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The Ca^{2+}-activated, large conductance K^+-channel (BK_{Ca}) is a player in the LH/hCG signaling cascade in testicular Leydig cells ☆

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ABSTRACT

In Leydig cells, hormonal stimulation by LH/hCG entails increased intracellular Ca^{2+} levels and steroid production, as well as hyperpolarization of the cell membrane. The large-conductance Ca^{2+}-activated K^+-channel (BK_{Ca}) is activated by raised intracellular Ca^{2+} and voltage and typically hyperpolarizes the cell membrane. Whether BK_{Ca} is functionally involved in steroid production of Leydig cells is not known. In order to explore this point we first investigated the localization of BK_{Ca} in human and hamster testes and then used a highly specific toxin, the BK_{Ca} blocker iberiotoxin (IbTx), to experimentally dissect a role of BK_{Ca}. Immunohistochemistry and RT-PCR revealed that adult Leydig cells of both species are endowed with these channels. Ontogeny studies in hamsters indicated that BK_{Ca} becomes strongly detectable in Leydig cells only after they acquire the ability to produce androgens. Using purified Leydig cells from adult hamsters, membrane potential changes in response to hCG were monitored. HCG hyperpolarized the cell membrane, which was prevented by the selective BK_{Ca} blocker IbTx. Steroidogenic acute regulatory (StAR) mRNA expression and testosterone production were not affected by IbTx under basal conditions but markedly increased when hCG, in submaximal and maximal concentration or when db-cAMP was added to the incubation media. A blocker of K_V4-channels, expressed by Leydig cells, namely phrixotoxin-2 (PhTx-2) was not effective. In summary, the data reveal BK_{Ca} as a crucial part of the signaling cascade of LH/hCG in Leydig cells. The hyperpolarizing effect of BK_{Ca} in the Leydig cell membrane appears to set in motion events limiting the production of testosterone evoked by stimulatory endocrine mechanisms.

1. Introduction

The Ca^{2+}-activated K^+-channel BK_{Ca} is characterized by a high single channel conductance (Latorre, 1994). It is activated by voltage and Ca^{2+} and consists of a pore-forming α-subunit and a modulatory β-subunit, which confers the channel with a higher calcium sensitivity. Until now, four types of β-subunits (β1–β4) have been reported (Vergara et al., 1998; Behrens et al., 2000). This channel has a prominent role in the cessation of Ca^{2+}-induced cellular responses by repolarizing the plasma membrane in excitable cells and thus is recognized to be involved in a variety of physiological processes (Hu et al., 2001).

BK_{Ca} channels were described in several mammalian endocrine cells (White et al., 1993; Prakriya et al., 1996). In non-excitable, endocrine cells producing steroids, namely human granulosa cells (GCs), BK_{Ca} was found to be functional (Kunz et al., 2002; Traut et al., 2009). Previous studies showed that in GCs, intra-ovarian signaling molecules (e.g. acetylcholine and oxytocin) increase intracellular Ca^{2+}, activate BK_{Ca} and hyperpolarize the membrane. These studies also link BK_{Ca} to alterations of steroidogenesis. However, while changes in intracellular Ca^{2+} levels, activation of BK_{Ca} and membrane hyperpolarization occur in the range of seconds, alterations in steroidogenesis are observed within hours. Thus, the mechanism by which ion channels are involved in the regulation of steroid production in human ovarian steroidogenic cells remains to be clarified.

In all steroid-producing cells Ca^{2+} is required for several steps of the steroidogenic pathway (Guagliardo et al., 2012). Recently, in steroid producing adrenal zona glomerulosa cells, a model for the

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interplay of the AT1-receptor, changes in membrane potential and intracellular Ca^{2+} levels was proposed (Guagliardo et al., 2012). According to this model, the changes in electrical excitability are due to the coordinated activities of several channel conductances. The activation of the AT1-receptor causes a temporary blockage of K^+-channels and an increase in the frequency of oscillations in membrane potential, which leads to Ca^{2+} influx via voltage-dependent Ca^{2+} channels and depolarization of the plasma membrane thus promoting steroid production. The activities of high voltage-dependent and Ca^{2+} activated K^+ conductances then hyperpolarize the membrane and restore the baseline membrane potential, returning voltage-dependent Ca^{2+}-channels to a closed state from which they can open again during the next oscillatory cycle (Guagliardo et al., 2012).

Whether this intriguing model is fully applicable for other steroidogenic cells depends on the repertoire of ion channels expressed and remains to be shown for each cell type. Leydig cells of the testis are another prominent steroidogenic endocrine cell type and intracellular Ca^{2+} levels are likewise crucial for effective androgen production by Leydig cells (Rossato et al., 2001). Interestingly, the hormones LH/hCG, via activation of LH-receptor, not only stimulate testosterone production, elevate cAMP but also increase intracellular Ca^{2+} levels and cause membrane hyperpolarization (Kumar et al., 1994; Carnio and Varanda, 1995; Costa and Varanda, 2007). The LH-induced increase of intracellular Ca^{2+} concentrations depends on the presence of Ca^{2+} in the extracellular medium, which enters the cells via open Ca^{2+}-channels (Sullivan and Cooke, 1986). Influx of Ca^{2+} can occur through transiently activated T-type Ca^{2+}-channels located in the plasma membrane of Leydig cells and/or through long-lasting activated L-type Ca^{2+}-channels (Lee et al., 2010, 2011). The specific involvement of the different Ca^{2+}-channel types in Leydig cells or ovarian steroid producing cells is not resolved, partly due to the lack of highly selective Ca^{2+}-channel blockers (Agoston et al., 2004; Perez-Reyes, 2003) and the dissociation of very rapid actions of ion channels and the slow process of steroid production.

Yet the use of blockers as tools to dissect the molecular mechanisms have led to an important insight, namely that the activation of L-type Ca^{2+}-channels is also apparently responsible for an inhibition of steroidogenesis. In spite of the many stimulatory roles of Ca^{2+} in the process of steroid production (Guagliardo et al., 2012), this surprising and counter-intuitive result is concluded from studies in MA-10 mouse Leydig tumor cells. This study employed nifedipine to pharmacologically block L-type Ca^{2+}-channels and results obtained revealed that the Ca^{2+} influx through L-type Ca^{2+}-channels normally inhibits c-AMP-stimulated steroidogenic acute regulatory protein (StAR) and steroidogenesis. Nifedipine consequently markedly enhanced cAMP-induced StAR expression and progesterone production and reduced the threshold of cAMP-induced StAR gene expression in Leydig cells (Pandey et al., 2010). Thus, the activity of L-type Ca^{2+}-channels in conjunction with intracellular Ca^{2+} appear to be highly relevant to steroidogenesis.

Ca^{2+}-channels are often co-localized with BK_{Ca}, at least in brain (Berkefeld et al., 2006). The expression of BK_{Ca} and its components at the molecular level in Leydig cells has to our knowledge not been reported, although a conductance with the characteristics of BK_{Ca} was detected in mouse and rat Leydig cells (Kawa, 1987; Joffre et al., 1984; Carnio and Varanda, 1995). Electrophysiological experiments showed that this assumed BK_{Ca}-channel was activated upon hCG treatment (Carnio and Varanda, 1995), entailing membrane hyperpolarization, (Joffre et al., 1984). Later on, a report by Gong et al. (2002) described the recording of a voltage- and Ca^{2+}-dependent, outwardly rectifying current in spermatogonia and primary spermatocytes with gating and pharmacological properties resembling those of BK_{Ca}. Furthermore, in the testis, BK_{Ca} β3/β4-subunits were detected (Behrens et al., 2000; Brenner et al., 2000).

In this study we focused on BK_{Ca} and explored first whether human Leydig cells express BK_{Ca}. Functional studies cannot be performed on human testicular biopsies. We therefore turned to the hamster, examined BK_{Ca} expression during sexual maturation and used primary cultures of adult Leydig cells to investigate the involvement of BK_{Ca} in the signaling cascade initiated by LH-receptor activation that leads to steroid production. We specifically took advantage of the fact that IbTx is a highly specific blocker of BK_{Ca} and we therefore used it as a tool to experimentally dissect the role of BK_{Ca}.

2. Materials and methods

2.1. Human testicular samples

The samples used for immunohistochemistry were similar to the ones used in other studies (Schell et al., 2010; Spinnler et al., 2010; Adam et al., 2011) and are archival, diagnostic biopsies from infertility patients with obstructive and non-obstructive azoospermia. The ones used for RT-PCR were also used a previous study (Kampfer et al., 2012). We focused on samples with normal spermatogenesis (four patients with obstructive azoospermia). The local ethics committee approved the study.

2.2. Animals

Male Syrian hamsters (*Mesocricetus auratus)* were raised in the animal care unit (Charles River descendants, Animal Care Lab., IBYME, Buenos Aires, Argentina). In this study, we used hamsters aged 10, 18, 36, 46, 60, and 90 days, which were kept in rooms at 23 ± 2 °C under a long day photoperiod (14 h light, 10 h dark; lights on 07.00–21.00 h). Animals had free access to water and Purina formula chow. Hamsters were killed by asphyxia with carbon dioxide (CO_2) according to protocols for animal laboratory use, approved by the Institutional Animal Care and Use Committee (IBYME-CONICET), following the NIH guidelines. Testes were dissected, fixed for at least 48 h in Bouin's fixative or formaldehyde fluids followed by dehydration, and then embedded in paraffin wax for histological and immunohistochemical studies. In other groups of adult animals, testes were used for Leydig cell purification.

In vitro incubations of Leydig cells followed by immunocytochemistry, determination of testosterone and mRNA expression (by RT-PCR or RT-qPCR) were performed.

For membrane potential measurements, hamsters were obtained from Charles River Laboratories, Germany GmbH, and kept for 3–4 weeks in the animal care unit at the Experimental Research Surgery Unit, Großhadern, Munich, under a long photoperiod with free access to food and water. Testis were dissected and used for Leydig cell purification. Afterwards changes in membrane potential (by $DiBAC_4(3)$-mediated fluorescence) were monitored and immunocytochemistry was performed.

2.3. Hamster Leydig cell purification and "in vitro" incubations

Syrian hamster testes were used to isolate Leydig cells. In brief, decapsulated testes were incubated in a shaking water-bath at 34 °C for 5 min in the presence of 0.2 mg/ml collagenase type I (Sigma Chemical Co, St. Louis, MO, USA). At the end of the incubation, collagenase activity was stopped by adding Medium 199, and the tubules were allowed to settle for 1 min. Supernatants were transferred to 25 cm^2 sterile flasks, and placed in an incubator at 37 °C under a humidified atmosphere with 5 % CO_2 for 10 min.

All unattached cells were then recovered by swirling, followed by a gentle washing with Medium 199, and filtered by a 100 μm Nylon cell strainer. More than 95% of attached cells were macrophages positive for Indian Ink, ED-1 antigen and ED-2 antigen. Filtered cells were employed for Leydig cell isolation under sterile conditions using a discontinuous Percoll density gradient as previously described by Frungieri et al. (2006). Cells that migrated to the 1.06–1.12 g/ml density fraction were collected and suspended in Medium 199. An aliquot was incubated for 5 min with 0.4% Trypan-blue and used for cell counting and viability assay in a light microscope. Viability of Leydig cells preparations was 97.5–98.5%. In order to evaluate purity of Leydig cells, 3 β-hydroxysteroid dehydrogenase (3 β-HSD) activity was measured, as previously described by Levy et al. (1959). Cell preparations were 87–90% enriched with hamster Leydig cells. Less than 0.006% of the contaminating cells were macrophages positive for Indian Ink, ED-1 and ED-2 antigens, whereas mast cells were not detected. The mouse monoclonal antibodies ED-1 and ED-2 (BD Pharmingen, San Diego, CA, USA), were used in an attempt to identify testicular non-resident and resident macrophages, respectively. The remaining cell types had the morphology of either peritubular cells or endothelial cells. Petri dishes containing 3×10^5 cells (for RT-PCR, RT-qPCR and testosterone production assay) or 8×10^4 cells (for determination of changes in membrane potential) were incubated at 37 °C under a humidified atmosphere with 5% CO_2. Cell were incubated in the presence or absence of 5 or 100 mIU/ml hCG (Ayerst, Princeton, NJ, USA; specific activity, 59 IU/mg), 0.5 mM dibutyryl-cAMP (db-cAMP; Sigma); 50–100 nM IbTx (Alomone Labslabs, Jerusalem, Israel) and 50–100 nM Phrixotoxin-2 (PhTx-2; Alomone labs), a peptid blocker of non-related K^+-channels, $K_V4.2$, also expressed by hamster Leydig cells (not shown), which served as a control. After incubations, cells in media were transferred to tubes and centrifuged at 1.200*g* for 10 min. Media were frozen at −70 °C until testosterone concentrations were determined by radioimmunoassay (RIA). Other batches of cells were used for measurement of the membrane potential, or for RNA extraction followed by RT-PCR or RT-qPCR.

2.4. Histological, immunohistochemical and immunocytochemical analyses

In addition to human samples, testes from immature (10- and 18-day-old) pre-pubertal (36-day-old), early pubertal (46-day-old), late pubertal (60-day-old) and adult (90-day-old) hamsters exposed to a LD photoperiod, were examined by histological and immunohistochemical assays. Groups of eight to ten animal testes were evaluated. After fixation, tissues were dehydrated and embedded in paraffin wax, and 5 μm sections obtained from three different levels were used. Formaldehyde-fixed purified Leydig cells from adult hamsters were also used. Sections were deparaffinzed and antigen retrieval was performed by heating in a microwave in 10 mM sodium citrate buffer. Endogenous peroxidase reactivity was quenched by a 20 min pretreatment with 10% methanol, 0.3% H_2O_2 in 0.01 M phosphate-buffered saline (PBS, pH 7.4). Then, sections and cells were permeabilized by a 5 min incubation with 0.5% saponin for detection of BK_{Ca} in sections and 0.05% saponin for immunodetection of BK_{Ca} and P450 side chain cleavage (P450scc) in formaldehyde-fixed Leydig cells. Non-specific proteins were blocked by subsequent incubation with 5% normal serum in PBS for 30 min. After several wash steps the incubation with the antiserum (polyclonal rabbit anti-BK_{Ca}, 1:100, Alomone Labs or polyclonal rabbit anti P450scc, 1:100, Chemicon Inc., Temecula, CA) diluted in incubation buffer (2% normal serum in PBS) was carried out overnight in a humidified chamber at 4 °C. On the second day, cells and testicular sections were washed and incubated with biotinylated secondary antiserum (goat anti-rabbit IgG; 1:500, Vector Lab, CA, USA for BK_{Ca} and for P450scc) for 2 h at room temperature. Finally, immunoreaction was visualized with 0.01 % H_2O_2 and 0.05% 3,3-diaminobenzidine (DAB) solution (in 0.05 M Tris–HCl, pH 7.6) and an avidin–biotin-peroxidase system (Vector Lab). For control purposes, either the first antiserum was omitted or incubation was carried out with normal non-immune sera. Some sections were counterstained with hematoxylin.

2.5. RT-PCR and qRT-PCR analyses

RNA was obtained from hamster Leydig cells using the QIAGEN RNeasy mini kit (QIAGEN Inc., Valencia, MO, USA). RNA was extracted from two human biopsies and RT-reactions were performed using dN6 random primers as described (Kampfer et al., 2012). A cDNA amount corresponding to 400 ng total RNA was used for each RT-PCR and RT-qPCR reaction.

RT-PCR conditions were 95 °C for 5 min, followed by cycles of 94 °C for 1 min, 54–60 °C (annealing temperature) for 1 min and 72 °C for 1 min, and a final incubation at 72 °C for 5 min. The number of cycles used (30–35 cycles) was previously validated in order to avoid saturation of the band intensities. PCR products were separated on 2% agarose gels, and visualized with ethidium bromide. The identity of the cDNA products was confirmed by sequencing using a fluorescence-based dideoxy-sequencing reaction and an automated sequence analysis on an ABI 373A DNA sequencer. The oligonucleotide primers used in RT-PCR assays are summarized in Table 1.

The qRT-PCR assays were performed using oligonucleotide primers for 18S rRNA (5′-ACACGGACAGGATTGACAGATT; 5′-CGTTCGTTATCGGAATTAACCA) and StAR (5′-AACGGGGATGAGGTGCTGAG; 5′-CCACTCTCCCATTGCCTC). 18S rRNA was chosen as the housekeeping gene. Reactions were conducted using SYBR Green PCR Master Mix and the ABI PRISM 7500 sequence detector System (Applied Biosystems, Foster City, CA, USA). The reaction conditions were as follows: 10 min at 95 °C (one cycle), followed by 40 cycles of 15 s at 95 °C, 30 s at 55 °C and 1 min at 60 °C for 18S, or by 40 cycles of 15 s at 95 °C, 30 s at 55 °C and 1 min at 60 °C for StAR. Following the mathematical model of Pfaffl (2001), the fold change of mRNA StAR expression was determined for each sample by calculating $(\text{E target})^{\Delta Ct(\text{target})}/(\text{E housekeeping})^{\Delta Ct(\text{housekeeping})}$, where *E* is the efficiency of the primer set, *Ct* the cycle threshold, and $\Delta Ct = Ct(\text{normalization cDNA}) - Ct(\text{experimental cDNA})$. The amplification efficiency of each primer set was calculated from the slope of the standard amplification curve of *Ct* value vs log microliters of cDNA per reaction over at least four orders of magni-

Table 1
Information about oligonucleotide primers and PCR conditions.

Target	Primers		Length (bp)	Annealing temperature (°C)
	Sense	Antisense		
BK_{Ca}-α (human)	5′-CCT-GAT-CCT-TGC-CAA-CAA-GT-3′	5′-AGC-TCG-GGA-TGT-TTA-GCA-GA-3′	158	56
BK_{Ca}-α (hamster)	5′-ATG-GAG-GTG-CCG-TGC-GAC-A-3′	5′-TCT-GCC-AGT-CAG-TGT-CTG-3′	312	60
BK_{Ca}-β1	5′-AGA-CCA-ACA-TCA-GGG-ACC-AG-3′	3′-GCT-CTG-ACC-TTC-TCC-ACG-TC-3′	205	59

tude ($E = 10^{-(1/slope)}$; E 18S rRNA = 1.94, E StAR = 1.94 vs hamster Leydig cells cDNA).

2.6. Testosterone assay

Testosterone levels were determined in the incubation media by RIA according to the method described by Frungieri et al. (2005) without extraction using antibodies obtained from Immunotech Diagnostic (Montreal, Canada). Testosterone was measured using an antibody to testosterone-7-butyrate-BSA, which is known to have 35% cross-reactivity with dihydrotestosterone. The minimal detectable assay concentration was 0.215 pmol/ml. Intra- and interassay coefficients of variation were less than 12% and less than 15%, respectively. *In vitro* testosterone production from Leydig cells is expressed in terms of pmol per 10^6 Leydig cells.

2.7. Evaluation of membrane potential changes

2.7.1. Evaluation of membrane potential changes

For measurements of membrane potential changes in response to hCG, we used the anionic fluorescent bisoxonol dye bis(1,3-dibutylbarbituric acid)-trimethine oxonol (DiBAC4(3), Molecular Probes, Invitrogen, Karlsruhe, Germany). Depending on membrane potential the dye is distributed across the plasma membrane yielding a fluorescence intensity increase with depolarization due to its negative charge (Langheinrich and Daut, 1997; Yamada et al., 2001; Baczko et al., 2004). Purified hamster Leydig cells were placed on glass cover slips. Cells were incubated for 30 min at 37 °C in DiBAC4(3) (500 nM in extracellular fluid (EC) solution, consisting of 140 mM NaCl, 3 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM HEPES and 10 mM Glucose). Afterwards cover slips were placed into a recording chamber and observed with a Zeiss Axioskop 2 FS plus microscope with a heated stage. In this set-up, medium with or without drugs was constantly added and removed at the same rate, causing a constant flow in the incubation chamber. DiBAC4(3) (500 nM in EC solution) was applied for 10 min to ensure equilibration Then a test solution with hCG 100 mIU/ml (in EC with 500 nM DiBAC4(3)) was applied for 15 min, followed by solution with a raised potassium concentration (66 mM) for 10 min, working as a positive control. Changes in fluorescence intensity were monitored for 30 min by sampling every min at excitation and emission wavelength of 488 and 520 ± 20 nm, respectively. To block BK_{Ca}, cells were incubated in EC solution with 500 nM DiBAC4(3) and iberiotoxin (IbTX; 100 nM) for 60 min before the experiment. Each experiment with about 30 cells was analyzed separately because of variations of absolute fluorescence intensity values. The slopes for each cell during the three different application periods were determined by linear regression.

2.7.2. Statistical analyses

Statistical analyses of qRT-PCR and testosterone assays were performed using ANOVA followed by Student–Newman–Keuls test for multiple comparisons. Data of membrane potential imaging were analyzed by repeated measures ANOVA and Bonferroni post test. Data are expressed as mean + S.E.M. or SD as indicated.

3. Results

3.1. Identification of BK_{Ca}-channels in human and hamster testes

Immunohistochemistry revealed that interstitial cells of the adult human testis express the BK_{Ca} α-subunit. Staining in germ cells was also noted (Fig. 1A). No reaction was observed in human testicular sections incubated only with normal, non-immune serum and the conjugated secondary antibody (Fig. 1B). RT-PCR followed by sequencing of human testicular samples, revealed the identity of the pore-forming BK_{Ca}-channel α-subunit (Fig. 1C).

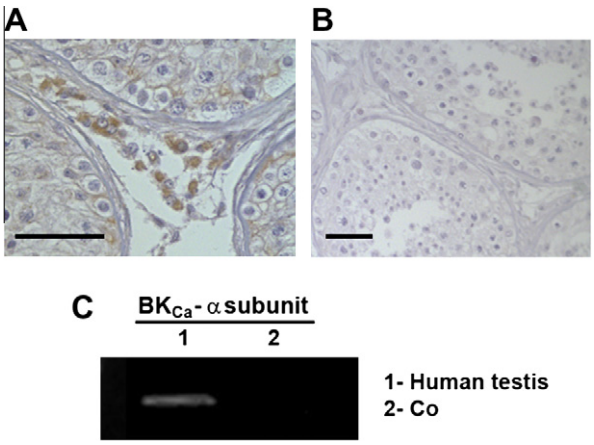

Fig. 1. Identification of BK_{Ca} in the human testis. Immunoreactive BK_{Ca} α-subunit was detected in interstitial cells in a testicular biopsy from a patient with normal spermatogenesis. Note the weak staining of germ cells (A). Hematoxylin counterstain. Bar, 60 μm. No reaction was observed in testis sections incubated only with normal non-immune serum and the conjugated antibody (B). Bar, 20 μm. Ethidium-bromide stained agarose gels showing a cDNA, which was sequenced and corresponds to the BK_{Ca} α-subunit in the human testicular sample; Co: control reaction (C).

Immunoreactive BK_{Ca} α-subunits were detected in peritubular myoid cells and a few interstitial cells in testes from 10-day-old (Fig. 2A) and 18-day-old immature hamsters (data not shown). In contrast, immunohistochemical analyses showed the expression of BK_{Ca}-channels in the cytoplasm of interstitial cells with the characteristic punctuate chromatin pattern of Leydig cells in prepubertal (36-day-old, Fig. 2B), early pubertal (46-day-old; data not shown), late pubertal (60-day-old; data not shown) and adult (90-day-old; Fig. 2C) hamster testes. In the adult, positive staining was also observed in some germ cells, namely spermatogonia and early spermatocytes. BK_{Ca} immunostaining was not found when hamster testes sections were incubated only with non-immune serum instead of the specific antibody (Fig. 2C, inset).

Immunocytochemical and RT-PCR experiments (Fig. 2D–G), followed by sequencing, using purified Leydig cells further substantiated that these cells express the BK_{Ca} α – and β 1-subunits. Leydig cells stained with an antibody against BK_{Ca} (Fig. 2D) and P450scc (Fig. 2E). No reaction was observed in hamster Leydig cells, when BK_{Ca} and P450scc antibodies, respectively, were replaced by normal non-immune serum (Fig. 2F). The obtained partial hamster BK_{Ca} α-sequence (from three independently derived identical sequences; 73 bp) has a 95.9% identity with human, rat, and mouse at the nucleotide level (TCTGGCGAACGCTCAAGTACCTGTGGACCG TTTGTTGCCACTGCGGGGGCAAGACGAAGGAGGCCCAGAAGAT). The hamster partial BK_{Ca} β 1-subunit sequence (two independently derived identical sequences, 26 bp) has a 96.2% identity with human and rat, and 92.3% identity with mouse at the nucleotide level (GTGCTGCCCCTCTACCAGACAAGCGT).

3.2. Effect of hCG and Iberiotoxin (IbTx) on membrane potential in hamster Leydig cells

Fluorescence intensity changes were monitored in three independent experiments using different Leydig cell preparations with a total of 53 control and 64 IbTx-preincubated cells, respectively (Fig. 3). In all experiments an initial intensity rise was observed. The application of hCG (100 mIU/ml) caused a stop of this overall depolarization (Fig. 3A and C). In Fig. 3A three individual cells are

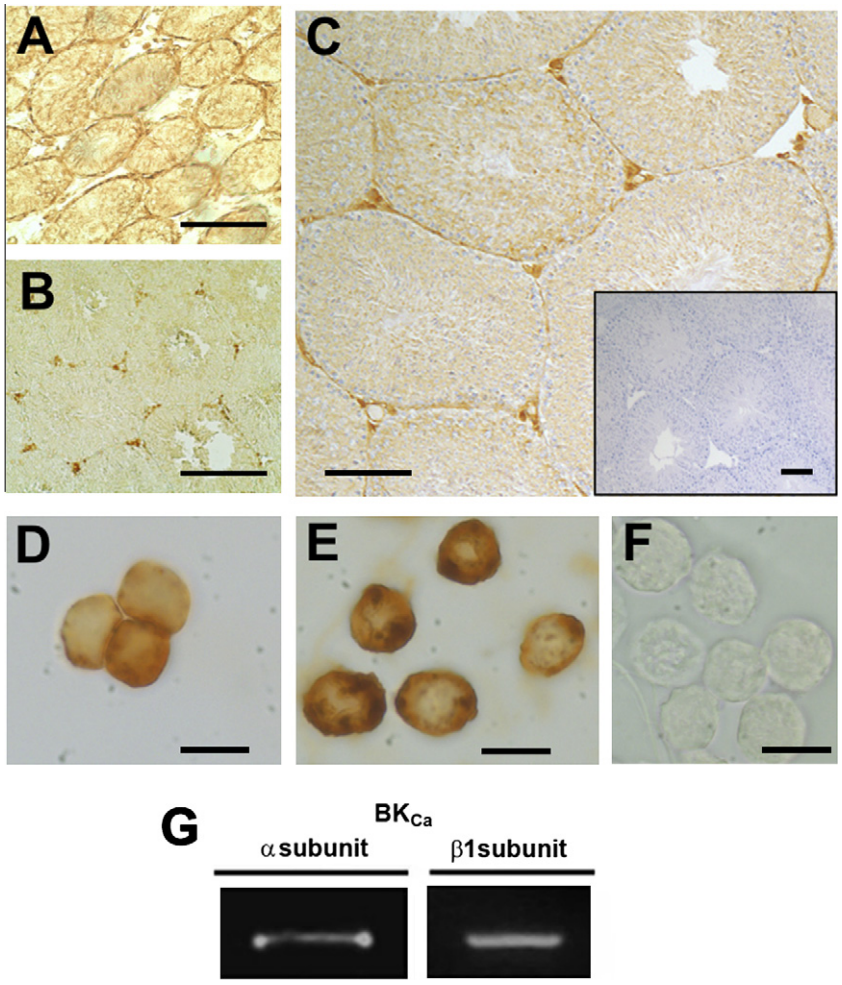

Fig. 2. BK_{Ca} immunolocalization in Syrian hamster testes: influence of age. and expression by isolated adult Leydig cells. BK_{Ca} immunostaining was localized to peritubular cells and to some interstitial cells in testes from immature (10-day-old) hamsters (A). Positive reaction was found in interstitial cells with the characteristic punctuate chromatin pattern of Leydig cells from prepubertal (36-day-old; B) and adult (90-day-old; C) male Syrian hamsters kept under a long-day photoperiod. In this section germ cells also stain. No reaction was observed in testis sections from adult hamsters incubated only with the conjugated secondary antibody (inset in C). Hematoxylin counterstain. Bars, 100 μm. (D and E). BK_{Ca} and P450scc expression in Syrian hamster Leydig cells. Immunocytochemical studies were performed in Leydig cells purified from adult hamsters kept under a long-day photoperiod. BK_{Ca} (D) and P450scc (E) were detected in these cells. No reaction was observed in hamster Leydig cells when BK_{Ca} and P450scc antibodies were replaced by normal non-immune serum (F). Bar, 10 μm. (G). Ethidium-bromide stained agarose gels showing cDNA fragments which, after sequencing, were proven to correspond to BK_{Ca} α- and β1-subunits in adult hamster Leydig cells.

shown and in Fig. 3C the mean ± SD of normalized fluorescence intensities for all cells in this experiment ($n = 27$). The mean slope prior to addition of hCG was 0.0230 ± 0.0010 min^{-1}, and changed upon hCG to −0.0003 ± 0.0011 min^{-1}. Upon shifting K^+ reversal potential by addition of a high potassium concentration solution (66 mM), fluorescence increased (mean slope 0.0420 ± 0.0005 min^{-1}), corresponding to a depolarization of the cell membrane. All slopes are significantly different (ANOVA, $P < 0.0001$; Bonferroni's post test, $P < 0.01$). When Leydig cells were preincubated with 100 nM IbTx (Fig. 3B and D), the same initial depolarization was observed. As this effect of unknown cause is IbTx-insensitive, it cannot be related to any action mediated by BK_{Ca} channels. The application of hCG in the presence of IbTx does not stop the initial depolarization as in the control experiments. Therefore, the hCG-induced hyperpolarization in Fig. 3A and C was attributed to hCG-caused activation of BK_{Ca} channels. In the experiment shown 26 cells were monitored, of which three individual ones are shown in Fig. 3B and the mean ± SD of normalized fluorescence intensities in Fig. 3D. The mean slope prior to addition was 0.0355 ± 0.0045 min^{-1} and upon hCG addition it changed to 0.0431 ± 0.0039 min^{-1} (not significantly different; Bonferroni's post test, $P > 0.1$). An external solution with high potassium concentration (66 mM) caused a significant further depolarization, but no change in slope (mean slope 0.0666 ± 0.0070 min^{-1}; significantly different to prior slopes; Bonferroni's post test, $P < 0.01$). In repetitions, the changes were similar, yet the absolute fluorescence intensities varied considerably between the experiments and were therefore not combined. Due to the blockage of the hCG-induced hyperpolarization by IbTx, the cells show a significantly higher Di-BAC fluorescence intensity at the end of the hCG treatment. This certainly may affect their ability to further depolarize when shifting the K^+ reversal potential by a higher extracellular K^+ concentration.

3.3. Effect of IbTx on StAR mRNA expression and testosterone production from hamster Leydig cells

As expected, a 3 h incubation in the presence of a maximally stimulatory concentration of hCG (100 mIU/ml) resulted in a significantly increased StAR mRNA expression and testosterone production from hamster Leydig cells (Fig. 4A and B, respectively). The BK_{Ca} blocker IbTx (50 nM – data not shown and 100 nM) did

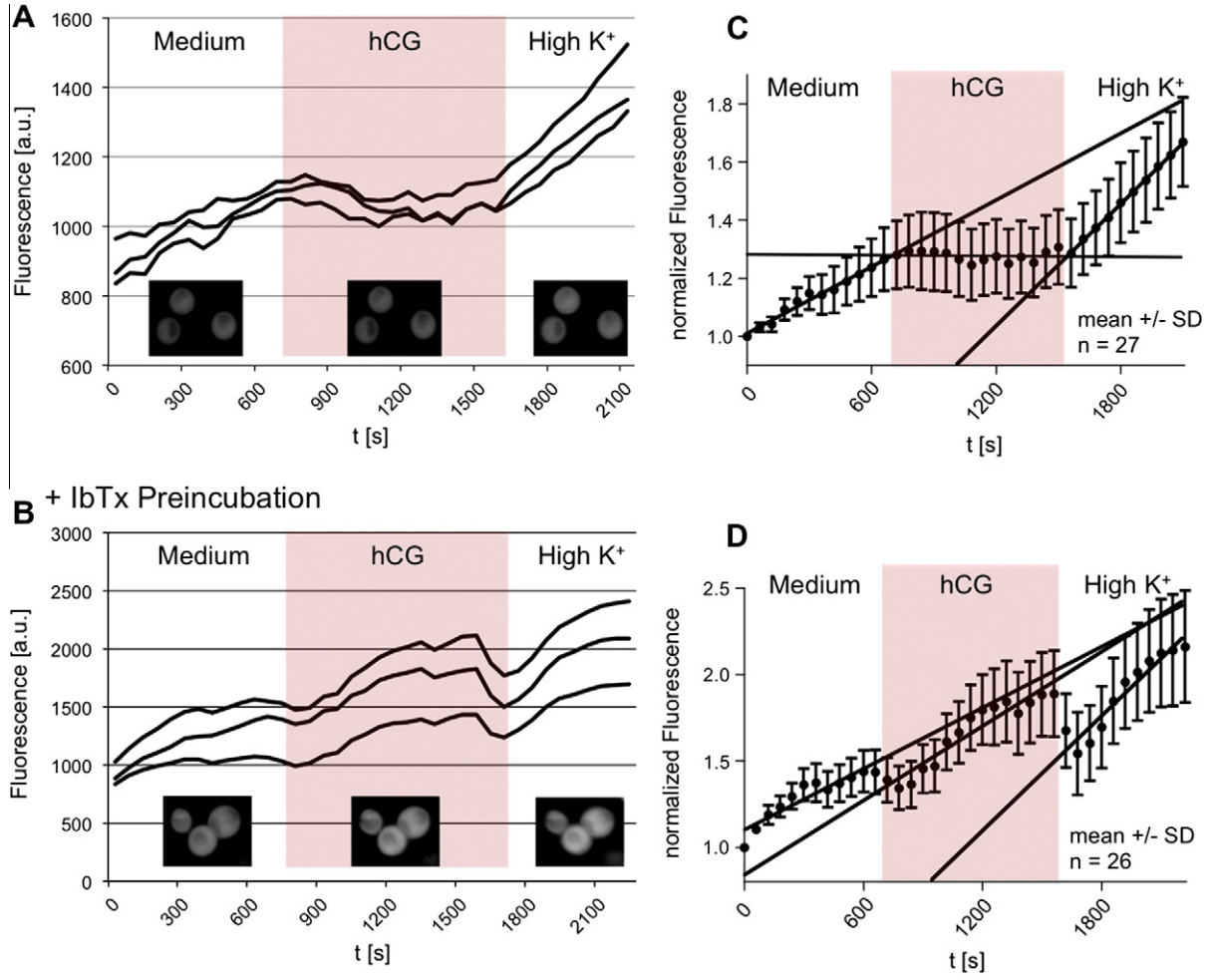

Fig. 3. Monitoring of membrane potential changes by fluorescence imaging. A and B: Representative examples of membrane potential changes in three Leydig cells monitored during stimulation with hCG. Hamster Leydig cells were loaded with $DiBAC_4(3)$ dye and fluorescence intensity changes were measured over time. The Leydig cells depicted in A show hyperpolarization upon stimulation with hCG. Depolarization was induced by the high K^+ solution. The Leydig cells in B were imaged after 60 min preincubation with 100 nM IbTx. In contrast to A, the slope remained unchanged by hCG stimulation, indicating that hyperpolarization was not induced by hCG. As in A, a strong depolarization (positive slope) was induced by the high K^+ solution. Note that slope scales differ between A and B. C and D: Mean fluorescence intensity values (±S.D.) normalized to starting values for one control experiment with 27 individual cells (C) and one experiment with 26 cells preincubated with IbTx (D). The depicted lines correspond to the results of linear regression for the three different treatment periods with medium, hCG and high K^+.

not alter StAR mRNA expression and testosterone production under basal conditions, but significantly further stimulated hCG-induced StAR expression and testosterone release into the incubation media (Fig. 4A and B, respectively). When the submaximal concentration of 5 mIU/ml of hGC was used, IbTX also increased the effects of hCG (Fig. 4C). When hCG was replaced by db-cAMP the increase in testosteron was also further stimulated by IbTx (Fig. 5A). No significant changes were observed in the basal and hCG-stimulated production of testosterone when hamster Leydig cells were incubated in the presence of 50 nM PaTx-2, which is a peptide toxin selectively blocking $K_V4.2$- and $K_V4.3$-channels (Fig. 5B).

4. Discussion

Binding of LH/hCG to the LH-receptor transmits a signal through G-proteins to adenylate cyclase to increase cytosolic cAMP, which would then stimulate steroidogenesis. However, several steps of steroid formation require sufficiently high intracellular levels of Ca^{2+} and indeed LH-receptor activation also entails increases in Ca^{2+} influx, via activation of voltage dependent Ca^{2+}-channels (Kumar et al., 1994; Costa and Varanda, 2007). Furthermore, activation of the LH-receptor of Leydig cells, causes changes in the membrane potential, namely both depolarization and hyperpolarization events, by a coordinated action of several ion channel types.

Previous electrophysiological studies in mouse and rat Leydig cells revealed conductances that strongly suggested the existence of both, voltage dependent Ca^{2+}-channels and large-conductance Ca^{2+}-dependent K^+-channels, corresponding to BK_{Ca} (Kawa, 1987; Joffre et al., 1984; Carnio and Varanda, 1995). Both were functionally interlinked and activated upon hCG treatment (Joffre et al., 1984; Carnio and Varanda, 1995). Based on these reports, our study explored the specific contribution of the prominent Ca^{2+}-dependent K^+-channel BK_{Ca} to steroidogenesis of Leydig cells.

First we identified BK_{Ca} at the molecular level by immunohistochemistry and then by PCR in Leydig cells of the human and the hamster testis. Although some interstitial cells are positively stained in testes of immature hamsters, BK_{Ca} becomes strongly expressed in Leydig cells only after they acquire the ability to produce androgens, i.e. androstane-3α, 17β-diol and testosterone, respectively (Frungieri et al., 1999). Thus BK_{Ca} was clearly identified in hamster Leydig cells from prepuberty to adulthood and in human Leydig cells, suggesting a potential role of these channels in the production of androgens. In addition, in the adult hamster

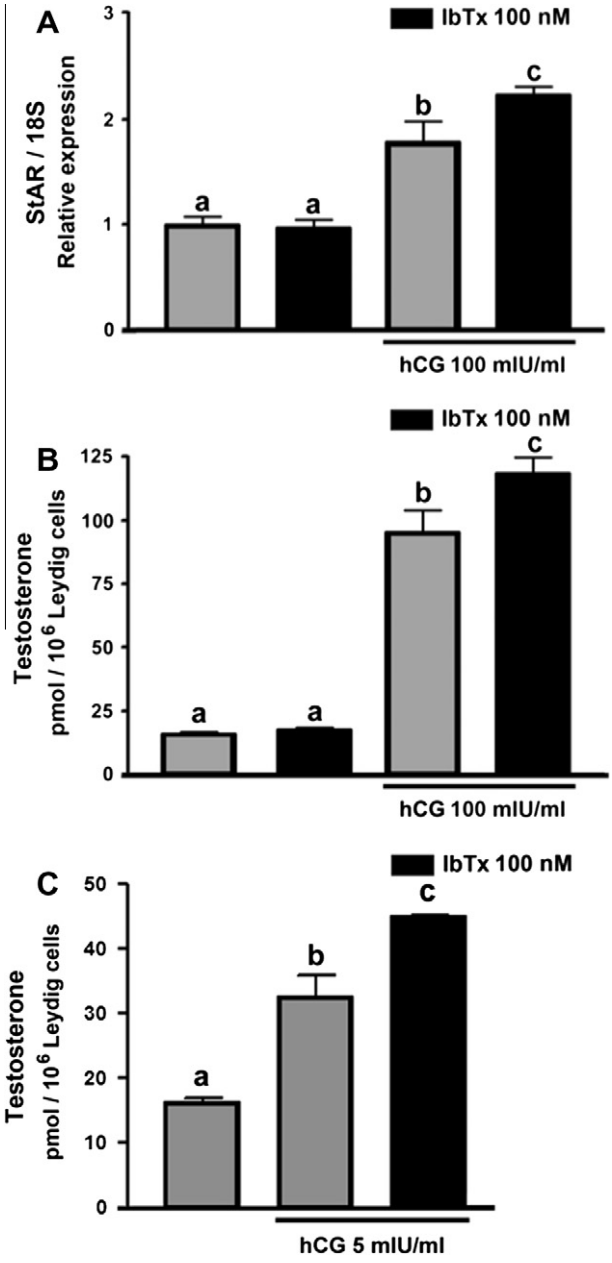

Fig. 4. Participation of BK_{Ca} in StAR mRNA expression and *in vitro* production of testosterone in adult hamster Leydig cells: effect of the selective BK_{Ca} blocker, IbTx. Effects of IbTx on basal and hCG-stimulated StAR mRNA expression (A) and testosterone production (B and C) in Leydig cells isolated from adult hamsters. In (A and B) a maximal hCG concentration (100 mIU/ml) was used, whereas in (C), a submaximal hCG dose (5 mIU/ml) was employed. Incubation time was 3 h. Bar plots show the mean ± SEM from three to five independent experiments (five to six replicates per experiment) performed in different cell preparations. All groups were compared; different letters above the bars denote a statistically significant difference between the groups ($P < 0.05$).

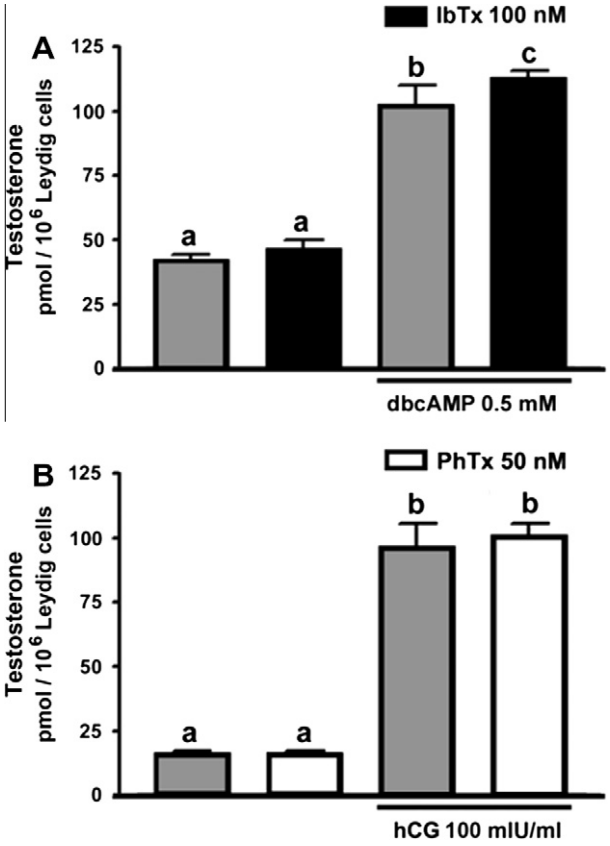

Fig. 5. Impact of IbTx vs PhTx-2 on *in vitro* production of testosterone stimulated by db-cAMP or hCG in adult hamster Leydig cells. Effects of IbTx on basal and db-cAMP-stimulated testosterone production (A) in Leydig cells isolated from adult hamsters. Effects of PhTx on basal and hCG-stimulated testosterone production (B) in Leydig cells isolated from adult hamsters. Incubation time was 3 h. Bar plots show the mean ± SEM from five to six replicates per experiment. All groups were compared; different letters above the bars denote a statistically significant difference between the groups ($P < 0.05$).

also germ cells stained for BK_{Ca}, in agreement with an electrophysiological study (Gong et al., 2002).

The molecular characterization of BK_{Ca} in Leydig cells by PCR and sequence analyses revealed the α-subunit, which forms the channel pore and the regulatory β1-subunit. The latter makes BK_{Ca} fully sensitive to nanomolar concentrations of IbTx (Meera et al., 2000). Thus at the molecular level BK_{Ca} of the hamster Leydig cells consists of the pore and at least one additional subunit.

Next we explored whether and how BK_{Ca} may be involved in the function of Leydig cells using the specific toxin IbTx as a tool. IbTx did not alter membrane potential, basal testosterone output or StAR levels, implying that BK_{Ca} in unstimulated Leydig cells is not active. Yet upon activation of the LH-receptor, significant contributions of BK_{Ca} to the regulation of the Leydig cell membrane potential and surprisingly to testosterone production and StAR became apparent. The use of IbTx enabled us to pinpoint these contributions. The toxin PhTx-2, which targets voltage dependent K^+-channels expressed by Leydig cells (our unpublished results) served as a control in some of these studies and absence of actions further support the conclusions of a specific role of BK_{Ca}.

We used a fluorescence dye to monitor the membrane potential in Leydig cells. This dye does not allow to monitor rapid membrane potential changes, but enabled us to monitor many Leydig cells simultaneously. We found that hCG treatment resulted in the robust hyperpolarization of the membrane of Leydig cells in hamster Leydig cells. This is in line with previous data in mice and rats (Joffre et al., 1984; Carnio and Varanda, 1995). Hyperpolarization is a typical consequence of BK_{Ca} activation, as previously described in steroid-producing human GCs (Kunz et al., 2002) and like in those cells it was IbTx-sensitive in Leydig cells.

Ca^{2+}-dependent K^+-channels are hardly activated unless the internal Ca^{2+} concentration increases. Therefore, in Leydig cells, activation of Ca^{2+}-dependent K^+-channel has been postulated to be coupled to open Ca^{2+}-channels and to the influx of Ca^{2+} (Kawa, 1987; Joffre et al., 1984; Carnio and Varanda, 1995).

We did not monitor level of intracellular Ca^{2+}, which based on previous results may occur through at least two types of voltage-

dependent Ca^{2+}-channels (Desaphy et al., 1996; Pandey et al., 2010; Lee et al., 2010, 2011). We assume however that raised intracellular Ca^{2+} most likely activated BK_{Ca} resulting then in hyperpolarization of the Leydig cell membrane. This in turn may result in the inactivation of voltage-regulated Ca^{2+}-channels and thus in the cessation of Ca^{2+} influx. A summary view of the assumed interaction between LH-receptor-induced depolarization, BK_{Ca}-induced hyperpolarization, Ca^{2+} and steroidproduction in Leydig cells is depicted in Fig. 6.

An interplay of a membrane receptor (LH-receptor), setting in motion events resulting in regulation of ion channel activity, intracellular Ca^{2+} and steroid production, is in agreement with a recently postulated model for an oscillatory electrical regulation of adrenal steroidogenic zona glomerulosa cells (Guagliardo et al., 2012). To what degree the postulated model for adrenal cells may be applicable to testicular Leydig cells, cannot yet be decided. Missing crucial components must first be identified and specific characteristics of Leydig cells must be considered, including the significant contribution of a Na^+/K^+ pump to the generation of the resting membrane potential (del Corsso and Varanda, 2003). Also it remains to be shown, how LH-receptor activation leads to membrane depolarization, which would be required to open voltage-regulated Ca^{2+}-channels, and which ones are involved.

Clearly, the specific involvement of Ca^{2+}-channels to steroidogenesis in Leydig cells remains to be elucidated. Blockage of T-type Ca^{2+}-channels by mibefradil in mouse primary Leydig cells reduced the augmenting actions of hCG on StAR and testosterone (Lee et al., 2010). Assuming an influx of Ca^{2+} via this route, this would be in agreement with a favoring role of intracellular Ca^{2+} in the process of steroid formation.

Yet the pharmacological blockage of L-type Ca^{2+}-channels by nifedipine increased c-AMP stimulated StAR and progesterone production in MA-10 Leydig tumor cells (Pandey et al., 2010). Although not tested, consequently the influx of Ca^{2+} should be blocked or reduced and BK_{Ca} would not be activated. The sustained depolarization due to inactive BK_{Ca} could then further promote steroid production, as our results show. We indeed found increased StAR expression and testosterone production in primary hamster Leydig cells, when IbTx was added to either a low and a maximum-stimulatory concentration of hCG, i.e. when BK_{Ca} was blocked. Whether in such a scenario an influx of Ca^{2+} may occur via T-type Ca^{2+}-channels, remains to show.

Taken together, a close coordinated interaction between voltage-dependent, presumably L-type Ca^{2+}-channels and BK_{Ca} in Leydig cells is strongly suggested and blockage of either one favors steroidogenesis and StAR expression. Although details of the assumed channel interactions remain to be shown, the results imply an unexpected and important role of dynamic membrane potential changes, in the regulation of steroid production.

Patch-clamp studies, which identified BK_{Ca} in Leydig cells, had also revealed that cAMP can substitute for hCG, i.e. LH-receptor activation, and can activate the typical BK_{Ca} conductance (Carnio and Varanda, 1995). Our results show that db-cAMP-treatment in the presence of IbTX evokes the same effect as hCG/IbTX and enhances testosterone production. This is in line with the ability of cAMP to activate BK_{Ca}. It is possible, for example that cAMP may open an unknown ion channel, possibly a Cyclic nucleotide-gated (CNG) ion channel (Biel and Michalakis, 2009), thereby depolarizing the membrane. A thorough investigation addressing this mode of action is, however, beyond the scope of the present investigation.

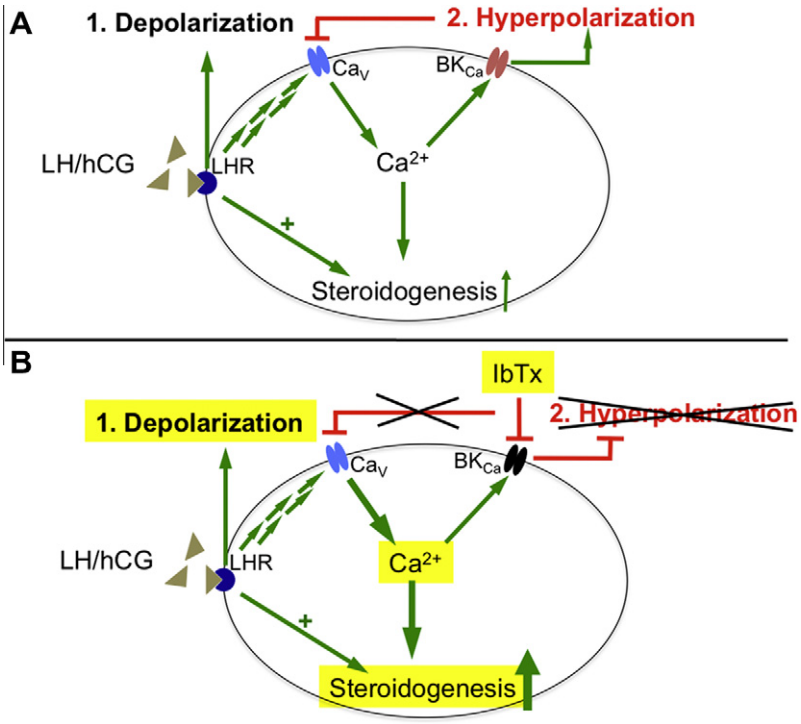

Fig. 6. A summary view of the assumed interactions between LH-receptor, ion channels, Ca^{2+} and steroidogenesis in Leydig cells, pinpointing a role of BK_{Ca}. (A) Activation of the LH-membrane receptor sets in motion events, which result in a dynamic, presumably cyclic regulation of ion channel activities. In a first step (1) depolarization of the membrane, evoked by unknown mechanisms, occurs and causes influx of Ca^{2+} via open voltage-dependent Ca^{2+}-channels. Elevations of intracellular Ca^{2+} is important for steroid production and, in addition, activates BK_{Ca}, resulting in (2) hyperpolarization of the membrane. This causes cessation of influx of Ca^{2+} via voltage-dependent Ca^{2+}-channels. This second step consequently slows steroidogenic processes. However, voltage-dependent Ca^{2+}-channels can become active again in the next cycle, which starts with membrane depolarization. (B) Hypothetical model, based on the experimental results of the present study: Blockage of BK_{Ca} by IbTx abolishes the second step, membrane hyperpolarization and increases testosterone output. A possible explanation is that the lack of hyperpolarization allows the continued influx of Ca^{2+} resulting in increased steroid output. This pinpoints an important role of BK_{Ca} and hyperpolarization in steroid production of Leydig cells.

In conclusion, activation of BK_{Ca} is a hitherto unrecognized part of the complex signaling pathway triggered by LH-receptor activation in hamster Leydig cells. Most likely, through the interplay with Ca^{2+}-channels and Ca^{2+}, BK_{Ca} contributes significantly to Leydig cell steroidogenesis. Finally, although we identified for the first time BK_{Ca} in Leydig cells of the human testis, it remains unclear whether the results obtained in Leydig cells of the Syrian hamster can be extrapolated to the regulation of the steroidogenic process in the human testis. If so, changes in ion channels of Leydig cells, including variations, mutations as well as their numbers, may be related to impaired Leydig cell function, often seen in infertile men (Giagulli and Vermeulen, 1988).

Disclosure

The authors have nothing to disclose.

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