and after sequential superfusion with penfluridol, ω-conotoxin, and nimodipine, each at 500 nM. A, *Left*, low threshold currents activated by test potentials to −20 mV in saline and after steady state block had been reached with each drug. Temporal sequence of four traces is from largest to smallest. *Right*, high threshold currents activated by test pulses to +20 mV, as described for traces at *left*. B, Peak current-voltage relationships obtained by application of test potentials in 10-mV increments from a holding potential of −80 mV, in the presence of the indicated antagonists. C, *Left*, current-voltage curves for the inactivating component of current (peak − maintained). *Right*, current-voltage curves for maintained current component. Test depolarizations were applied at 0.1 Hz.

and after sequential superfusion with penfluridol, ω-conotoxin, and the L channel-specific antagonist nimodipine, each at a concentration of 500 nM. These agents were superfused individually in this order. Because inhibition by penfluridol or ω-conotoxin was not measurably reversed by washing in our experiments, additive effects of the drugs were observed. Penfluridol inhibited 82% of low threshold current activated by test pulses to −20 mV. Subsequent superfusion of ω-conotoxin, followed by nimodipine, produced little additional block. With stronger depolarizations, most of the Ca^{2+} current passed through high threshold channels and was less sensitive to penfluridol. Only 20% of the peak Ca^{2+} current activated by test pulses to +10 mV was blocked by penfluridol. ω-Conotoxin and nimodipine combined to reduce the Ca^{2+} current by an additional 40%. Although a large fraction of high voltage-activated current was inhibited, almost 40% of the current remained unblocked after exposure to all three antagonists (Fig. 3, A and B). Current-voltage plots of transient and sustained components of Ca^{2+} currents in this experiment showed clearly that penfluridol preferentially blocked low threshold inactivating current, whereas the other two antagonists inhibited high threshold sustained currents (Fig. 3C, *left* and *right*, respectively).

Our pharmacological studies indicated that high threshold currents in rat C cells result from Ca^{2+} entry through N, L, and possibly other Ca^{2+} channels. Although L and N currents are not easily separated in whole-cell recordings, we attempted to obtain an estimate of the potency of penfluridol as an inhibitor of these two individual channel subtypes. N current in C cells includes inactivating and maintained components, both of which are ω-conotoxin sensitive (18). Current-voltage plots of only inactivating current in many cells were clearly biphasic, reflecting the presence of T and N current. Fig. 4A shows that the low threshold component of inactivating current was much more sensitive to inhibition by penfluridol. At concentrations of 0.5 and 5 μM, penfluridol inhibited 74 and 91%, respectively, of the low threshold inactivating current. By comparison, high threshold transient current was inhibited by 31 and 61% in the same cell. In a total of 20 cells, 500 nM penfluridol reduced the low and high threshold inactivating components by 75 ± 2.9 and 35 ± 4.9%, respectively. Current available for activation from −40 mV was primarily nimodipine-sensitive, noninactivating, L-type current. At a concentration of 500 nM, nimodipine blocked 91 ± 6% (n = 3) of this current. By comparison, penfluridol inhibited 50 ± 2.9% (n = 3) of this current (Fig. 4B).

It appeared that a large fraction of high voltage-activated current elicited from a holding potential of −80 mV was insensitive to penfluridol concentrations much greater than those sufficient to block all T-type current. A small number of C cells (<10%) possessed little or no T-type current; the effects of penfluridol on high voltage-activated Ca^{2+} currents could be observed most clearly in these cells. In four such cells, 5 μM penfluridol blocked 52 ± 3.9% of peak Ca^{2+} current.

Characteristics and mechanism of T-type Ca^{2+} current block. The inhibition of T-type Ca^{2+} current by penfluridol included several distinctive characteristics. When cells were superfused with 500 nM penfluridol while test pulses were applied at 0.1 Hz, from a holding potential of −80 mV, block

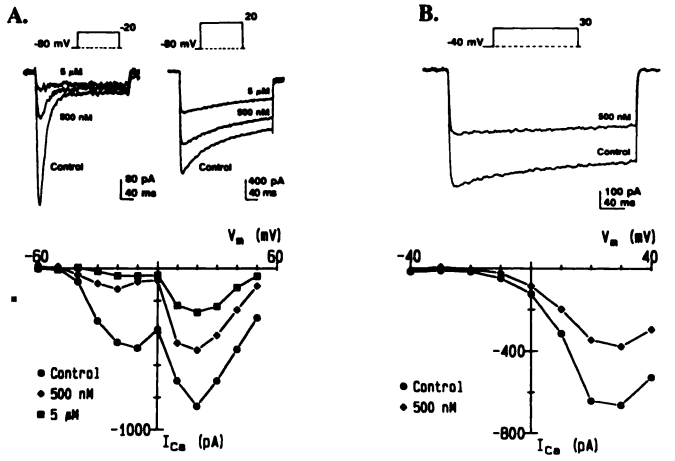

Fig. 4. Inhibition of separate current components by penfluridol. A, Low and high threshold inactivating currents. Ca^{2+} currents were activated by depolarizing steps of increasing size in 10-mV increments, applied at 0.1 Hz, from a holding potential of −80 mV, before and after exposure of the cell to 500 nM and 5 μM penfluridol. *Top left*, low threshold current records in saline and penfluridol, as indicated. *Top right*, high threshold currents. *Bottom*, current-voltage plots of biphasic inactivating current. Inactivating current was measured as peak minus maintained current at each test voltage. High threshold inactivating component has been corrected for contaminating T current by subtraction of an extrapolated T current value at these extrapolated values. B, L-type current. Ca^{2+} currents were activated by test pulses of increasing size in 10-mV increments, from a holding potential of −40 mV, before and after exposure of the cell to 500 nM penfluridol. *Top*, current records before and after drug. *Bottom*, current-voltage plots of peak currents.

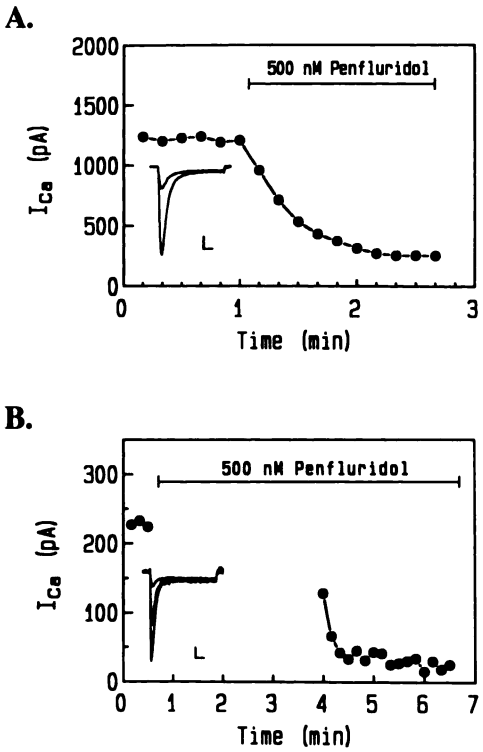

Fig. 5. Time- and stimulus-dependent block of T current. T-type Ca^{2+} current was activated by 60-mV test pulses applied at 0.1 Hz, from a holding potential of −80 mV, either continuously while penfluridol was superfused (A) or discontinuously with a 3.5-min pulse-free gap during the superfusion of drug, as indicated (B). Peak current amplitude is plotted against time. *Insets*, current records in saline and after steady state block with continuous test pulses (A) or in saline and after block before and after test pulses (B). *Scale bars*, 200 pA and 40 msec (A) or 30 pA and 40 msec (B).

typically reached a steady state value within 1–1.5 min (Fig. 5A). Upon superfusion of cells with penfluridol in the absence of test pulses, a smaller percentage of Ca^{2+} channels was blocked, even with markedly longer exposures to the drug. In Fig. 5B, T-type Ca^{2+} current was inhibited by 43% after a 3-min pulse-free exposure to penfluridol. Upon resumption of test pulses, block increased rapidly, reaching a maximum of 87% within 1 min. In five similar experiments, superfusion of cells for 3–4 min in the absence of test pulses inhibited T current by an average of 42 ± 3.2%. After six test pulses, block increased to a maximum steady state value of 76 ± 5.0%.

The accelerated and enhanced block of T current by penfluridol observed with test pulses at a frequency of 0.1 Hz indicated a significant use-dependent action of the drug. However, when test pulses were applied at frequencies below 0.03 Hz, no facilitation of inhibition occurred. At these low frequencies, inhibition typically approached, within several minutes, a steady state level that was not different from that observed in the absence of test pulses. Upon increase of the rate of stimulation up to maximum levels consistent with full recovery from inactivation, additional block developed rapidly. In Fig. 6, test pulses were first applied at 10/min in normal saline, with no measurable reduction in current amplitude. Then, a 4-min exposure to 250 nM (Fig. 6A) or 500 nM (Fig. 6B) penfluridol while test pulses were applied at 1/min inhibited T current by 24% and 36%, respectively. Increase of the stimulation frequency again, to 10/min, resulted in additional block, which reached 52% (Fig. 6A) and 83% (Fig. 6B) within 30 sec. This frequency-dependent component of inhibition was largely reversible when the rate of stimulation was again reduced (Fig. 6). Reversal was maximum with a 1-min pulse-free interval and was not further enhanced by longer times or more negative holding potentials.

During the course of a standard 300-msec test pulse to −20 mV, virtually all T-type Ca^{2+} channels that opened went on to inactivate. To determine whether channel inactivation was necessary for development of use-dependent block, we compared the effects of long and short test pulses on T current inhibition by penfluridol. Fig. 7A shows tail current records in control saline and after steady state block was obtained by application of 300-msec test pulses at 0.1 Hz in the presence of 500 nM penfluridol. A 2-min pulse-free period restored approximately 40% of the blocked current (Fig. 7B, *trace 1*), but reblock occurred rapidly with the application of 10-msec depolarizing test pulses applied at 0.1 Hz, reaching its previous steady state amplitude within 1 min.

Inhibition by some organic Ca^{2+} antagonists appears to be strongly influenced by G proteins (23). To determine whether activation of G proteins modulated block of T-type Ca^{2+} channels by penfluridol in C cells, we studied the inhibition of Ca^{2+} current after addition to the pipet of guanosine nucleotides that activate or inhibit G proteins. The GTP analog GTPγS (200 μM), which irreversibly activates G proteins, did not significantly affect the inhibition of Ca^{2+} currents by penfluridol. With GTPγS in the pipet, penfluridol (500 nM) inhibited 74 ± 5.7% ($n = 3$) of the Ca^{2+} current. No enhancement of this

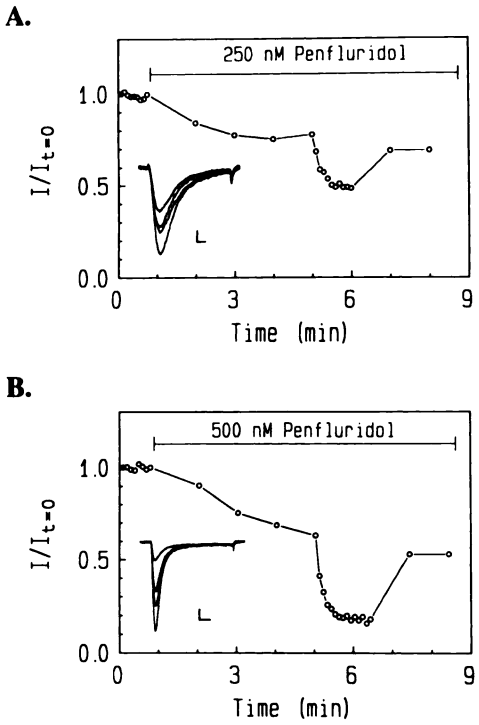

Fig. 6. Frequency dependence and reversal of T current block. Ca^{2+} currents were activated by test pulses to −20 mV, from a holding potential of −80 mV, at frequencies of 0.16 or 0.016 Hz. After recording of currents at the higher stimulation rate for 1 min, cells were superfused with 250 (A) or 500 nM (B) penfluridol while test pulses were continued at 0.016 Hz. After 4 min, the stimulus rate was again switched to the higher frequency, until a new steady state block was achieved. Recovery from block was observed at 0.016 Hz. *Scale bars*, 80 pA and 10 msec (A) or 50 pA and 40 msec (B).

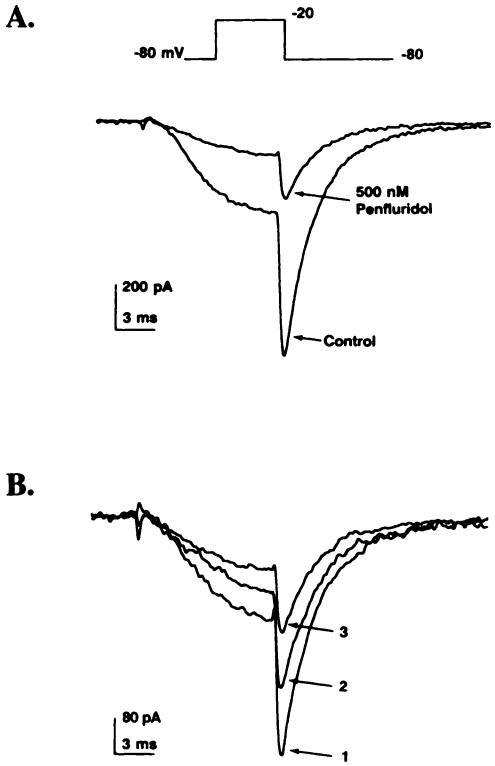

Fig. 7. Block of T channels with short test pulses. A, Tail currents in control saline and after steady state block had been reached with 500 nM penfluridol, while 300-msec test pulses were applied at 0.1 Hz. Tail currents were recorded at −80 mV after 10-msec depolarizing test pulses to −10 mV. B, Tail current records in same cell as A, after a 4-min pulse-free period. Test pulses were applied at 0.1 Hz. Currents were recorded 10 (*trace 1*), 30 (*trace 2*), and 120 sec (*trace 3*), after test pulses were resumed.

current was observed at any time. Inhibition by penfluridol was also unaffected by the presence in the pipet of the guanosine diphosphate analog GDPβS (500 μM), which prevents activation of G proteins by GTP.

Block of human C cell T current. The human medullary thyroid carcinoma TT cell line expresses only low voltage-activated T-type Ca^{2+} channels (19). Penfluridol blocked T-type Ca^{2+} currents in human C cells with a potency and temporal pattern similar to those observed in rat C cells. Reduction of the external [Ca^{2+}] from 10 to 2 mM enhanced the potency of penfluridol. With 2 mM external Ca^{2+}, 100 nM penfluridol blocked approximately 70% of the Ca^{2+} current (Fig. 8).

Other neuroleptics. Preferential block of T-type Ca^{2+} channels was a characteristic of other DPBP antipsychotics. The DPBP fluspirilene blocked Ca^{2+} channels with characteristics similar to those of penfluridol. In the experiment illustrated in Fig. 9, 1 μM fluspirilene inhibited 90% of T-type current activated by test pulses to −24 mV (Fig. 9, *top*), whereas the maximum peak current at a test potential of +10 mV was inhibited by only 27% (Fig. 9, *bottom*). Inhibition by fluspirilene differed from that observed with penfluridol in that it was readily reversed with washing.

Non-DPBP antipsychotics, including clozapine (a dibenzodiazepine), haloperidol (a butyrophenone), and thioridazine (a phenothiazine), also inhibited T-type Ca^{2+} channels but were 20–100-fold less potent than the DPBPs. In Fig. 10A, 3 and 9 μM clozapine reduced T-type current by 23 and 40%, respectively. Inhibition was readily reversible upon washing. For comparison, subsequent superfusion of this same cell with 1

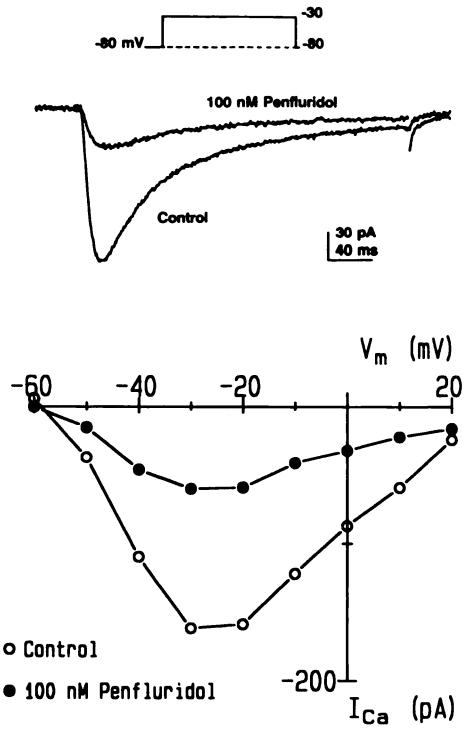

Fig. 8. Inhibition of Ca^{2+} current by penfluridol in a human medullary thyroid carcinoma (TT) cell line. Whole-cell Ca^{2+} currents were recorded in TT cells by application of test pulses of increasing size at 0.1 Hz, from a holding potential of −80 mV, before and after superfusion of 100 nM penfluridol. *Top*, current records in response to test potentials to −30 mV before and after penfluridol, as indicated. *Bottom*, current-voltage relationships; peak current is plotted against test potential. External [Ca^{2+}] was 2 mM.

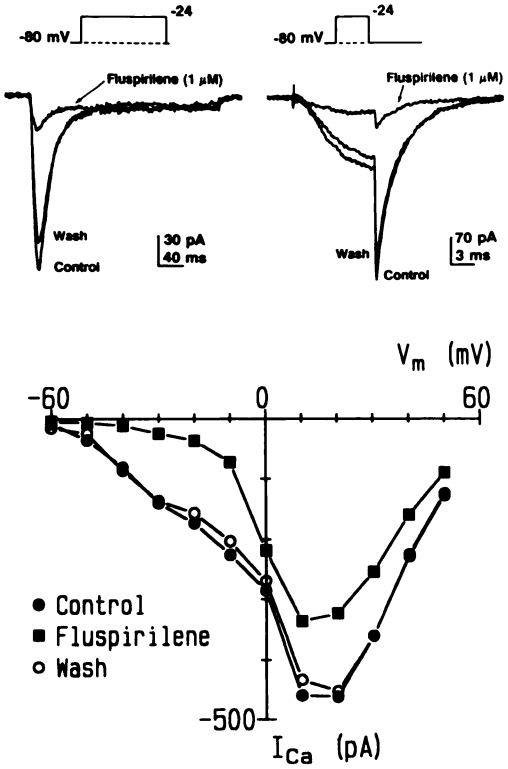

Fig. 9. Block of Ca^{2+} currents by fluspirilene. Ca^{2+} currents were activated by depolarizing steps of varying size applied at 0.1 Hz, from a holding potential of −80 mV, before and after superfusion of 1 μM fluspirilene. *Top left*, low threshold current activated by test pulses to −24 mV in control saline, after block by fluspirilene, and after wash, as indicated. *Top right*, tail currents from the same cell, under conditions as described above. *Bottom*, current-voltage curves; peak currents are plotted against test potentials for the three conditions described above, as indicated.

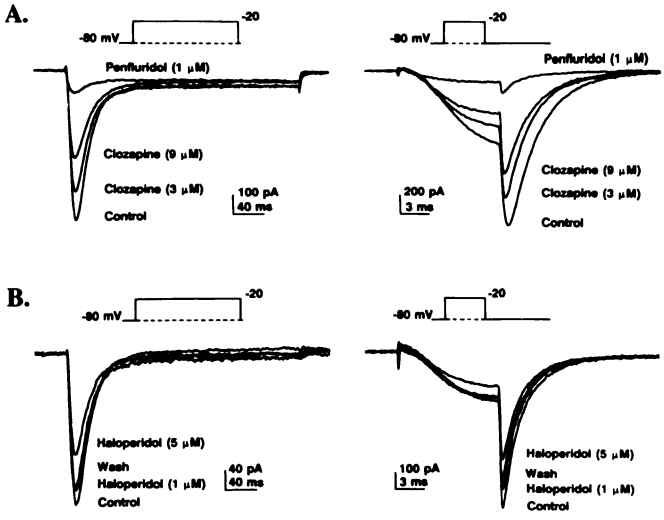

Fig. 10. Inhibition of T Ca^{2+} currents by clozapine and haloperidol. Current through T channels was activated by depolarizing test pulses to −20 mV applied at 0.1 Hz, from a holding potential of −80 mV, in control saline and during the superfusion of drugs. Tail currents were recorded under steady state conditions. A, Clozapine. Ca^{2+} current records in saline, after steady state block by 3 and 9 μM clozapine, and after 1 μM penfluridol, after reversal of clozapine block by washing. B, Haloperidol. Ca^{2+} current records in saline, after steady state block by 1 and 5 μM haloperidol, and again after reversal of block in control saline.

μM penfluridol reduced T current by approximately 88%. The estimated IC_{50} for clozapine inhibition of T current was approximately 10 μM. Haloperidol reversibly blocked T-type Ca^{2+} current with a potency similar to that of clozapine (Fig. 10). At a concentration of 5 μM, haloperidol reduced T-type Ca^{2+} current by 31 ± 4% (n = 4). The phenothiazine thioridazine was even less potent as a T channel blocker, reducing this current by approximately 25% at a concentration of 10 μM (data not shown). This drug was more effective as an inhibitor of high voltage-activated channels, with >80% of high voltage-activated current being inhibited at a concentration of 10 μM.

Discussion

In the present study, we found that DPBP neuroleptics are unique among organic Ca^{2+} channel blockers in potently and preferentially blocking T-type Ca^{2+} channels in rat and human neural crest-derived C cell lines. Inhibition by the DPBP penfluridol was enhanced by depolarizing test pulses, in a frequency-dependent manner. Non-DPBP neuroleptics from several other classes blocked T-type Ca^{2+} channels but were at least 20–100-fold less potent. T-type current in the rat C cell line was readily isolated by its voltage dependence of activation and kinetics of inactivation and deactivation. Consequently, inhibition of this specific current could be measured unambiguously in our experiments. Because T-type current in C cells was relatively stable over periods of many minutes, contamination by "rundown" was not a problem. When currents were recorded for periods longer than 10 min, some rundown of high voltage-activated currents may have occurred. A sequence of control currents was always recorded, to ensure that currents were stable, before application of drugs. Nevertheless, our estimates of the selectivity of block by DPBPs are conservative and may slightly underestimate the selectivity of these agents as T channel antagonists.

Similarities in voltage dependence and kinetics of L- and N-type Ca^{2+} currents in C cells made it difficult to determine accurately the potency of DPBPs as antagonists of these separate Ca^{2+} channel subtypes. In a previous study, we found that N-type current in C cells, as in neurons, consists of inactivating and maintained components (18). The inactivating component of high threshold current in C cells is largely ω-conotoxin sensitive and presumably represents Ca^{2+} entry through N channels. The inhibition of this component of current by penfluridol serves as our best estimate of its potency as an N channel antagonist. The block of L-type current measured at a holding potential of −40 mV may overestimate the potency of penfluridol at more negative voltages; we have observed voltage-dependent block of L channels by DPBPs in pituitary and heart (13, 17). The possibility that C cells might express additional types of Ca^{2+} channels that may be more or less sensitive to DPBPs cannot be excluded. Regardless, a large fraction of high voltage-activated Ca^{2+} current in C cells is not blocked by penfluridol at concentrations 10-fold higher than those that block all T-type current.

Although the inhibition by penfluridol was only partly reversible with washing, additional reversibility with prolonged hyperpolarization indicated that a toxic effect of the drug was not involved. Further, T current inhibition by the DPBP fluspirilene was readily reversible, a disparity that we cannot currently explain. Penfluridol was used in most of our experiments because of solubility problems experienced with other DPBPs in the external solution.

T-type Ca^{2+} current in many types of excitable cells is relatively insensitive to the major organic Ca^{2+} antagonists (for reviews, see Refs. 1 and 2). In those cells where inhibition of T-type current has been reported, it typically occurs at drug concentrations much higher than those required to block L-type channels in the same cells. The dihydropyridine felodipine blocks T-type Ca^{2+} channels in atrial cells at low concentrations, but no preferential inhibition has been reported (24). The Ca^{2+} antagonist flunarizine has been reported to block T-type Ca^{2+} channels in hypothalamic neurons at submicromolar concentrations (25). However, significantly higher concentrations were required to inhibit T channels in guinea pig ventricular myocytes (26). We found that flunarizine blocked T-type Ca^{2+} current in rat C cells with an IC_{50} of approximately 10 μM.[1] Several other drugs, including amiloride, diphenylhydantoin, and ethosuximide, have been reported to block T channels with some selectivity (10–12). However, each of these is 50–100 times less potent than the DPBPs in our study. The specificity of these agents at such high concentrations is questionable. Other agents, including octanol and tetramethrin, may be more selective T channel antagonists (8, 27).

The DPBPs were identified as potential Ca^{2+} antagonists when it was discovered that these agents blocked binding of dihydropyridines to their receptors in brain and inhibited depolarization-dependent Ca^{2+} uptake and contraction in muscle (28). Since then, voltage-clamp studies have shown the DPBPs to be relatively potent blockers of L-type Ca^{2+} channels in endocrine and muscle cells, including heart and pituitary (13, 17). The present study demonstrates that the DPBPs potently block T-type channels in neural crest-derived cells. Importantly, the potency of these agents as antagonists of low and high voltage-activated channels is reversed, compared with the major organic blockers, and T channels are preferentially inhibited. Penfluridol also blocked T-type Ca^{2+} channels in human C cells. The pharmacology of low voltage-activated Ca^{2+} channels in human excitable cells has not been described. Considerable variability exists among T channels in different cell types, with respect to pharmacology. A 50-fold difference has been reported for T channels in heart and pituitary cells, with respect to their sensitivity to felodipine (24).

Block of L-type Ca^{2+} channels by many organic Ca^{2+} antagonists is characterized by voltage and use dependence and typically is enhanced by repeated or prolonged depolarization (29, 30). According to the modulated receptor hypothesis, this type of block indicates that the antagonist preferentially binds to and blocks Ca^{2+} channels in conformations other than those represented by the inactive state (31, 32). Previously, we have shown that block of L-type Ca^{2+} channels in heart and pituitary cells by DPBPs is enhanced by depolarization and partially reversed by prolonged hyperpolarization (13, 17). In the present study, we discovered a significant use-dependent component of T channel block by the DPBPs. Among organic Ca^{2+} antagonists, marked use-dependent block of L-type Ca^{2+} channels occurs with verapamil and D600, tertiary amines that are predominantly charged at physiological pH (30). Neutral drugs such as nitrendipine show much less use dependence. A similar pattern exists between charged and uncharged local anesthetics, with respect to use-dependent block of Na^+ channels

[1] J. J. Enyeart, B. Mlinar, and B. Biagi, unpublished observations.

(31, 33). Penfluridol, a tertiary amine with a pK_a of 8.9, would be predominantly charged at pH 7.4. The use-dependent block of T channels that we observed is consistent with the pattern described above and suggests that T-type Ca^{2+} channels must first open for charged penfluridol molecules to reach their binding site. Apparently, inactivation of T channels was not required for development of use-dependent block, because block was observed with very short test pulses, where little or no inactivation occurs. However, penfluridol did not accelerate T current inactivation kinetics,[1] a characteristic of agents that block open channels. The reversibility of the stimulus-dependent component of block during pulse-free periods indicates that penfluridol can reach and leave its receptor through more than one physical route, such as the hydrophobic and hydrophilic pathways proposed by Hille (33).

Because of their potent and preferential block of T-type Ca^{2+} channels, the DPBP antipsychotics will be a useful new class of drugs for defining clearly the function of T-type Ca^{2+} channels in excitable cells. We have used them to explore the regulation of prolactin gene expression by Ca^{2+} channels in pituitary cells (17). At low concentrations, DPBPs should be valuable for determining how these channels affect electrical activity in spontaneously active cells. Previous studies on heart, brain, and endocrine cells have identified high affinity binding sites for DPBPs on the L-type Ca^{2+} channel (15, 16). The present study indicates that a binding site with equal or higher affinity exists on T-type Ca^{2+} channels of rat and human cells. DPBPs may be useful in quantitating and/or isolating these channels.

Neuroleptic drugs of many classes share the ability to block D_2 dopamine receptors. Potent block of T-type Ca^{2+} channels does not appear to be a characteristic shared by the various classes of neuroleptic drugs. Consequently, interaction with these channels may be unrelated to their therapeutic actions. However, our finding that penfluridol very potently blocks T-type Ca^{2+} channels in human C cells indicates that these channels, as well as D_2 dopamine receptors, could be occupied at clinically relevant doses. This would be especially likely in rapidly firing cells, where potency is enhanced.

The only known pharmacological action that distinguishes DPBPs from the butyrophenone neuroleptics is their Ca^{2+} antagonist activity. In clinical trials, the DPBPs produced therapeutic effects clearly different from those of other antipsychotics, relieving the negative as well as the positive symptoms of schizophrenia. It has been suggested that L-type Ca^{2+} channels could be involved in these distinctive actions of the DPBPs (15, 34). Our results indicate that T-type Ca^{2+} channels are also potential targets for these agents.

Specific blockers of T-type Ca^{2+} channels could potentially be useful in treating diseases that involve inappropriate electrical activity originating in spontaneously active cells, like that which occurs in epilepsy or arrhythmias. Ethosuximide, a drug that is useful in the treatment of absence seizures, blocks T-type Ca^{2+} channels in thalamic neurons with an IC_{50} of 200 μM, a high but clinically relevant concentration (35). DPBPs with reduced affinity for D_2 dopamine receptors would be interesting candidates as potential antiepileptic drugs.

The DPBPs may be unique among Ca^{2+} channel antagonists in their ability to interact with many different Ca^{2+} channel subtypes. It will be interesting to determine their potency as blockers of other neuronal Ca^{2+} channels, including the recently discovered P-type channel (3, 5). Through their interactions with neuronal Ca^{2+} channels as well as dopamine receptors, the DPBPs might be expected to produce complex effects on neuronal and central nervous system function. Recently, we demonstrated that DPBPs could potently inhibit prolactin gene expression through action on pituitary Ca^{2+} channels (17). It will be interesting to determine whether these drugs have similar effects on gene expression in neurons.

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