FEBS 22951 FEBS Letters 462 (1999) 171–176

Voltage-dependent Ca^{2+} channel subunit expression and immunolocalization in mouse spermatogenic cells and sperm

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Received 31 August 1999; received in revised form 14 October 1999

Abstract Though voltage-dependent Ca^{2+} channels contribute to the orchestratation of sperm differentiation and function, many questions remain concerning their molecular architecture. This study shows that α_{1A} and α_{1C} Ca^{2+} channel pore-forming subunits are expressed in spermatogenic cells. In addition, it provides what is to our knowledge the first evidence for the presence of the Ca^{2+} channel β auxiliary subunits in spermatogenic cells and sperm. Using RT-PCR we demonstrated the expression of all four known genes encoding the β subunits in spermatogenic cells. Specific antibodies detected three of these proteins in spermatogenic cells and sperm. In spermatogenic cells both α_1 and β subunits are diffusely distributed throughout the cytoplasm while in sperm they appear to be regionally localized.

Key words: Ca^{2+} channel; Voltage-dependent Ca^{2+} channel; α_1 Subunit; β Subunit; Spermatogenic cell; Sperm

1. Introduction

Voltage-dependent Ca^{2+} channels (VDCC) are transmembrane proteins involved in orchestrating diverse physiological processes including spermatogenesis and the sperm acrosome reaction [1,2]. Therefore, understanding their molecular composition and distribution in spermatogenic cells and sperm is very important. In general, two different types of VDCC have thus far been identified in mammalian cells on the basis of their voltage activation threshold: low voltage- and high voltage-activated channels (LVA and HVA, respectively). Four subtypes of HVA Ca^{2+} channels (named L, N, P/Q, and R) and one subtype of LVA Ca^{2+} channels (known as T) have been defined [3]. HVA channels consist of at least three subunits: α_1, β, and $\alpha_2\delta$. Molecular cloning has revealed seven HVA channel α_1 genes (A, B, C, D, E, F and S) encoding proteins responsible for ion conduction, voltage sensing, and binding of specific drugs and toxins [4]. Recently, the cloning of three novel α_1 subunits (G, H and I) has given initial insight into the molecular structure of the LVA channels [5–7]. In addition, a number of β subunits (β_1–β_4) have also been cloned [8]. These β subunits do not cross the plasma membrane, but interact directly with the α_1 pore-forming subunit and appear to be important for determining the kinetic properties of the channel [3,4,8].

Despite the crucial role of the VDCC in the physiology of spermatozoa [1,2,9,10], their definitive identification remains elusive. The size, complex geometry and highly differentiated and motile nature of sperm has precluded their systematic electrophysiological characterization [11]. In addition, the inability of sperm to synthesize proteins impedes the use of standard molecular approaches to learn about their ion channels. For these reasons, more recent efforts have focused on the germ-line cells from which sperm arise. Patch-clamp studies have revealed the presence of only T-type Ca^{2+} currents in spermatogenic cells [12–14]. However, transcripts for a number of VDCC α_1 subunits have been identified in these cells, including α_{1A}, α_{1E} and α_{1C} encoding HVA [15–17], as well as α_{1G} and α_{1H} [17] encoding T-type channels. Evidence for expressed α_1 proteins was not provided until recently, when immunocytochemical data showed that three Ca^{2+} channel α_1 subunits (A, C and E) are present and regionally localized in mammalian mature sperm [18,19]. Though not yet reported, the expression of auxiliary subunits in spermatogenic cells and sperm is anticipated, considering the observations stated above. In the present study we have examined the expression and distribution of several HVA Ca^{2+} channel β subunits in both mouse spermatogenic cells and mature sperm.

2. Materials and methods

2.1. RNA isolation and RT-PCR experiments

RNA from isolated spermatogenic cells [15] or total tissue (brain and testis) was extracted using RNAzol (Life Technologies Inc., Frederick, MD, USA) according to the manufacturer's instructions. The Superscript system (Life Technologies) was used for reverse transcription polymerase chain reaction (RT-PCR). Total RNA was digested with RNase-free DNase and 5 μg was reverse transcribed using random or polydT primers. The resulting cDNA was used for amplification of the four different known genes for the Ca^{2+} channel β subunits. A set of degenerated β_{common} primers were used: forward1 (PF1; 5′-AAYGAYTGGTGGATHGGNCGN), and reverse1 (PR1; 5′-CATCATRTCNGTNACYTCRTA). An additional set of β_4-specific primers was also designed: forward2 (PF2; 5′CGGATGCCTGTGAACATCTGG) and reverse2 (PR2; 5′CAGTCCGGGTAATCTTCTTCC). cDNA fragments amplified by PCR were analyzed in a 1% agarose gel, cloned into pBluescript SK^+ (Stratagene, La Jolla, CA, USA) and sequenced by the dideoxy chain termination method with a Sequenase Ver 2.0 DNA Sequencing Kit (United States Biochemicals, Cleveland, OH, USA).

2.2. Immunolocalization

Aliquots (100 μl) of washed, diluted (1×10^6 cell/ml) mouse spermatogenic cells and sperm obtained as described previously [11,13] were dispensed onto glass slides. Settled cells were fixed with formaldehyde (5% final), rinsed in phosphate buffered saline (PBS), and blocked with either (in PBS): 2% bovine serum albumin, 5% non-fat milk or 10% gelatin for 1–18 h. Samples were then incubated overnight at room temperature with primary antibodies at appropriate dilutions (Table 1). After rinsing with PBS, samples were incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC) or Alexa (Molecular Probes Inc., Eugene, OR, USA) conjugated secondary antibodies (Table 1). Mature sperm were processed in the same manner except for permeabilization with 0.1% Triton X-100 in PBS for 10 min after fixation. Control experiments were processed likewise

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PII: S0014-5793(99)01518-5

except that the primary antibody was replaced with peptide-blocked antibody. Fluorescence images were acquired with a Bio-Rad MRC-600 Kr-Ar confocal microscope attached to a Zeiss Axioscope equipped with a 100× objective (1.3 numerical aperture) using Comos 7.0 software (Bio-Rad Microscience, Hercules, CA, USA). To enable comparison, all images were recorded at the same adjustments of laser power and photomultiplier sensitivity. Images shown are representative of at least three separate experiments in each condition and were processed using identical values for contrast and brightness.

3. Results and discussion

Previously it was shown that at least three subtypes of HVA Ca^{2+} channel α_1 subunits (A, C and E) are expressed in mouse mature sperm [18,19], though their presence in spermatogenic cells was not examined. These ion-conducting subunits comprise four hydrophobic homologous domains (I–IV) linked by intracellular loops. These domains exhibit a high degree of sequence conservation between α_1 subunit subtypes, but the connecting loops and the large intracellular carboxy-terminus are highly variable and characteristic of each subtype. These latter regions have been used to produce specific site-directed anti-peptide antibodies. Hence, a polyclonal antibody made against a peptide corresponding to residues 1691–1707 of the rat cardiac α_{1C} subunit [22], and a set of commercial antibodies against α_{1A}, α_{1B}, α_{1C} and α_{1D} (Alomone Labs Ltd., Jerusalem, Israel) were used to characterize the expression and cell distribution of these subunits in spermatogenic cells and sperm. Inasmuch as comparatively similar results were obtained in pachytene spermatocytes, round and condensing spermatids (cells at different stages of differentiation used in this study), we refer to them collectively as spermatogenic cells hereafter. Confocal images from spermatogenic cells stained with anti α_{1A} and α_{1C} antibodies clearly showed that these pore-forming subunits are not restricted to surface membranes but are also diffusely distributed in the cytoplasm (Fig. 1A,C, respectively). The nuclei excluded α_1 stain. Control experiments using antibodies blocked by exposure to the peptide antigen exhibited very low residual staining (Fig. 1B,D). No specific signals were obtained when antibodies against α_{1B} and α_{1D} were used in spermatogenic cells or sperm (data not shown). These results are in agreement with RT-PCR experiments performed in spermatogenic cells [15], where transcripts for these subunits were not detected. Taken as a whole, these data demonstrate that mouse spermatogenic cells indeed possess at least two different VDCC Ca^{2+} channel pore-forming α_1 subunits (α_{1A} and α_{1C}). As previously reported [18,19] and corroborated in our laboratory (not shown), the same two ion-conducting subunits are also present in mature sperm, indicating that the Ca^{2+} channel α_1 subunits are expressed early in spermatogenesis and retained up to the last stages in this process. Notably, a major fraction of α_1 immunoreactivity is cytoplasmic in spermatogenic cells, suggesting that these proteins may serve other functions in early development or might require association with targeting factors for their incorporation into the plasma membrane in late spermatogenesis. It would be possible also that the membrane localization of HVA Ca^{2+} channels subunits is restrained in early development and somehow promoted in the final stages of this process.

It is generally assumed that native HVA Ca^{2+} channels contain auxiliary subunits. Having shown that different HVA Ca^{2+} channel α_1 subunits are present in spermatogenic cells and sperm, it seemed necessary to determine which β subunits that have been proved to be fundamental in determining the native properties of all known HVA Ca^{2+} channels are expressed. The presence of the corresponding mRNAs in enriched populations of these cells was investigated using RT-PCR. A set of degenerate oligonucleotides was designed to amplify the four known mammalian β genes (Fig. 2A; see Section 2). These β_{common} primers (PF1 and PR1) indeed amplified all four genes, as confirmed in control experiments using mouse brain cDNA. When these primers were probed with cDNA from spermatogenic cells at different stages of development, several bands were obtained in agarose gel analysis (Fig. 2B). Cloning and DNA sequencing of these bands revealed the presence of β_1, β_2 and β_3 in all cell types tested, although no evidence for the expression of the β_4 subunit was found. However, another set of β_4-specific primers (PF2 and PR2) revealed a 329 bp PCR product in brain, testis and mouse round spermatids (Fig. 2C). The sequence of this fragment was consistent with that of β_4, indicating that spermatogenic cells indeed express all four known Ca^{2+} channel β subunits.

As depicted in Fig. 2A, a comparison of the amino acid sequence between the four β subunit gene products and splice variants has defined two high (conserved domains II and IV) and three low homology domains (the N- and C-terminal domains and the linker domain III). Taking advantage of

Table 1
Specific Ca^{2+} channel subunit antibodies

First antibody	Source	First antibody dilution		Second antibody	Second antibody dilution
		Spermatogenic cells	Sperm		
α_{1A}	rabbit[a]	1/60	1/60	Alexa[d]	1/100
α_{1B}	rabbit[a]	1/200	1/200	Alexa[d]	1/100
α_{1C}	rabbit[a]	1/200	1/200	Alexa[d]	1/100
α_{1C}	rabbit[b]	1/800	1/800	Alexa[d]	1/100
α_{1D}	rabbit[a]	1/200	1/200	Alexa[d]	1/100
β_1	rabbit[c]	1/800	1/500	Fluorescein[e]	1/80
β_2	rabbit[c]	1/500	1/100	Fluorescein[e]	1/80
β_3	sheep[c]	1/500	1/50	Fluorescein[f]	1/80
β_4	rabbit[c]	1/400	1/130	Fluorescein[e]	1/80

[a]Alomone Labs Ltd. (Jerusalem, Israel).
[b]Gurnett et al. [22].
[c]Liu et al. [20].
[d]Molecular Probes Inc. (Eugene, OR, USA).
[e]Sigma Chemical Co. (St. Louis, MO, USA).
[f]Calbiochem (La Jolla, CA, USA).

this feature, specific sequences in the C-terminal domain of the four β subunits have been utilized for the production of site-directed anti-peptide polyclonal antibodies. These antibodies, whose specificity has been tested previously [20,21], were used to investigate the expression of the distinct β subunits at the protein level in mouse spermatogenic cells and sperm. Fig. 3A shows a confocal immunofluorescence image of spermatogenic cells treated with antibodies directed to the β_1 subunit. Immunostaining is diffusely distributed throughout the cytoplasm, but lightly concentrated in a tubular/reticular perinuclear region, presumably the endoplasmic reticulum. These data give a true picture of the β_1 subunit localization since using the specific primary antibody blocked with the peptide antigen greatly decreased fluorescence staining (Fig. 3B). Virtually identical results were obtained when the β_2 subunit antibody was used (data not shown). Fig. 3C shows a representative immunoimage of β_1 staining in the head and flagellum of a mature sperm. Specific immunoreactivity was punctate presumably corresponding to clusters of β_1 subunits. β_1 puncta were distributed mostly to the apical tip and the acrosomal crescent of the sperm head (arrows). Since flagellar immunostaining was only partially blocked by the corresponding antigen fusion protein (Fig. 3D, asterisks) β_1 seemed to be expressed at a very low density, or not at all, in this region. Fig. 3E displays a typical β_3 subunit immunoimage in spermatogenic cells. The staining patterns are quite dependent on the optical plane of the confocal section examined. Although in some cells the 'nuclei' are apparently labeled (arrows), analysis of optical sections obtained at focal planes of increasing distance from the proximal surface (*z*-series) indicate that nuclei actually excluded staining. The cell in the left upper corner is an example of a more central optical slice showing the nucleus. Control optical sections stained with the β_3 antibody preincubated with the antigen fusion protein demonstrated that staining is specific (Fig. 3F). Fig. 3G shows β_3 subunit immunoreactivity in mature sperm, where mainly the flagellum is labeled with a punctate pattern (arrow). Puncta are specifically distributed along the dorsal and ventral regions of the principal piece (the distal segment of the tail). The dense spot localized in the apical tip probably corresponds to non-specific staining since it was not consistently blocked by incubation with the corresponding antigen fusion protein (Fig. 3H, asterisk). Although the use of specific primers indicated expression of the β_4 subunit mRNA, no conclusive immunocytochemical evidence for the expression of this protein was found (not shown).

This study provides what is to our knowledge the first evidence for the presence of the Ca^{2+} channel β auxiliary subunit in spermatogenic cells and sperm. Interestingly, co-expression studies of the cloned cDNAs have demonstrated that all four β auxiliary subunits can interact with a given α_1 ion-conducting subunit to determine Ca^{2+} channel activity, but differ in their relative effectiveness [3,4,8]. Hence, Ca^{2+} channel composition is important to determine their biophysical properties; however, beyond these functional aspects, the combination of a given β subunit with distinct α_1 subunits, at least in the final stages of sperm development, may affect the subcellular localization of the channels and consequently their abil-

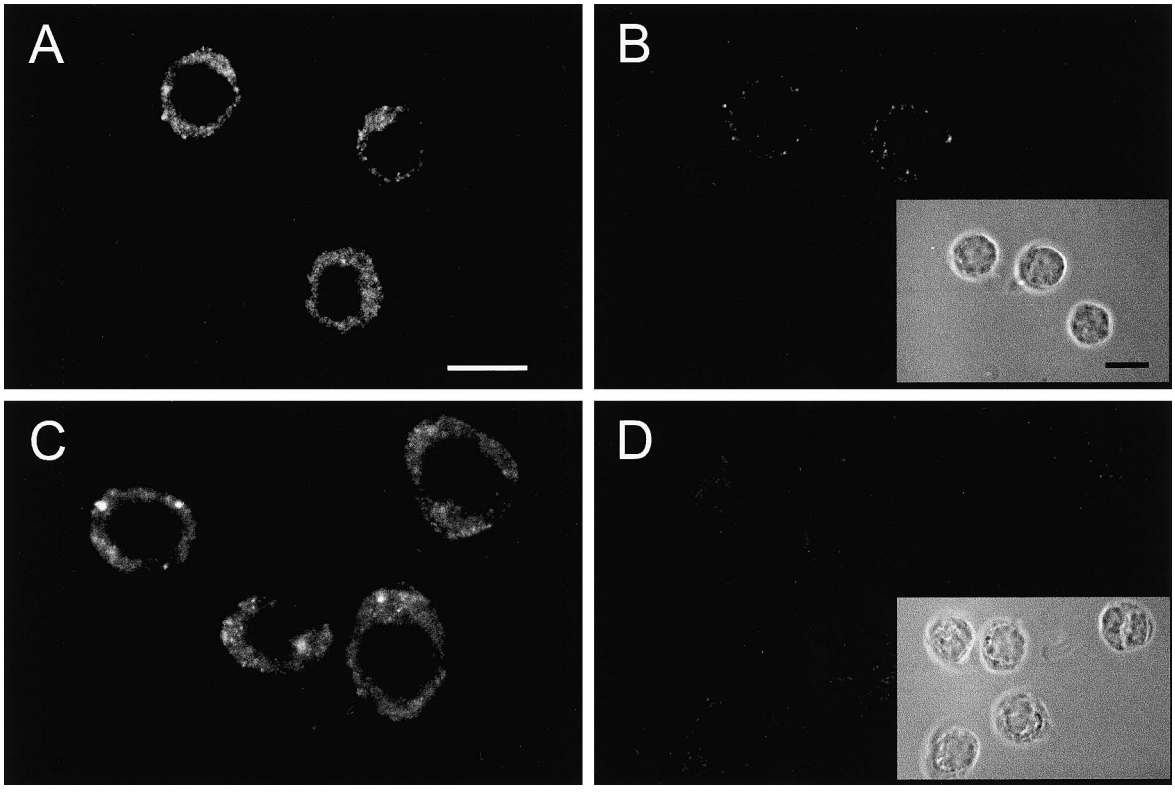

Fig. 1. Immunolocalization of Ca^{2+} channel α_1 subunits. A, C: Confocal immunofluorescence images of spermatogenic cells stained with anti-α_{1A} and anti-α_{1C} subunit antibodies, respectively, illustrating the diffuse and punctate immunoreactivity observed in these cells. Scale bar is 10 μm. B, D: Control sections showing that specific staining is blocked by its corresponding antigen fusion protein. Insets represent the corresponding phase contrast images of the control experiments.

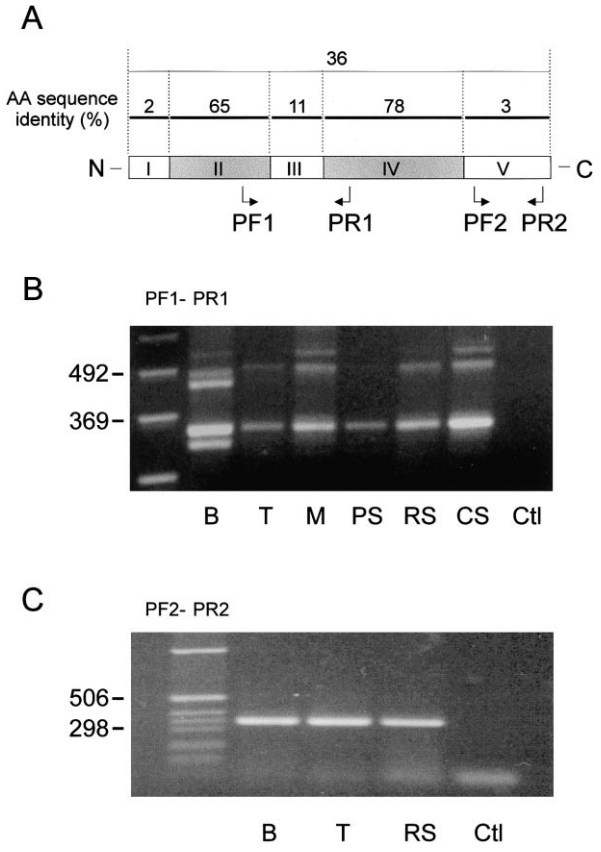

Fig. 2. Ca^{2+} channel β subunit gene expression. A: Schematic representation of a generic VDCC β subunit divided into five structural domains. Amino acid (AA) sequence identity in the five domains among all β subunits is denoted with the number above each domain. Regions with the highest homology are shown with identical fills. The overall sequence homology between the β subunits is also indicated with the number above the thinner line extending the drawing. The arrows show regions where primers used in RT-PCR experiments are located. PF1 and PR1 represent β_{common} primers, while PF2 and PR2 designate primers specific for the β_4 subunit. B: RT-PCR using primers PF1 and PR1 with cDNA of mouse brain (B), testis (T), a mixture of spermatogenic cells (M), pachytene spermatocytes (PS), round spermatids (RS) and condensing spermatids (CS). Lane 1 contains a 123 bp DNA ladder and lane 8 is the negative control (Ctl). C: RT-PCR using the β_4-specific primers PF2 and PR2 with brain (B), testis (T), and round spermatid (RS) cDNAs as templates. Molecular weight markers (1 kb ladder) are on the left, and lane 5 contains the negative control (Ctl).

ity to interact with other signaling proteins, contributing to the determination of their precise functional profile. Although no information on colocalization using double staining is yet available, the possibility exists that a given β subunit may associate with different α_1 subunits in an anatomical region-dependent manner. Corroboration of Ca^{2+} channel composition and cell distribution in these cells is an interesting topic for future studies.

In summary, there is evidence indicating that rises of intracellular Ca^{2+} in spermatogenic cells are key signals for cell division, differentiation and maturation [23]. Though intracellular Ca^{2+} increases as maturation progresses [24], little is known about the transport systems involved. Patch-clamp whole-cell analysis of Ca^{2+} currents in pachytene spermatocytes and round spermatids reveals mainly T-type currents [14,15]. The present article shows that most of the HVA Ca^{2+} channel subunits are cytoplasmic at these stages. This distribution pattern may help to explain why most of the voltage-dependent Ca^{2+} macroscopic current has been found to be of the T-type. Alternatively, although a fraction of these α_1 subunits could reach the plasma membrane, they could give rise to currents that resemble the T-type as reported for recombinant HVA Ca^{2+} channel pore-forming subunits expressed under particular conditions [25]. In addition, transcripts for at least two novel ion-conducting α_1 subunits (α_{1G}, α_{1H}) are present in spermatogenic cells and may encode T-type channels [17], and interestingly enough, in a recent report Dolphin and colleagues demonstrated that HVA Ca^{2+} channel auxiliary subunits including β_1 directly interact with α_{1G} to increase membrane localization of functional recombinant α_{1G} channels expressed in COS-7 cells and *Xenopus* oocytes [26]. Moreover, most of what we know regarding the physiology of Ca^{2+} channels in spermatozoa has been obtained in studies employing pachytene spermatocytes and round spermatids which are not the developmental stages immediately preceding mature sperm. Round spermatids, however, at the initial stage of spermiogenesis synthesize proteins that will be required for the development of mature sperm [23]. During the last step of this sperm maturation process, condensing spermatids undergo substantial changes in morphology, rearrangements in the composition and function of a number of proteins and changes in the intracellular concentration of Ca^{2+}. These proteins might include targeting factors for the incorporation of HVA Ca^{2+} channels to the plasma membrane, or alternatively, de-repression factors to release the cytoplasmic confinement of the HVA channels in early stages of development.

Knowledge of Ca^{2+} channel structure and distribution in spermatogenic cells and sperm will contribute to our understanding of the diversity in form and function of these unique proteins from spermatogenesis to fertilization. In addition, it will also provide the initiative for future studies directed to the investigation of pathophysiological and therapeutic aspects of several important conditions such as human male infertility linked to defective Ca^{2+} influx.

Acknowledgements: This work was supported by grants from CONACyT, DGAPA (UNAM), HHMI and ICGEB to A.D. C.J.S. was the recipient of a DGEP (UNAM) predoctoral fellowship. We thank Dr. K.P. Campbell for his generous gift of Ca^{2+} channel antibodies. We are grateful to Dr. G. Biddelcome for helpful discussions as well as to X. Alvarado, J. Monroy, P. Gaytán, E. López, E. Mata, S. González and G. Cabeza for expert technical assistance.

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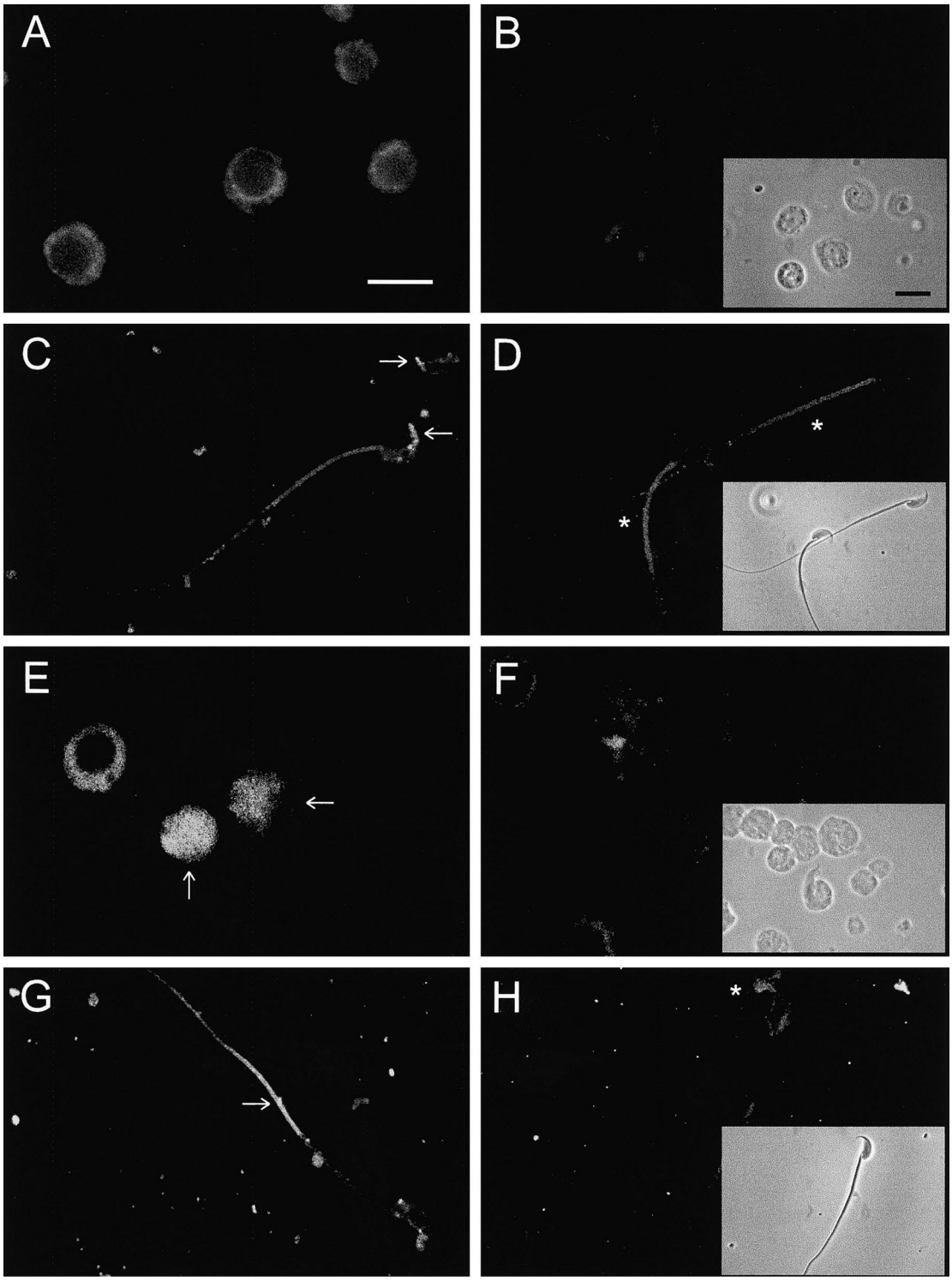

Fig. 3. Immunolocalization of Ca^{2+} channel β subunits. A: Confocal immunofluorescence image of spermatogenic cells labeled with anti-β_1 antibodies illustrating the perinuclear pattern of staining. Scale bar: 10 μm. C: Representative optical section of a mature sperm stained with the same anti-β_1 antibody indicating specific staining in the sperm head (arrows). E: Spermatogenic cells treated with antibodies to the β_3 subunit showing smooth specific staining throughout the cytoplasm of the cells. Top confocal section of two cells (arrows) and a more central slice are depicted. G: A typical mature sperm illustrating the pattern of β_3 immunoreactivity in these cells. Arrow points to specific staining in the principal piece of the flagellum. B, D, F, H: Control optical sections of spermatogenic cells and sperm stained with the anti-Ca^{2+} channel subunit antibodies preincubated with its corresponding antigen fusion protein to demonstrate that specific staining is blocked by the fusion protein. Asterisks denote residual staining after exposure to the peptide-blocked antibody prepared by incubation with its corresponding fusion protein. Insets represent the corresponding phase contrast images in control experiments.

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