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ZD7288 inhibits low-threshold Ca^{2+} channel activity and regulates sperm function

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Abstract

In this study, ZD7288, a blocker of hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels, has been found to inhibit the mouse sperm acrosome reaction (AR). HCN channels have not yet been either recorded or implicated in mouse sperm AR, but low-threshold (T-type) Ca^{2+} channels have. Interestingly, ZD7288 blocked native T-type Ca^{2+} currents in mouse spermatogenic cells with an IC_{50} of about 100 μM. This blockade was more effective at voltages producing low levels of inactivation, suggesting a differential affinity of ZD7288 for different channel conformations. Furthermore, ZD7288 inhibited all cloned T-type but not high-threshold N-type channels heterologously expressed in HEK-293 cells. Our results further support the role of T-type Ca^{2+} channels in the mouse sperm AR.

Keywords: Acrosome reaction; Ca^{2+} channels; HCN channels; ZD7288

ZD7288 (4-(*N*-ethyl-*N*-phenylamino)-1,2-dimethyl-6-(methylamino) pyridinium chloride), a sinoatrial node modulating agent, produces a selective decrease in the heart rate [1,2]. This drug blocks a hyperpolarization-activated cation current (I_h) that plays a major role in the initiation and regulation of pacemaker depolarizations in some central nervous system (CNS) mammalian neurons and other cells [3–6]. The ion channels underlying I_h are hyperpolarization and cyclic nucleotide-gated (HCN) channels [7]. ZD7288 targets mouse HCN1 channels and the sea urchin sperm homologue of I_h channels, SpHCN [8]. Though transcripts of a homologue of the SpHCN channel have been detected in mouse testis [9], the role of such channels in mammalian sperm physiology is unknown. Thus, we explored the possible effects of ZD7288 in the mouse sperm acrosome reaction (AR) and found that it inhibits this process.

The potency of this compound to inhibit mouse sperm AR found in the present work is significantly less than the one it displays towards HCN pacemaker channels. Because T-type channels are expressed in rodent male germ cells [10–12] and are believed to be pivotal in the induction of the AR in mouse sperm [13], we asked ourselves whether ZD7288 could antagonize these currents and thus explain its ability to inhibit AR. Sperm are tiny cells and it still has not been possible to directly record their T-type Ca^{2+} channels. These currents have been studied in mouse spermatogenic cells [10–12]. Interestingly, we have found that ZD7288 can block the T-type whole-cell currents of mouse spermatogenic cells, which is consistent with their proposed role in the AR [13].

Perez-Reyes and co-workers [14] have cloned a set of α_1 subunits (named $Ca_V3.1$–3.3, formerly known as to α_{1G}, α_{1H}, and α_{1I}, respectively) that yields T-type currents when expressed in heterologous expression systems. RT-PCR studies have indicated the expression of the three different genes coding T-type Ca_V ion-conducting α_1 subunits in mouse and human spermatogenic

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cells and sperm [15–17]. In the current report we found, as anticipated, that ZD7288 effectively blocks recombinant low-threshold Ca^{2+} channels, though it does not significantly change the properties of cloned N-type high-threshold channels.

Materials and methods

Assay for acrosome reaction. Caudal epididymal mouse sperm were mechanically collected from CD1 mice and placed in capped 1.5-ml microcentrifuge tubes containing medium 199 supplemented with BSA (0.1% wt/vol), Na^+ pyruvate (30 mg/L), and $NaHCO_3$ (2.2 g/L) at 37 °C. The percentage of AR was measured using the Coomassie blue method. In brief, sperm were incubated 30 min at 37 °C followed by the addition of 5 *zona pellucida* (ZP) eq/μl. Subsequently, an equal volume of 2× fixative (10% formaldehyde in phosphate-buffered saline) was added. Following fixation, 10 μl aliquots of the sperm suspension were spread onto glass slides and air-dried. The slides were stained with 0.22% Coomassie blue R-250 in 50% methanol and 10% glacial acetic acid for ~5 min, rinsed, and mounted with 50% (v/v) glycerol in phosphate-buffered saline. To calculate the percentage of AR, at least 100 sperm were assayed per experimental condition.

Spermatogenic cell preparation and electrophysiology. Spermatogenic cells were obtained following the procedure described by Espinosa et al. [11]. Ca^{2+} currents were recorded using the whole-cell configuration of the patch-clamp technique [18]. Cells were bathed in a solution containing (in mM): $CaCl_2$ 10; NaCl 130; KCl 3; $MgCl_2$ 2; $NaHCO_3$ 1; NaH_2PO_4 0.5; Hepes 5; and glucose 10 (pH 7.3). The internal (patch pipette) solution consisted of (mM): $CsMeSO_3$ 110; CsF 10; CsCl 15; $CaCl_2$ 4.6; EGTA 10; Hepes 5; ATP-Mg_2 4; and phosphocreatine 10 (pH 7.3). Currents were sampled at 10 kHz and recorded (following filtering at 5 kHz) by a patch-clamp amplifier (Axopatch 200A; Axon Instruments) interfaced to a personal computer via an A-to-D board (DigiData 1200A; Axon) using computer-driven software (pCLAMP 6.0.3 "Clampex"; Axon). Unless otherwise noted, capacity transients were electronically compensated, and linear leak and residual capacity currents were subtracted on-line using a P/4 protocol. ZD7288, purchased from Tocris Cookson (UK), was dissolved in distilled water and directly added to the external recording solution.

T-type Ca^{2+} channel expression and electrophysiology. Human embryonic kidney (HEK-293) cells stably expressing the human α_{1G} channel, $Ca_V3.1$ (GenBank Accession No. AF190860); the human α_{1H} channel, $Ca_V3.2$ (GenBank Accession No. AF073931); and the human α_{1I} channel, $Ca_V3.3$ (GenBank Accession No. AL022312) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with G418 (1 g/L), 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2–95% air humidified atmosphere. Whole-cell Ca^{2+} currents were recorded according to the patch-clamp technique using an Axopatch 200B amplifier, a Digidata 1320A/D converter, and pCLAMP 8.0 software (Axon). Currents were digitized at 5 kHz and filtered at 2 kHz. Cells were bathed in a solution containing (in mM): $CaCl_2$ 5; tetraethylammonium (TEA) chloride 155; and Hepes 10 (pH 7.4). The internal solution contained (in mM): CsCl 135; EGTA 10; Mg-ATP 4; Na_3GTP 0.3; and Hepes 10 (pH 7.3).

N-type Ca^{2+} channel expression and electrophysiology. HEK-293 cells were grown in DMEM-high glucose supplemented with 10% equine serum, 2 mM L-glutamine, 110 mg/L sodium pyruvate, and 50 μg/ml gentamicin at 37 °C in a 5% CO_2–95% air humidified atmosphere. Transfections were performed using the Lipofectamine Plus reagent (Gibco-BRL) with 1 μg plasmid cDNA encoding the rabbit brain N-type Ca^{2+} channel α_{1B}, $Ca_V2.2$ pore-forming subunit (GenBank Accession No. D14157); 1 μg plasmid cDNA coding the rat brain Ca^{2+} channel $\alpha_2\delta$-1 accessory subunit (GenBank Accession No. M86621), and 0.1 μg of a plasmid cDNA encoding the green fluorescent protein (pGreen Lantern-1; Gibco-BRL) to select cells that expressed channels. After two days, positively transfected cells were subjected to the whole-cell mode of the patch-clamp technique as described above. The bath solution contained (in mM) 10 $BaCl_2$, 125 TEA-Cl, 10 Hepes, and 15 glucose (pH 7.3). The internal solution consisted of (mM) 110 CsCl, 5 $MgCl_2$, 10 EGTA, 10 Hepes, and 4 Na-ATP (pH 7.3).

Results

The bradycardic agent ZD7288 has been shown to produce a voltage-controlled blockade of pacemaker channels [8]. Though the functional relevance of HCN channels in sperm is not known, we sought to resolve whether this drug might affect the fertilizing capability of the mammalian male gametes. Fig. 1 shows that treatment with different concentrations of ZD7288 (1–1000 μM) decreased the fraction of acrosome-reacted cells in a concentration-dependent manner by as much as 70%. An IC_{50} of ~430 μM can be estimated from these data which is far from that reported for recombinant HCN channels (~40 μM) [8]. Since T-type Ca^{2+} channels have been postulated to regulate the Ca^{2+} influx that triggers acrosomal exocytosis, we determined if ZD7288 could block these channels. As reported earlier, whole-cell patch-clamp recordings in mouse spermatogenic cells indicated that the voltage-dependent Ca^{2+} currents are mainly of the T-type [10–12]. Peak current (recorded in 10 mM Ca^{2+}) was obtained when cells were depolarized from a holding potential (HP) of −90 mV to a test potential of −30 mV, and therefore,

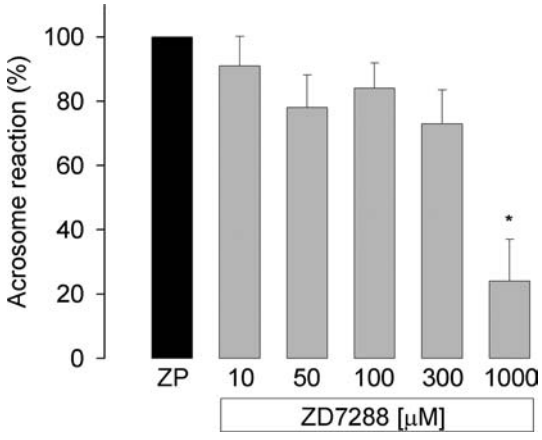

Fig. 1. ZD7288 inhibits the sperm AR. Cells were incubated in the presence of increasing concentrations of ZD7288. After capacitation, the %AR was monitored by Coomassie blue staining. Spontaneous AR after capacitation was 19.2 ± 3.7% and 35 ± 6.3% after ZP application. The ZP-induced AR values were normalized with respect to the control corrected for spontaneous AR. Average values for ZD7288 treated cells were: 88 ± 16, 78 ± 9, 81 ± 13, 71 ± 10, and 31 ± 8 (for 10, 50, 100, 300, and 1000 μM, respectively). Data represent means ± SD of 3–5 independent experiments, (*) denotes a significant difference ($P < 0.05$).

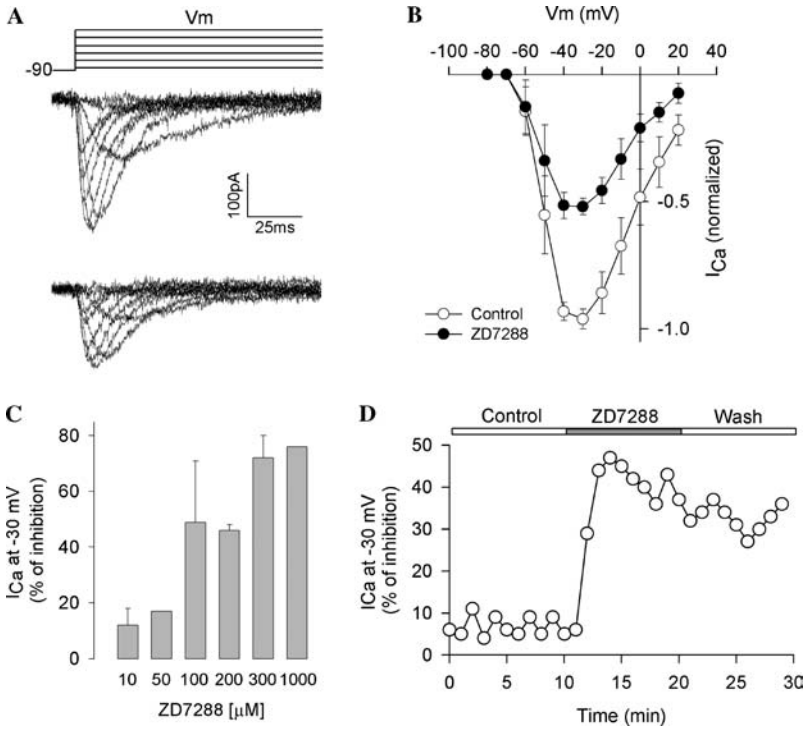

Fig. 2. ZD7288 inhibits Ca^{2+} T-currents in mouse spermatogenic cells. (A) Traces in upper panel illustrate Ca^{2+}currents evoked by 200 ms depolarizations from a HP of −90 mV to test potentials ranging from −70 to −10 mV with 10 mV increase in the pulse amplitude per step. Following addition of ZD7288 (100 μM), a significant reduction in current amplitude was observed (lower panel). (B) Average normalized peak current plotted against test potential (V_m) before and 3 min after drug application. (C) Inhibition of T-channel activity by ZD7288 was dose dependent. Peak currents were normalized by their value before the cells were exposed to the drug. (D) Time course of current traces in the presence of ZD7288 (100 μM) recorded in response to 200 ms depolarizing pulses from −90 to −30 mV.

the effects of ZD7288 were initially determined at this potential (Fig. 2). During 10-min control experiments, the rundown of the current was <10%. Extracellular application of ZD7288 (200 μM) resulted in a statistically significant reduction of peak current amplitude at almost all potentials tested (Figs. 2A and B). The decrease in the peak current was concentration-dependent in the 1–1000 μM range (Fig. 2C) and only partially reversed upon washout of the drug (Fig. 2D). Lastly, the percent block of current measured at the peak was plotted against ZD7288 concentration, and a nonlinear least-squares fit of the concentration–response equation to the data points (not shown) yielded a K_D of 107 μM.

Though these results could be consistent with the participation of the T-type Ca^{2+} channels in the mammalian sperm AR, ZD7288 blocked the AR with much less potency than native T-type channels in spermatogenic cells. Experimental differences such as membrane potential could contribute to explain this apparent discrepancy. Ca^{2+} currents were recorded in spermatogenic cells at a HP of −90 mV while the AR assays were performed at mouse sperm resting potentials ranging from ∼−50 mV before capacitation [19] to ∼−65 mV after sperm capacitation in vitro [20]. Consequently, we studied possible state-dependent actions of ZD7288. To this end, patch-clamp whole-cell experiments were carried out in spermatogenic cells held at −50 mV. As expected, at this voltage most channels were inactivated and no channel opening was detectable in response to a test pulse to −30 mV, however a brief hyperpolarizing pulse to −90 mV could overcome this. Therefore, we tested the effects ZD7288 on Ca^{2+} channel activity using this hyperpolarizing pre-pulse protocol. Fig. 3A shows that application of the drug under these conditions did not affect substantially T-currents. ZD7288 treatment caused on average a ∼20% current inhibition, in contrast to the ∼70% observed when the HP was kept at −90 mV (Fig. 3B), suggesting that the drug has less effects when the channels are inactivated.

It is still a matter of debate which of the T-type channel isoforms constitute the pore of the native sperm T-type channels [10,15–17]. Since ZD7288 blocks the T-currents of spermatogenic cells, it could differentially affect the various Ca_V3 isoforms and thus serve as a tool to identify them. Fig. 4 illustrates patch-clamp studies in HEK-293 cells stably expressing the different recombinant low-threshold Ca^{2+} channels in the presence and absence of ZD7288. Unfortunately, the drug inhibited the three recombinant Ca_V3 isoforms in virtually the same proportion. The block of recombinant low-threshold channels was dose-dependent and often reversible (not shown). To further test the drug selectivity,

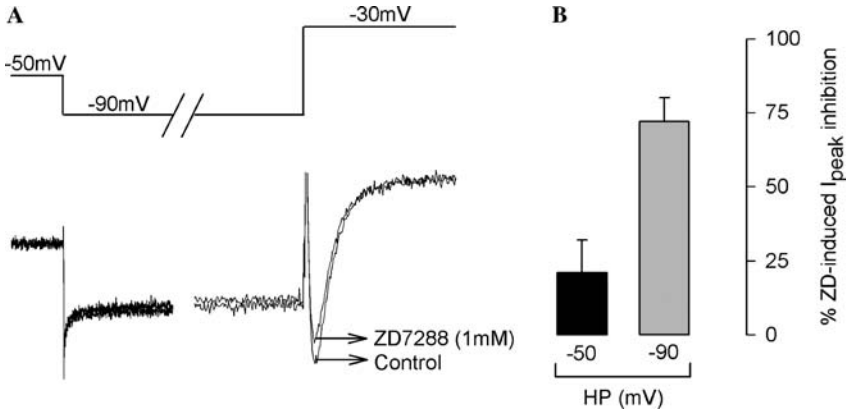

Fig. 3. Block of T-type Ca^{2+} current in spermatogenic cells by ZD7288 is state-dependent. (A) Superimposed current records obtained in mouse spermatogenic cells. ZD7288 was applied for 5 min in cells held at −50 mV and the voltage was changed to −90 mV for 300 ms to remove inactivation of the channels. A second activating pulse at −30 mV was applied to test the blockade of the channels by the drug. For the control, the voltage protocol was applied without blocker present. Leak subtraction was not performed in these recordings. (B) Comparison of peak inhibition after application of ZD7288 (1 mM) in cells kept at the two different holding potentials as indicated ($n = 6$–12).

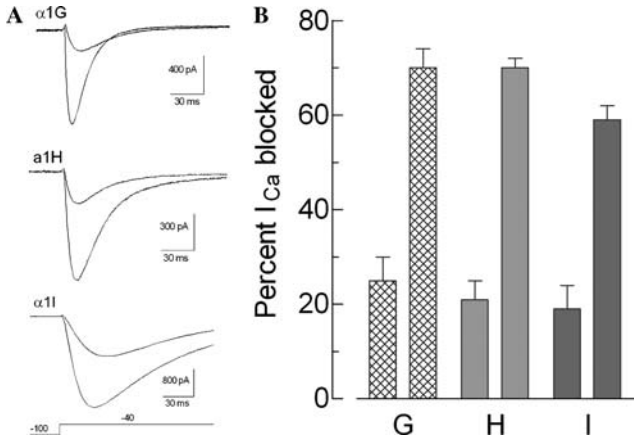

Fig. 4. ZD7288 blocks recombinant T-type Ca^{2+} channels. (A) Ca^{2+} currents recorded from HEK-293 cells expressing the indicated α_1 subunits in the absence (bigger traces) and in the presence of 1 mM ZD7288 (smaller traces). (B) Bars represent the combined data of percent block of Ca^{2+} current at −40 mV for the three T-type calcium α_1 subunits (small bars 100 μM ZD7288; large bars 1 mM ZD7288). Letters below the bars refer to the type of currents shown on the right. Average values for 1 mM ZD7288 were: $\alpha_1$3.1 (α_{1G}) 70 ± 4 ($n = 5$), $\alpha_1$3.2 (α_{1H}) 70 ± 2 ($n = 3$), and $\alpha_1$3.3 (α_{1I}) 59 ± 3 ($n = 3$), while values for 100 μM were: $\alpha_1$3.1 25 ± 5 ($n = 4$), $\alpha_1$3.2 21 ± 4 ($n = 3$), and $\alpha_1$3.3 19 ± 5 ($n = 3$).

we explored its effects on high-threshold Ca^{2+} channels of the N-type heterologously expressed in HEK-293 cells. Notably, ZD7288 had no effect on the recombinant expressed channels. The Ba^{2+} current (I_{Ba}) through Ca^{2+} channels elicited by a voltage step from −80 to +20 mV was virtually identical before and 5 min after drug application (Figs. 5A and C). In contrast, significant current reduction was observed after applications of Cd^{2+} and La^{3+}, two well-known blockers of Ca^{2+} channels (Fig. 5B). In Fig. 5C, the data points illustrate the representative time course of the I_{Ba} recorded under control conditions and during the application of ZD7288 and Cd^{2+}. In these experiments, the I_{Ba} current was activated every 20 s by a −80 to +20 mV voltage step and normalized to the control current before treatment.

Discussion

Previous work has indicated that ZD7288, an I_h channel blocker in mammalian heart, inhibits SpHCN channel activity acting from the intracellular side [8]. SpHCN (formerly SPIH) is a channel cloned from sea urchin testis with characteristics similar to those of mammalian I_h channels that have been involved in sperm function [21]. Therefore, ZD7288 could be used as a pharmacological tool to provide evidence for the presence of HCN channels in mammalian sperm. When tested on mouse sperm, ZD7288 inhibited the AR (Fig. 1) with an IC_{50} of ~430 μM, a value >10-times higher to that obtained by Shin et al. [8] to block HCN channels heterologously expressed in HEK-293 cells. These data disagree with the view that the blockade of HCN channels could be a likely mechanism for the inhibition caused by the drug on the mammalian sperm AR.

In contrast, our results demonstrate that ZD7288 inhibits the T-type Ca^{2+} current in mouse male germ cells and effectively decreases the activity of cloned T-Ca^{2+} channels stably expressed in HEK-293 cells. It is well established that the activation of voltage-gated T-type Ca^{2+} channels plays a key role in the induction of the mammalian AR [12,13,22]. This is the first study of ZD7288 block on native and recombinant low-threshold Ca^{2+} channels. All block by the drug was concentration-dependent and partially reversible. The concentration range for the inhibitory effects of ZD7288 on Ca^{2+} channels, and the time required to reach saturation

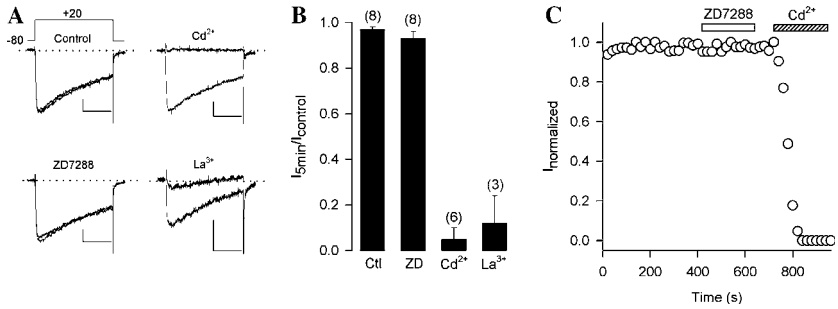

Fig. 5. ZD7288 did not affect high-threshold Ca^{2+} channels. (A) Superposition of $\alpha_1 2.2/\alpha_2\delta$-1 Ba^{2+} current (I_{Ba}) traces in HEK-293 cells in the absence (control) or the presence of ZD7288 and two Ca^{2+} channel blockers, Cd^{2+} and La^{3+}. Scale bar: 50 pA, 50 ms. (B) Relative I_{Ba} amplitude in the control and 5 min after treatment with ZD7288 (1 mM), Cd^{2+} (500 μM) and La^{3+} (300 μM). The numbers of recorded cells are indicated in parentheses. (C) Plotted is peak current amplitude against time in the same cell. The cell was depolarized to +20 mV from a HP of −80 mV. Current was normalized to control. ZD7288 (1 mM) and Cd^{2+} (500 μM) were applied to the cell, as indicated by the bars above data points.

(4–5 min), was similar to that found in studies on HCN channels. This is also consistent with the explanation that the drug enters the cytosol due to its partial lipophilic nature and blocks the channel from the inside [3,5].

Our findings also show that the potency of ZD7288 to affect the sperm AR is less than that required to inhibit native or recombinant T-type Ca^{2+} channels. Inhibition of channel activity has an IC_{50} of ~100 μM, while at this concentration the compound only decreases the ZP-induced AR by ~16%. The reason for this discrepancy may arise from the fact that the channels can be blocked by ZD7288 more effectively at negative voltages producing low levels of inactivation. The patch-clamp experiments were carried out at a HP of −90 mV, while the resting potential of capacitated sperm is between −50 and −65 mV, which still allows a significant amount of inactivation. We suspect that this voltage dependence arises from preferential binding of the drug to non-inactivated conformations of the channel. This is in agreement with the preferential closed state blockade model of ZD7288 that has been suggested for mHCN1 channels. This model states that the opening of the channel is required for blockade and that the blocker can be trapped in the closed state of the channel [8]. Interestingly, blockers that appear to bind in the pore but have a closed state preference have been described also for CNG channels [23] and voltage-gated K^+ channels [24].

Undoubtedly, elucidating the mechanism for ZD7288 blockade of T-type Ca^{2+} channels is an interesting topic for future studies. T-currents contribute to determine rhythmic activity of different cell types involved in a host of physiological processes such as the beating of the heart, the cycle of sleep and wakefulness, breathing, and the release of hormones. Therefore, drugs that inhibit T-type channels such as ZD7288 may both be therapeutically useful in the treatment of certain diseases, as well as powerful tools for understanding the molecular mechanisms regulating pacemaker depolarizations.

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References

[1] R.E. BoSmith, I. Briggs, N.C. Sturgess, Inhibitory actions of ZENECA ZD7288 on whole-cell hyperpolarization activated inward current (I_f) in guinea-pig dissociated sinoatrial node cells, Br. J. Pharmacol. 110 (1993) 343–349.

[2] I. Briggs, R.E. BoSmith, C.G. Heapy, Effects of Zeneca ZD7288 in comparison with alinidine and UL-FS 49 on guinea pig sinoatrial node and ventricular action potentials, J. Cardiovasc. Pharmacol. 24 (1994) 380–387.

[3] N.C. Harris, A. Constanti, Mechanism of block by ZD 7288 of the hyperpolarization-activated inward rectifying current in guinea pig substantia nigra neurons in vitro, J. Neurophysiol. 74 (1995) 2366–2378.

[4] G. Maccaferri, C.J. McBain, The hyperpolarization-activated current (I_h) and its contribution to pacemaker activity in rat CA1 hippocampal stratum oriens-alveus interneurones, J. Physiol. 497 (1996) 119–130.

[5] S. Gasparini, D. DiFrancesco, Action of the hyperpolarization-activated current (I_h) blocker ZD 7288 in hippocampal CA1 neurons, Pfügers Arch. 435 (1997) 99–106.

[6] M. Biel, A. Schneider, C. Wahl, Cardiac HCN channels. structure, function, and modulation, Trends Cardiovasc. Med. 12 (2002) 206–213.

[7] U.B. Kaupp, R. Seifert, Molecular diversity of pacemaker ion channels, Annu. Rev. Physiol. 63 (2001) 235–257.

[8] K.S. Shin, B.S. Rothberg, G. Yellen, Blocker state dependence and trapping in hyperpolarization-activated cation channels: evidence for an intracellular activation gate, J. Gen. Physiol. 117 (2001) 91–101.

[9] R. Seifert, A. Scholten, R. Gauss, A. Mincheva, P. Lichter, U.B. Kaupp, Molecular characterization of a slowly gating human hyperpolarization-activated channel predominantly expressed in thalamus, heart, and testis, Proc. Natl. Acad. Sci. USA 96 (1999) 9391–9396.

[10] A. Liévano, C.M. Santi, J. Serrano, C.L. Treviño, A.R. Bellve, A. Hernández-Cruz Darszon, T-type Ca^{2+} channels and α_{1E} expression in spermatogenic cells and their possible relevance to the sperm acrosome reaction, FEBS Lett. 388 (1996) 150–154.

[11] F. Espinosa, I. Lopez-Gonzalez, C. Munoz-Garay, R. Felix, J.L. De la Vega-Beltran, G.S. Kopf, P.E. Visconti, A. Darszon, Dual regulation of the T-type Ca^{2+} current by serum albumin and β-estradiol in mammalian spermatogenic cells, FEBS Lett. 475 (2000) 251–256.
[12] C. Arnoult, R.A. Cardullo, J.R. Lemos, H.M. Florman, Egg-activation of sperm T-type Ca^{2+} channels regulates acrosome reactions during mammalian fertilization, Proc. Natl. Acad. Sci. USA 93 (1996) 13004–13009.
[13] A. Darszon, C. Beltrán, R. Felix, T. Nishigaki, C.L. Treviño, Ion transport in sperm signaling, Dev. Biol. 240 (2001) 1–14.
[14] E. Perez-Reyes, Molecular physiology of low-voltage-activated T-type calcium channels, Physiol. Rev. 83 (2003) 117–161.
[15] F. Espinosa, I. López-González, C.J. Serrano, G. Gasque, J.L. de la Vega-Beltrán, C.L. Treviño, A. Darszon, Anion channel blockers differentially affect T-type Ca^{2+} currents of mouse spermatogenic cells, α1E currents expressed in *Xenopus* oocytes and the sperm acrosome reaction, Dev. Genet. 25 (1999) 103–114.
[16] S. Jagannathan, E.L. Punt, Y. Gu, C. Arnoult, D. Sakkas, C.L. Barratt, S.J. Publicover, Identification and localization of T-type voltage-operated calcium channel subunits in human male germ cells. Expression of multiple isoforms, J. Biol. Chem. 277 (2002) 8449–8456.
[17] J.Y. Park, H.J. Ahn, J.G. Gu, K.H. Lee, J.S. Kim, H.W. Kang, J.H. Lee, Molecular identification of Ca^{2+} channels in human sperm, Exp. Mol. Med. 35 (2003) 285–292.
[18] A. Marty, E. Neher, Tight-seal whole-cell recording, in: B. Sakmann, E. Neher (Eds.), Single-Channel Recording, Plenum Press, New York, 1995, pp. 31–52.
[19] F. Espinosa, A. Darszon, Mouse sperm membrane potential: changes induced by Ca^{2+}, FEBS Lett. 372 (1995) 119–125.
[20] C. Munoz-Garay, J.L. De la Vega-Beltran, R. Delgado, P. Labarca, R. Felix, A. Darszon, Inwardly rectifying K^+ channels in spermatogenic cells: functional expression and implication in sperm capacitation, Dev. Biol. 234 (2001) 261–274.
[21] R. Gauss, R. Seifert, U.B. Kaupp, Molecular identification of a hyperpolarization-activated channel in sea urchin sperm, Nature 393 (1998) 583–587.
[22] I. Lopez-Gonzalez, T. Olamendi-Portugal, J.L. De la Vega-Beltran, J. Van der Walt, K. Dyason, L.D. Possani, R. Felix, A. Darszon, Scorpion toxins that block T-type Ca^{2+} channels in spermatogenic cells inhibit the sperm acrosome reaction, Biochem. Biophys. Res. Commun. 300 (2003) 408–414.
[23] A.A. Fodor, K.D. Black, W.N. Zagotta, Tetracaine reports a conformational change in the pore of cyclic nucleotide-gated channels, J. Gen. Physiol. 110 (1997) 591–600.
[24] G.E. Kirsch, J.A. Drew, Gating-dependent mechanism of 4-aminopyridine block in two related potassium channels, J. Gen. Physiol. 102 (1993) 797–816.