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Pharmacological Properties of the T-Type Ca^{2+} Current of Mouse Spermatogenic Cells

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ABSTRACT

The effects of pharmacological agents on the T-type Ca^{2+} current were studied in dissociated spermatogenic cells from the mouse. Ca^{2+} currents were elicited by depolarization in 10 mM Ca^{2+} and recorded in the whole-cell configuration of the patch clamp technique. The T-type current was inhibited by the following compounds: PN200–110 ($IC_{50} = 4 \times 10^{-8}$ M) > nifedipine ($IC_{50} = 4 \times 10^{-7}$ M) > pimozide ($IC_{50} = 4.6 \times 10^{-7}$ M) > mibefradil ($IC_{50} = 5 \times 10^{-6}$ M) > Ni^{2+} ($IC_{50} = 3.4 \times 10^{-5}$ M) > verapamil ($IC_{50} = 7 \times 10^{-5}$ M) > amiloride ($IC_{50} = 2.4 \times 10^{-4}$ M) > Cd^{2+} ($IC_{50} = 2.8 \times 10^{-4}$ M). However, the agents differed in the reversibility and the use dependence of their effects. Currents recovered rapidly and completely after removal of Ni^{2+}, Cd^{2+}, amiloride, or mibefradil, whereas recovery from verapamil block was rapid but incomplete. In contrast, we observed little recovery after the removal of pimozide and of the dihydropyridines (PN200–110, nifedipine). Moreover, mibefradil and pimozide exhibit a strongly use-dependent inhibition of current that is due to selective interaction of these drugs with the open state and the inactivated state of the channel, respectively, rather than with the resting state. These properties of the spermatogenic T-type Ca^{2+} channel differ from those of somatic cell T channels and suggest a molecular diversity of low voltage-activated Ca^{2+} channels.

Two classes of voltage-sensitive Ca^{2+} currents are defined based on their biophysical and pharmacological properties. The high voltage-activated class of currents share a requirement for a strong depolarization to evoke opening. This broad class is composed of L-, N-, P-, Q-, and R-type subclasses, many of which exhibit characteristic pharmacological properties. For example, L-type currents are selectively inhibited by low concentrations (nanomolar) of 1,4-dihydropyridines, N-type currents by ω-conotoxin GVIA, P-type currents by low concentrations of ω-conotoxin MVIIC and by high concentrations (micromolar) of agatoxin IVA, and Q-type currents by low concentrations of agatoxin IVA. These inhibitory signatures permit the identification of high voltage-activated currents based on pharmacological properties and facilitate the rational design of antagonists.

In contrast, a T-type low voltage-activated current has been identified in a variety of tissues. This current is evoked by weak depolarizations and contributes to diverse physiological processes, including cardiac pacemaker activity (Irisawa *et al.*, 1993), spontaneous oscillatory activity in thalamic bursting neurons (Huguenard and Prince, 1992), cortisol secretion (Enyeart *et al.*, 1993), spontaneous activity during neuron development (Gu and Spitzer, 1993), and the control of mammalian sperm acrosome reaction during fertilization (Arnoult *et al.*, 1996a).

An understanding of the structure and function of this channel is limited by the absence of potent antagonists that inhibit T currents with high specificity. The usefulness of available antagonists is limited by (1) low specificity, as in the case of amiloride; (2) limited selectivity, as in the case of ethosuximide (Coulter *et al.*, 1989) and other agents that act only on a subset of T currents; or (3) by a complex pharmacology, as in the case of the 1,4-dihydropyridines, which have no effect on some T currents (Fox *et al.*, 1987) while inhibiting others with high potency [$IC_{50} \leq 1$ μM (hypothalamic neurons, Akaike *et al.*, 1989a; aorta smooth muscle, Akaike *et al.*, 1989b; CA1 pyramidal neurons, Takahashi and Akaike, 1991; dorsal root ganglion, Richard *et al.*, 1991; atrial myocytes, Cohen *et al.*, 1992; and spermatogenic cells, Arnoult *et al.*, 1996a, Santi *et al.*, 1996)] yet others with lower potency [$IC_{50} \sim 10$ μM (Bean, 1985)]. Recently, it was suggested that pimozide, a diphenylbutylpiperidine, and mibefradil, a benzimidazolyl-substituted tetraline derivative, inhibit T-type Ca^{2+} currents under conditions in which high voltage-acti-

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ABBREVIATIONS: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

vated Ca^{2+} currents are unaffected. However, these studies focused on a limited array of cell types; for example, the effects on T currents of pimozide and mibefradil have been described in the adrenal zona fasciculata cells (Enyeart *et al.*, 1993) and on smooth muscle (Mishra and Hermsmeyer, 1994), respectively. It is necessary to examine the effects of these agents on a broader range of preparations to assess their use as T channel antagonists.

We studied the role of sperm T channels in fertilization. The sperm acrosome reaction is a Ca^{2+}-dependent secretory event that must be completed before fertilization (Yanagimachi, 1994). In mammals, acrosome reactions are initiated by sperm contact with the extracellular matrix of the egg, or zona pellucida. The signal transducing mechanism activated by the zona pellucida includes an essential induction of a T-type Ca^{2+} current, and the secretion of acrosome is inhibited by T channel antagonists (Arnoult *et al.*, 1996a). Moreover, it has been reported that the 1,4-dihydropyridines antagonists of T- and L-type Ca^{2+} channels may have a male contraceptive effect (Benoff *et al.*, 1994; Hershlag *et al.*, 1995). Although this channel is central to fertilization and provides a new target for contraceptive intervention, an extensive pharmacological analysis of the T-type Ca^{2+} current of male germ cells has not been performed. Here, we report the effects of a range of T channel antagonists on this current, focusing particularly on pimozide and mibefradil. There were three objectives of this study: (1) to identify the potent inhibitors of the T-type Ca^{2+} current in spermatogenic cells; (2) to begin to evaluate the possibility of effects on the germ cell T channel, and hence a possible antifertility effect, after clinical use of these drugs; and (3) to examine the use dependence of pimozide and mibefradil.

Materials and Methods

Cell preparation. Seminiferous tubules were isolated from the testes of CD-1 mice (16 weeks old; Charles River Laboratories, Wilmington, MA) and incubated at 37° for 30 min in 3 ml of a solution containing 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NaH_3PO_4, 12 mM $NaHCO_3$, 11 mM D-glucose, pH 7.3, and collagenase type IA (1 mg/ml; Sigma Chemical, St. Louis, MO). Tubules were rinsed twice in collagenase-free medium and cut into 2-mm sections. Spermatogenic cells were obtained by manual trituration and attached to culture dishes coated with Cell-Tak (Collaborative Biomedical Products, Bedford, MA). Pachytene spermatocytes and round spermatids are the prominent cell types obtained from the diploid meiotic and haploid postmeiotic stages of spermatogenesis, respectively. These cells are readily distinguished based on cellular and nuclear morphology (Romrell *et al.*, 1976; Arnoult *et al.*, 1996a). These stages are routinely used for electrophysiological recording; similar results were obtained with both stages, and the data are pooled for presentation.

Electrophysiological recordings. Ca^{2+} currents were recorded in the whole-cell configuration of patch-clamp technique and analyzed using Biopatch (BioLogic, Claix, France). Pipettes were pulled from Corning 7052 glass (Gardner Glass, Claremont, CA), coated with Sylgard 184 (Dow Corning, Midland, MI), and fire polished. Pipette resistance was 5–7 MΩ. Currents were obtained with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). All traces were corrected for leak and capacitance currents, filtered at 2 kHz, and digitized every 0.1 msec. Other details of the voltage protocols used here are provided in Results. Data typically are expressed as peak current amplitude values. However, in some cases, current densities were calculated from measured current amplitude, based on measured cell diameters and assuming spherical shape.

The pipette solution was designed to eliminate all K^+ currents and consisted of 130 mM Cs-glutamate, 5 mM D-glucose, 10 mM HEPES, 2.5 mM $MgCl_2$, 20 mM TEA-Cl, 4 mM Mg_2ATP, and 10 mM EGTA-Cs, pH 7.2 (adjusted with 1 N CsOH). For experiments, the bath solution was changed to a recording solution that consisted of 100 mM NaCl, 5 mM KCl, 10 mM $CaCl_2$, 1 mM $MgCl_2$, 26 mM TEA-Cl, 6 mM Na-lactate, 10 mM HEPES, and 3.3 mM D-glucose, pH 7.4 (adjusted with 1 N NaOH). The cells are isolated in a 1-ml chamber and perfused at a rate of 4–8 ml/min. All experiments are done at room temperature (~25°).

Stock solutions of nifedipine, PN200–110 (Novartis, East Hanover, NJ), and fluspirilene were prepared in dimethylsulfoxide, and pimozide stocks were prepared in ethanol. Dimethylsulfoxide concentrations were <0.01% in all cases. Control experiments demonstrated that this solvent had no effect on Ca^{2+} current amplitude even at concentrations of 0.02% (seven experiments). Free Ni^{2+} and Cd^{2+} concentrations were corrected for binding by lactate using the ALEX program (Vivaudou *et al.*, 1991). Mibefradil was a gift from J.-P. Clozel and S. Bottari.

Results

A T-type Ca^{2+} current is the only Ca^{2+} current that is detected in dissociated mouse spermatogenic cells using the whole-cell configuration of the patch-clamp (Hagiwara and Kawa, 1984; Arnoult *et al.*, 1996a; Lievano *et al.*, 1996; Santi *et al.*, 1996). The biophysical characteristics of this current were described previously (Arnoult *et al.*, 1996a) and may be summarized as follows: during depolarization from holding potentials below −80 mV, the current has an activation threshold of −60 mV and a peak current at −20 to −30 mV and exhibits pronounced voltage-dependent inactivation ($V_{1/2}$ = −70 mV). The amplitude of this T-type Ca^{2+} current also is subject to positive modulation by voltage- and tyrosine phosphatase-dependent mechanisms and to negative modulation by a tyrosine kinase-dependent mechanism (Arnoult *et al.*, 1997).

T current inhibition: potency studies. The effects of inhibitors of both T- and L-type currents of somatic cells on the spermatogenic cell T current were determined (Fig. 1). T-type current inhibitors included Ni^{2+}, Cd^{2+}, amiloride, pimozide, fluspirilene, and mibefradil, whereas L-type current inhibitors included 1,4-dihydropyridines (PN200–110, nifedipine) and verapamil. Whole-cell currents were recorded in 10 mM Ca^{2+} during 100-msec depolarizations from a holding potential of −90 mV to a test potential of −20 mV (frequency, 0.1 Hz). During 10-min control experiments, the rundown of peak current was frequently undetectable and always <15%, even at stimulation rates of 1 Hz.

The spermatogenic cell Ca^{2+} current is inhibited by Ni^{2+} and Cd^{2+}. The respective IC_{50} values of 34 and 285 μM (Fig. 1A) are characteristic of somatic cell T currents (Fox *et al.*, 1987; Herrington and Lingle, 1992). The germ cell current is also inhibited by amiloride (IC_{50} 245 μM; Fig. 1B). Somatic cell T currents vary widely in their sensitivity to amiloride, with reported IC_{50} values ranging from <50 μM (Tang *et al.*, 1988) to ~1 mM (Herrington and Lingle, 1992), but many T currents exhibit a sensitivity similar to that of the channel in spermatogenic cell (Hirano *et al.*, 1989; Behe *et al.*, 1990; Tytgat *et al.*, 1990). Thus, the spermatogenic cell T-type Ca^{2+} channel is similar to those in many somatic tissues with regard to the effects of these agents.

Pimozide is a diphenylbutylpiperidine that acts as a neuroleptic agent. Its therapeutic use is principally due to its

effects on dopaminergic transmission. However, pimozide also inhibits T currents in some somatic cell preparations and spermatogenic cells (IC_{50} = 0.46 μM, Fig. 1C; Arnoult *et al.*, 1996a). A related diphenylbutylpiperidine, fluspirilene, also inhibits T currents in spermatogenic cells (Fig. 2D). The observed inhibitory potency of diphenylbutylpiperidines is similar to that detected in adrenal zona fasciculata cells (IC_{50} for pimozide ~ 0.5 μM; Enyeart *et al.*, 1993) and thyroid carcinoma cells (IC_{50} for penfluridol ~ 0.2 μM; Enyeart *et al.*, 1992). In contrast, T currents from GH3 clonal pituitary cells are relatively insensitive to diphenylbutylpiperidines (Herrington and Lingle, 1992).

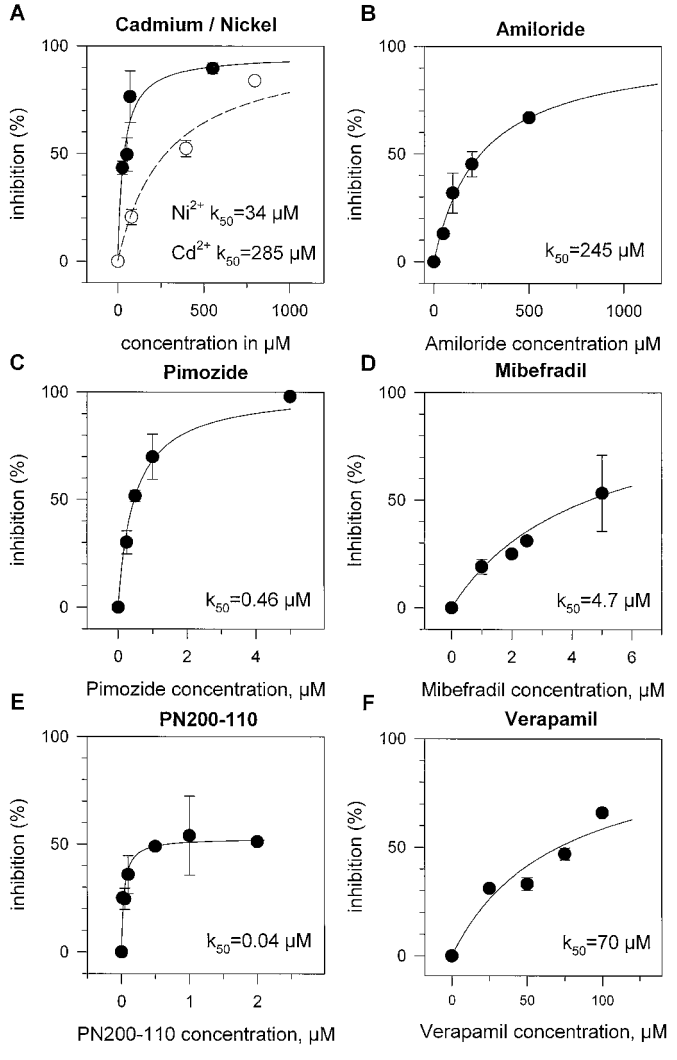

Fig. 1. Dose-response relationships for the inhibition of peak current amplitude by T channel antagonists. Data represent the mean ± standard error inhibition of one to eight challenges and were fit with the equation $I = (I_{max} \times [C])/(k_{50} + [C])$, where I is the inhibition of current (percent of control), I_{max} is the maximum inhibition, k_{50} is the inhibitory constant, and [C] is the inhibitor concentration. Calculated k_{50} values are indicated. All data are obtained by depolarizing cells from a holding potential of −90 mV to a test potential of −20 mV at a rate of 0.1 Hz in an external solution containing 10 mM Ca^{2+}. A, Nickel (●) and cadmium (○). B, Amiloride. C, Pimozide inhibition was determined from inhibition of the peak current density after incubation of the cells with the drug for ≥15 min. Current density is 5.20 ± 0.25 μA/cm² (27 experiments) in control solution and 3.63 ± 0.28 (5 experiments), 2.52 ± 0.14 (8 experiments), 1.5 ± 0.55 (5 experiments), and 0.05 ± 0.05 (5 experiments), in presence of 0.25, 0.5, 1, and 5 μM pimozide, respectively. D, Mibefradil. E, PN200–110. F, Verapamil.

The kinetics of diphenylbutylpiperidine effects are complex and are determined by both drug concentration and stimulation rate. For example, at a concentration of 5 μM, 100% of the current is inhibited within 3 min (Fig. 2D) but 10 min is required to reach the steady state inhibition after the application of a <1 μM concentration of these drug. Fig. 3A illustrates the slow binding of 200 nM pimozide at a rate of depolarization of 0.2 Hz, under these conditions, up to 8 min is required for a steady state inhibition. We have found that some rundown of current occurs during the time course of inhibition when using low concentrations of pimozide (<500 nM). This may complicate assessment of drug-dependent inhibition. To avoid such complications, we determined IC_{50} values from inhibition of the peak current density after incubation of the cells with the drug for ≥15 min.

Recently, the results of studies on Ca^{2+} currents in vascular smooth muscle have suggested that mibefradil may inhibit T-type currents with an IC_{50} value of ~0.1 μM, whereas 10–100-fold higher concentrations are required to inhibit L-type, high voltage-activated Ca^{2+} channels (Mishra and Hermsmeyer, 1994a). However, a limited range of cell preparations have been characterized, and the reported potency of this drug varies widely. In the case of thyroid carcinoma cells, mibefradil inhibits T current with lower potency (IC_{50} ~ 2.7 μM; Mehrke *et al.*, 1994) and cannot discriminate between T-type and L-type currents. As shown in Fig. 1D, relatively high concentrations of mibefradil are required to inhibit the T current of mouse spermatogenic cells (IC_{50} ~ 4.7 μM). At these concentrations, mibefradil also inhibits high voltage-activated Ca^{2+} channels (Bezprozvanny and Tsien, 1995).

The 1,4-dihydropyridine class of Ca^{2+} antagonists, which accomplish their therapeutic action principally by inhibiting the L-type high voltage-activated current, also are known to inhibit T-type currents in both somatic (Akaike *et al.*, 1989a, 1989b; Richard *et al.*, 1991; Takahashi and Akaike, 1991; Cohen *et al.*, 1992) and male germ (Arnoult *et al.*, 1996a; Lievano *et al.*, 1996; Santi *et al.*, 1996) cells. PN200–110 produces a half-maximal inhibition of the germ cell current at 40 nM and had a maximal effect at ~200 nM (Fig. 1E), whereas nifedipine produced half-maximal and maximal inhibition at 0.5 and 2 μM, respectively (not shown). These agents block T currents slowly, with 3–4 min required to reach steady state inhibition (Fig. 2C). The time course of inhibition is not dependent on 1,4-dihydropyridine concentration (data not shown), unlike the case of diphenylbutylpiperidines. However, the inhibition produced by PN200–110 and nifedipine is complex, and even at higher drug concentrations, both agents reduced the spermatogenic cell T current by a maximum of only 50% (Fig. 1E). The basis for this partial inhibition is not understood.

Verapamil is an arylalkylamine Ca^{2+} antagonist that acts principally by inhibiting L-type high voltage-activated Ca^{2+} currents. However, this agent is similar to the 1,4-dihydropyridines in that it also inhibits the T current of spermatogenic cells (IC_{50} ~ 70 μM; Fig. 1F).

T current inhibition: reversibility. The experimental and clinical use of Ca^{2+} antagonists is dependent on recovery of current after drug removal. In the case of the T channel antagonists, this is particularly relevant with regard to evaluating the possibilities of an antifertility effect. Antagonists that dissociate slowly may block the sperm acrosome reaction

induced by egg contact, resulting in compromised fertility. In this regard, we recently demonstrated that PN200–110, a 1,4-dihydropyridine, produces a sustained inhibition of the germ cell T current after drug removal (Arnoult *et al*., 1996a). We suggested that the resulting inhibition of the acrosome reaction (Arnoult *et al*., 1996a) may account for the reported infertility of men treated with these drugs (Benoff *et al*., 1994; Hershlag *et al*., 1995).

We therefore determined the reversibility of spermatogenic cell T currents after drug removal. Three broad groups of antagonists were identified based on these reversibility studies. The first group is composed of agents in which recovery of current is complete and includes Ni^{2+}, Cd^{2+} (not shown), amiloride (Fig. 2A), and mibefradil (Fig. 2B). Although the time courses of recovery vary among these compounds (see Fig. 2, A and B), in all cases there is complete reversal of inhibition. In this regard, complete recovery of current after removal of amiloride also is a characteristic of somatic cell T currents (Tang *et al*., 1988; Tytgat *et al*., 1990).

A second group of antagonists produces a sustained inhibition of T current in which recovery either is not observed or occurs very slowly. This class includes the 1,4-dihydropyridines, PN200–110 (Arnoult *et al*., 1996a) and nifedipine (Fig. 2C), and the diphenylbutylpiperidines, fluspirilene (Fig. 2D) and pimozide (not shown). The lack of recovery from fluspirilene (3 experiments) and pimozide (10 experiments) treatment after extensive washing was in marked contrast to the response in certain somatic cells, in which complete recovery is observed (Enyeart *et al*., 1992). This rate of recovery was not affected by membrane hyperpolarization (not shown). Finally, a third pattern of recovery is illustrated by verapamil. A fraction (~50%; five experiments) of the T current

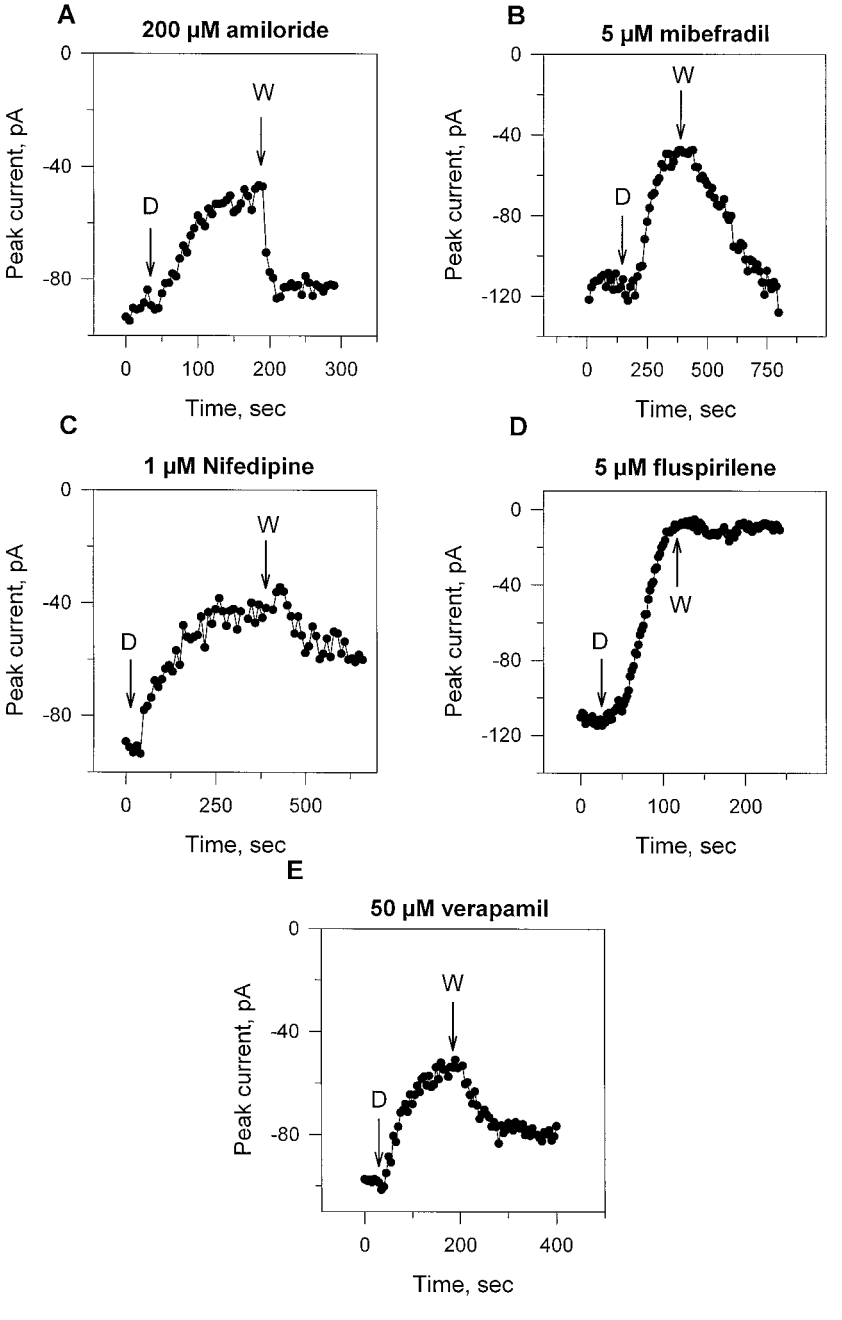

Fig. 2. Kinetics of onset and reversibility of current inhibition by T channel antagonists. All data were obtained by depolarizing the cell from a holding potential of −90 mV to a test potential of −20 mV in an external solution containing 10 mM Ca^{2+}. Rate of depolarization was 0.1 Hz for mibefradil and nifedipine, 0.2 Hz for amiloride and verapamil, and 0.5 Hz for fluspirilene. *D arrows*, starting time of cell perfusion by the drug solution. *W arrows*, end of the cell perfusion by washing the drug solution with the recording standard solution.

recovers rapidly ($t_{1/2} \sim 40$ sec; Fig. 2E), whereas there is little recovery of the remaining current during a 10-min wash.

Use-dependent block by pimozide and mibefradil. It is understood that drug potency may be modified by a variety of factors, including charge carrier concentration and rate of stimulation. Among the drugs shown in Fig. 1, only mibefradil, verapamil, and diphenylbutylpiperidines were characterized by their use-dependent inhibition. The inhibition of the T current by dihydropyridines was not enhanced by increasing the rate of stimulation, unlike with L-type Ca^{2+} channels (Bean, 1984; Kamp *et al.*, 1989). We therefore examined the use-dependency of two T channel antagonists: pimozide and mibefradil. In these experiments, peak current amplitude was determined after depolarization from a holding potential of −90 mV to a test potential of −20 mV in the presence or absence of T channel antagonists.

A stable, inward Ca^{2+} current is evoked by low frequency depolarization (0.1–0.2 Hz) of spermatogenic cells (Fig. 3, A and B; Arnoult *et al.*, 1996a, 1997). There is considerable variation in the amplitude of this current between spermatogenic cells, as illustrated by a comparison of Fig. 3A (90–100 pA) and 3B (130–140 pA). These differences in amplitude may be due to several factors, including (1) cell size, which decreases as cells progress through spermatogenesis (Romrell *et al.*, 1976), and (2) T channel modulation state (Arnoult *et al.*, 1997). However, the current evoked in a cell by low frequency depolarization (~0.1 Hz) is highly reproducible.

The addition of 0.2 μM pimozide (Fig. 3A) or 2.5 μM mibefradil (Fig. 3B) produced a progressive inhibition of the T current. However, increasing the frequency of depolarization resulted in an enhanced rate of current inhibition. In the case of pimozide, the level of inhibition increased from 45% to 70% at a depolarization frequency of 0.5 Hz, and mibefradil-dependent inhibition increased from 45% to 70% at a depolarization frequency of 1 Hz. The enhanced inhibitory potency of these agents was reversed when depolarization frequency was subsequently reduced to 0.1 Hz (Fig. 3, A and B, *arrows*). Control experiments demonstrated that depolarization frequency at rates ≤2 Hz had no effect on the amplitude of the current in absence of drug (data not shown). Dose-response studies indicate that this enhanced inhibition of current is due to an increase in drug potency. As shown in Fig. 4, the IC_{50} value of mibefradil decreases from 4.71 to 0.85 μM on increase of depolarization frequency from 0.1 to 1 Hz.

The use dependence of T current inhibition by diphenylbutylpiperidines, such as pimozide, has been described in other tissues (Enyeart *et al.*, 1992). In contrast, mibefradil has complex effects on Ca^{2+} currents in somatic tissues. The inhibition of T currents in vascular smooth muscle (Mishra and Hermsmeyer, 1994a) and of the L-type high voltage-activated current in smooth muscle (Mishra and Hermsmeyer, 1994b) exhibits no use dependence. However, a use-dependent inhibition by mibefradil has been observed for L-type currents that are produced by the expression of the α1C channel in *Xenopus* oocytes (Bezprozvanny and Tsien, 1995).

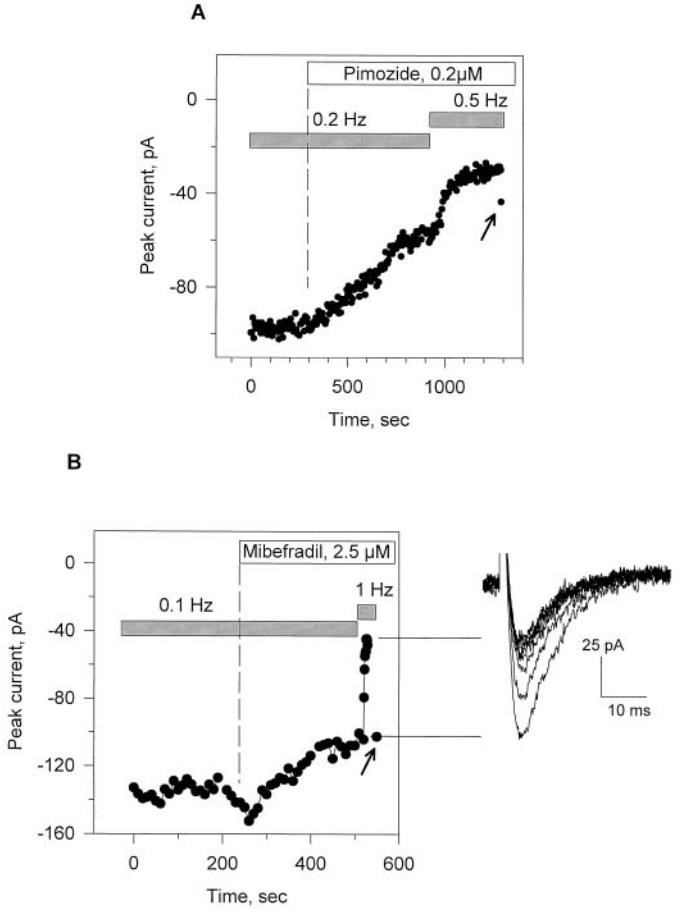

Fig. 3. Use dependence of T current inhibition by pimozide and mibefradil. A, Cell was depolarized from a holding potential of −90 mV to a test potential of −20 mV at a rate of 0.2 Hz in an external solution containing 10 mM Ca^{2+}. A reproducible peak T current is evoked by application of this voltage protocol in the absence of pimozide. Pimozide is added by perfusion (*shaded bar*) and leads to an inhibition of T current that reaches a steady state level of 45% inhibition by 400 sec. Switching depolarization frequency to 0.5 Hz (*bar*) results in further inhibition of T currents to a final level of ~70%. A partial reversal of this use-dependent inhibition is noted when stimulation is curtained for 10 sec (*arrow*). B, An experimental protocol similar to that shown in A was carried out with 2.5 μM mibefradil. Inhibition of T current increased from 31% during 0.1 Hz stimulation to 70% at 1 Hz stimulation. Complete reversal of use-dependent inhibition occurred during 10-sec recovery period (*arrow*). Current traces adjacent to B illustrate the use dependence of T current inhibition as stimulation frequency is increased from 0.1 Hz (*lowest trace*) to 1 Hz.

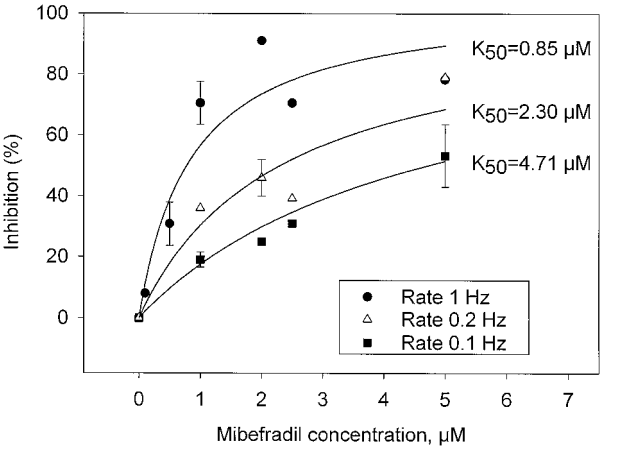

Fig. 4. Effects of stimulation frequency on potency of T current inhibition by mibefradil. Cells are depolarized in presence of mibefradil from a holding potential of −90 mV to a test potential of −20 mV at rates of 0.1, 0.2, and 1 Hz. The external solution contained 10 mM Ca^{2+}. Data were fit with the equation $I = (I_{max} \times [C])/(k_{50} + [C])$, where I is the inhibition of T current (percent of control current), I_{max} is the maximum inhibition, k_{50} is the inhibitory constant, and [C] the inhibitor concentration. Calculated k_{50} values are indicated.

Use-dependent action typically reflects drug selectivity for either the open or inactivated state of a channel, rather than for the closed state. To assess the influence of channel functional state on inhibitor action, we examined the effects of mibefradil and pimozide as a function of the duration of depolarization. Voltage-dependent inactivation of the T channel occurs during sustained depolarization (45–375 msec), whereas channels can deactivate from the open state directly to the closed state after brief depolarizations (10–15 msec; Fig. 5A).

Spermatogenic cell T current amplitude was monitored as a function of pulse duration during depolarization at 1 Hz. Fig. 5B shows that 0.5 μM pimozide has only a minor inhibitory effect on T currents evoked by 9.3-msec pulses: peak current amplitude was decreased by ~10% within 10 pulses. However, the inhibitory efficacy of this agent increased as pulse duration was lengthened from 9.3 to 375 msec, such that peak current amplitude was decreased by 60–70% after 10 pulses of 375 msec. Pimozide acts with a similar time course at all pulse durations, and the enhancement of inhibition at prolonged pulse durations reflects an increase in the maximal degree of inhibition (Fig. 5B). The effects of pulse duration on the inhibitory efficacy of 2 μM mibefradil are shown in Fig. 5C. Mibefradil produced a 40–50% inhibition of T current amplitude even during brief pulse duration (9.3 msec). Maximal inhibition was observed with pulse durations of 47 msec, and only a minor enhancement was observed as pulse lengths were increased to 375 msec.

Differences between the use dependence of inhibition by pimozide and mibefradil were explored in a second series of experiments. Spermatogenic cells were incubated with 0.5 μM pimozide or 1 μM mibefradil (Fig. 6). A steady state level of inhibition was established during low frequency depolarization (0.1 Hz) from holding potential (−90 mV) to a −20-mV test potential. T current amplitude inhibition then was determined during a series of 10 pulses of 47-msec du-

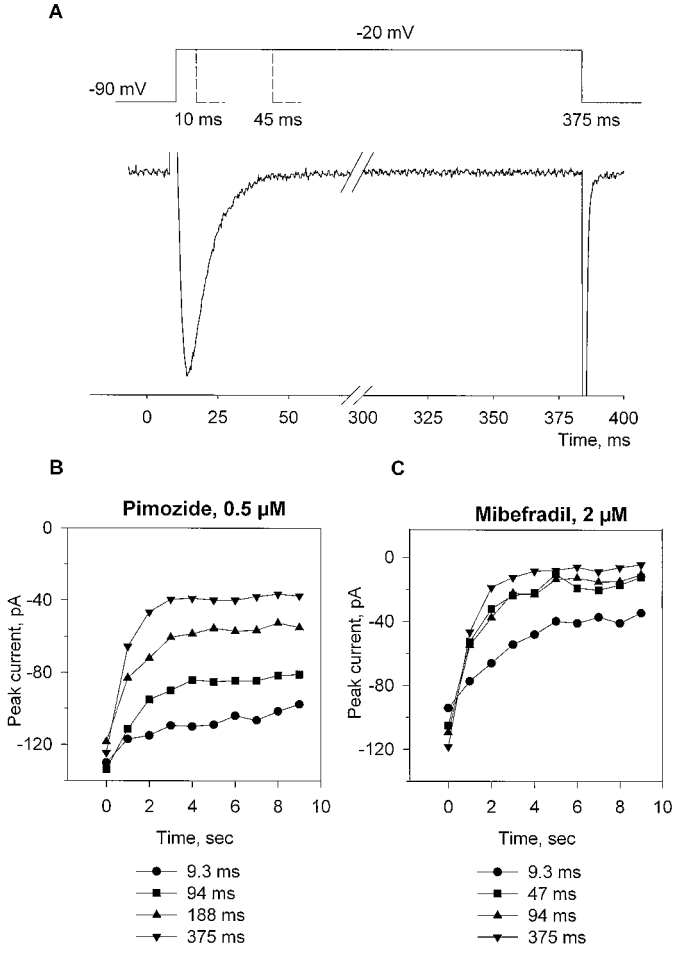

Fig. 5. Effects of pulse duration on the inhibition of spermatogenic cell T currents by pimozide and mibefradil. Cells are depolarized from a holding potential of −90 mV to a test potential of −20 mV. A, Representative Ca^{2+} current trace showing kinetics of voltage-dependent activation and inactivation of the T current. Current is elicited by a depolarizing pulse of 375 msec from a holding potential of −90 mV to a test potential of −20 mV. Voltage-dependent inactivation of the T channel occurs during sustained depolarization (45–375 msec), whereas channels can deactivate from the open state directly to the closed state after brief depolarizations. B, Peak current amplitudes during depolarization at 1 Hz in presence of 0.5 μM pimozide. The duration of depolarizing pulses is switched from 9.3 to 375 msec, as indicated. C, Same experiment carried out with 2 μM mibefradil.

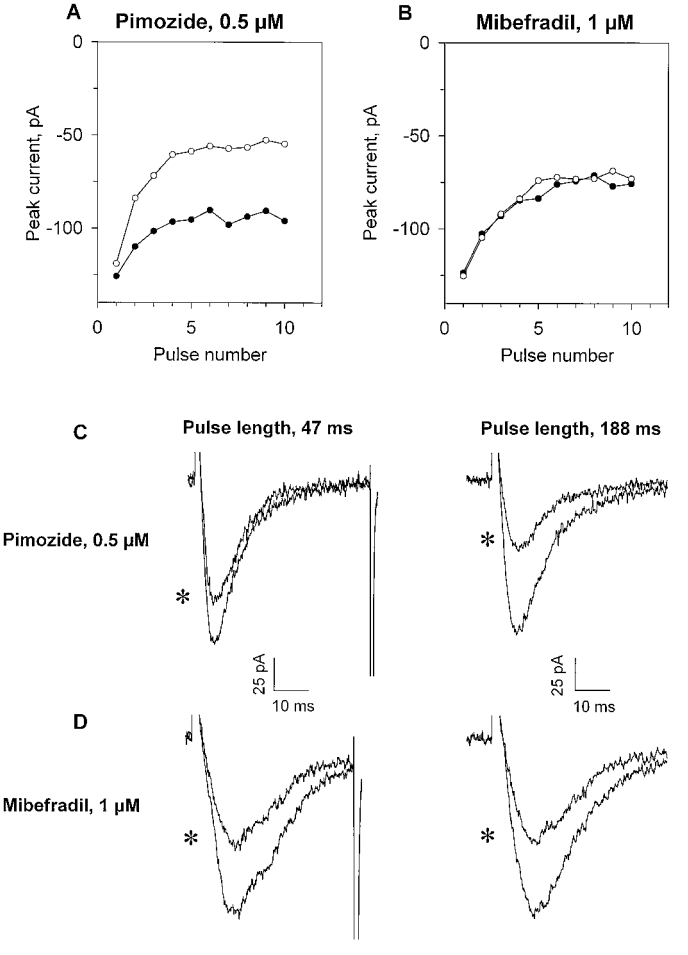

Fig. 6. Effects of pulse duration on the inhibition of spermatogenic cell T currents by pimozide and mibefradil. Cells are depolarized from a holding potential of −90 mV to a test potential of −20 mV. A and B, Test cells are incubated with (A) 0.5 μM pimozide or (B) 1 μM mibefradil, and steady state inhibition was established by stimulation at 0.1 Hz. Cells then are stimulated by a train of 10 pulses of 47-msec duration at a frequency of 1 Hz, allowed to recover during 20 sec, and stimulated with a train of 10 pulses of 188-msec duration at a frequency of 1 Hz. The degree of pimozide inhibition is greater with 188-msec pulses (○) than with 47-msec pulses (●); however, pulse duration has no effect on inhibition by mibefradil. C and D, Montage of the first and last (*) current traces obtained during a train of 10 pulses (1 Hz) of 47- and 188-msec duration in the presence of (A) 0.5 μM pimozide or (B) 1 μM mibefradil.

ration at a frequency of 1 Hz (Fig. 6, A and B, *closed symbols*). After a 20-sec recovery period, this protocol was repeated on the same cell, although the pulse duration was lengthened to 188 msec (Fig. 6, A and B, *open symbols*).

The inhibitory effects of both drugs were maximal after four or five pulses. However, the degree of pimozide inhibition was greater when the longer pulse duration protocol was used. This is shown in Fig. 6C, which compares the current traces after the first and the 10th (*) depolarizing pulses in these voltage trains. Pimozide inhibited the T current by ~60% during trains of 188-msec depolarizing pulses but had little effect (<25%) during 47-msec pulse trains. In contrast, the inhibitory effects of 1 μM mibefradil are not altered by this 4-fold increase in pulse duration (Fig. 6, B and D). These observations strongly suggest that use-dependence inhibition of pimozide is likely due to the presence of a high affinity site on the inactivated state of the channel with the drug and that use-dependence inhibition of mibefradil is principally due to the presence of a high affinity site on the open state of the channel. In addition, mibefradil binds to the inactivated state of channel, with lower affinity (Fig. 5B).

Discussion

Mouse spermatogenic cells express a T-type Ca^{2+} current, but high voltage-activated Ca^{2+} currents are not detected (Hagiwara and Kawa, 1984; Arnoult *et al.*, 1995, 1996a; Lievano *et al.*, 1996; Santi *et al.*, 1996). This preparation provides a relatively simple model in which to examine the regulation and function of T channels. The present study provides the first analysis of the actions of T channel antagonists in this new model system.

Diphenylbutylpiperidines and 1,4-dihydropyridines are potent inhibitors of the spermatogenic cell T current, whereas the current is less sensitive to inhibition by mibefradil, amiloride, or Cd^{2+}. The rank order of potency for the inhibition of the T current of spermatogenic cells is PN200–110 > pimozide $\gg$ mibefradil. In particular, 1,4-dihydropyridines are potent inhibitors of the spermatogenic cell, with PN200–110 and nifedipine producing half-maximal effects at 40 nM and <1 μM, respectively. Under saturation conditions, drugs of this class reduce the spermatogenic cell T current by only 50%. Currently, the mechanisms that underlie these complex inhibitory effects of 1,4-dihydropyridines are not well understood. These features differ in certain respects from those anticipated at T-type channels, whereas mibefradil and the diphenylbutylpiperidine pimozide are expected to act as potent, high affinity antagonists with IC_{50} values for current inhibition of 100 nM (Mishra and Hermsmeyer, 1994) and 250 nM (Enyeart *et al.*, 1992, 1993), respectively, and 1,4-dihydropyridines are low affinity antagonists (IC_{50} = 1–10 μM; (Akaike *et al.*, 1989a, 1989b; Richard *et al.*, 1991; Takahashi and Akaike, 1991; Cohen *et al.*, 1992).

A second unanticipated feature of the pharmacology of T channels in spermatogenic cells is that the 1,4-dihydropyridines and diphenylbutylpiperidines act as irreversible or slowly reversible antagonists. We have shown previously that the T channel of spermatogenic cells is retained on sperm after differentiation and is activated by adhesive contact with the extracellular matrix of the egg during induction of the sperm acrosome reaction (Arnoult *et al.*, 1996a). Because acrosome reactions must be completed before fertilization (Yanagimachi, 1994) and T channel antagonists inhibit the egg-induced acrosome reaction (Arnoult *et al.*, 1996a), it follows that such channel blockers may have a contraceptive effect. In this regard, a contraceptive action in males has been ascribed to 1,4-dihydropyridine Ca^{2+} antagonists (Benoff *et al.*, 1994; Hershlag *et al.*, 1995), and it is plausible that these agents function by inhibiting sperm T channels (Arnoult *et al.*, 1996a).

The therapeutic application of 1,4-dihydropyridines as antagonists of L-type high voltage-activated Ca^{2+} currents requires plasma concentrations of 50–500 nM (Opie, 1997). In contrast, T currents from several somatic tissues are inhibited by 1–10 μM 1,4-dihydropyridines. Consequently, this class of drugs is thought to act principally through inhibition of the L-type channel. However, the mouse spermatogenic cell T channel is inhibited by PN200–110 and nifedipine at concentrations that are within the typical therapeutic dose range in humans. Given the low rate of recovery of T current after removal of 1,4-dihydropyridines, it follows that a channel block imposed by these agents within the male reproductive tract could be sustained during the several hours required for sperm transport, capacitation, and fertilization within the female (Yanagimachi, 1994; Arnoult *et al.*, 1996a). In contrast, the spermatogenic cell T current is relatively insensitive to mibefradil, where therapeutic plasma doses of ~1 μM (Clozel *et al.*, 1991) would be predicted to reduce currents by only 20% (Fig. 1D). Similarly, therapeutic doses of 200–800 nM verapamil block L channels (Opie, 1997), and at these concentrations, there is no detectable inhibition of the germ cell T current (Fig. 1F). Thus, nondihydropyridine agents may provide a means of imposing an antihypertensive effect without potentially compromising male fertility.

Finally, we have found that the inhibitory effects of both mibefradil and pimozide are use dependent. Voltage-dependent inactivation is a character of Ca^{2+} channels, including the spermatogenic cell T-type Ca^{2+} channel (Arnoult *et al.*, 1996a; Lievano *et al.*, 1996; Santi *et al.*, 1996). When spermatogenic cells are stimulated with brief depolarization pulses of 9.3 msec, a duration approximately equal to that required for peak T channel opening (Fig. 5A), channels accumulate in the open state but do not inactivate extensively. In contrast, longer depolarizing pulses permit a greater degree of voltage-dependent inactivation. The results presented in Fig. 5–6 are consistent with a model in which mibefradil and pimozide selectively interact with the open and inactivated states of the spermatogenic cell T channel, respectively. This is in contrast to previous reports in somatic cells that diphenylbutylpiperidines, such as pimozide, selectively bind to the open state of T channels in neural crest-derived cell lines (Enyeart *et al.*, 1992) and that the inhibitory effects of mibefradil are not use dependent (Mishra and Hermsmeyer, 1994a).

The potential pharmacological use of a use-dependent inhibition can be considered with regard to the physiology of mammalian sperm. Sperm differentiate within the testicular seminiferous epithelium, are transported to the epididymides, and are stored before release within the lumen of the cauda epididymides. The Na^+/K^+ ratio in epididymal plasma is 1 of 2 (Hinton and Palladino, 1995), and media of this composition depolarize sperm membrane potential (Zeng *et al.*, 1995). It is likely that sperm within the cauda epididymides are very sensitive to T channel inhibition by pimozide.

Mammalian sperm must complete an activation process known as capacitation before fertilization *in vivo* and *in vitro* (Yanagimachi, 1994). During capacitation, the membrane potential of sperm populations hyperpolarizes from −25 to −60 mV, as reported by potentiometric fluorescent probes (Zeng *et al*., 1995). The germ cell T channel is partially inactivated at membrane potentials equivalent to that of capacitated sperm (Arnoult *et al*., 1996a) and hence may be particularly susceptible to inhibition by antagonists that selectively recognize the inactivated state.

Finally, contact of capacitated sperm with ZP3, a glycoprotein constituent of the zona pellucida of the egg, leads to membrane depolarization (Arnoult *et al*., 1996b) and the activation of T channels (Arnoult *et al*., 1996a). T channel activation in turn is required for the initiation of the sperm acrosome reaction, a secretory event that must be completed before fertilization (Arnoult *et al*., 1996a). Sperm remain bound to the zona pellucida for several minutes before the acrosome reaction. It is unknown at present whether ZP3 provides a single depolarizing signal or a train of impulses. In the latter case, it is likely that T channel inactivation occurs during induction of the acrosome reaction. The determination of impulse pattern provided by ZP3 will be essential in an effort to design channel-based antifertility agents.

In conclusion, the T-type Ca^{2+} channel of the male germ lineage differs from somatic cell T channels in several regards, including a relatively low sensitivity to inhibition by the nondihydropyridine Ca^{2+} antagonist mibefradil. Moreover, both mibefradil and pimozide inhibit this channel in a use-dependent manner that differs from that reported in neurons and smooth muscle. These observations support the notion that T-type channel are heterogeneous, provide essential preliminary information for the rational design of channel-based contraceptive agents, and offer a rationale for avoiding potential antifertility effects that may be associated with antihypertensive agents.

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