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 × 10$^{-8}$ M) > nifedipine (IC$_{50}$ = 4 × 10$^{-7}$ M) > pimozide (IC$_{50}$ = 4.6 × 10$^{-7}$ M) > mibefradil (IC$_{50}$ = 5 × 10$^{-6}$ M) > Ni$^{2+}$ (IC$_{50}$ = 3.4 × 10$^{-5}$ M) > verapamil (IC$_{50}$ = 7 × 10$^{-5}$ M) > amiloride (IC$_{50}$ = 2.4 × 10$^{-4}$ M) > Cd$^{2+}$ (IC$_{50}$ = 2.8 × 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 $\omega$-conotoxin GVIA, P-type currents by low concentrations of $\omega$-conotoxin MVIIIC 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$_{50}$ = 1 nM (hypothalamic neurons, Akaïke et al., 1989a; aorta smooth muscle, Akaïke et al., 1989b; CA1 pyramidal neurons, Takahashi and Akaïke, 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$_{50}$ = 10 nM (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 mibebradil have been described in the adrenal zona fasciculata cells (Enyeart et al., 1993) and on smooth muscle (Mishra and Hermansmeyer, 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 mibebradil. 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 mibebradil.

**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 Ca\(^{2+}\), 1 mM Mg\(^{2+}\), 1 mM NaH\(_2\)PO\(_4\), 12 mM NaHCO\(_3\), 1 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 at least 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).

**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 determined 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 mibebradil, 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).

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 drugs. 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 mibebradil 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 Hermmsmeyer, 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, mibebradil 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 mibebradil are required to inhibit the T current of mouse spermatogenic cells (IC_{50} ~ 4.7 μM). At these concentrations, mibebradil 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 aryalkylamine 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 Ni²⁺, Cd²⁺ (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 Ca²⁺. 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.
recover 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 mibebradil. 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 mibebradil, 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 mibebradil. 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; Arnout 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 (Arnout et al., 1997). However, the current evoked in a cell by low frequency depolarization ($\sim 0.1$ Hz) is highly reproducible.

The addition of 0.2 $\mu$M pimozide (Fig. 3A) or 2.5 $\mu$M mibebradil (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 mibebradil-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 $\lesssim 2$ Hz had no effect on the amplitude of the current in the 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 mibebradil decreases from 4.71 to 0.85 $\mu$M 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, mibebradil has complex effects on Ca$^{2+}$ currents in somatic tissues. The inhibition of T currents in vascular smooth muscle (Mishra and Hermensmeyer, 1994a) and of the L-type high voltage-activated current in smooth muscle (Mishra and Hermensmeyer, 1994b) exhibits no use dependence. However, a use-dependent inhibition by mibebradil has been observed for L-type currents that are produced by the expression of the a1C channel in Xenopus oocytes (Bezprozvanny and Tsien, 1995).

![Fig. 3. Use dependence of T current inhibition by pimozide and mibebradil. 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 $\sim 70\%$. A partial reversal of this use-dependent inhibition is noted when stimulation is curtailed for 10 sec (arrow). B. An experimental protocol similar to that shown in A was carried out with 2.5 $\mu$M mibebradil. 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.](http://example.com/fig3)

![Fig. 4. Effects of stimulation frequency on potency of T current inhibition by mibebradil. Cells are depolarized in presence of mibebradil 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 $I = (I_{\text{max}} \times [C])/k_{s0} + [C]$, where I is the inhibition of T current (percent of control current), $I_{\text{max}}$ is the maximum inhibition, $k_{s0}$ is the inhibitory constant, and [C] the inhibitor concentration. Calculated $k_{s0}$ values are indicated.](http://example.com/fig4)
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 mibebradil 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 mibebradil are shown in Fig. 5C. Mibebradil 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 mibebradil were explored in a second series of experiments. Spermatogenic cells were incubated with 0.5 μM pimozide or 1 μM mibebradil (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-

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**Fig. 5.** Effects of pulse duration on the inhibition of spermatogenic cell T currents by pimozide and mibebradil. Cells are depolarized from a holding potential of −90 mV to a test potential of −20 mV. A, Representative Ca²⁺ 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 mibebradil.

**Fig. 6.** Effects of pulse duration on the inhibition of spermatogenic cell T currents by pimozide and mibebradil. 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 mibebradil, 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 mibebradil. 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 mibebradil.
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 \( \sim 60\% \) during trains of 188-msec depolarizing pulses but had little effect (<25%) during 47-msec pulse trains. In contrast, the inhibitory effects of 1 \( \mu \text{M} \) 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 > mibefradil. In particular, 1,4-dihydropyridines are potent inhibitors of the spermatogenic cell, with PN200—110 and nifedipine producing half-maximal effects at 40 \( \text{nM} \) and <1 \( \mu \text{M} \), 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 \( \text{nM} \) (Mishra and Hermmsmeyer, 1994) and 250 \( \text{nM} \) (Enyeart et al., 1992, 1993), respectively, and 1,4-dihydropyridines are low affinity antagonists (IC\(_{50}\) = 1–10 \( \mu \text{M} \); (Akaite et al., 1989a, 1989b; Richard et al., 1991; Takahashi and Akaite, 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 (Bennett et al., 1994; Herslag 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 \( \text{nM} \) (Opie, 1997). In contrast, T currents from several somatic tissues are inhibited by 1–10 \( \mu \text{M} \), 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 \( \sim 1 \mu \text{M} \) (Clozel et al., 1991) would be predicted to reduce currents by only 20% (Fig. 1D). Similarly, therapeutic doses of 200–800 \( \text{nM} \) 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-dependenent 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 Hermmsmeyer, 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 Ca2+ 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 Ca2+ antagonist mibefradil. Moreover, both mibebradil and pimozone 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.

References


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