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Localization and age-dependent expression of the inward rectifier K^+ channel subunit Kir 5.1 in a mammalian reproductive system

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Abstract Kir 5.1 is a member of the inward rectifier potassium channel superfamily which does not form functional channels when expressed by itself in *Xenopus laevis* oocytes. rt-PCR reveals high levels of Kir 5.1 mRNA expression in testis but the function of this channel remains unknown. To determine the cell-specific expression of this channel in the testis we raised a polyclonal antibody against an external epitope of Kir 5.1 and tested its specificity in *Xenopus* oocytes expressing several cloned Kir subunits. Strong immunoreactivity for Kir 5.1 was found in seminiferous tubules of rat testis and, particularly, in spermatogonia, primary and secondary spermatocytes, spermatids and in the head and body of spermatozoa. The intensity of Kir 5.1 immunofluorescence, quantified using laser scanning microscopy, increased with age at every stage in the development of sperm from spermatogonia and reached a peak in 60-day-old rats. In contrast, the immunofluorescence decreased in 90-day-old animals and was detected mostly in spermatozoa. The results demonstrate that Kir 5.1 expression in the testis is localised to cells involved in spermatogenesis, showing a temporal pattern of expression during sexual maturity.

Key words: Potassium channel; Spermatozoon; Immunohistochemistry; Immunofluorescence; Testis; Confocal microscopy

1. Introduction

Virtually every cell type expresses inwardly rectifying potassium channels (Kir) which maintain the resting membrane potential of the cell near the K^+ reversal potential (E_K), regulate their excitability and K^+ homoeostasis [1–8]. The degree of inward rectification varies amongst the many cloned members of this potassium channel superfamily [9–14]. Generally, the inward flow of potassium ions is greater at potentials more negative than the E_K, but at more positive potentials the outward flow is inhibited and the membrane potential is free to change [15]. The rectifying nature of the conductance is due to a voltage-dependent block of the intracellular mouth of the pore by cytoplasmic polyamines and Mg^{2+} ions [16–21]. Like voltage-gated potassium channels Kir channels are tetramers which may be comprised of homomeric as well as heteromeric subunits [22–26]. Kir 5.1 appears to require the coassembly of additional inward rectifier potassium channel subunits to produce functional channels in *Xenopus* oocytes. To date, Kir 4.1 is the only cloned subunit which has been shown to co-assemble specifically with Kir 5.1 in a heterologous expression system [27]. Interestingly, the heteromeric channels formed display macroscopic current kinetics and single channel properties dependent upon the relative positions occupied by the two types of subunits [27]. Kir 5.1 mRNA distribution, analysed by rt-PCR, indicates that this subunit is expressed in the rat testis [13], but the cellular localisation of Kir 5.1 protein remains unknown.

Here we show that Kir 5.1 subunits are expressed in a markedly age-dependent fashion in all proliferating and differentiating cells of rat testis. Spermatogenesis represents a major reproductive function tightly regulated by several hormonal and cellular events. Thus, the identification and functional characterisation of proteins implicated in sperm maturation, activation and sperm-egg interaction are of fundamental importance. Several ion channels such as voltage-dependent calcium channels [28–31], cyclic nucleotide-gated channels [32] and pH-sensitive potassium channels [33] have been cloned and described in testis where they may have an important role in spermatogenesis and reproduction.

2. Materials and methods

2.1. Molecular biology

The subunits were concatenated as dimers and the in vitro mRNAs were generated and microinjected in *Xenopus* oocytes as described [27].

2.2. Immunohistochemistry

A portion of the extracellular loop linking the first transmembrane domain to the pore region of Kir 5.1 subunit was chosen as a immunogen peptide (see boxed sequence shown in Fig. 1) and synthesised in vitro (kindly provided by Dr M. Salmona). The peptide was adsorbed to a poly-L-lysine network in order to increase its immunogenicity before subcutaneous injection in rabbits. The polyclonal antiserum was purified by ion exchange chromatography through DEAE-Sephacell (Pharmacia). The IgG fraction thus obtained was affinity-purified using the antigenic peptide coupled to CNBr-activated Sepharose 4B (Fluka Chemie AG, Buchs, Switzerland). The final concentration of the antibody was obtained by determining the absorbance of the eluate at 280 nm.

The presence and specificity of the anti-Kir 5.1 subunit IgGs have been tested by means of dot-blot analysis [34]. Briefly, the rabbit antiserum, obtained 15–20 days after the second immunisation, specifically reacted with immunogen peptide in a concentration-dependent fashion. In contrast, no reactivity of the rabbit antiserum obtained before immunisation was observed. After an incubation step with a blocking solution (Tris-saline buffer containing 3% bovine serum al-

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                    TM1                    L                    P-region
Kir 1.1 WFLFGLLWYVVAYVHKDLPEFY--PPDNRTP--------CVENINGMTSAFLFSLETQVTIGYG
Kir 2.2 WLLFGIIFWVIAVAHGDLEPA---EGRGRTP--------CVLQVHGFMAAFLFSIETQTTIGYG
Kir 3.1 WLFMASMWWVIAYTRGDLNKA---HVGNYTP--------CVANVYNFPSAFLFFIETEATIGYG
Kir 3.4 WLFFGFIWWLIAYVRGDLDHV---GDQEWIP--------CVENLSGFVSAFLFSIETETTIGYG
Kir 4.1 WFLFGVVWYLVAVAHGDLLELG--PPANHTP--------CVVQVHTLTGAFLFSLESQTTIGYG
Kir 5.1 WLIFGSIFWLIALHHGDLLSD---PD--ITP--------CVDNVHSFTAAFLFSLETQTTIGYG
Kir 6.1 WLLFAIMWWLVAFAHGDIYAY---MEKGITEKSGLESAVCVTNVRSFTSAFLFSIEVQVTIGFG
Kir 7.1 WLVFAVLWYVLAEMNGDLELDHDAPPENHTI--------CVKYITSFTAAFSFSLETQLTIGYG
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Fig. 1. Deduced amino acid sequence alignment of the indicated Kir channels. The boxed region shows the amino acid sequence chosen to synthesise the immunogenic peptide. The proposed transmembrane domain (TM1), extracellular loop (L) and the pore region (P-region) are overlined; dashes represent gaps introduced to optimise the alignment.

bumin (BSA), pH 7.4), different concentrations of the immunogen peptide (10, 5, 1, 0.5 μg/ml) and BSA (5, 2.5, 1 μg/ml) were incubated with three different dilutions of the rabbit serum (1/100, 1/200, 1/500 in Tris-saline buffer) and with the rabbit serum before immunisation (1/100 in Tris-saline buffer) as a negative control. The sheet of nitrocellulose was then incubated with a secondary goat anti-rabbit IgG peroxidase-conjugated (1/500 in Tris-saline buffer) and finally, with the reaction buffer (50% chloronaphthol in 20 ml cold methanol with 100 ml Tris-saline buffer). The development of the colorimetric reaction was blocked by several washes in Tris-saline buffer. After each incubation step, the sheet of nitrocellulose was washed three times with Tris-saline buffer containing 0.025% Tween 20. Tris-HCl, BSA, Tween 20, chloronaphthol, were purchased by Sigma Chemical Co. (St. Louis, MO, USA). Nitrocellulose transfer membrane was purchased by Schleicher and Schuell Inc. (Keene, NH, USA). Goat anti-rabbit IgG peroxidase-conjugated antibody was purchased from Calbiochem Co. (La Jolla, CA, USA). Rat tissues and *Xenopus* oocytes were sectioned and immunostained using the avidin-biotin complex (ABC method) as previously described [35]. Briefly, male Sprague-Dawley rats were deeply anaesthetised with Nembutal (40 mg/kg i.p.); tissues were washed and fixed by intracardiac perfusion with sodium phosphate buffer (PBS 0.1 M, pH 7.4) and with 10% buffered formalin, respectively. Testes from rat of different ages were dissected, post-fixed in 10% formalin for 24 h at room temperature, dehydrated and paraffin-embedded. *Xenopus* oocyte dissection and injections were performed as described [36]. Tissue blocks were cut with a microtome (Ernst Leitz GmbH, Austria) and 5 μm sections were pre-treated with 0.3% H_2O_2 in ethanol at 4°C for 15 min to inhibit endogenous peroxidase activity and washed with PBS (0.1 M, pH 7.4) containing 0.05% Tween 20. Sections were incubated with 50% normal goat serum (NGS) for 10 min at 37°C, to reduce non-specific immunostaining, then with anti-Kir 5.1 polyclonal antibody (0.15 μg/ml) in 1% NGS at 4°C overnight. Detection of anti-Kir 5.1 antibody was accomplished using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Sections were incubated with an anti-rabbit IgG biotin-conjugated (Calbiochem) (1:100) in 50% NGS for 30 min at room temperature and finally incubated with the ABC reagent for 30 min at room temperature. After each incubation step, sections were carefully washed with PBS (0.1 M, pH 7.4). The localisation of anti-Kir 5.1 was visualised using 3,5′-diaminobenzidine (DAB) (0.04% in 0.1 M PBS) containing (0.33‰) H_2O_2, and tissues were counterstained with methylene blue. Negative control sections were processed in the same way using the anti-Kir 5.1 antibody pre-adsorbed with an excess of the antigenic peptide or 1% NGS, instead of the anti-Kir 5.1 antibody. The specificity of the anti-Kir 5.1 antibody was tested on sections of formalin-fixed, paraffin-embedded *Xenopus* oocytes injected with mRNAs encoding: Kir 5.1, Kir 4.1, Kir 3.2 and dimeric Kir 3.1-3.4, Kir 4.1-5.1 constructs. Uninjected *Xenopus* oocytes were used as negative controls. Photomicrographs were taken using a Zeiss Axiophot microscope (Carl Zeiss Inc., Oberkochen, Germany).

2.3. Immunofluorescence

Tissue sections were obtained as described above. For immunofluorescence analysis, samples were first incubated with NH_4Cl (50 mM in 0.1 M PBS) for 15 min at room temperature to reduce autofluorescence of tissues, then with a blocking solution containing 0.5% BSA, 0.05% saponin in 0.1 M PBS for 30 min at room temperature. Tissues were incubated with anti-Kir 5.1 (0.15 μg/ml) in blocking solution, for 1 h at room temperature, carefully washed with 0.1 M PBS and incubated with Cy3-labelled anti-rabbit IgG (Sigma) (1:400) in blocking solution for 1 h at room temperature in darkness.

2.4. Laser scanning confocal microscopy (LSCM)

After immunostaining, sections were mounted in Mowiol 4-88 (Calbiochem, La Jolla, CA, USA), and imaged using an INSIGHT PLUS laser scanning confocal microscope system (Meridian, Oketos, MI, USA) equipped with an Olympus IMT-2 inverted microscope. Five groups of optical *Z*-section serial slices from each experiment were taken with 0.3 μm *Z*-steps from the top to the bottom of the specimen. Fluorescent images were recorded using a Dage CCD camera, and stored directly on computer using a frame grabber and INSIGHT-IQ software. The integrated fluorescence of each image in the stack was calculated using the *Z*-quantitation part of INSIGHT-IQ software. The optical slice with highest intensity from each series was selected as the most representative for morphometric analysis. Using INSIGHT-IQ software, the average fluorescence of selected tissue sites was calculated.

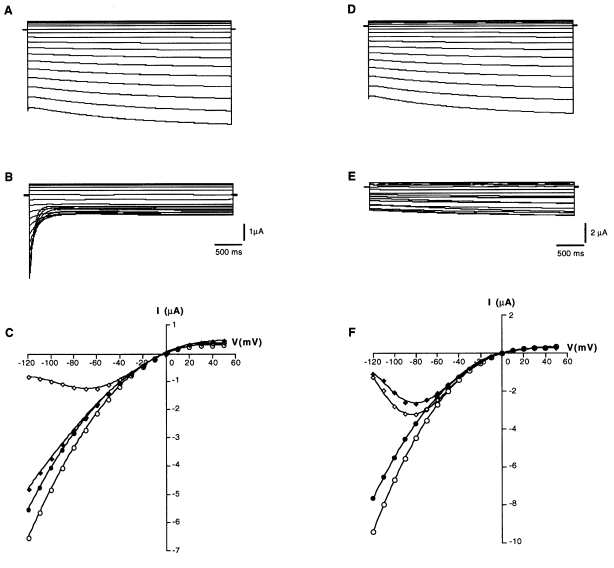

Fig. 2. Heteromeric Kir 4.1-5.1 channel expression in *Xenopus* oocytes and block by barium and cesium ions. Representative current traces recorded from oocytes expressing Kir 4.1-5.1 channels before (A, D) and after the application of 30 μM barium (B) or 300 μM cesium (E). Currents were evoked in 90 mM extracellular potassium by voltage commands from +50 to −120 mV, every 10 mV decrements, from a holding potential of −10 mV. Current-voltage relationships were obtained from the shown traces by plotting the steady-state current and the instantaneous currents as a function of membrane potential before and after the application of Ba^{2+} (C), or Cs^{+} (F) (open circles: steady-state currents; closed circles: instantaneous currents in control conditions; open diamonds: steady-state currents; closed diamonds: instantaneous currents after the application of the blocking cations).

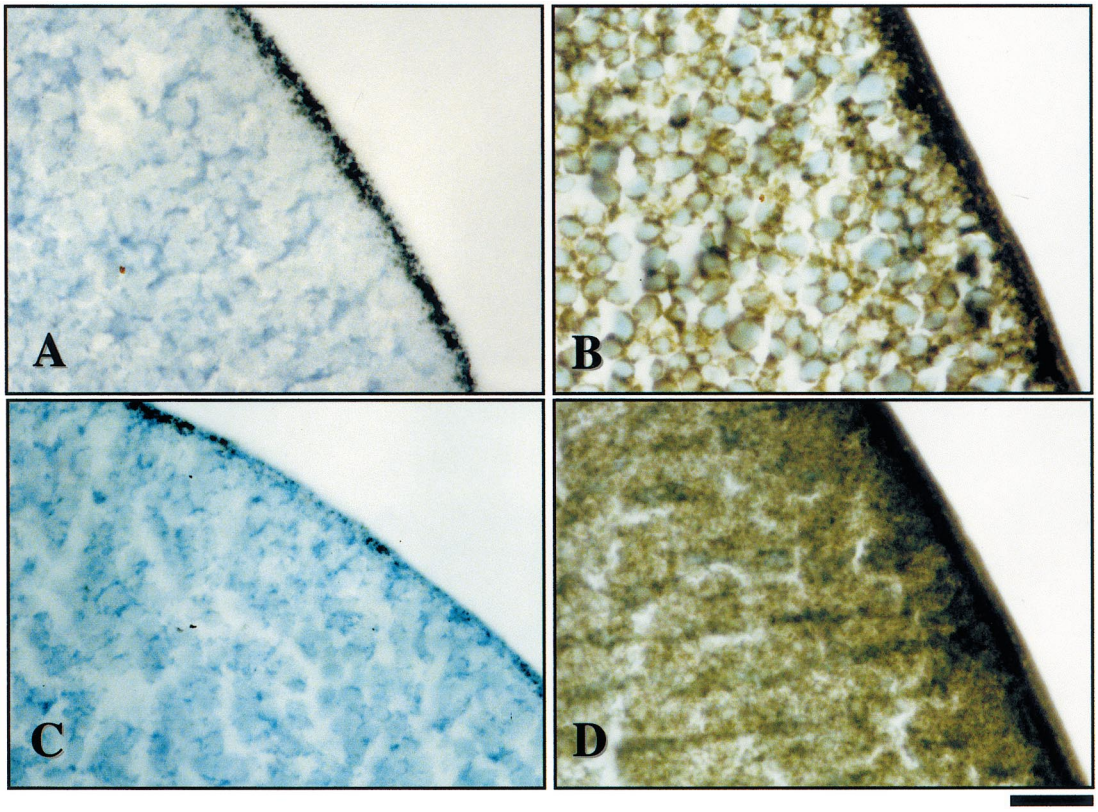

Fig. 3. Anti-Kir 5.1 antibody specificity. The anti-Kir 5.1 antibody immunoreactivity was tested in 5 μm slices dissected from formalin-fixed, paraffin-embedded *Xenopus* oocytes expressing Kir 4.1 (A), Kir 5.1 (B), Kir 3.1-3.4 (C) and Kir 4.1-5.1 linked subunits (D). Positive immunoreactivity is revealed by a brown staining. Note that the oocyte plasma membrane appears partially black (A, C) due to the presence of pigment vesicles in the animal pole of the cell (bar = 25 μm).

3. Results

Kir subunits are comprised of two transmembrane domains (TM), a pore segment (P-region) with the N- and C-termini residing inside the cell [25,26]. The extracellular loop (L) linking TM1 to the P-region of Kir 5.1 subunit shows low sequence similarity compared to several cloned Kir channels (Fig. 1) and displays a high immunogenic property (analysis not shown). Hence, a peptide identical to a portion of this Kir 5.1 loop region (Fig. 1) was synthesised to generate in rabbits a polyclonal antibody against Kir 5.1 subunits. To test the specificity of the affinity-purified antibody for Kir 5.1 subunits, *Xenopus laevis* oocytes were injected with mRNAs encoding several inward rectifier potassium channel subunits.

The injection into oocytes of Kir 5.1 mRNA by itself did not give rise to electrophysiologically detectable currents, as previously reported ([13,27]; not shown). In contrast, the expression of the tandemly linked subunits Kir 4.1-5.1 in *Xenopus* oocytes gave rise to functional channels (Fig. 2) [27]. Kir 4.1-5.1 potassium currents, recorded in two-electrode voltage-clamp configuration, displayed a slow activation component upon membrane hyperpolarisation and a current-voltage relationship with strong inward rectifying properties (Fig. 2A,C). The extracellular application of 30 μM barium (Fig. 2B) or 300 μM cesium (Fig. 2E) provoked a marked inhibition of Kir 4.1-5.1 currents which was steeply time-dependent (Fig. 2B,E). The analysis of the instantaneous and steady-state current-voltage relationships also revealed a steep voltage dependence of Ba^{2+} and Cs^{+} block which was more pronounced at more hyperpolarised potentials (Fig. 2C,F). These results indicate that the pore of the heteromeric Kir 4.1-5.1 channel retains its susceptibility to the block by extracellular cations, a characteristic feature of inward rectifier potassium channels.

After the electrophysiological recordings, the oocytes were collected, processed for immunocytochemical analysis and several 5 μm sections were used to determine the immunoreactivity of the anti-Kir 5.1 antibody. A positive dark brown immunostaining was detected in oocytes expressing Kir 4.1-5.1 heteromeric channels (Fig. 3D). Surprisingly, both the cytoplasm and the plasma membrane of oocytes injected with Kir 5.1 mRNA by itself were labelled, suggesting that Kir 5.1 subunits are normally synthesised and transferred on the membrane (Fig. 3B). In contrast, immunoreactivity was observed neither in sections dissected from uninjected oocytes, which endogenously express Xir channels [37] (not shown), nor in oocytes heterologously expressing Kir 4.1 (Fig. 3A), Kir 3.2 (not shown) or Kir 3.1-3.4 heteromeric channels (Fig. 3C). The specificity of the anti-Kir 5.1 subunit IgGs was also confirmed by means of dot-blot analysis according to Harlow and Lane [34]. These results strongly suggest that the anti-Kir 5.1 antibody specifically recognises Kir 5.1 subunits and the heteromeric complexes in which this subunit is present.

By using this generated polyclonal anti-Kir 5.1 antibody we determined the cellular localisation of Kir 5.1 subunits. A positive Kir 5.1 immunoreactivity was observed testis but

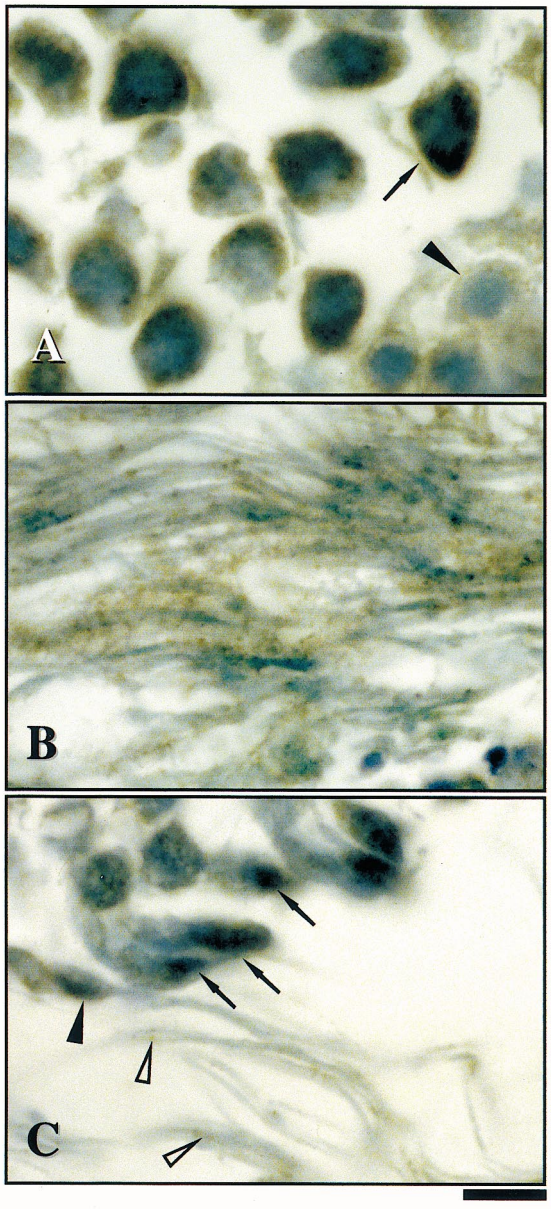

Fig. 4. Detailed localisation of Kir 5.1 subunits in seminiferous tubules of 60-day-old rat testis. Positive ABC immunoperoxidase staining showing the localisation of Kir 5.1 subunits (A) in spermatogonia (arrowhead), primary spermatocytes (arrow) and (B) in the head and body of mature sperm. C: Kir 5.1 subunits were also detected in secondary spermatocytes (arrows), spermatids (closed arrowhead) and spermatozoa (open arrowhead). Bar = 10 μm.

not in the ovary (not shown). In 60-day-old rats testis Kir 5.1 subunits were localised in the seminiferous tubules and, particularly, in spermatogonia, primary and secondary spermatocytes, spermatids and in the head and body of spermatozoa (Figs. 4 and 5). Neither Sertoli cells nor the interstitial Leydig cells were stained by the anti-Kir 5.1 antibody (Figs. 5 and 6).

To examine the developmental profile of Kir 5.1 protein appearance in rat testis tissues were dissected from animals at postnatal day 10 (P10), P20, P30, P60 and P90. Negligible positive immunoreactivity was detected in P10 and P20 rat testis (Fig. 5A). In contrast, a significant labelling was differentially detected in the seminiferous tubules of P30, P60 and P90 rats (Fig. 5B–D). Each experiment was repeated three times on specimens collected from three animals of different ages and gave similar results.

In order to quantify the Kir 5.1-specific signals at different developmental stages, the fluorescent images of several serial slices were recorded and analyzed by LSCM. Bright rings of immunofluorescence were observed in all developing cells of the seminiferous tubules suggesting that Kir 5.1 protein is mostly localised on the plasma membrane (Fig. 6B–D). Moreover, the morphometric analysis of specimens from P10 to P90 revealed that Kir 5.1 protein appearance in testis increased with age and reached a peak in 60-day-old rats (Fig. 6E). In contrast, the immunofluorescence decreased in 90-day-old animals to levels comparable to P30 and was mostly localised in spermatozoa (Fig. 6D,E). These results strongly suggest that Kir5.1 subunits follow a specific developmental pattern of expression related to spermatogenesis and to the sexual maturity of the animal.

4. Discussion

A polyclonal antibody raised in rabbits by using a distinct highly immunogenic peptide specifically recognises Kir 5.1 subunits and the heteromeric channels comprised of Kir 4.1 and Kir 5.1 subunits. Interestingly, experiments performed with *Xenopus* oocytes injected with Kir 5.1 mRNA by itself reveal the ability of these cells to synthesise and to properly translocate the Kir 5.1 protein to the plasma membrane. However, no channel activity could be recorded electrophysiologically from oocytes expressing Kir 5.1 alone. These observations suggest that Kir 5.1 subunits may form homomeric channels in the plasma membrane but require an additional, still unknown component(s) other than Kir 4.1, capable of activating the channel. On the other hand, tandemly linked Kir 4.1-5.1 subunits give rise to potassium currents which display strong inward rectifying properties and an instantaneous component followed by a slowly activating phase, whose kinetics are conferred by the C-terminal region of Kir 5.1 subunits [27,38]. Barium ions block in a time- and voltage-dependent fashion preferably the slowly activating component of Kir 4.1-5.1 currents implying that these ions act as open channel pore blockers. In contrast, cesium ions equally block both components suggesting a more complex interaction with the ion conducting pore.

We focused our interest on the immunolocalisation analysis of rat testis and showed that Kir 5.1 subunits can be detected in spermatogonia, primary and secondary spermatocytes, spermatids and in the head and body of spermatozoa but not in Sertoli cells or Leydig cells. These observations strongly suggest that inward rectifier potassium channels comprised of Kir 5.1 subunits may regulate important physiological functions such as the resting membrane potential, excitability and K^+ homeostasis of cells related to spermatogenesis. Indeed, it has been shown that an increase in extracellular potassium concentrations causes a reduction of sperm motility [39]. This observation suggests that Kir channels, by setting the resting membrane potential of sperm near the K^+ reversal potential, may play an important role in regulating the free-swimming properties of spermatozoa. Moreover, the G protein-gated *Girk2* channel, a member of the inward rectifier potassium channel superfamily, has been reported to be expressed in testis and to play a pivotal role in spermatogenesis.

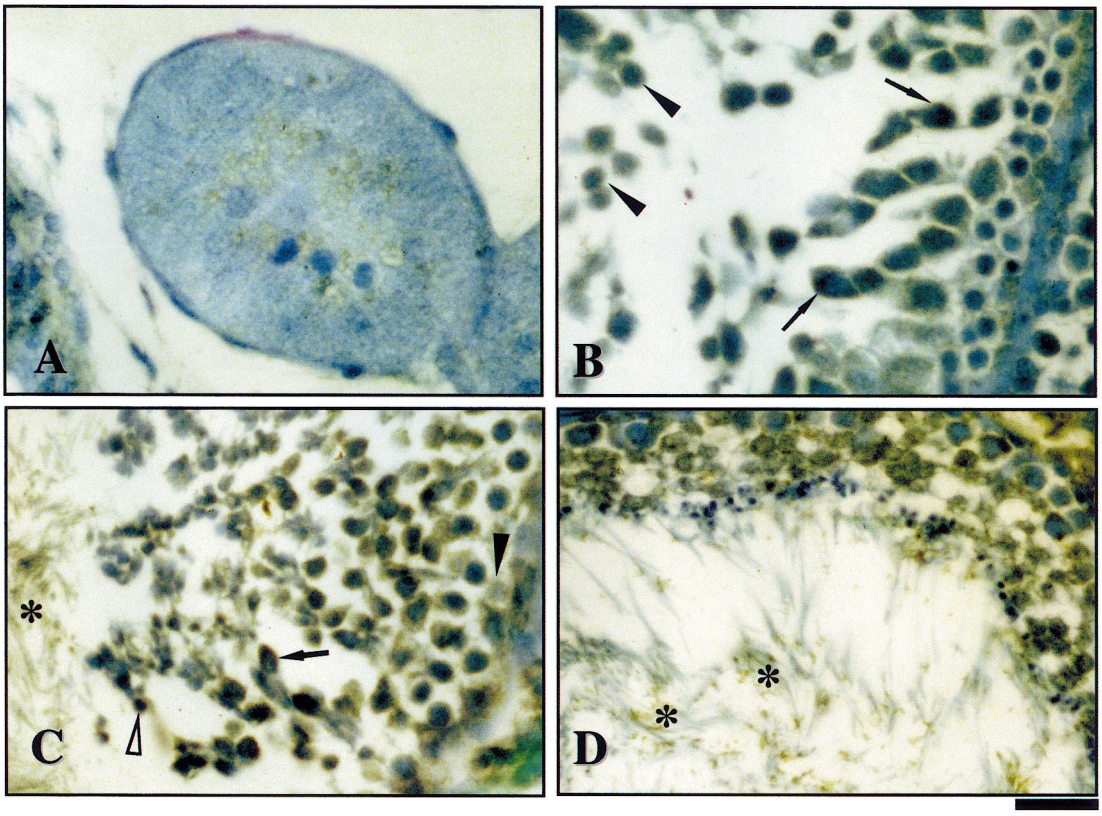

Fig. 5. Age-dependent localisation of Kir 5.1 subunits. A: 5 μm cross-section of a P10 seminiferous tubule showing a very low immunoreactivity of the anti-Kir 5.1 antibody. Positive immunostaining can be observed in sections dissected from P30 (B), P60 (C), and P90 (D) rat testis. Kir 5.1 subunits are localised in all stages of spermatogenesis and are indicated in B: primary (arrows) and secondary spermatocytes (closed arrowheads); in C: primary (arrows) and secondary spermatocytes (open arrowheads); in D: spermatozoa (*). Note that Sertoli cells (closed arrowhead in C) are not positively stained. Bar = 25 μm.

In fact, a genetic mutation in the coding sequence of the *Girk2* channel disrupts the selectivity filter of these channels, blocks neuronal differentiation and causes male sterility in *weaver* mice [40–42].

Kir 4.1 subunits have been detected in rat testis by rt-PCR analysis, but the cellular localisation of these subunits remains unknown [13]. Our previous and present results demonstrate that Kir 5.1 does not form functional channels by itself but requires the specific co-assembly of Kir 4.1 subunits [27]. If both subunits are expressed in the same cells, it would be plausible to hypothesise the formation of heteromeric channels comprised of Kir 5.1 and Kir 4.1 subunits in rat testis. Therefore, if both Kir 5.1 and Kir 4.1 subunits show similar patterns of expression in rat testis they may form heteromeric channels which are expected to possess electrophysiological properties and cation-blocking features similar to Kir4.1-5.1 dimers. Alternatively, spermatozoa and germinal cells may express, as yet unidentified proteins, capable of activating Kir 5.1 homomeric channels.

Both the ABC immunoperoxidase staining and the immunofluorescence LSCM analysis showed that Kir 5.1 protein may be detected on the plasma membrane and to a lesser extent in the cytoplasm of cells located in the seminiferous tubules. Moreover, the expression of Kir 5.1 subunits is strongly related to the development of a mammalian species, being highest during the sexual maturity of the animal. However, the role of this temporally defined pattern of Kir 5.1 expression in sperm motility or male fertility remains to be determined.

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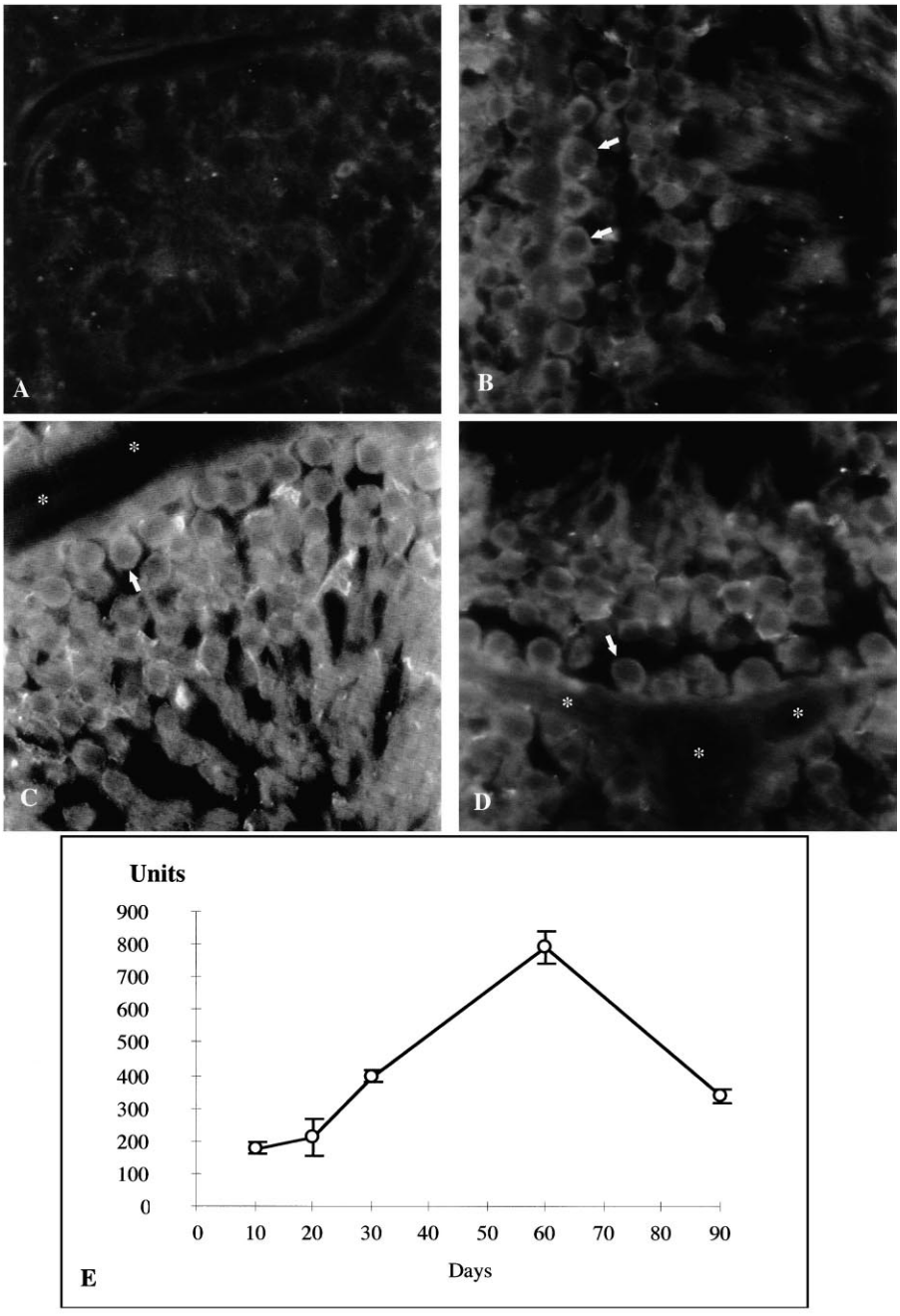

Fig. 6. Estimation of Kir 5.1 age-dependent expression by LSCM analysis. Kir 5.1 immunofluorescence was extremely low in P10 (A) or P20 sections (not shown) while it becomes detectable in P30 (B) cells located within the seminiferous tubules. The highest intensity of labelling was detected in P60 sections (C) and decreased to lower levels in P90 specimens (D). Arrows indicate that Kir 5.1 signal is mostly localised on plasma membrane and no labelling can be detected in interstitial cells (see asterisks in C and D). E: Morphometric analysis showing the average intensity of immunofluorescence, expressed in arbitrary units, as a function of days after birth (mean ± S.E.M.; $n = 3$).

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