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$Ca_v2.3$ (α_{1E}) Ca^{2+} channel participates in the control of sperm function

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Abstract To know the function of the Ca^{2+} channel containing $\alpha_1 2.3$ (α_{1E}) subunit ($Ca_v2.3$ channel) in spermatozo we analyzed Ca^{2+} transients and sperm motility using a mouse strain lacking $Ca_v2.3$ channel. The averaged rising rates of Ca^{2+} transients induced by α-D-mannose–bovine serum albumin in the head region of $Ca_v2.3-/-$ sperm were significantly lower than those of $Ca_v2.3+/+$ sperm. A computer-assisted sperm motility assay revealed that straight-line velocity and linearity were greater in $Ca_v2.3-/-$ sperm than those in $Ca_v2.3+/+$ sperm. These results suggest that the $Ca_v2.3$ channel plays some roles in Ca^{2+} transients and the control of flagellar movement.

Key words: $Ca_v2.3$ (α_{1E}) channel; Voltage-dependent Ca^{2+} channel; Zona pellucida; α-D-Mannose–bovine serum albumin; Flagellar movement

1. Introduction

The voltage-dependent Ca^{2+} channel (VDCC) is one of the important pathways of Ca^{2+} influx into sperm [1,2], though the nature of VDCCs in sperm is not fully documented yet. VDCCs are classified into several distinct groups in terms of electrophysiological and pharmacological characteristics, and have been named: L-, N-, P-, Q-, R- and T-types [3,4]. A channel from each class is composed of α_1, α_2-δ, β, and γ subunits. Among these subunits, α_1 subunit is essential for channel conductance and determines fundamental channel properties, which provide a rationale for VDCC classification [5]. So far, 10 α_1 cDNAs have been cloned, and their properties have been studied extensively. In spite of these extensive studies, the properties of the Ca^{2+} channel containing $Ca_v2.3$ subunit ($Ca_v2.3$ channel) remain elusive [6–10]. This channel was initially considered to be responsible for low-voltage-activated currents, however, at least a part of the high-voltage-activated R-type current is thought to be coded by this channel [11,12].

Several kinds of the cloned α_1 subunits have been suggested to exist in mammalian spermatozoa. As for the channel protein, immunocytochemical studies suggest the presence of $Ca_v1.2$, $Ca_v2.1$, $Ca_v2.3$ [13], and $Ca_v2.2$ [14] proteins both in the head and flagellum of mouse sperm. The presence of N- and R-type currents in mature sperm was suggested pharmacologically [14]. The $Ca_v2.3$ channel was one of the leading candidates for the low-voltage-activated Ca^{2+} current in immature spermatocytes [15]. Recently, the function of $Ca_v2.3$ channel in mature spermatozoa has become more enigmatic as no apparent contribution of this channel to the LVA current was observed in pachytene spermatocytes [16]. Instead, $Ca_v2.3$ channel is now expected to play roles in the control of acrosome reaction, capacitation and flagellar movement as inferred from its localization in mature sperm [13]. However, no definite functions of the $Ca_v2.3$ channel in sperm have been identified yet partly because of the limited availability of its specific blocker.

As an approach to elucidate the physiological function of $Ca_v2.3$ channel, we established a mouse strain lacking $Ca_v2.3$ channel and reported that $Ca_v2.3$ channel plays some roles in the control of pain responses [17] and in the formation of the accurate spatial memory [18]. Although male mice lacking the $Ca_v2.3$ channel were found to be fertile, we searched for possible abnormalities in functions of spermatozoa from the mutant mice. We explored the contribution of this channel to intracellular Ca^{2+} transient related to acrosome reaction and its contribution to sperm flagellar movement. We have found that the averaged rising rates of Ca^{2+} transients induced by α-D-mannose–bovine serum albumin (mannose–BSA) in the head region of $Ca_v2.3-/-$ sperm were significantly lower than that of $Ca_v2.3+/+$ sperm, and that linearity of the movement was apparently increased in $Ca_v2.3-/-$ sperm. These results suggest that the $Ca_v2.3$ channel is functional in spermatozoa, playing some roles in Ca^{2+} transients and the control of flagellar movement.

2. Materials and methods

2.1. Chemicals

Fura-2 AM (acetoxymethyl ester) and Cs-BAPTA were obtained from Molecular Probes (Eugene, OR, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA) unless otherwise stated.

2.2. Animals

Mice lacking $\alpha_1 2.3$ subunit of the VDCC were generated as previ-

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Abbreviations: VDCC, voltage-dependent Ca^{2+} channel; BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration

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ously reported [17]. Male wild-type ($Ca_v2.3+/+$) or homozygous mutant ($Ca_v2.3-/-$) mice with a hybrid background of C57BL/6 and 129/Sv (aged 8–30 weeks) were used in all the experiments in a blind manner.

2.3. Imaging and recordings of Ca^{2+} transients

Epididymal sperm were released into TYH medium (94.5 mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 5.6 mM glucose, 28 mM lactic acid sodium salt and 0.5 mM pyruvic acid) supplemented with 4 mg/ml BSA. The pH was adjusted to 7.4 by 5% CO_2 saturation. The sperm were allowed to swim up for 30 min at 37°C. To measure changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of individual sperm, the sperm were first incubated for 15 min with 5 μM fura-2 AM [19], and immobilized on a coverslip coated with laminin [20]. The coverslip was then placed on a microscope stage (Nikon TMD300, Japan) and the temperature was kept at 35–36°C by a controller (DTC300, Diamedical Co., Japan). The Ca^{2+} influx was evoked by exposure of the sperm to solubilized zona pellucida (two zonae equivalents/μl), mannose–BSA (10 μg/ml) or K8.6 (135 mM KCl, 5 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM TAPS (*N*-tris [hydroxymethyl] methyl-4-aminobutanesulfonic acid), 10 mM glucose, 10 mM lactic acid sodium salt and 1 mM pyruvic acid, pH 8.6) [14]. Zonae pellucidae were prepared from oocytes of superovulated ICR mice and solubilized as described previously by Shirakawa [21]. A glass micropipette with a tip opening of 4–5 μm was filled with solubilized zona pellucida. With the aid of a 40× objective lens, the micropipette was applied to the surface of the coverslip. Digitonin (0.01%), prepared with the medium that was used in zona pellucida solubilization, was applied to the sperm as a positive control to confirm that the application route was appropriate. Before observing the Ca^{2+} transients caused by zona pellucida and mannose–BSA, TYH medium was perfused at a rate of 3 ml/min for a few minutes to wash out the extracellular Ca^{2+} indicator. In the measurement of depolarization-induced Ca^{2+} transients, HS medium (135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM HEPES, 10 mM glucose, 10 mM lactic acid sodium salt and 1 mM pyruvic acid, pH 7.4) fortified with 15 mM $NaHCO_3$ was used as a control. Excitation wavelengths of 360 and 380 nm were used to excite fura-2. Calcium transients were imaged via a low pass 510 nm filter using a 40× NA 1.3 oil immersion lens. Images were captured using a C2400 intensified CCD camera (Hamamatsu Photonics, Japan). $[Ca^{2+}]_i$ was estimated by the ratio F_{360}/F_{380}, where F_{360} and F_{380} represent fluorescence intensities at excitation wavelengths of 360 nm and 380 nm, respectively. The fluorescence intensities were recorded every 2.5 s and analyzed by an Argus 50 Calcium imaging system (Hamamatsu Photonics, Japan). In this study, only spermatozoa that were stuck mildly to the coverslip at the head but were still beating the tail were analyzed. Rising rates of the F_{360}/F_{380} ratio (s^{-1}) were defined as the slopes of regression lines calculated for the linear part of the $[Ca^{2+}]_i$ increase.

2.4. The characteristics of sperm motility

For the analysis of sperm motility, epididymal sperm were dispersed into TYH, HS or modified-HS medium, each of which was supplemented with 4 mg/ml BSA. Modified-HS medium was composed of: 108 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM HEPES, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, 10 mM lactic acid sodium salt and 1 mM pyruvic acid (pH 7.4 with 5% CO_2 saturation). The sperm suspension was placed in a 100 μm deep cannula that was set on a microscope stage and kept at 37°C. Sperm ($n > 500$) were examined in total for 15 fields of view, and all measurements were completed within 30 min. In order to characterize sperm motility, tracks of the individual sperm were traced at 30 Hz by a computer-assisted sperm assay (CASA) system with a sperm motility analyzer (IVOS Ver 10.7; Hamilton-Thorne Research, Beverly, MA, USA). Three indices were obtained in terms of velocity (μm/s). Straight-line velocity (VSL) was calculated from the straight-line distance between the beginning and the end of the track (Fig. 2A). Curvilinear velocity (VCL) was calculated from the total length along the swimming track, and path velocity (VAP) was obtained from the path length of the smoothed tracks obtained by moving average. Additionally two indices were also used to describe the swimming pattern: (1) linearity (LIN), defined as VSL/VCL, indicated the similarity of the cell track to a straight line and (2) straightness (STR), defined as VSL/VAP, which indicated the closeness to the straight line but based on the cell's gross movement. The parameters used to obtain motility indices were set as described previously by Huang et al. [22].

2.5. Statistical analysis

Data were expressed as mean ± S.E.M. For statistical analyses, an unpaired *t*-test was used, and the difference was considered significant when the *P* value was less than 0.05.

3. Results

3.1. Ca^{2+} transients in the sperm head

To investigate whether the $Ca_v2.3$ channel contributes to $[Ca^{2+}]_i$ rise in the sperm head, we measured Ca^{2+} transients induced by agonists for acrosome reaction. Firstly, solubilized zona pellucida was used as the physiological agonist. This stimulation induced rapid changes in $[Ca^{2+}]_i$ in the sperm head (Fig. 1A,D) in 61.3% (38/62) and 60% (42/70) of spermatozoa of $Ca_v2.3+/+$ and $Ca_v2.3-/-$ mice, respectively. In each experiment, F_{360}/F_{380} reached its peak within 12.5 s from the onset of the response, and the peak level continued for at least 1 min. The rising rate of $[Ca^{2+}]_i$ in sperm from $Ca_v2.3-/-$ mice (0.091 ± 0.008 s^{-1}, $n = 42$) was lower than that from $Ca_v2.3+/+$ mice (0.105 ± 0.008 s^{-1}, $n = 38$), although not reaching a statistically significant level (Fig. 1G).

Secondly, by application of 10 μg/ml mannose–BSA, 97.1% (66/68) and 92.3% (72/78) of spermatozoa responded in $Ca_v2.3+/+$ and $Ca_v2.3-/-$ mice, respectively (Fig. 1B,E). The response occurred within 165 s after the start of mannose–BSA application and the average onset of the response was 50.1 ± 4.2 s and 55.3 ± 4.6 s in $Ca_v2.3+/+$ and $Ca_v2.3-/-$ mice, respectively. In each trial, F_{360}/F_{380} reached the peak within 10 s from the onset of the response, and the peak level was maintained for a few minutes. The rising rates of $[Ca^{2+}]_i$ in sperm from $Ca_v2.3-/-$ mice (0.066 ± 0.004 s^{-1}, $n = 72$) were significantly lower than that from $Ca_v2.3+/+$ mice (0.078 ± 0.004 s^{-1}, $n = 66$, $P = 0.034$) (Fig. 1H).

Finally, a Ca^{2+} transient in the head region was also induced by application of a high potassium solution, K8.6. With this stimulation, $[Ca^{2+}]_i$ reached its peak within 10 s after the start of K8.6 perfusion (Fig. 1C,F) and recovered slowly after washing with the control solution. In this stimulation protocol, however, the averaged rising rate in $Ca_v2.3-/-$ sperm (0.098 ± 0.006 s^{-1}, $n = 49$) was higher than that in $Ca_v2.3+/+$ sperm (0.089 ± 0.004 s^{-1}, $n = 57$, Fig. 1I).

3.2. Characteristics of sperm motility

Expression of the $Ca_v2.3$ channel has been shown in the tail region of the sperm [13]. Therefore, we next examined whether the $Ca_v2.3$ channel played any roles in sperm motility: we analyzed the characteristics of sperm movement in HS medium using CASA. Representative traces of spermatozoa from both genotypes are shown (Fig. 2B). VSL from $Ca_v2.3-/-$ sperm (80.8 ± 2.7 μm/s) was significantly higher than that from $Ca_v2.3+/+$ sperm (72.7 ± 1.8 μm/s, $P = 0.026$, Fig. 3A). The VAP also showed a similar tendency to be higher in $Ca_v2.3-/-$ sperm (115.7 ± 2.5 μm/s) than in $Ca_v2.3+/+$ sperm (109.6 ± 1.8 μm/s, $P = 0.074$). The values of VCL were 235.2 ± 5.4 μm/s in $Ca_v2.3-/-$ sperm and 229.3 ± 3.5 μm/s in $Ca_v2.3+/+$ sperm. The analysis also revealed that linearity (LIN = VSL/VCL) was significantly greater in $Ca_v2.3-/-$ sperm ($35.1 \pm 1.0\%$) than in $Ca_v2.3+/+$ sperm ($32.3 \pm 0.8\%$, $P = 0.045$) (Fig. 3B). Straightness (STR = VSL/

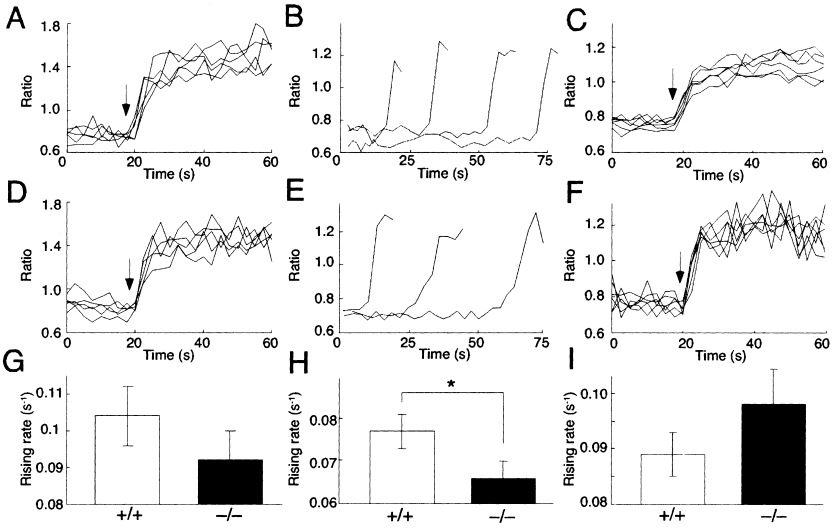

Fig. 1. transients recorded in the sperm head. A, D, and G: Ca^{2+} transient induced by solubilized zona pellucida. Typical records of Ca^{2+} transients induced by solubilized zona pellucida application in sperm from $Ca_v2.3+/+$ (A) and $Ca_v2.3-/-$ (D) mice. Each line represents data from independent cells. Arrows indicate the point when zona pellucida was applied. G: The average rising rate in sperm from $Ca_v2.3+/+$ mice (clear bar) was higher than that from $Ca_v2.3-/-$ mice (black bar), though this was not statistically significant. B, E, and H: Ca^{2+} transient induced by mannose–BSA. Typical records of Ca^{2+} transients induced by mannose–BSA application in sperm from $Ca_v2.3+/+$ (B) and $Ca_v2.3-/-$ (E) mice. Each line represents data from independent cells. Mannose–BSA was applied at time 0 s. Ca^{2+} transients were detected after various delays. H: The average rising rate in sperm from $Ca_v2.3+/+$ (clear bar) was significantly higher than that from $Ca_v2.3-/-$ mice (black bar, $P = 0.034$). C, F, and I: Ca^{2+} transient induced by K8.6. Typical records of Ca^{2+} transients induced by K8.6 application in $Ca_v2.3+/+$ (C) and $Ca_v2.3-/-$ sperm (F). Arrows indicate the point when K8.6 medium was applied. I: The average rising rate in sperm from $Ca_v2.3+/+$ (clear bar) was lower than that from $Ca_v2.3-/-$ mice (black bar), though this was not statistically significant.

VAP) also displayed a tendency to be greater in $Ca_v2.3-/-$ sperm ($69.3 \pm 1.6\%$) than in $Ca_v2.3+/+$ sperm ($65.9 \pm 1.4\%$). Other movement parameters such as the beat cross frequency and the amplitude of lateral head displacement did not show any particular tendency (data not shown). On the other hand, when the assays were performed in TYH medium, no apparent differences for VSL, VAP, VCL, LIN and STR were observed (Fig. 3C,D). In order to clarify why no apparent differences were observed between the sperm from two genotypes in TYH medium, we modified the HS medium with the addition of 25 mM $NaHCO_3$ and 1.19 mM KH_2PO_4. In this modified-HS medium, the differences that were observed between $Ca_v2.3+/+$ and $Ca_v2.3-/-$ sperm motility in HS medium were not evident (Fig. 4A,B).

4. Discussion

The principal finding obtained in our study on the $Ca_v2.3$ channel function in spermatozoa is that this channel participates in the control of swimming behavior in sperm. In HS medium, VSL and LIN were greater in sperm from $Ca_v2.3-/-$ mice than those from $Ca_v2.3+/+$ mice. $Ca_v2.3$ channels are distributed along the dorsal and ventral aspects of the proximal segment of the principal piece of mouse sperm [13]. Based on this localization pattern, the $Ca_v2.3$ channel is supposed to control Ca^{2+} influx to produce an asymmetry in flagellar beat, which is observed in hyperactivation and is expected to occur in chemotaxis, though the nature of the endogenous attractants is not identified in mammals [13]. In sea urchin sperm, the involvement of VDCC in chemotactic responses [23,24] and in flagellar responses to electrical stimulation [25] has been well documented. It is generally accepted that an increase in $[Ca^{2+}]_i$ enhances flagellar waveform asymmetry, which leads to the decrease in straight motion and ultimately to inhibition of the movement [26,27]. Although the exact mechanisms underlying these changes in the motility parameters remain elusive, our result is compatible with the above-mentioned idea, because the lack of $Ca_v2.3$ channel resulted in an increase of straight movements. This confirms the participation of a VDCC in the control of sperm motility in mammalian species as well.

However, this effect of the $Ca_v2.3$ channel deficiency was not so evident in TYH medium as in HS medium. Sperm behave differently in these two media. In fact, when sperm from $Ca_v2.3+/+$ mice were assayed in the two media, an apparent difference in flagellar beat frequency was observed (TYH: 32.2 ± 0.8 Hz, HS: 26.6 ± 1.2 Hz, $P = 0.0009$). The situation was the same in sperm from $Ca_v2.3-/-$ mice (TYH: 30.9 ± 0.6 Hz, HS: 26.6 ± 0.7 Hz, $P = 0.0002$). This suggested the existence in the TYH medium of some substance that affects sperm flagellar movement. One possible candidate for such a substance is the bicarbonate ion, and the possible reason why the effect of the $Ca_v2.3$ channel deficiency is dependent on the medium may be as follows. It is known that the bicarbonate ion, which is a component of TYH but not of HS medium, enters sperm through an anion transporter in the plasma membrane, activates the soluble adenylyl cyclase and increases cAMP production [28]. Increased levels of cAMP activate hyperpolarization-activated channel, and the resultant depolarization is suggested to lead to Ca^{2+} influx through T-type Ca^{2+} channels [29]. cAMP is also thought to activate L-type VDCC [30] and the cyclic nucleotide-gated Ca^{2+} channel [31], leading to higher level of $[Ca^{2+}]_i$. Therefore, it is possible that these mechanisms rescue the attenuation of Ca^{2+} increase due to the lack of $Ca_v2.3$ channel in $Ca_v2.3-/-$ sperm. In addition, protein kinase A, a classic target of cAMP, catalyzes

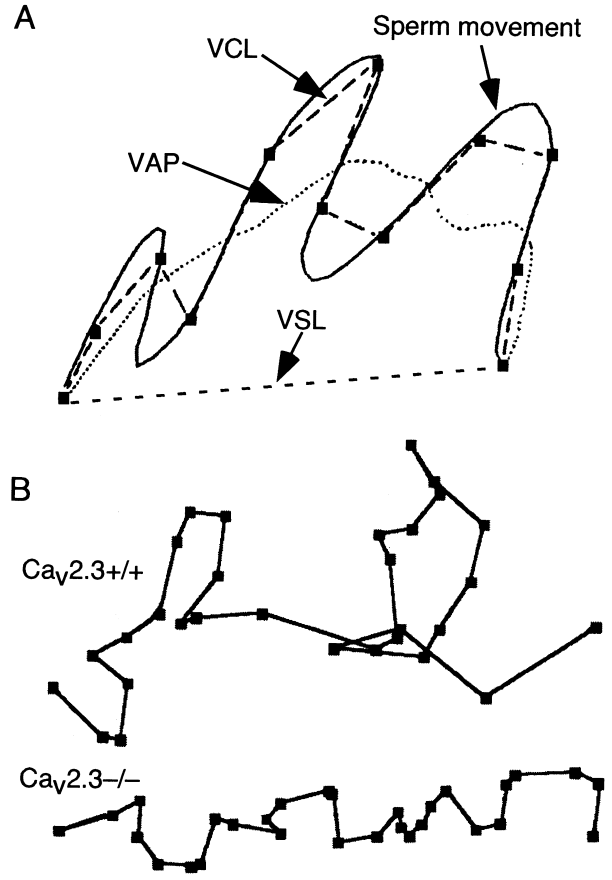

Fig. 2. Parameters used in CASA assays (A) and representative traces of sperm from both genotypes (B). The position of each sperm head is recognized by phase-contrast microscopy every 1/30 s, stored and analyzed automatically. Closed squares represent the position of sperm head at each moment. A: Linearity is defined by VSL/VCL, and straightness is defined by VSL/VAP. B: Upper and lower traces represent movements of $Ca_v2.3+/+$ and $Ca_v2.3-/-$ spermatozoa, respectively. The trace of movement of $Ca_v2.3-/-$ spermatozoa shows a linear pattern in comparison with the randomized motility pattern of $Ca_v2.3+/+$ spermatozoa.

the phosphorylation of several flagellar proteins [29] and modulates the flagellar movement to mask the difference caused by $Ca_v2.3$ channel deficit. These possibilities are suggested by the result that in modified-HS medium containing bicarbonate ions, the differences observed in HS medium were not obvious. The reasons why movement parameters were low in modified-HS medium are not clear at present.

Mammalian sperm motility is modified by the exposure to complicated environments in female reproductive tracts [32]. It would be important to investigate how the $Ca_v2.3-/-$ sperm behave in the in vivo environment. The randomized motility pattern characterized in normal sperm may have an advantage in searching for chemoattractants. It is possible that $Ca_v2.3$ channel is required for such a randomized pattern of swimming.

In this study, we also investigated whether $Ca_v2.3$ channel is involved in Ca^{2+} transient leading to acrosome reaction. Several ligand (glycan)–receptor interactions are thought to be involved in gamete interaction and successful fertilization [33]. The binding of mannose to sperm-surface mannosidase is one of the candidates for these multiple interactions between zona pellucida and sperm [34], and VDCCs such as T-type [35,36], and L-type channels [37] are thought to be involved in this process. Our results suggest the additional contribution of $Ca_v2.3$ channel to Ca^{2+} transient induced by mannose–BSA. In this study, ZP induced a slightly slower tendency in Ca^{2+} transient of $Ca_v2.3-/-$ sperm, whereas mannose–BSA induced a significantly slower Ca^{2+} transient in $Ca_v2.3-/-$ sperm. This suggests that $Ca_v2.3$ channel is involved in the Ca^{2+} transient induced by mannose–BSA, but this channel is not indispensable to the Ca^{2+} transient induced by zona pellucida. In contrast to these agonists, Ca^{2+} transient caused by KCl depolarization tended to be higher in $Ca_v2.3-/-$ sperm than $Ca_v2.3+/+$ sperm. It could be speculated that some other VDCCs might compensate for the loss of $Ca_v2.3$ channel.

In conclusion, $Ca_v2.3$ channel is functional in spermatozoa, being involved in Ca^{2+} transient induced by mannose–BSA and flagellar movement. Further elucidation of the physiological role of sperm VDCCs may shed light on the pathologic feature in male infertility.

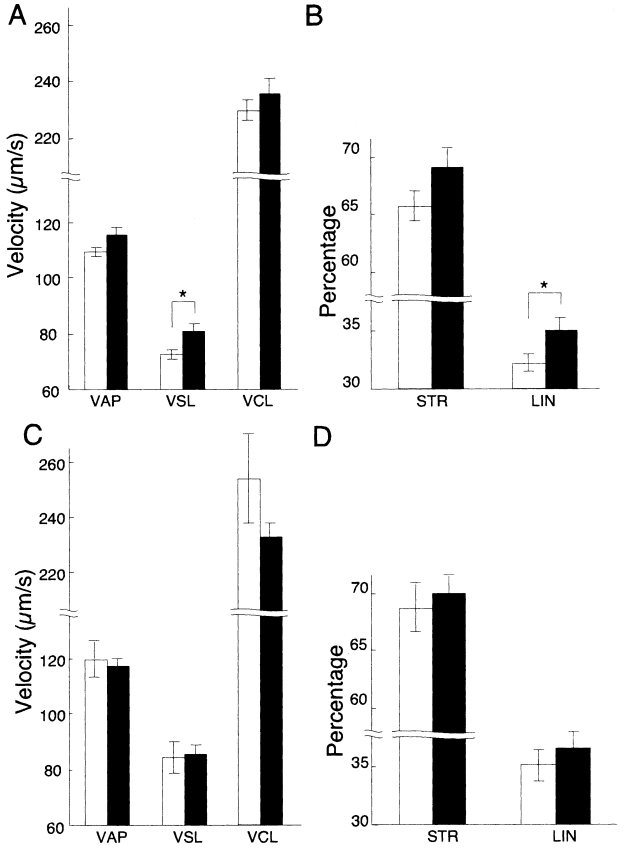

Fig. 3. Analyzed parameters from the CASA assay in HS medium (A and B) and TYH medium (C and D). Clear bar, $Ca_v2.3+/+$; black bar, $Ca_v2.3-/-$ in all panels. A: Out of the three velocity parameters, VSL is higher in $Ca_v2.3-/-$ sperm than in $Ca_v2.3+/+$ sperm ($P=0.026$). VAP shows a similar tendency (P+0.07). B: LIN (linearity) is greater in sperm from $Ca_v2.3-/-$ mice than from $Ca_v2.3+/+$ ($P=0.045$). STR (straightness) also tends to be greater in sperm from $Ca_v2.3-/-$ mice than from $Ca_v2.3+/+$ ($P=0.12$). $n=8$ for $Ca_v2.3+/+$ mice, and $n=9$ for $Ca_v2.3-/-$ mice. C: VSL, VAP and VCL did not show particular tendency between both genotypes. D: STR and LIN display no apparent differences. $n=10$ for $Ca_v2.3+/+$ mice, and $n=13$ for $Ca_v2.3-/-$ mice.

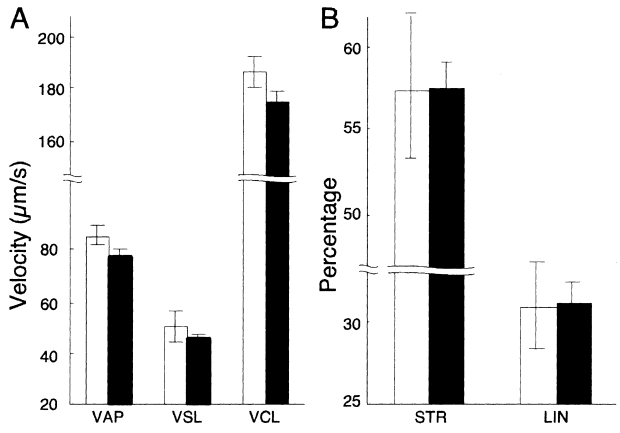

Fig. 4. Analyzed parameters from the CASA assay in modified-HS medium. Clear bar, $Ca_v2.3+/+$; black bar, $Ca_v2.3-/-$ in both panels. The tendency observed in HS medium was not evident in modified-HS medium. STR and LIN display no apparent tendency. $n=4$ for $Ca_v2.3+/+$ mice, and $n=5$ for $Ca_v2.3-/-$ mice.

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