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A remarkable increase in the pHi sensitivity of voltage-dependent calcium channels occurs in human sperm incubated in capacitating conditions

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Abstract

Human sperm are endowed with voltage-dependent calcium channels (VDCC) that produce increases in $[Ca^{2+}]_i$ in response to depolarization with KCl. These channels are stimulated during ''capacitation'', a complex biochemical process, accompanied by a slight pHi alkalization, that sperm must accomplish to acquire the ability to fertilize the egg. The stimulation can be explained in part by the fact that in non-capacitated sperm, calcium influx through VDCC is stimulated by pHi alkalization in the range of pHi observed during capacitation. In this work, we explored the effect of pHi on VDCC in capacitated sperm loaded with fura ff. Strikingly, the pHi sensitivity of VDCC increased ~7-fold when sperm was capacitated, as compared with non-capacitated sperm. This finding suggests that the pHi sensitivity of VDCC can be modulated during capacitation so that a combined effect of pHi alkalization and biochemical regulation enhances calcium influx through these channels.

Keywords: Human sperm; Capacitation; Intracellular pH; Intracellular calcium; Fura ff; VDCC

In sperm of mammalian species, the modulation of the intracellular calcium concentration $[Ca^{2+}]_i$ is fundamental to understand the mechanism involved in specific aspects of sperm physiology, such as the so-called ''capacitation'' and the subsequent acrosome reaction (AR) [1]. Capacitation is a calcium-dependent process that occurs in hours in the female genital tract, or in vitro in defined media, and is related to a myriad of biochemical changes [2]. These changes result in outstanding increases in cAMP content and the ensuing PKA activation [3], and in an increase in tyrosine kinase activity [4]. During the process, there is also a small but consistent alkalization of the intracellular pH (pHi) of ~0.15 pHi units [5]. Once capacitated, sperm is capable to respond to ZP3, an egg zona pellucida glycoprotein, increasing the $[Ca^{2+}]_i$ transiently and consequently undergoing the AR, a process that permits sperm to cross the zona pellucida and fuse the egg.

The calcium entry mechanisms induced by ZP3 have been extensively studied in mouse sperm. ZP3 would trigger a still unidentified ionic flux that would produce depolarization in capacitated sperm and consequently, gating of T-type voltage-dependent calcium channels (VDCC), which have been detected by patch clamp in mouse spermatocytes [6,7]. The gating of the T-type VDCC would produce a transient (milliseconds) peak of calcium in the micromolar range detected in mag-fura loaded capacitated mouse sperm [8]. In seconds, ZP3 would stimulate IP3 production that would empty the calcium content of the acrosome and hence, activate store-operated calcium channels [9] present in the plasma membrane near to the acrosome. As a result, a small but sustained $[Ca^{2+}]_i$ increase would be established causing AR.

In human sperm, we have detected putative VDCC [10] that would reasonably play the role of the T-type VDCC

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proposed for mouse sperm. These channels have been functionally detected in fura 2-loaded sperm via the increase in intracellular calcium induced by potassium depolarization in the presence of the potassium ionophore valinomycin [10]. These putative channels are insensitive to nifedipine [10], but sensitive to mibefradil [11] and nickel [10,12]. Furthermore, they inactivate in ~90 s in calcium deprived medium [10] and are stimulated by pHi alkalization in a narrow range of pHi [12]. The calcium influx through these channels are stimulated during sperm capacitation, a phenomenon that may have an important physiological significance [12,13]. This stimulation may be explained in part by the alkalization observed in human sperm during capacitation. Indeed, according to the pHi sensitivity of human sperm VDCC, the increase in pHi alkalization observed during capacitation (of ~0.15 pHi units) would contribute to ~30% of the stimulation observed during this process [12]. This suggests that besides the pHi alkalization, other biochemical regulators that activate during capacitation may modify the channels so that the calcium permeation increases.

In this work, we examined the pHi sensitivity of VDCC of human sperm incubated in capacitating medium. To do this, we loaded the cells with fura ff, a low affinity calcium detector that allowed to detect reliable intracellular calcium increases that occurred in the micromolar range. We report that small pHi alkalization pulses remarkably stimulated VDCC in capacitated sperm and that the pHi-sensitivity of these channels increased nearly 7-fold, as compared with non-capacitated cells. This effect suggests that biochemical regulators activated during capacitation act on VDCC producing an increase in their sensitivity to pHi in human sperm.

Materials and methods

Materials and media. Fura ff-AM was obtained from Molecular probes. The other reagents were obtained from SIGMA. Hepes-buffered human sperm medium (H-HSM), originally proposed by Suarez et al. [14], had the following composition (in mM): NaCl 117.5, KCl 8.6, $CaCl_2$ 2.5, NaH_2PO_4 0.3, $MgCl_2$ 0.49, Na–pyruvate 0.3, Na–lactate 19, glucose 2, and 25 Hepes–Na (pH 7.6). Capacitating medium (cap-HSM) had the same salt composition except that Hepes was lowered to 15 mM and it was supplemented with 25 mM $NaHCO_3$ and 3 mg/ml BSA (Sigma, fraction V). The pH was adjusted to 7.6 with NaOH.

Sperm purification, dye loading and capacitation. Human semen was obtained from a panel of seven, 18–31-year-old, donors. Sperm purification was performed using Percoll gradients according to [14]. For intracellular calcium measurements, purified sperm ($0.6–1.5 \times 10^8$ cells) were loaded with 5 μM fura ff in 1 ml H-HSM medium at 36 °C for 40 min. The sample was washed by centrifugation and the cells scattered in disposable centrifuge tubes at $\sim 1 \times 10^7$ cells in 3 ml of either H-HSM (for non-capacitated sperm) or cap-HSM (for capacitated sperm) and maintained at 36 °C. Sperm incubated in non-capacitating medium (H-HSM) were immediately used for fluorescence recordings. To capacitate, sperm diluted in cap-HSM were incubated for 4 h at 36 °C.

To detect pHi, fura ff-loaded sperm were loaded with 1 μM BCECF-AM for 30 min. For pHi measurements in non-capacitated sperm, BCECF-AM was added during sperm incubation with fura ff-AM, 10 min after starting incubation with fura ff-AM. As for capacitated cells, BCECF-AM was added during capacitation of fura ff-loaded sperm, 30 min before completing the 4 h incubation time in cap-HSM medium.

Measurement and calibration of $[Ca^{2+}]_i$. An aliquot of fura ff-loaded sperm (containing $\sim 1 \times 10^7$ cells), incubated either in capacitating or in non-capacitating medium, was centrifuged and the pellet added to the fluorescence cell containing 2.5 ml H-HSM, supplemented with 5 mM $NaHCO_3$ [12], at 36 °C and under constant magnetic stirring. Fluorescence was measured with a PTI fluorometer (Photon technology International) with a 488 nm optical interference filter, exciting alternately at 340 and 380 nm with the PTI monochromator. Ratios 340/380 were acquired and digitalized at 0.86 Hz. The ratios were converted to intracellular calcium concentration by using the Grynkievicz equation [15], using a $K_d = 5.5$ μM (value provided by the manufacturer, Molecular probes).

Measurement and calibration of pH_i. Sperm loaded with BCECF and fura ff were poured into the fluorescence cell following the procedure described for intracellular calcium measurements. The fluorescence was detected at 550 nm, exciting alternately at 500 and 439 nm. Calibration was performed according to [12]. Briefly, at the end of each trace, 0.1% Triton X-100 was added to permeabilize the cells, increasing the ratio values to a value corresponding to pH 7.6 (the pH of the medium) (not shown). Then, the pH was acidified with different amounts of HCl and the measured pH values (determined with a conventional pH meter) were compared with the corresponding ratio values. These data were analyzed with the Felix software of PTI to convert ratios to pH values.

Assessment of voltage-dependent calcium influx. Voltage-dependent calcium influx was triggered by adding 30 mM KCl, 1 min after the potassium ionophore valinomycin (0.8 μM). Valinomycin makes the membrane potential mainly dependent on potassium permeability and, according to the Nernst potential for *K* distribution, brings the membrane potential from resting ~−40 mV to ~−71 mV [10]. Addition of 30 mM KCl depolarizes the membrane to ~−30 mV and induces a transient calcium influx. The difference of $[Ca^{2+}]_i$ at the peak minus resting was considered calcium influx through VDCC [12].

Results

The effect of intracellular alkalization on the $[Ca^{2+}]_i$ increase induced by depolarization, from ~−71 to ~−30 mV, in non-capacitated and capacitated sperm is shown in Fig. 1. In non-capacitated cells, at physiological pHi (no ammonium added), the calcium influx induced by depolarization was easily detected in spite of the fact that the K_d of fura ff-calcium complex ($K_d = 5.5$ μM) is 20-fold higher as compared with fura 2 ($K_d = 260$ nM), the calcium detector that we have previously used [10,12,13]. The voltage-dependent calcium influx detected with fura ff was 1.18 ± 0.14 μM, a value that was much higher than that detected in fura 2-loaded cells (close to 0.35 μM) [12]. Furthermore, the stimulation of the intracellular calcium increase induced by depolarization in capacitated cells was also higher than expected (Figs. 1A and B). In previous experiments made with fura 2-loaded cells, the estimated stimulation reached values around 0.6–0.9 μM [12,13] whereas that in experiments reported here in fura ff-loaded cells the calcium influx was 4.89 ± 0.53 μM ($n = 5$, SE). These discrepancies may reflect that K_d values for fura ff and/or fura 2 within sperm are inaccurate. However, the use of fura ff evidently improved the measurement at high intracellular calcium values (micromolar range) and also provided reasonable calibration values at a low range $[Ca^{2+}]_i$. Given that the results obtained with fura ff were qualitatively similar to those obtained with fura 2, we considered convenient to continue the use of fura ff to detect the effect of pHi alkalization in capacitated sperm.

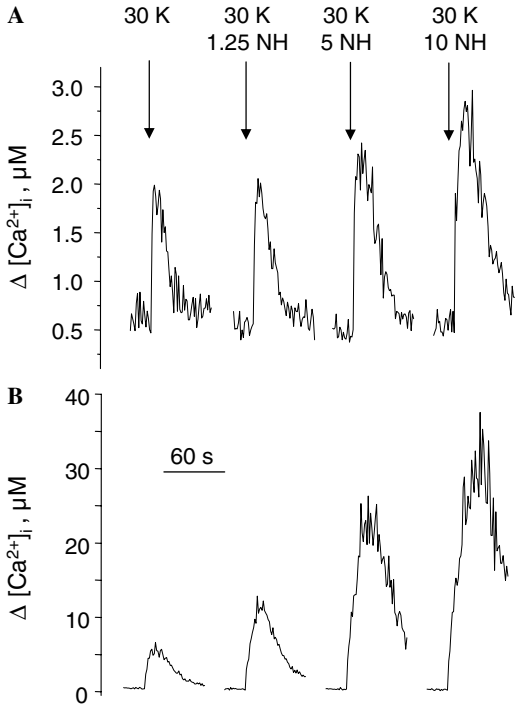

Fig. 1. Effect of ammonium on the calcium influx induced by depolarization in both non-capacitated (A) and capacitated (B) human sperm. Fura ff-loaded sperm were poured in 2.5 ml H-HSM supplemented with 5 mM $NaHCO_3$, thermostatized at 36 °C and under constant magnetic stirring. The sample was incubated for 1 min to allow stabilization and then 0.8 μM valinomycin was added to make the membrane potential sensitive to potassium (not shown in trace). One minute later, voltage-dependent calcium influx (from ~−71 to ~−30 mV) was triggered with 30 mM KCl (K) or 30 mM KCl + 1.25, 5.0, or 10 mM, NH_4Cl (NH), as indicated. The traces are representative of five different human sperm samples (see statistics in Fig. 3). The series of traces, both in non-capacitated and capacitated cells, were performed in a lapse of time of ~25 min.

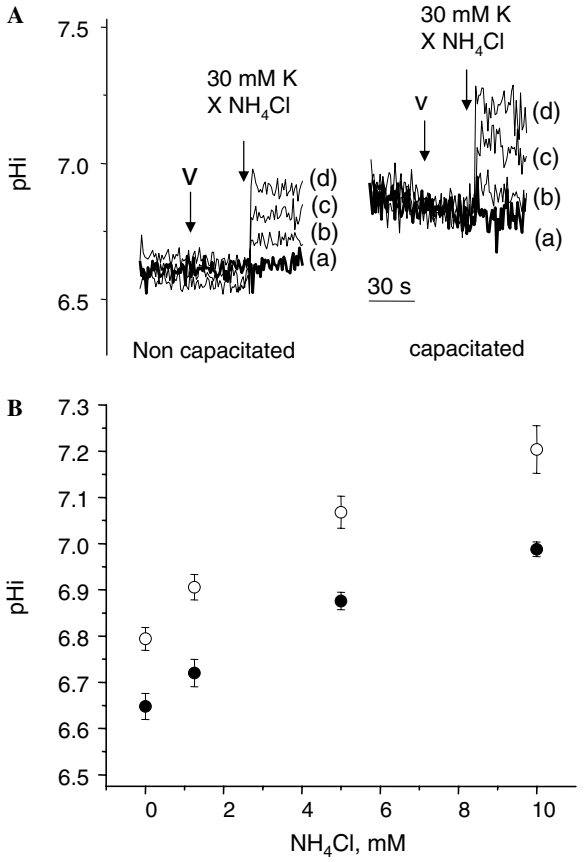

Fig. 2. Effect of NH_4Cl on the pHi of non-capacitated and capacitated human sperm. Either non-capacitated or capacitated fura ff-loaded sperm were loaded with BCECF-AM as described in Materials and methods. Once washed, sperm pellets were poured in the fluorescence cell containing 2.5 ml H-HSM medium + 5 mM $NaHCO_3$. In (A) 0.8 μM valinomycin followed by 30 mM KCl (traces a, in bold) or by mixtures of 30 mM KCl + 1.25 (traces b), 5.0 (traces c), or 10.0 mM (traces d) NH_4Cl was added to non-capacitated (traces at left) or capacitated (traces at right) human sperm. In (B) pHi reached upon NH_4Cl-KCl additions as a function of [NH_4Cl] in non-capacitated (closed circles) and capacitated (open circles) human sperm. The pHi was the average of 30 s recording (A) collected after NH_4Cl–KCl additions. All data are means ± SE, $n = 5$ individuals.

The effect of pHi on sperm VDCC was compared by co-adding, 1 min after valinomycin, 30 mM KCl plus either 1.25, 5.0, or 10 mM NH_4Cl. These concentrations site on the nearly linear relationship between voltage-dependent calcium influx and pHi (that has an apparent p*K*a ~7.00) observed in non-capacitated sperm [12]. In non-capacitated cells, the stimulation of the calcium influx depended on the ammonium added and was nearly duplicated by the addition of 10 mM NH_4Cl, an effect that was consistent with previous experiments made with fura 2-loaded cells [12] (Fig. 1A). Strikingly, in capacitated sperm the effect of identical amounts of NH_4Cl produced a marked over stimulation of the voltage-dependent calcium influx (Fig. 1B).

To compare the effect of pHi alkalization on the voltage-dependent calcium influx in capacitated and non-capacitated sperm, we determined the effect of ammonium on the pHi on both group of cells. Figs. 2A and B show that ammonium produced a higher alkalization in capacitated cells as compared with non-capacitated cells. This effect could be related to the fact that, in agreement with previous studies [5,12], the resting pHi in capacitated cells (6.80 ± 0.02, $n = 5$ SE) was 0.15 pHi units more alkaline than in non-capacitated cells (6.65 ± 0.03, $n = 5$ SE). Fig. 3 shows the effect of pHi on VDCC of capacitated and non-capacitated sperm. The curves show that the effect of pHi on sperm VDCC is notably enhanced in sperm incubated in capacitating conditions. In capacitated cells, the voltage-dependent calcium influx over intracellular pHi was approximately ~49 μM/pHi unit whereas that in non-capacitated sperm was ~6.7 μM/pHi unit. Thus, in human sperm the sensitivity of VDCC to pHi increased nearly 7-fold after 4 h incubation in capacitating conditions.

Discussion

Results presented here suggest that calcium influx through VDCC can be greatly potentiated by a combined effect of capacitation and pHi alkalization in human sperm.

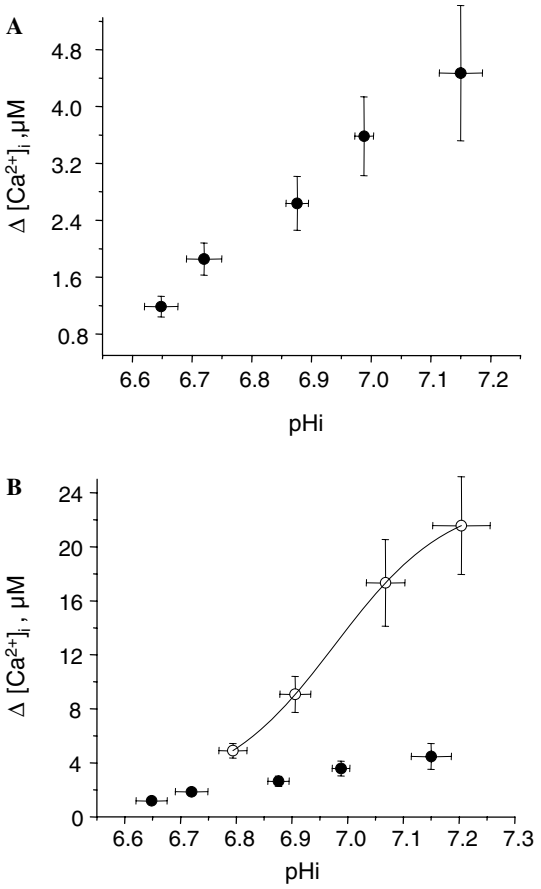

Fig. 3. Voltage-dependent calcium influx as a function of pHi in non-capacitated and capacitated human sperm loaded with fura ff. The $\Delta[Ca^{2+}]_i$ was obtained from the difference between the calcium peak induced by 30 mM KCl or by 30 mM KCl + 1.25, 5.0, or 10 mM NH_4Cl minus resting $[Ca^{2+}]_i$ obtained from records depicted in Fig. 1. The pHi and $\Delta[Ca^{2+}]_i$ values were compared using samples of the same sperm donor. (A) Curve corresponding to non-capacitated sperm samples. An additional experiment (mean ± SE, $n = 4$), made with 20 mM NH_4Cl (pHi ~7.15), was included to the plot to better compare the effect of pHi on both groups of sperm (see next). The rest of data are means ± SE with $n = 5$ individuals. (B) Curve corresponding to the same samples incubated in capacitating conditions (open circles). Non-capacitated sperm values (closed circles) from (A) are included for comparison.

It is important to note that the type of the calcium channels gated during the *K*-induced depolarization has not been determined yet. Pharmacological evidence has not been sufficient to establish, undoubtedly, what kind of channels participate in the response. Nevertheless, immunochemical evidence suggests the presence of T-type VDCC in human sperm [16]. Furthermore, mammalian sperm are endowed with CatSper 1, a peculiar form of VDCC identified in mouse sperm that is required for hyperactivation of mouse sperm motility [17,18] and that is also related to motility deficiency of human sperm [19]. Thus, it is reasonable that the calcium influx induced by depolarization with potassium would activate these channels. It is important to note that T, N, and L-type VDCC, detected by patch clamp in other cells, are sensitive to pHi, stimulating their activity at alkaline values [20–24], an effect that may involve the β subunit (in L-type channels) [20]. Here, we show evidence that the pHi sensitivity of VDCC may depend on the physiological condition of the cell. It is possible that biochemical regulators, such as cAMP, PKA or tyrosine kinases, which increase during sperm capacitation [3,4], produce changes in these channels resulting in the observed increase in pHi sensitivity.

Given the proposed participation of VDCC in the AR induced by the egg zona pellucida ZP3, we consider that the increase in pHi sensitivity of VDCC reported here may be significant in the mechanism by which ZP3 induces AR only in capacitated sperm.

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References

[1] A. Darszon, T. Nishigaki, C. Wood, C.L. Trevino, R. Felix, C. Beltran, Calcium channels and Ca^{2+} fluctuations in sperm physiology, Int. Rev. Cytol. 243 (2005) 79–172.
[2] E. Baldi, M. Luconi, L. Bonaccorsi, G. Forti, Signal transduction pathways in human spermatozoa, J. Reprod. Immunol. 53 (2002) 121–131.
[3] M. Luconi, I. Porazzi, P. Ferruzzi, S. Marchiani, G. Forti, E. Baldi, Tyrosine phosphorylation of the a kinase anchoring protein 3 (AKAP3) and soluble adenylate cyclase are involved in the increase of human sperm motility by bicarbonate, Biol. Reprod. 72 (2005) 22–32.
[4] J.E. Osheroff, P.E. Visconti, J.P. Valenzuela, A.J. Travis, J. Alvarez, G.S. Kopf, Regulation of human sperm capacitation by a cholesterol efflux-stimulated signal transduction pathway leading to protein kinase A-mediated up-regulation of protein tyrosine phosphorylation, Mol. Hum. Reprod. 5 (1999) 1017–1026.
[5] N.L. Cross, P. Razy-Faulkner, Control of human sperm intracellular pH by cholesterol and its relationship to the response of the acrosome to progesterone, Biol. Reprod. 56 (1997) 1169–1174.
[6] C.M. Santi, A. Darszon, A. Hernandez-Cruz, A dihydropyridine-sensitive T-type Ca^{2+} current is the main Ca^{2+} current carrier in mouse primary spermatocytes, Am. J. Physiol. 271 (1996) C1583–C1593.
[7] C. Arnoult, R.A. Cardullo, J.R. Lemos, H.M. Florman, Activation of mouse sperm T-type Ca^{2+} channels by adhesion to the egg zona pellucida, Proc. Natl. Acad. Sci. USA 93 (1996) 13004–13009.
[8] C. Arnoult, I.G. Kazam, P.E. Visconti, G.S. Kopf, M. Villaz, H.M. Florman, Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation, Proc. Natl. Acad. Sci. USA 96 (1999) 6757–6762.
[9] C.M. O'Toole, C. Arnoult, A. Darszon, R.A. Steinhardt, H.M. Florman, Ca(2+) entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drives the acrosome reaction, Mol. Biol. Cell. 11 (2000) 1571–1584.
[10] L. Linares-Hernández, A.M. Guzman-Grenfell, J.J. Hicks-Gomez, M.T. González-Martínez, Voltage dependent calcium influx in human sperm assessed by simultaneous detection of intracellular calcium and membrane potential, Biochim. Biophys. Acta 1372 (1998) 1–12.
[11] P.F. Blackmore, S. Eisoldt, The neoglycoprotein mannose-bovine serum albumin, but not progesterone, activates T-type calcium channels in human spermatozoa, Mol. Hum. Reprod. 5 (1999) 498–506.
[12] J.J. Fraire-Zamora, M.T. González-Martínez, Effect of intracellular pH on the depolarization-evoked calcium influx in human sperm, Am. J. Physiol. 287 (2004) C1688–C1696.

[13] M.T. González-Martínez, M.A. Bonilla-Hernández, A.M. Guzmán-Grenfell, Stimulation of voltage dependent calcium channels during capacitation and by progesterone in human sperm, Arch. Biochem. Biophys. 408 (2002) 205–210.
[14] S.S. Suarez, D.P. Wolf, S. Meizel, Induction of the acrosome reaction in human spermatozoa by a fraction of human follicular fluid, Gamete Res. 14 (1986) 107–121.
[15] G. Grynkievicz, M. Poenie, R.Y. Tsien, A new generation of calcium indicators with greatly improved fluorescent properties, J. Biol. Chem. 260 (1985) 3440–3450.
[16] C.L. Trevino, R. Felix, L.E. Castellano, C. Gutierrez, D. Rodriguez, J. Pacheco, I. Lopez-Gonzalez, J.C. Gomora, V. Tsutsumi, A. Hernandez-Cruz, T. Fiordelisio, A.L. Scaling, A. Darszon, Expression and differential cell distribution of low-threshold Ca(2+) channels in mammalian male germ cells and sperm, FEBS Lett. 563 (2004) 87–92.
[17] A.E. Carlson, R.E. Westenbroek, T. Quill, D. Ren, D.E. Clapham, B. Hille, D.L. Garbers, D.F. Babcock, CatSper1 required for evoked Ca2+ entry and control of flagellar function in sperm, Proc. Natl. Acad. Sci. USA 100 (2003) 14864–14868.
[18] T.A. Quill, S.A. Sugden, K.L. Rossi, L.K. Doolittle, R.E. Hammer, D.L. Garbers, Hyperactivated sperm motility driven by CatSper2 is required for fertilization, Proc. Natl. Acad. Sci. USA 100 (2003) 14869–14874.
[19] P. Nikpoor, S.J. Mowla, M. Movahedin, S.A. Ziaee, T. Tiraihi, CatSper gene expression in postnatal development of mouse testis and in subfertile men with deficient sperm motility, Hum. Reprod. 19 (2004) 124–128.
[20] K. Schuhmann, C. Voelker, G.F. Hofer, H. Pflugelmeier, N. Klugbauer, F. Hofmann, C. Romanin, K. Groschner, Essential role of the beta subunit in modulation of C-class L-type Ca^{2+} channels by intracellular pH, FEBS Lett. 408 (1997) 75–80.
[21] L. Kiss, S.J. Korn, Modulation of N-type Ca2+ channels by intracellular pH in chick sensory neurons, J. Neurophysiol. 81 (1999) 1839–1847.
[22] G.C. Tombaugh, G.G. Somjen, Differential sensitivity to intracellular pH among high and low-threshold Ca^{2+} currents in isolated rat CA1 neurons, J. Neurophysiol. 77 (1997) 639–653.
[23] M. Yamakage, K.S. Lindeman, C.A. Hirshman, T.L. Croxton, Intracellular pH regulates voltage-dependent Ca^{2+} channels in porcine tracheal smooth muscle cells, Am. J. Physiol. 268 (1995) L642–L646.
[24] C.M. Santi, T. Santos, A. Hernández-Cruz, A. Darszon, Properties of a novel pHi-dependent Ca^{2+} permeation pathway present in germ cells with possible roles in spermiogenesis and mature sperm function, J. Gen. Physiol. 112 (1998) 33–53.