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Infertile Spermatozoa of c-*ros* Tyrosine Kinase Receptor Knockout Mice Show Flagellar Angulation and Maturational Defects in Cell Volume Regulatory Mechanisms[1]

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ABSTRACT

Homozygous c-*ros* knockout male mice that lack prepubertal differentiation of the epididymal initial segment are healthy but sterile, despite normal sperm production and mating. Detailed computerized analysis of the motility of spermatozoa maturing in the epididymis revealed only minor defects. However, the majority of motile mature sperm released from the cauda epididymidis showed various extents of flagellar angulation that could not be corrected by raising extracellular osmolality. Measurement of the osmolality of cauda epididymal fluid showed no difference from the wild type. Studies in wild-type mice indicated a maturational change in the ability of motile sperm to maintain straight flagella during incubation, but angulation was induced in cauda sperm by the volume-sensitive ion channel blockers quinine, 5-nitro-2-(3-phenylpropylamino)-benzoic acid and $BaCl_2$, or by exposure to hypotonic media. Flagellar angulation, induced in the wild type or intrinsic to the knockout, was relieved upon demembranation by Triton X-100, confirming that it was a cell swelling phenomenon. A lack of response of immature wild-type sperm and mature knockout sperm to the channel blockers suggests that there is normally a development of the volume regulatory mechanisms upon maturation that is defective in sperm from the knockout animal. The resultant flagellar angulation may account for the reduction in sperm numbers in the oviduct of mated females and the failure to fertilize in vivo.

INTRODUCTION

In mammals, spermatozoa are produced in the testis; they enter the epididymis, where they undergo maturational changes by which they attain the ability to perform the sequence of fertilization processes eventually occurring in the female tract [1]. At the end of their epididymal sojourn, mature sperm cells are stored for a variable period of time in the cauda epididymidis until they are ejaculated. Over the last four decades, increasing efforts have been invested in the study of the sperm maturation processes and the role of the epididymis in them [2]. Despite the characterization of sperm properties acquired in the epididymis [3], little is known about the mechanisms involving defined epididymal factors in regulating acquisition of the fertilizing capacity by epididymal spermatozoa. The c-*ros* knockout male mice, which are healthy with normal sperm production and mating behavior but are sterile [4], provide the first transgenic animal model, and a unique one, for the study of sperm maturation in the epididymis [5].

The protooncogene c-*ros* is an orphan receptor with an intracellular tyrosine kinase domain and a long extracellular domain [6, 7]. The original c-*ros* knockout mice were generated with the intention of examining the role of c-*ros* in morphogenesis, since it is expressed in specific temporal patterns in the embryonic epithelia of the kidney, lung, and intestine [7, 8]. It is also expressed in the wolffian duct, the embryonic genital tract. Whereas the expression of c-*ros* in other organs rapidly disappears in neonatals, that in the epithelium of the proximal epididymis (caput) is up-regulated during prepubertal development, before the arrival of spermatozoa from the testis, and persists in adults [4]. This coincides with the differentiation of the proximal caput epididymidis into the so-called initial segment. In the homozygous knockout mice, c-*ros* is not detected at the mRNA or protein level and there is no differentiation of the initial segment, which is the only abnormal phenotype found in these infertile animals. Heterozygous transgenic mice are indistinguishable from the wild type.

To elucidate the relationship between the specific epididymal abnormality and the infertility of these transgenic animals, it is necessary to characterize the sperm defects in these mice. Previous findings of reduced numbers of sperm in the oviduct of mated wild-type females despite normal mating and uterine deposition of sperm, and of successful reproduction using these sperm in conventional in vitro fertilization and embryo transfer techniques [4], suggest that sperm from the homozygous mice may have defective motility compromising their transport through the female tract. Therefore the maturation of sperm kinematics in the epididymis was examined in detail in the present study. Preliminary observations of abnormal forms of the sperm flagellum [5] were also investigated systematically. Angulation or coiling of the sperm tail is well recognized as a phenomenon of sperm cell swelling (e.g., [9–11]). There are several reports of infertility associated with the presence of coiled sperm in the ejaculates of stallions, boars, and bulls in which coiling originates in the epididymis [12], but the mechanism of volume regulation in spermatozoa has received hardly any attention. Since mammalian spermatozoa are stored in a hypertonic environment in the epididymis [13] and would experience relative hypoosmotic conditions upon ejaculation, consideration of regulatory volume decrease (RVD) is particularly pertinent. The present work is the first to characterize the maturational development of RVD in mammalian sperm and the involvement of ion channels, and to reveal defects in them that are possibly linked to infertility.

MATERIALS AND METHODS

All reagents were from Sigma (Deisenhofen, Germany) except glibenclamide (Molecular Probes, Leiden, the Netherlands) and 5-nitro-2-(3-phenylpropylamino)-benzoic acid

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(NPPB; Calbiochem-Novabiochem, Bad Soden, Germany). The basal medium used for incubation of spermatozoa contained 123 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 0.4 mM $MgSO_4$, 25 mM $NaHCO_3$, 5 mM glucose, 12.5 mM sodium lactate, 0.25 mM pyruvate, 8 μg/ml phenol red, and 12 mg/ml BSA, with a pH of 7.4 and a measured osmolality of 302–310 mmol/kg. Media of differing osmolality were made by adjusting the amount of NaCl to achieve 230–510 mmol/kg. The Ca^{2+}-free medium was made by replacing $CaCl_2$ with NaCl, and 1 mM EGTA was added.

Animals and Sperm Preparation

Homozygous and heterozygous transgenic mice with targeted deletions of c-*ros* were produced in the Max-Delbrück Center. Since the deletion of either the intracellular kinase domain or the extracellular domain resulted in complete infertility in homozygous mice [4], the two genotypes were used indiscriminately in the present study. Wild-type mice of the same strain (C57/BL6) were obtained for control experiments from Charles River (Sulzfeld, Germany). Animals were anesthetized with ethyl carbamate, and several loops of tubule were excised from each defined region of the epididymis. Sperm were gently expressed into a drop of experimental medium, transferred into an Eppendorf (Hamburg, Germany) tube kept in an incubator at 37°C with 5% CO_2, and examined at various times.

Measurement of Motility and Flagellar Angulation of Diluted Sperm

Sperm were obtained from 5 regions of the epididymis: the initial segment (or the topologically equivalent zone in the homozygous mice), distal caput, proximal corpus, and proximal and distal cauda. After dilution and dispersion for 1 min at 37°C, the sperm were loaded onto a 40-μm-deep siliconized chamber and examined on a microscope stage warmed to 37°C. One hundred cells were classified as motile (flagellating) or immotile, and the tail form was categorized as straight, angled, or hairpin (see *Results* for description). A video recording was made from several microscopic fields over 3 sec and analyzed later by tracking 100–200 motile cells for 30 video frames at a rate of 25 Hz, using the computerized sperm analysis system (HTM-C version 10.6; Hamilton Thorne Research, Beverly, MA) as described previously [14]. Kinematic parameters measured included curvilinear velocity (VCL), straight-line velocity (VSL), linearity (LIN = 100% × VSL/VCL), and amplitude of the lateral displacement of sperm head from the averaged swim path (ALH). Median values of the kinematic parameters were obtained for each sample.

Examination of Sperm Flagellar Angulation In Situ

To determine whether sperm were angulated in the epididymal lumen or whether the phenomenon appeared only when sperm were released from the lumen and diluted, the entire epididymis was fixed by immersion in 5% glutaraldehyde in PBS (Gibco, Eggerstein, Germany). The fixed tubule segments from 7 regions were coarsely minced, and the sperm were dispersed by mild sonication (0.5 sec at 50 W; Zinsser Analytic, Frankfurt, Germany). The form of sperm tails was examined in wet preparations or through preparation of smears and staining by the modified Papanicolaou method [15].

Effect of Ion Channel Blockers on Flagellar Angulation

Sperm from the distal caput and the mid cauda epididymidis were released into the basal medium, and each of the blockers (quinine, 1 mM; NPPB, 50 μM; $BaCl_2$, 5 mM; ouabain, 0.1 mM; glibenclamide, 100 or 250 μM) was added separately to the sperm suspension after dispersion, as described above. The sperm were incubated for 20 min, and an aliquot was analyzed for motility and flagellar angulation. To test whether the kinking of the flagellum was a result of cell swelling, Triton X-100 (0.1% v:v) was added to another aliquot to remove the restraint due to the cell membrane so the axoneme could unbend, and the form of the sperm tail was reexamined.

Measurement of the Osmolality of Luminal Epididymal Fluid

Osmolality was examined using a vapor pressure osmometer (Vapro, model 5520; Wescor, Schlag GmbH, Bergisch Gladbach, Germany), which can measure 2- to 10-μl samples between 100 and 3000 mmol/kg with an error of about ± 3 mmol/kg; measurements of dew-point depression are not affected by sample viscosity or the presence of suspended particulates [16]. The vas deferens was cannulated and perfused in retrograde with PBS. The undiluted viscous luminal contents exuded from a tubule cut in the proximal cauda epididymidis were drawn up a positive displacement pipette. Luminal contents (3 μl) were transferred onto a 2 × 1.5-mm strip of Kimwipe (Kimberly-Clark Corp., Irving, TX) tissue paper in the loading chamber of the vapor pressure, and readings were taken after a delay of 10 and 15 min (to ensure saturation of the chamber as reflected by a difference of < 2 mmol/kg between the two readings). Calibration of the osmometer was performed using standard osmolality solutions of 290, 400, and 1000 mmol/kg via similar procedures before and after measurement of the samples.

Statistics

Comparisons between sperm kinematic parameters of wild-type and knockout animals were made with Student's *t*-test. Statistics on percentages were obtained after arcsine transformation. Mean values and upper standard errors (mean + SEM) given in the paper were retransformed into percentages for presentation. Differences between wild-type and knockout animals, and between treatments, were tested for statistical significance ($P < 0.05$) using one-way ANOVA followed by the Student-Newman-Keuls test.

RESULTS

Epididymal Development of Sperm Motility

Only a few spermatozoa were recovered from the initial segment. These cells from mice of all genotypes were straight; they exhibited no regular flagellation, with about 10% showing barely discernible twitching of the tail. Motility potential developed along the length of the epididymis with the acquisition of flagellation and then forward progression. The only difference in the percentage motile sperm between the homozygous transgenic and the heterozygous/wild-type groups was found in the corpus region after 3-h incubation, but this slight deficiency in the sperm of the knockout mice was not persistent through maturation, since there was no difference in cauda sperm. Detailed analysis of kinematics revealed only slight decreases in ve-

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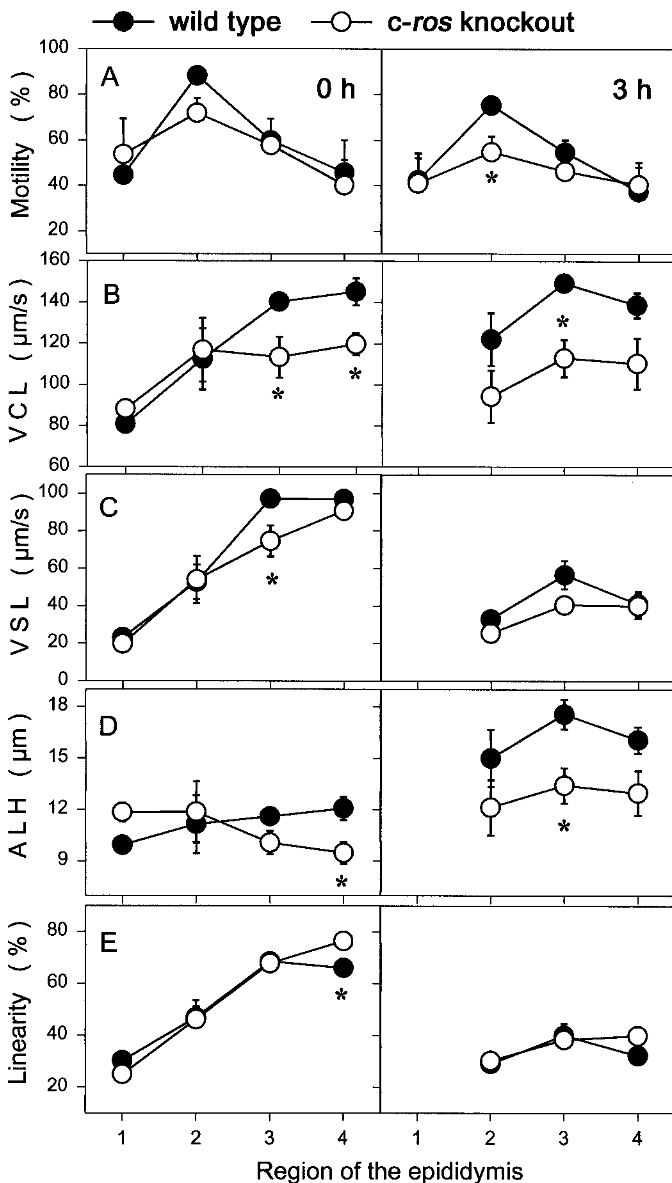

FIG. 1. Motility parameters of spermatozoa released from various regions of the epididymis: 1) distal caput, 2) proximal corpus, 3) proximal cauda, and 4) distal cauda from wild-type and homozygous c-*ros* knockout mice, before (left) and after (right) 3-h incubation in fully supplemented medium at 310 mmol/kg. **A**) Percentage motile cells, **B**) curvilinear velocity, **C**) straight-line velocity, **D**) amplitude of lateral head displacement, **E**) linearity (ratio of straight-line to curvilinear velocity × 100%). Values are mean ± SEM; n = 5; asterisks indicate a difference between the knockout and the corresponding value of the wild type.

locities of the mature spermatozoa, with concomitant increases in linearity and decreases in lateral head displacement (Fig. 1). Kinematic deficiencies of the same extents were shown in the knockouts when glycolysis, which is essential for fertilization processes, was made compulsory using medium containing only glucose as substrate (data not shown).

Flagellar Angulation in Maturing Sperm

In the heterozygous and wild-type mice, about 10% of the epididymal sperm displayed flagellar angulation when freshly released and dispersed in the basal medium (310 mmol/kg). These bent forms showed various extents of kinking of the tail at the location of the cytoplasmic droplet, up to the extreme of hairpin forms (Fig. 2). Upon incubation, there was a slight increase in bent forms for cauda sperm and a greater increase for corpus sperm, whereas the majority of caput sperm showed angulation. Hence in the normal epididymis, the phenomenon of flagellar angulation in immature sperm incubated in vitro subsided on maturation (Fig. 3). Angulation was manifested by both motile and immotile cells and was more commonly found in the immotile than in the motile sperm with the exception of caput sperm. For instance, after 3-h incubation, 20 + 5% (mean + SEM) of the total but only 5 + 1% of the motile cauda sperm, and 26 + 8% of the total but 15 + 13% of the motile corpus sperm, were in bent forms. Since interpretation of angulation in immotile sperm could be complicated if cell shape were to change on dying, only data on the motile population are presented unless stated otherwise.

In contrast to observations in the wild type, the large majority of cauda sperm from the knockout mice were bent upon release, although the percentage decreased to about 40% on incubation (Fig. 3). Fresh corpus and caput sperm also showed higher percentages of bent forms than the wild type but upon incubation exhibited the same extents of further increase as did controls.

Effects of Osmolality on Flagellar Angulation

Since flagellar angulation is a phenomenon of hypotonic swelling of spermatozoa, responses of mature sperm to changes in tonicity of the medium were compared between the knockout and the wild-type groups. As expected, 70% of the wild-type cauda sperm were in the bent form when released into the 230 mmol/kg medium. Flagellar angulation decreased drastically with increasing osmolalities to barely 2% at 430 mmol/kg (Fig. 4, bottom). On the other hand, there was no such large decrease over the same range of osmolalities in the knockout group (only from 86% to 56%, Fig. 4), with the majority retaining the bent form even at 510 mmol/kg (53 + 11%; mean + SEM, n = 3). The percentage motility remained unchanged for all osmolalities tested for both wild type and knockouts (Fig. 4, top).

Flagellar Angulation In Situ and Osmolality of Luminal Fluid

In the wild-type mice, about 80% of sperm in the initial segment possessed straight tails. The occurrence of kinked sperm slightly increased as they passed through the caput before these forms gradually decreased such that only about 10% retained their flagellar angulation in the distal epididymis (Fig. 5). In the knockout mice, sperm in the proximal epididymis showed the same percentages of straight tails; yet the initial trend of decrease was not reversed as in the wild type but continued up to the proximal corpus region, such that only 60% of mature sperm had straight tails. Among the bent sperm, one third to one half showed only a very slight kink at the site of the cytoplasmic droplet; the other flagella were bent into either a conspicuous angle or a hairpin bend. This in situ angulation in the mature sperm, compared to that exhibited in vitro upon release and dilution with the basal medium, occurred less frequently (40 + 9% vs. 55 + 5% bent, respectively) and to a lesser extent (9 + 3% vs. 25 + 6% in hairpin form).

Cauda fluid from the wild-type epididymides had an osmolality of 415 ± 12 mmol/kg (mean ± SEM, n = 11), whereas that from the c-*ros* knockout mice was 427 ± 27

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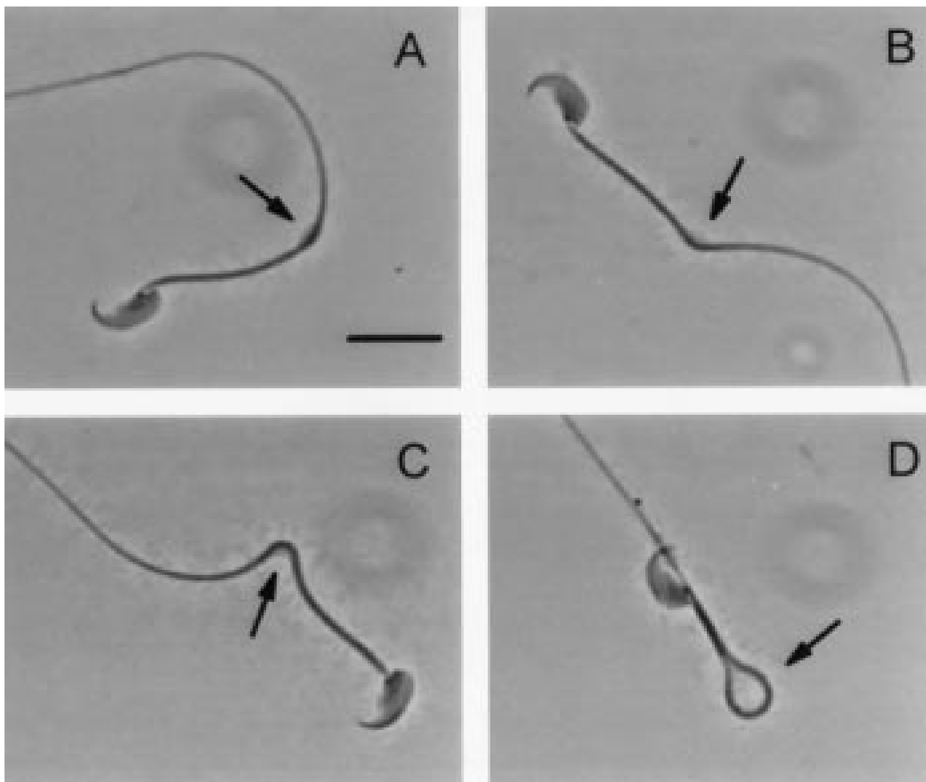

FIG. 2. Spermatozoa fixed within the cauda epididymidis of the c-*ros* knockout mice in situ before release, showing the various forms of flagellar angulation at the site of the cytoplasmic droplet (arrows): **A**) straight (no angulation), **B**) slight angulation, **C**) acute angulation, **D**) hairpin. Bar = 10 μm.

mmol/kg (n = 7), indicating no difference between the two genotypes.

Sperm Volume Regulation and Responses to Ion Channel Blockers

The concentrations of reagents used were those shown to have maximal effects in somatic cells; they did not affect the percentages of motile sperm. NPPB and $BaCl_2$ (Cl^-- and K^+-channel blockers, respectively) significantly induced flagellar angulation in the cauda sperm from the wild type, and quinine showed a stronger effect, with only 38% of motile sperm maintaining straight tails compared to 91% in the control basal medium (Fig. 6A). In all these cases the majority of kinked flagella were hairpin forms. In the knockout group, 40% of motile sperm were straight and 60% were bent, as shown above, but none of the blockers caused any further angulation (Fig. 6B). This lack of response of the cauda sperm in the knockout male was similar to that shown by the immature caput sperm of the wild type (Fig. 7A). Caput sperm from the c-*ros* knockout also did

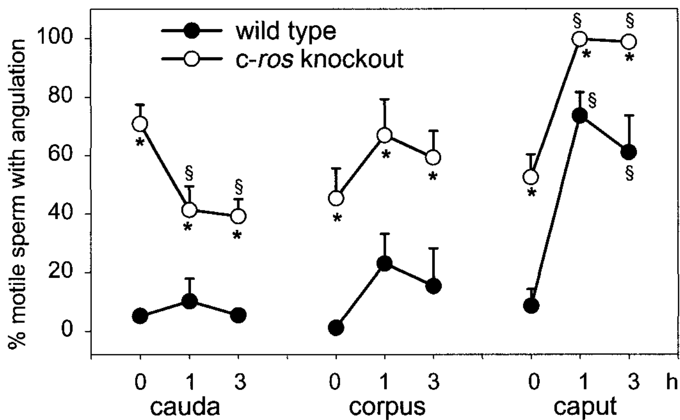

FIG. 3. Percentages of motile spermatozoa showing flagellar angulation (as in Fig. 2, B–D) at 0-, 1-, and 3-h incubation after release from the cauda, corpus, and caput epididymidis of wild-type (n = 5) and c-*ros* knockout mice (n = 7). * Indicates a difference between the knockout and the corresponding value of the wild type; § indicates a difference from 0 h at the same osmolality.

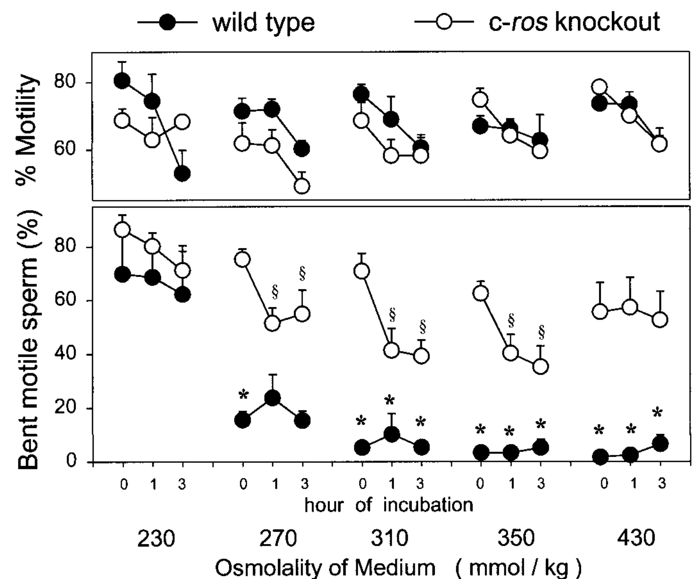

FIG. 4. Spermatozoa from the cauda epididymidis of wild-type and c-*ros* knockout mice at 0, 1, and 3 h of incubation in media of increasing osmolality, showing similar % motility (top) but different extents of flagellar angulation (as in Fig. 2, B–D) in the motile sperm (bottom). Values are mean + SEM; n = 5; * indicates a difference between the knockout and the corresponding value of the wild type; § indicates differences from 0 h at the same osmolality.

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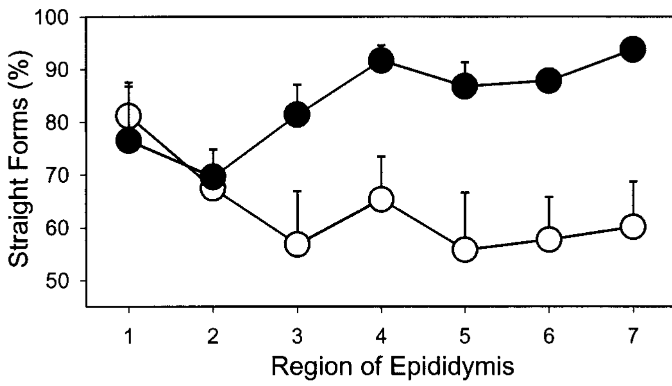

FIG. 5. Percentages of flagella of spermatozoa in straight forms, which were fixed within various regions of the epididymis of the wild-type (solid symbols) and c-*ros* knockout mice (open symbols); n = 7–9.

not respond to NPPB or $BaCl_2$, and when treated with quinine they showed a reverse effect with a decrease of hairpin tails resulting in an increase in straight motile sperm (Fig. 7B).

Evidence for Cell Swelling as a Cause of Angulation

To verify that flagellar angulation was a manifestation of cell swelling, percentages of all sperm in straight or kinked forms were compared in the absence and presence of Triton X-100 used to perforate the cell membrane. Flagellar angulation in the cauda sperm of both the wild type (induced by quinine) and knockout (in basal medium) largely disappeared in Triton X-100 (from 62 + 6% and 60 + 8% to 11 + 2% and 20 + 5%, respectively). The hairpins in the knockout caput sperm opened up (51 + 7% vs. 4 + 1% hairpin in the absence and presence of Triton, respectively) into the angled form but did not straighten further, resulting in only 50% with straight tails when demembranated compared to 6% while intact. Quinine-treated caput sperm from both wild type and knockouts straightened out in Triton X-100 to 85 + 5% and 82 + 3%, respectively.

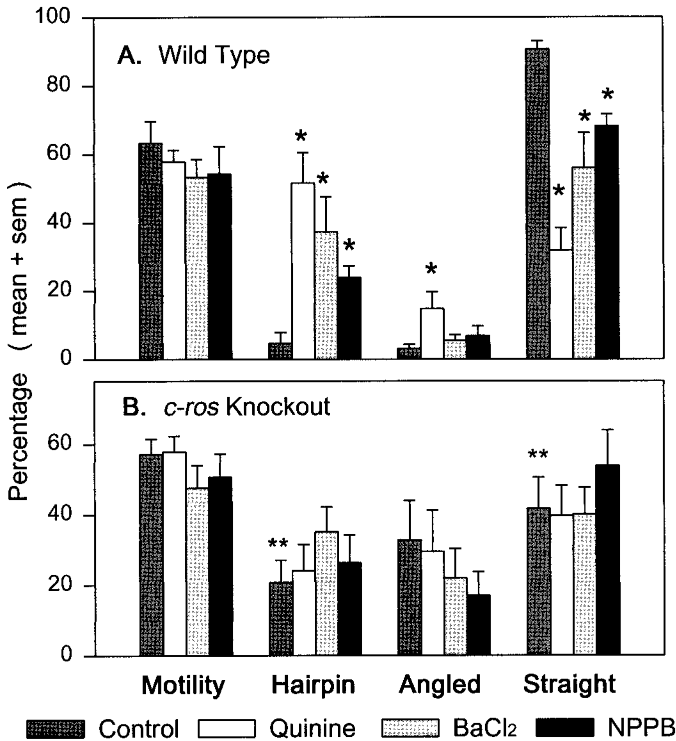

FIG. 6. Motility (% motile cells) and the percentages of flagella of motile spermatozoa in hairpin, angled, or straight (with no angulation) forms of mature sperm from the cauda epididymidis of **A**) wild-type (n = 5) and **B**) knockout mice (n = 7), in basal medium (310 mmol/kg) in the absence (control) or presence of ion channel blockers (1 mM quinine, 5 mM $BaCl_2$, or 50 μM NPPB). * Indicates differences within the same genotype from the control medium; ** indicates differences between the wild-type and knockout group in the same medium.

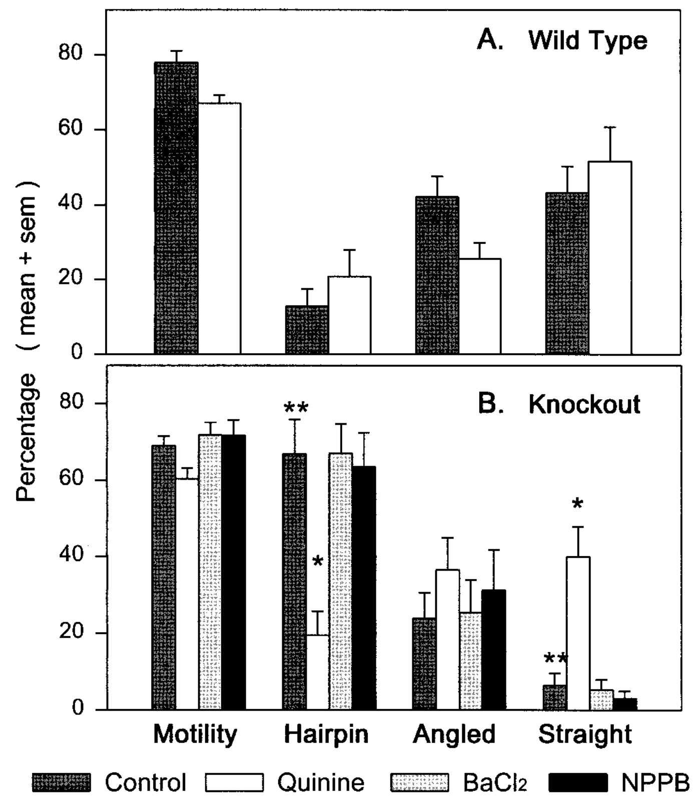

FIG. 7. Motility (% motile cells) and the percentages of flagella of motile spermatozoa in hairpin, angled, or straight (no angulation) forms of immature sperm from the caput epididymidis of **A**) wild-type (n = 5) and **B**) knockout mice (n = 7) in basal medium (310 mmol/kg) in the absence (control) or presence of ion channel blockers (1 mM quinine, 5 mM $BaCl_2$, or 50 μM NPPB). * Indicates differences within the same genotype from the control medium; ** indicates differences between the wild-type and knockout group in the same medium.

Further Characterization of RVD in Wild-Type Mature Sperm

Ouabain did not alter the motility or the form of the motile cauda sperm (90 + 3% straight forms vs. 92 + 1% in controls; mean + SEM, n = 6); neither did glibenclamide at 100 μM (Fig. 8) or at a higher concentration at which motility was suppressed (250 μM; data not shown).

When Ca^{2+} was removed from the medium, beat amplitude decreased markedly (not quantified), although the percentage of motile sperm remained unchanged. Flagellar angulation gradually increased with incubation time, such that by 2 h only 3% of the feebly flagellating motile sperm still retained a straight form (Fig. 8). Whereas the increased angulation was first detected largely in angled forms at 20 min, 85% and 92% of kinked flagella were in hairpin forms by 1 and 2 h of incubation, respectively. In Ca^{2+}-free medium, the effect of quinine was reduced (Fig. 8, histograms)

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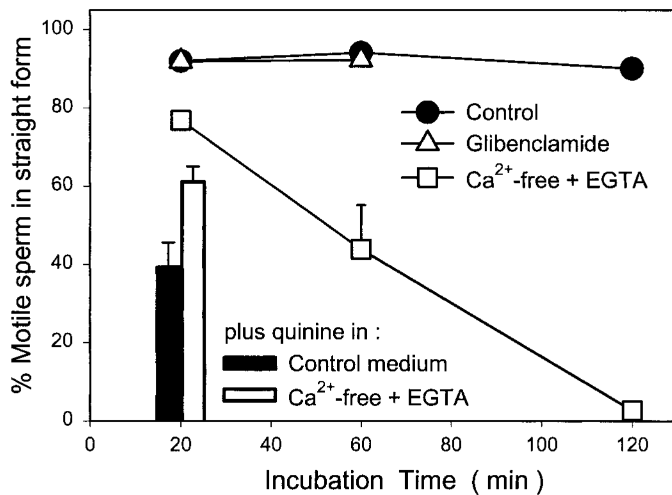

FIG. 8. Effects of adding 0.1 mM glibenclamide to, or removing Ca^{2+} from, the basal medium (control) over 2 h of incubation on the flagellar form of wild-type motile cauda spermatozoa (shown by the line plots); and effects of 1 mM quinine in the presence (control) and absence of Ca^{2+} (given by the histograms). Values are mean + SEM of 5 animals.

such that the decrease in straight forms was only 16 percentage points compared with 53 in the control medium. Significantly fewer hairpin forms were observed when quinine treatment was accompanied by the absence of Ca^{2+} (6 + 2% vs. 20 + 6% in the presence of Ca^{2+}).

DISCUSSION

*Major Defect of the Infertile Sperm of c-*ros *Knockout Mice*

One possible explanation for the absence or reduced number of sperm in the oviduct of wild-type females after mating with the infertile homozygous c-*ros* knockout mice is a reduction in sperm motility that compromises passage in the female tract. However, the present findings of only a 20% decrease in velocities and lateral head displacement, and no decrease in percentage motility of mature sperm, are unlikely to account for the complete infertility. The slight kinematic differences were probably a consequence of the kinked sperm tails, which could compromise the efficiency of propulsion. It is interesting to note the implication that the initial segment, which is not developed in the knockout mice, was not essential for the development of flagellating ability of immature sperm. The nearly normal velocities and forward progression of the more mature sperm are consistent with the finding that these kinematic changes occur mostly downstream in the corpus epididymidis [14], which appeared normal in the knockouts.

In contrast to the slight abnormality in motility, a marked defect of the motile mature (cauda) sperm from the homozygous transgenic mice was revealed as flagellar angulation. Although sperm are transported passively as far as the uterotubal junction, there is no en masse passage through the junction into the oviduct. It has been observed in rats [17] that motile sperm pass through individually at intervals of a few minutes, implying that the junction is a selection barrier. The kinked sperm would have difficulty passing through the uterotubal junction or the cumulus mass to reach the oocytes. Spermatozoa are exempted from these barriers in the conventional in vitro fertilization procedure, which revealed no difference in the fertilization rate between the wild-type and the knockout sperm [4].

The observed tail angulation was an indication of cell swelling and not an intrinsic axomenal abnormality, since it was largely reduced by demembranation using detergent and since wild-type sperm showed the same phenomenon to an increasing extent when exposed to media of decreasing tonicity. Osmolarity of epididymal luminal fluids from laboratory animals ranges from 330 to 420 mmol/kg and is highest in the corpus [13]. The present study documents for the first time that the osmolality of the cauda epididymal fluid is 415 mmol/kg in the wild-type mouse, and the same as in the knockouts. This means that the dilution of epididymal sperm upon release with the commonly used experimental media (290–310 mmol/kg) presents a similar hypotonic stress to sperm from both the wild type and knockouts. That flagellar angulation was not seen in the majority of mature sperm from the wild-type mice suggests that there is normally a rapid occurrence of volume regulation.

The present observations of kinking of the mutant sperm upon dilution might reflect an in vitro response as a consequence of prior exposure to some abnormal luminal components not experienced by normal fertile sperm. Indeed, abnormal flagellar angulation was already detectable in situ. In the wild-type epididymis, caput sperm fixed in situ showed the highest level of kinking, which was no longer observed in sperm fixed in the distal half of the epididymis—indicating a development of volume regulation during normal epididymal maturation. In the homozygous knockout mice, slight kinking attained in the caput was further increased and maintained in the distal epididymis, suggestive of a lack of such volume regulation. Furthermore, upon release and dilution of the cauda sperm, the defect was immediately exaggerated by rapid swelling. Increasing the osmolality of the medium only slightly relieved the angulation, which persisted up to 510 mmol/kg. In comparison, wild-type sperm in hypotonic media showed swelling-induced kinking, as expected, which was drastically decreased to low levels at 310 mmol/kg and to a few percent at hyperosmolarity. This confirms that the defect in the knockouts is in sperm volume regulation rather than the osmolality of epididymal luminal fluid.

Sperm Volume Regulation in Wild-Type Mice

In wild-type epididymal sperm, the manifestation of flagellar angulation upon in vitro incubation, to decreasing extents with increasing maturity (caput > corpus > cauda), also indicates a normal maturational progress in the development of the volume regulation mechanisms.

Different pathways used by different cell types for RVD include K/Cl cotransport (best studied in erythrocytes and regulated by tyrosine kinase and phosphatase [18, 19]), Ca-sensitive K^+ channels [20], separate Cl and K channels [18, 21], voltage-gated Cl channels, and volume-sensitive organic osmolyte anion channels [22, 23]. The latter permit the efflux of amino acids, myoinositol, betaine, and glycerophosphocholine in renal epithelial cells, which are sensitive to anion blockers [24]. Interestingly, these substances or their derivatives are secreted by the epididymis in high amounts [13, 25]. The mechanism for the regulation of cell volume by sperm is virtually unknown. Early work on bovine ejaculated sperm demonstrated that extracellular Ca^{2+} is required for the stability of sperm volume as well as motility [26, 27]. The control of ejaculated bovine sperm volume involves quinine-sensitive K channels [28] and is modulated by oxidative metabolism and the Na/K pump [27]. Water permeability in ram sperm decreases upon in-

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hibition of glucose transport, but the water channel aquaporin CHIP28 cannot be found on the sperm surface [29]. Both cation and anion channels with various putative roles, such as chemotaxis, acrosomal exocytosis, and flagellar motion, have been reported in spermatogenic or sperm cells [30–32]. However, it is not known whether any of these are involved in RVD, except for a Cl channel (pI_{Cln}) [33] suggested to regulate cell volume-induced Cl^- efflux [34]. It is currently debated, however, whether this is a swelling-sensitive chloride channel or a channel regulator or a cation channel [35–37]. Although the protein has been immunocytochemically localized in abundance in the rat testis, epididymal epithelium, and sperm [38], there is as yet no direct evidence that this protein regulates cell volume of sperm.

In the present study, the ion channels involved in normal sperm RVD were probed by means of various commonly used blockers on sperm from wild-type animals. RVD was found to be inoperative in immature sperm, since there was no response to any of the blockers tested. In wild-type mature sperm, RVD was rapidly inhibited by quinine, NPPB, and $BaCl_2$. Quinine is well known as an inhibitor of many types of K channels involved in RVD [39, 40] and volume-sensitive anion channels [23, 25]. NPPB has been used as a blocker of the swelling-activated Cl channel, mostly showing maximal inhibition at 0.1 mM [22, 23]. It also blocks anion channels for organic osmolytes, such as the swelling-induced efflux of taurine, inositol, and gluconate [41, 42]. Ba^{2+} is a specific K-channel blocker frequently used to study K^+ currents involved in RVD [40, 43]. The inhibition of sperm RVD to the same extent by NPPB and $BaCl_2$, and to a greater extent by quinine, suggests that for spermatozoa, the mechanisms involve a K channel and a Cl or an organic anion channel. Further characterization showed no effect on RVD by the Na-channel blocker ouabain, or by glibenclamide up to 0.25 mM. The latter is a sulfonylurea effective in blocking the ATP-sensitive inwardly rectifying K channels, the Na-activated voltage-dependent K channels [40], and a Ca-independent CFTR-like Cl channel [44]. The present findings suggest no involvement of these channels in sperm RVD.

The involvement of Ca^{2+} in RVD is controversial and may vary for various channels and cell types. Intracellular Ca^{2+} release augmented by influx of extracellular Ca^{2+} has been implicated in some epithelial cells (e.g., [20, 45, 46]). On the other hand, it has been demonstrated that extracellular or intracellular Ca^{2+} is not involved in the activation of certain types of volume-sensitive Cl channels in submandibular gland cells [44]. RVD involving organic osmolyte efflux or K^+ efflux in certain cell types is also Ca-independent [47, 48]. In leukocytes, prolonged depletion of extracellular Ca^{2+} leads to inhibition of RVD [49], but it is not clear whether this is secondary to the resultant decreased energy metabolism, which can cause gradual swelling of bull sperm [27]. Inhibition of volume-sensitive organic anion channels by mitochondrial inhibitors has been demonstrated [42]. The slow effect of Ca^{2+} depletion in inducing flagellar angulation in the present study, accompanied by a reduction in the vigor of flagellation, may reflect such an indirect mechanism.

*Defect of RVD Mechanism in Spermatozoa from c-*ros *Knockout Mice as the Probable Cause of Infertility*

The failure of sperm from the knockout mouse to respond to quinine, NPPB, and $BaCl_2$ with further angulation suggests that the relevant ion channels were not functioning normally. Since the present findings showed the acquisition of the ability for RVD, and a development on maturation in the wild-type epididymis of the responsiveness to the relevant ion channel blockers by the immature sperm, it can be hypothesized that some epididymal factors normally present in the lumen may interact with maturing spermatozoa in the development of their RVD mechanisms. Flagellar angulation should greatly compromise the hydrodynamics of motile sperm in their migration through the restrictive uterotubular junction and could explain the absence of sperm from the oviduct in 40% of the wild-type females, and the reduced numbers in the others, after mating with the homozygous c-*ros* knockout males [4]. Penetration of the intact cumulus to approach the zona pellucida of the oocyte with the sperm head at the appropriate angle for binding would also be difficult. Therefore a defect in RVD is a plausible cause of the in vivo infertility, although other contributing factors cannot be ruled out. Since the knockout mice lack development of the initial segment, which is very active in resorption and secretion, including that of epididymal-specific proteins [50], the infertile sperm would probably have multiple defects. This study suggests that much effort is needed to define the epididymal factors leading to natural infertility, as well as the nature of the development of ion channels in regulating sperm cell volume, which seem to be important for normal sperm function in the female tract.

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REFERENCES

1. Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction, 2nd ed. New York: Raven Press; 1994: 189–317.
2. Orgebin-Crist M-C. The epididymis across 24 centuries. J Reprod Fertil Suppl 1998; 53:285–292.
3. Cooper TG. Epididymis. In: Neill JD, Knobil E (eds.), Encyclopedia of Reproduction, vol 2. New York: Academic Press; 1998: 1–17.
4. Sonnenberg-Riethmacher E, Walter B, Riethmacher D, Gödecke S, Birchmeier C. The c-*ros* tyrosine kinase receptor controls regionalization and differentiation of epithelial cells in the epididymis. Gene Dev 1996; 10:1184–1193.
5. Yeung CH, Sonnenberg-Riethmacher E, Cooper TG. Receptor tyrosine kinase c-*ros* knockout mice as a model for the study of epididymal regulation of sperm function. J Reprod Fertil Suppl 1998; 53: 137–147.
6. Birchmeier C, Birnbaum D, Waitches G, Fasano O, Wigler M. Characterization of an activated human *ros* gene. Mol Cell Biol 1986; 6: 3109–3116.
7. Sonnenberg E, Gödecke A, Walter B, Bladt F, Birchmeier C. Transient and locally restricted expression of the *ros*1 protooncogene during mouse development. EMBO J 1991; 10:3693–3702.
8. Tessarollo L, Nagarajan L, Parada LF. c-*ros*: the vertebrate homolog of the sevenless tyrosine kinase receptor is tightly regulated during organogenesis in mouse embryonic development. Development 1992; 115:11–20.
9. Drevius LO, Eriksson H. Osmotic swelling of mammalian spermatozoa. Exp Cell Res 1966; 42:136–156.
10. Drevius L. Osmotic behavior, membrane permeability and mobility mechanisms in bull spermatozoa. Acta Univ Ups Abstr Upps Diss Sci 1972; 195:1–20.
11. Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJD. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil 1984; 70:219–228.
12. Cooper TG. The epididymis as a site of contraceptive attack. In:

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Nieschlag E, Habenicht U-F (eds.), Spermatogenesis, Fertilization, Contraception. Molecular, Cellular and Endocrine Events in Male Reproduction. Berlin: Springer Verlag; 1992: 419–460.
13. Cooper TG. The Epididymis, Sperm Maturation and Fertilization. Berlin: Springer-Verlag; 1986:117–230.
14. Soler C, Yeung CH, Cooper TG. Development of sperm motility patterns in the murine epididymis. Int J Androl 1994; 17:271–278.
15. WHO (World Health Organisation). Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction, 3rd edition. Cambridge: Cambridge University Press; 1992.
16. Sweeney TE, Beuchat CA. Limitation of methods of osmometry: measuring the osmolality of biological fluids. Am J Physiol 1993; 264: R469-R480.
17. Gaddum-Rosse P. Some observations on sperm transport through the uterotubal junction of the rat. Am J Anat 1981; 160:333–341.
18. Sarkadi B, Parker JC. Activation of ion transport pathways by changes in cell volume (review). Biochim Boiphys Acta 1991; 1071:407–427.
19. Cossins AR, Gibson JS. Volume-sensitive transport systems and volume homeostasis in vertebrate red blood cells. J Exp Biol 1997; 200: 343–352.
20. McCarty NA, O'Neil RG. Calcium signaling in cell volume regulation. Physiol Rev 1992:1037–1061.
21. Macleod RJ, Hamilton JR. Separate K^+ and Cl^- transport pathways are activated for regulatory volume decrease in jejunal cells. Am J Physiol 1991; 260:G405–415.
22. Strange K, Emma F, Jackson PS. Cellular and molecular physiology of volume-sensitive anion channels. Am J Physiol 1996; 270:C711-C730.
23. Okada Y. Volume expansion-sensing outward-rectifier Cl-channel: fresh start to the molecular identity and volume sensor. Am J Physiol Cell Physiol 1997; 42:C755-C789.
24. Strange K, Jackson PS. Swelling-activated organic osmolyte efflux: a new role for anion channels. Kidney Int 1995; 48:994–1003.
25. Hinton BT, Palladino MA. Epididymal epithelium: its contribution to the formation of a luminal fluid microenvironment. Microsc Res Technique 1995; 30:67–81.
26. Bredderman PJ, Foote RH. Alteration of cell volume in bull spermatozoa by factors known to affect active cation transport. Exp Cell Res 1971; 66:190–196.
27. Bredderman PJ, Foote RH. Factors stabilising bull sperm cell volume and prolonging motility at high dilution. Exp Cell Res 1971; 66:458–464.
28. Kulkarni SB, Sauna ZE, Somlata V, Sitaramam V. Volume regulation of spermatozoa by quinine-sensitive channels. Mol Reprod Dev 1997; 46:535–550.
29. Curry MR, Millar JD, Watson PE. The presence of water channel proteins in ram and human sperm membranes. J Reprod Fertil 1995; 104:297–303.
30. Schreiber M, Wei AG, Yuan A, Gaut J, Saito M, Salkoff L. Slo3, a novel pH-sensitive K+ channel from mammalian spermatocytes. J Biol Chem 1998; 273:3509–3516.
31. Shi YL, Ma XH. Ion-channels reconstituted into lipid bilayer from human sperm plasma membrane. Mol Reprod Dev 1998; 50:354–360.
32. Wiesner B, Weiner J, Middendorff R, Hagen V, Kaupp B, Weyand I. Cyclic nucleotide-gated channels on the flagellum control Ca^{2+} entry into sperm. J Cell Biol 1998; 142:473–484.
33. Paulmichl M, Li Y, Wickman K, Ackerman M, Peralta E, Clapham D. New mammalian chloride channel identified by expression cloning. Nature 1992; 356:238–241.
34. Gschwentner M, Nagl UO, Wöll E, Schmarda A, Ritter M, Paulmichl M. Antisense oligonucleotides suppress cell-volume induced activation of chloride channels. Pfluegers Arch Eur J Physiol 1995; 430: 464–470.
35. Clapham DE. The list of potential volume-sensitive chloride currents continues to swell (and shrink). J Gen Physiol 1998; 111:623–624.
36. Strange K. Identity of the outwardly rectifying, swelling-activated anion channel: time to reevaluate pICln. J Gen Physiol 1998; 111:617–622.
37. Li CH, Breton S, Morrison R, Cannon CL, Emma F, SanchezOlea R, Bear C, Strange K. Recombinant pI(Cln) forms highly cation-selective channels when reconstituted into artificial and biological membranes. J Gen Physiol 1998; 112:727–736.
38. Nagl UO, Fürst J, Laich A, Dobson A, Pinggera GM, Gschwentner M, Schneider R, Paulmichl M. Immunocytochemical localization of ICln, a protein paramount for cell volume-regulated chloride transport. J Gen Physiol 1996; 108:27a.
39. Kuriyama H, Kitamura K, Nabata H. Pharmacological and physiological significance of ion channels and factors that modulate them in vascular tissues. Pharmacol Rev 1995; 47:387–573.
40. Quayle JM, Nelson MT, Standen NB. ATP-sensitive and inwardly rectifying potassium channels in smooth muscle. Physiol Rev 1997; 77:1165–1232.
41. Jackson PS, Strange K. Volume-sensitive anion channels mediate swelling-activated inositol and taurine efflux. Am J Physiol 1993; 265: C1203-C1209.
42. Patel AJ, Lauritzen I, Lazdunski M, Honore E. Disruption of mitochondrial respiration inhibits volume-regulated anion channels and provokes neuronal cell swelling. J Neurosci 1998; 18:3117–3123.
43. Miller C. 1990: Annus mirabilis of potassium channels. Science 1991; 252:1092–1096.
44. Zeng W, Lee MG, Muallem S. Membrane specific regulation of Cl^- channels by purinergic receptors in rat submandibular gland acinar and duct cells. J Biol Chem 1997; 272:32956–32965.
45. Woll E, Ritter M, Haller T, Volkl H, Lang F. Calcium entry stimulated by swelling of Madin-Darby canine kidney cells. Nephron 1996; 74: 150–157.
46. Wu X, Yang H, Iserovich P, Fischbarg J, Reinach PS. Regulatory volume decrease by SV40-transformed rabbit corneal epithelial cells requires ryanodine-sensitive Ca^{2+}-induced Ca^{2+} release. J Membr Biol 1997; 158:127–136.
47. Jørgensen NK, Lambert IH, Hoffmann EK. Role of LTD4 in the regulatory volume decrease response in Ehrlich ascites tumor cells. J Membr Biol 1996; 151:159–173.
48. Moran J, Morales-Mulia S, Hernandez-Cruz A, Pasantes-Morales H. Regulatory volume decrease and associated osmolyte fluxes in cerebellar granule neurones are calcium independent. J Neurosci Res 1997; 47:144–154.
49. Grinstein S, Foskett JK. Ionic mechanisms of cell volume regulation in leukocytes. Annu Rev Physiol 1990; 52:399–414.
50. Dufaure JP. Androgen regulation of gene expression in epididymis. In: Waites GMH, Frick J, Baker GWH (eds.), Current Advances in Andrology. Florence: Monduzzi Editore; 1997: 245–251.

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