Erg K$^+$ channels modulate contractile activity in the bovine epididymal duct

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Mewe M, Wulfse I, Schuster AME, Middendorff R, Glassmeier G, Schwarz JR, Bauer CK. Erg K$^+$ channels modulate contractile activity in the bovine epididymal duct. Am J Physiol Regul Integr Comp Physiol 294: R895–R904, 2008. First published January 9, 2008; doi:10.1152/ajpregu.00521.2007.—The expression and functional role of ether-à-go-go-related gene (erg) K$^+$ channels were examined in the bovine epididymal duct. Sperm transit through the epididymal duct relies on spontaneous phasic contractions (SC) of the peritubular smooth muscle wall. Isometric tension studies revealed SC-enhancing effects of the erg channel blockers E-4031, dofetilide, cisapride, and haloperidol and SC-suppressing effects of the activator NS-1643. In the corpus epididymidis, EC50 values of 32 nM and 8.3 nM were determined for E-4031 and NS-1643, respectively. E-4031 was also able to elicit contraction in epithelium-denuded segments, which lacked SC. In the cauda region, E-4031 and NS-1643 exerted effects on agonist-induced contraction similar to those observed in the proximal duct. Experiments with nifedipine and thapsigargin suggested that the excitatory effects of E-4031 depended mainly on external calcium influx and not on intracellular calcium release. Western blot and RT-PCR assays revealed the expression of both, erg1a and erg1b, in all duct regions. Because erg1b appears to predominate in the epididymal duct, patch-clamp experiments were performed on heterologously expressed erg1b channels to investigate the sensitivity of this splice variant to NS-1643. In contrast to its effects on erg1a, NS-1643 induced a concentration-dependent current increase mainly due to a marked leftward shift in erg1b channel activation by ~30 mV at 10 μM, explaining the inhibitory effect of the drug on epididymal SC. In summary, these data provide strong evidence for a physiological role of erg1 channels in regulating epididymal motility patterns.

IN SMOOTH MUSCLES, voltage-gated K$^+$ (Kv) channels play an important role in the control of contraction by contributing to the maintenance of the resting membrane potential and excitability (see Ref. 17 for review). Whereas in skeletal muscle cells the resting potential is mediated by classical inward-rectifying K$^+$ channels, in smooth muscle cells (SMC), ether-à-go-go-related gene (erg) K$^+$ channels are often involved (see Ref. 34 for review). Three erg channels have been cloned (erg1 (39), erg2, and erg3 (35); Kv11.1–3), forming a subfamily of the ether-à-go-go K$^+$ channels. There is accumulating evidence that erg1 channels modulate the electrical and contractile activities in smooth muscle and heart, whereas erg2 and erg3 are suggested to be widely “brain-specific” (35). Distinctive features of erg currents are marked inward rectification, a high sensitivity to methanesulfonanilides such as the class III antiarrhythmic agent E-4031, and an increased conductance in high extracellular K$^+$ (31, 36, 38). Recently, a specific activator of the human ortholog of erg1 (HERG) channels, 1,3-bis-(2-hydroxy-5-trifluoromethyl-phenyl)-urea (NS-1643), was described (11), which mainly acts via reduced channel inactivation (4).

The best-known function of an erg1 current is its contribution to the repolarization of the cardiac action potential (31). E-4031-sensitive currents have also been detected in SMC of the esophagus (1), portal vein (28), gallbladder (29), stomach (27) and intestine (8, 18, 20). It is assumed that, in SMC, erg currents serve the function of threshold currents; a pharmacological block of erg channels depolarizes the membrane potential, which is correlated with muscle contraction (see Ref. 34 for review). Within the intestinal musculature, erg channels are concentrated on the intestinal cells of Cajal and may play a crucial role in regulating their pacemaker frequency (41). Likewise, multiple erg1 isoforms may constitute the molecular basis of pacemaker activity in sinoatrial node myocytes (5).

In the bull, passage of sperm through the epididymal duct occurs by means of spontaneous (caput and corpus region) and induced (cauda) contractions of peritubular SMC (23). In the present study, the functional impact of erg channels on epididymal contractile activity was investigated. Using tension studies, RT-PCR, and immunological analyses, we demonstrate expression and functional activity of erg1 channels in SMC of all regions of the epididymal duct. This finding, in conjunction with the newly described pharmacological properties of heterologously expressed erg1b channels, suggests that erg1 currents form a crucial determinant of epididymal SMC membrane excitability and therefore sperm transport.

METHODS

Tissue preparation. Bovine epididymal tissue was obtained from sexually mature bulls in a local slaughterhouse. The epididymes were removed after exsanguination and immediately placed in ice-cold Ca$^{2+}$-free Hanks’ balanced salt solution (GIBCO-BRL, Karlsruhe, Germany) for transport to the laboratory. Freshly prepared tissue samples of different regions of the epididymis [caput-corpus-cauda (22)] were fixed in Bouin’s fluid for 24 h at 20°C for immunohistochemical analyses or frozen in liquid nitrogen for RT-PCR and Western blot experiments. For tension studies, segments of the epididymal duct were separated by carefully dissecting the surrounding tissue. For isolated muscle wall preparations, the epididymal epithelium was removed by perfusion of duct segments with 1% Triton X-100 (Merck-Calbiochem, Darmstadt, Germany) in PBS for 3 min

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using a Hamilton-syringe as described previously (23). Until use in
tissue bath assays, the segments were stored in Dulbecco’s minimal
essential medium (DMEM; GIBCO) at 4°C. For positive controls,
Lewis rats weighing 300–350 g were used. The epididymides were
quickly excised after anesthetizing the animals with an intraperitoneal
injection of sodium thiopental (100 mg/kg body wt) and killing them
by exsanguination. Animal care and experimental procedures were
reviewed and approved by the animal welfare committee of the
University Medical Center Hamburg-Eppendorf (UKE).

Cell culture. HEK-293 and Chinese hamster ovary (CHO) cells
were cultured in DMEM (GIBCO) supplemented with 1% penicillin-
 streptomycin-glutamine (GIBCO) and 10% FCS (Biotech, Kelkheim,
Germany) at 37°C in a humidified incubator (95% air-5% CO2).
The culture medium was changed every 2–3 days, and the cells were
passaged when they reached confluence.

Heterologous expression. HEK-293 cells were plated on poly-t-
lysine (Sigma, Deisenhofen, Germany)-coated glass cover slips in
35-mm plastic culture dishes (Nunc) and microinjected with an
Eppendorf Transjector 5246 (Eppendorf) with cDNA encoding rat
erg1b (20 ng/μl; GenBank database acc. no. AY669863; see Ref. 12)
cloned into the pcDNA3 vector (Invitrogen, Karlsruhe, Germany). To
allow detection of successfully expressing cells, 10 ng/μl enhanced green
fluorescent protein-N1 (Clontech, Heidelberg, Germany) was
cross-linked. For immunological assays, CHO cells were plated on 14-cm
plastic culture dishes and transfected with rerg1a (30 μg/plate; acc. no.
Z96106; see Ref. 3) or rerg1b (30 μg/plate) using lipofectamine 2000
(Invitrogen) according to the manufacturer’s instructions.

Tension studies. Segments of different regions of the epididymal
duct were mounted on two hooks in a double-jacketed organ bath
(volume, 15 ml) filled with modified Krebs bicarbonate Ringer (KBR).
Isometric contractions were measured at 33–34°C using a
SG4–90 force-displacement transducer (Hugo Sachs, Freiburg,
Germany). The output of the transducer was digitized at 0.5 Hz. Data
collection was carried out using the BIO PAC MP35 data acquisition
unit and the Biopac Student Lab PRO software (Biopac Systems,
Santa Barbara, CA). Further data processing was performed with
Excel (Microsoft, Redmond, WA) and Sigma Plot 7.101 (SPSS,
Chicago, IL). Segments from the cauda were stretched incrementally
to a preload tension of ~10 mN, and segments from the proximal ductal
regions (caput and corpus) were stretched to a preload tension of ~6
and 3 mN in the case of Triton X-100-prepared material. Prepara-
tions were allowed to equilibrate for 60 min and to relax to a
steady-state resting tension. Contraction frequency and amplitude
were averaged over periods of 5 min before and after application of
the substances.

RT-PCR. RNAs were extracted from the frozen tissues using
RNAzol B (AGS, Heidelberg, Germany). Total RNA (1 μg) was
employed for oligo(dT)-primed reverse transcription using Moloney
murine leukemia virus reverse transcriptase (Invitrogen). The cDNAs
were amplified with 1.25 units of Taq DNA polymerase (Stratagene,
Amsterdam, Netherlands), 1.5 mM MgCl2, and 0.2 mM of each dNTP
in 50 μl reaction assays using 5 pmol of forward and reverse
oligonucleotide primers specific for the different bovine erg (b-erg)
family members. For amplification, a predenaturation step at 94°C for
1 min was used followed by 40 cycles consisting of three temperature
steps (first step: 94°C; second step: annealing temperature different
for each primer pair; third step: 72°C; each temperature step lasted
for 1 min) terminated by an elongation step at 72°C for 5 min. The
annealing temperature was calculated for every primer pair from
the G/C and A/T content of the oligonucleotide primers used in the
reaction. Amplified DNA fragments were analyzed by agarose gel
electrophoresis and sequencing. For the PCR amplifications,
the following oligonucleotide primer sequences were employed (with the
GenBank database accession nos. in brackets): b-erg1: nucleotides
31947–34144 (acc. no. NW_001494963 region 249880–284452);
forward 5′-GGTGGGGGACCCCCCTGACCC-3′ and reverse 5′-
GTCTGAGCGCATGTCTGTCGA-3′; b-erg1a: nucleotides 26–355 (acc.
no. NM_001099101); forward 5′-ATGCCGTTGCGGAGGGGCGCA-3′
and reverse 5′-CACCAGCACAGGAGGACGC-3′; b-erg1b: nucleotides
24974–27976 (acc. no. NW_001494963 region 249880–284452);
forward 5′-ATGCCGCTCCGGTTGGGGA-3′ and reverse 5′-GCT-
TCCGGGCAACGTCACC-3′; b-erg2: nucleotides 542–836 (acc. no.
XM_867795): forward 5′-GGTCGGGGGACAGGGGCC-3′ and reverse
5′-CCTTGAATGGGGCTGATGC-3′; and b-erg3 nucleotides
85492–100676 (acc. no. NW_001494594 region 42079–67990): for-
ward 5′-AACAAGAAAGACTGCAGCC-3′ and reverse 5′-AACCT-
TGGGCACAATTG-3′. Analysis using H2O instead of template
served as negative control.

Western blotting. Membrane preparations of the bovine and rat
epididymis as well as rat erg1a- or erg1b-overexpressing CHO cells
were prepared as follows. Tissues or cells were washed with cold
PBS, collected, and homogenized in lysis buffer (25 mM Tris-HCl,
250 mM sucrose, 20 mM EDTA; protease inhibitors: 2 μg/ml leu-
peptin, aprotinin, and pepstatin A (Sigma), 0.1 mM phenylmethylsul-
fonyl fluoride (Sigma), 2 mM NaOAc (Merck-Calbiochem); pH 7.2)
on ice by the use of a glass-Teflon hand homogenizer (Braun,
Melsungen, Germany). After incubation on ice for 10 min, samples
were centrifuged for 5 min at 800 g at 4°C. Cell debris was removed,
and the supernatant was centrifuged again for 30 min at 48,000 g. The
pellets containing the cell membranes were resuspended in lysis
buffer and stored at ~80°C until SDS-PAGE separation and immu-
noblotting. Membrane and tissue samples (50 μg) were fractionated on
10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes
were blocked with 5% lowfat milk powder in buffer containing 20
mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20 and then incubated
with the specific antibody for erg1 (Chemicon, Hampshire, UK) in a
1:1000 dilution overnight. The anti-erg1 antibody is targeted against
the COOH terminus of HERG1 (amino acids 1145–1159), and rec-
ognizes bands at 175/205 kDa (representing glycosylated and
deglycosylated erg1a, respectively) as well as at 95 kDa (repre-
senting erg1b) (30). Detection of immunoreactivity was carried out
with the ECL Plus Western blotting detection system (Amersham
Pharmacia, Freiburg, Germany).

Immunohistochemistry. Immunohistochemical analyses were per-
formed essentially as described previously (25). In brief, paraffin
sections 5 μm thick were mounted on chrome-alum-coated slides and
incubated with the rabbit polyclonal anti-erg1 antibody (1:100)
night. For visualization of immunoreactivity, a combination of the
peroxidase-antiperoxidase technique and the avidin-biotin-peroxidase
complex method was used (6). Biotinylated anti-rabbit IgG (Dako,
Hamburg, Germany) was used as secondary antibody. Peroxidase
activity was visualized by the nickel glucose oxidase technique. For
negative controls, the primary antibody was omitted to indicate any
nonspecific binding of the secondary antibody.

Electrophysiology. Membrane currents in erg1b-expressing HEK-
293 cells were recorded in the conventional and the nystatin-perfo-
rated-patch whole cell configuration of the patch-clamp technique (10,
13). The patch electrodes were made from 1.5-mm-diameter borosili-
cate glass capillaries with resistances of 2.5–4 MΩ. Data were
low-pass filtered at 3 kHz and compensated for both fast- and
slow-capacity transients before the pulse protocols. The access resis-
tance ranged between 6 and 29 MΩ, and series resistance compensa-
tion was as high as possible (60–85%). Data were corrected for the
liquid junction potential error in perforated-patch experiments (~12
mV), but not in conventional whole cell experiments (~4 mV). All
experiments were performed at room temperature. Cells were contin-
uously perfused with 2 ml/min Ringer solution using a peristaltic
pump (custom made by T. Homfeldt, UKE). An EPC-9 patch-clamp
amplifier was used in combination with the PULSE stimulation and
data acquisition software (HEKA, Lampecht, Germany).

Solutions and chemicals. The modified KBR used in the tension
studies contained (in mM): 118 NaCl, 4.75 KCl, 1.2 KH2PO4, 1.2
MgSO4, 2.5 CaCl2, 25 NaHCO3, and 11 p-glucose continuously
gassed with carbogen to establish a pH of 7.3–7.4. For all electro-

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physiological experiments, the external Ringer solution contained (in mM): 140 NaCl, 5 KCl, 0.8 MgCl2, 1 CaCl2, 10 HEPES, and 5 glucose, with pH adjusted to 7.3 with NaOH. The standard pipette solution contained (in mM): 140 KCl, 2 MgCl2, 1 CaCl2, 10 HEPES, and 2.5 EGTA (EQCAL: 66 nM free Ca2+), with pH adjusted to 7.3 with KOH. For perforated-patch experiments, the whole amount of KCl was replaced by potassium gluconate (130 mM) and NaCl (10 mM). Nystatin [dissolved in dimethyl sulfoxide (DMSO)] was added to the pipette solution with a final concentration of 0.24 mg/ml. KCl was replaced by potassium gluconate (130 mM) and NaCl (10 mM). Stocks of NS-1643, dofetilide, and haloperidol were prepared in DMSO, cisapride, and thapsigargin in ethanol. The solvents had no effect on epididymal contractile activity in their highest final concentration.

Data analysis. Experimental data are given as means ± SE, with n representing the number of cells or tissue samples derived from different animals. Student’s two-tailed paired t-test was used to assess statistical significance (*P < 0.05 and **P < 0.01). Nonlinear regression analysis of isometric force data was used to create sigmoidal dose-response curves and to calculate EC50 values using GraphPad Prism 4 (GraphPad Software, San Diego, CA). Processing of the electrophysiological data was performed with PulseFit 8.65 (HEKA), Excel, and Sigma Plot 7.101. The voltage dependence of channel activation was fitted with a Boltzmann equation: y = 1/[1 + exp{−(V − V0.5)/k}], where V0.5 is the potential of half-maximal erg current amplitude and k is the slope factor.

RESULTS

Effects of E-4031 and NS-1643 on epididymal contractility. Isometric force recordings were performed to investigate the functional role of erg channels in the epididymal duct. Under resting tone conditions, application of the selective erg channel blocker E-4031 enhanced spontaneous phasic contractions (SC) in both the caput (Fig. 1B) and the corpus epididymidis (Fig. 1Aa). At a concentration of 500 nM, a mean increase in SC amplitude by 51.3 ± 3.8% (n = 6, P < 0.05) and a mean reduction in SC frequency by 38.1 ± 4.3% (n = 6, P < 0.01) was observed in the proximal duct, when a steady state was approached. In Fig. 1Ab, the concentration-response relationship for E-4031 in the corpus region is shown, yielding EC50 values of 32 and 88 nM for the increase in SC amplitude and the decrease in SC frequency, respectively (n = 4). The SC modulatory effects of E-4031 were always accompanied by an abrupt and transient increase in tone particularly pronounced at the level of the caput.

In normally “quiescent” stretch-relaxed segments from the midcauda [cauda (m)] region, 500 nM E-4031 elicited a monophasic contraction within 10 min after application in 6 of 10 experiments (data not shown). OT (500 nM), which was recently found to evoke strong contraction in the epididymal cauda region (24), was applied at the end of each experiment to check intactness of the epididymal musculature. To test the ability of E-4031 to affect contraction in the cauda (m), the channel blocker was applied after application of OT (500 nM). As demonstrated in Fig. 1C, E-4031 (500 nM) reinforced the OT-induced contraction. This effect was consistently observed and characterized by an increase in contraction amplitude by 46.7 ± 3.9% (P < 0.01) and a decrease in contraction frequency by 33.8 ± 5.1% (n = 4). Interestingly, E-4031 never induced an increase in basal tone in cauda (m) segments.

To verify that an inhibition of erg channels underlies the E-4031 effects in the epididymal duct, we employed the class III antiarrhythmic agent dofetilide, the gastrointestinal prokri-
netic agent cisapride, and the dopamine receptor blocker haloperidol. All of these drugs have been shown to inhibit HERG channels (see Ref. 32 for review), and actually mimicked the effect of E-4031 in the corpus (Fig. 2, Aa and Ab). At a concentration of 500 nM, the respective increases in SC amplitude and decreases in SC frequency were 32.8 ± 4.2% and 55.1 ± 2.1% for dofetilide (n = 3), 35.9 ± 2.9% and 42.2 ± 1.2% for cisapride (P < 0.01, n = 4), and 18.1 ± 2.7% and 33.2 ± 2.7% for haloperidol (P < 0.05, n = 3). In the cauda (m), application of 500 nM cisapride consistently induced phasic contractions without altering basal tone (n = 3; Fig. 2B).

To investigate whether the mechanisms of E-4031-induced contraction in the epididymal duct require epithelial factors, experiments were performed with epithelium-denuded corpus segments that lacked phasic activity, as previously shown (23). At a concentration of 500 nM, the drug was able to elicit temporary contraction in these muscle preparations (n = 3; Fig. 3A), indicating localization and functional relevance of erg channels in the proximal duct peritubular muscle wall. Further pharmacological analysis using thapsigargin and nifedipine was carried out in another series of experiments with intact corpus segments to test the action on SMC calcium handling by E-4031. Figure 3Ba demonstrates that the SC modulatory effects of the blocker (500 nM) after prior application of the sarcoplasmic reticulum (SR) Ca²⁺-ATPase (SERCA) pump inhibitor thapsigargin (2 μM, 20 min) were very similar compared with the control. The SC amplitude was increased by 52.8 ± 6.9% and the SC frequency decreased by 34.8 ± 2.9% (n = 4). However, E-4031 hardly affected the basal tone any more. The acute effects of thapsigargin consisted of a reduction in tone and SC amplitude, whereas cumulative application of the L-type Ca²⁺ channel blocker nifedipine (10 μM) had no effect on tone but totally suppressed phasic activity. Likewise, in the cauda (m), the excitatory effect of OT (500 nM)- and the E-4031 (500 nM)-induced amplification of OT-evoked contraction after prior application of thapsigargin (2 μM) resembled that in the control (Fig. 3Bb). The E-4031-induced increase in SC amplitude and decrease in SC frequency amounted to 52.8 ± 6.9% (P < 0.05) and 34.8 ± 2.9% (P < 0.05), respectively (n = 4). The channel blocker again did not affect basal tone; however, the OT-induced increase in tone was clearly reduced in the presence of the SR Ca²⁺ uptake inhibitor. Similar to its effect in the corpus, nifedipine (10 μM) totally suppressed rhythmic activity in the cauda (m) without significantly affecting tone. The same nifedipine effects were observed without prior application of thapsigargin (n = 4, data not shown).

In the following, we studied the effects of the HERG channel activator NS-1643 on epididymal spontaneous and agonist-induced contractions. As demonstrated in Fig. 4, Aa and B, 30 μM of the drug totally suppressed spontaneous contractility in both the corpus (n = 6) and the caput region (n = 4) within 10 min after application. The EC50 value for NS-1643-induced suppression of phasic contractile amplitude in the corpus was 8.3 μM (n = 4; see Fig. 4Ab). To test whether erg channel activation could also affect receptor-mediated contraction, we investigated the effect of NS-1643 on OT-pretreated cauda (m) segments. As shown in Fig. 4C, application of 30 μM NS-1643 resulted in an abrupt and persisting suppression of the OT (500 nM)-induced rhythmic activity, again accompanied by a decrease in basal tone.

**Epididymal expression of erg1 protein and transcripts.** Western blotting was performed to confirm the presence of erg1 proteins in the epididymal duct. An antibody against the COOH-terminal peptide fragment of erg1 described by Pond et al. (30) was used to detect putative erg1a and erg1b subunits. Comparative immunoblotting assays clearly demonstrated erg1 protein expression in membrane fractions of all duct regions (n = 3). As shown in Fig. 5Aa, the anti-erg1 antibody recognized bands at ~160 kDa and at ~135 kDa, consistent with the estimated sizes of glycosylated and unglycosylated erg1a, respectively. In the corpus preparation, an additional band at ~170 kDa was detected. Moreover, immunoreactivity for erg1 was associated with a protein of ~90 kDa, possibly representing the b-erg1b subunit, an NH2-terminal splice variant of the erg1. Because in the caput and corpus region the putative erg1b band was clearly more marked than those of erg1a, a predominate of the erg1a isofrom in the proximal epididymal duct is possible.

Membrane fractions of the rat epididymal caput and cauda region as well as CHO cells transfected with erg1a or erg1b cDNA were used as positive controls and markers for the expected protein size of the erg1 subunits (Fig. 5Ab). In preparations of the CHO cells, single protein bands at the expected sizes of partially glycosylated erg1a (155 kDa) and glycosylated erg1b (~105 kDa) were detected. In the rat epididymal fractions, a ~114-kDa protein, probably representing a glycosylation form of the erg1b splice variant, and again a ~170-kDa

![Fig. 2. Effects of the ether-à-go-go-related gene (erg) channel blockers dofetilide and cisapride on epididymal contractility. Typical traces illustrating the contractile effects of dofetilide (Aa) in the corpus region as well as of cisapride at the level of the corpus (Ab) and the cauda (m) (B).](image-url)
protein were found. In addition, the antibody detected single bands at positions expected for unglycosylated erg1a (~135 kDa) in the cauda and for partially glycosylated erg1a (~145 kDa) in the caput region.

Immunohistochemical analysis was used to investigate the cellular distribution of erg1 protein in cross sections from all bovine epididymal duct regions. As shown for the corpus in Fig. 5B, immunoreactivity against the anti-erg1 antibody could be localized to the epididymal smooth muscle and epithelial principal cells as well as to associated blood vessels all along the duct. No staining was seen when the primary antibody was omitted.

To verify the molecular identity of the erg channel subunits in the bovine epididymal duct, conventional RT-PCR was performed using cDNA preparations from the caput, corpus, and cauda region as template. First, specific primers for the bovine erg1a and erg1b splice variants, b-erg1a and b-erg1b, were applied. As demonstrated in Fig. 5C, mRNA transcripts for both subunits were detected in all duct regions. We then examined the epididymal expression of all three erg channels. Specific primer pairs designed to detect transcripts of the bovine erg genes (b-erg1, b-erg2, and b-erg3) were used. Figure 5D shows that mRNA transcripts of the erg1 gene (590 bp) were easily found in all duct regions, whereas erg2 and erg3 signals could not be detected in the epididymis.

**Effects of NS-1643 on rerg1b currents.** Because erg1b is highly expressed in the epididymis and NS-1643 effects have only been described for the erg1a isoform (4, 11) and most...
recently for erg2 (7), we examined the effects of the erg channel activator on erg1b currents using HEK-293 cells as a heterologous expression system. Electrophysiological investigation of native erg channels turned out to be not feasible, which might be due to the strong dissociation procedure necessary to free the epididymal contractile cells from the substantial amount of connective tissue present in the lamina propria.

With the use of the conventional whole cell technique, modulation of erg1b activation by NS-1643 was investigated by 4-s depolarizing pulses to +40 mV followed by repolarization to −40 mV for 5 s from a holding potential of −80 mV every 20 s (Fig. 6A). Application of 10 μM NS-1643 resulted in a huge current increase. At the end of the experiment, E-4031 (10 μM) was applied, which resulted in a complete blockage of the erg current, confirming that NS-1643 specifically affected erg1b. The tail currents induced upon repolarization to −40 mV are also shown on expanded time scales. They are due to the fast recovery from inactivation followed by slower deactivation of the erg channels (36). The time course of the NS-1643 effect is illustrated in Fig. 6B. The erg1b peak tail current amplitude before and after application of 10 μM NS-1643 is given as a function of time, demonstrating a considerable current increase within 10 min after application of the channel activator. Figure 6C shows that the tail currents increased at all tested concentrations of the stimulator. The percentage of increase was 70 ± 8.5% (n = 4, P < 0.05) with 1 μM, 286 ± 31.4% (n = 7, P < 0.01) with 10 μM, and 237 ± 26.9% (n = 3, P < 0.05) with 30 μM NS-1643, indicating saturation in erg1b current increment in the range of 10–30 μM. By contrast, the steady-state current at +40 mV was not consistently increased.

To determine the effect of NS-1643 on the voltage dependence of erg1b current activation, erg currents were elicited with varying depolarizing pulses of 4-s duration before and after drug application. Starting from a holding potential of −60 mV, the HEK cells were clamped to −80 mV for 1 s. Test pulses to potentials between −80 and 60 mV were followed by a constant pulse to −40 mV for 5 s (Fig. 7Aa). When using 30 μM NS-1643, the cells were held at −80 mV, and test pulses ranged from −100 to 60 mV, since at this concentration the activator already evoked erg currents at −80 mV. Representative examples of erg1b current families elicited under control conditions and in the presence of 1 and 30 μM NS-1643 are shown in Fig. 7, Aa and Ab, respectively. In control experiments, outward currents were activated by depolarization to potentials more positive than about −40 mV. The amplitude of these currents increased with depolarizations up to 10 mV and then declined, resulting in a crossing of current traces at more positive potentials. After drug application, the activation threshold was shifted to more negative potentials.

The tail current amplitudes recorded at the constant repolarizing pulse to −40 mV were normalized and plotted against the prepulse potential, yielding the isochronal activation curve of the erg1b current. A Boltzmann function was fitted to the data yielding the potential at which the erg1b channels were half maximally activated. At all tested activator concentrations, the current-voltage curves were significantly shifted to negative potentials (see Fig. 7, Ba and Bb). The V0.5 values and slope factors determined were −0.6 ± 1.8 and 10.5 ± 0.3 mV for
control (n = 14), −10.5 ± 2 and 12.2 ± 1.9 mV for 1 μM NS-1643 (n = 4), −29.5 ± 6 and 12.2 ± 2.3 mV for 10 μM NS-1643 (n = 7), and −62.8 ± 3.4 and 11 ± 3.7 mV for 30 μM NS-1643 (n = 3).

Perforated-patch recordings confirmed the effects of 10 μM NS-1643 on erg1b current activation measured in the conventional whole cell mode. As demonstrated in Fig. 6C, the mean peak tail current amplitude increased by 164 ± 82% (n = 6, P < 0.05). Likewise, the current-voltage curve was significantly shifted to negative potentials. The V0.5 values and slope factors determined were −3.2 ± 1.4 and 11.1 ± 0.4 mV in the control vs. −44.9 ± 4.6 and 9.8 ± 0.6 mV after application of the channel activator (n = 11, P < 0.01; Fig. 7B).

DISCUSSION

The present study provides strong evidence for the expression of erg1 currents in the bovine epididymal duct and suggests an important role for erg1 currents in the control of electrical and mechanical activity in epididymal smooth muscle. Functional significance of erg channels was demonstrated by the excitatory effect of E-4031 and other known HERG blockers as well as by the inhibitory effect of NS-1643 on contractility all along the duct. RT-PCR and immunological approaches allowed amplification of erg1 mRNA transcripts and confirmed functional translation and successful incorporation into the membrane of both erg1 isoforms, erg1a and erg1b, in all duct regions. Using HEK-293 cells as an expression system, erg1b currents showed to be activated by NS-1643 in a unique voltage- and concentration-dependent manner.

Effects of blocking and activating erg channels. The selective erg channel blocker E-4031 exerted excitatory effects on the SC amplitude and inhibitory effects on SC frequency in the epididymal duct. The half-maximal effects of the methanesulfonanilide in the epididymal corpus region were 32 and 88 nM, which is in the range of the EC50 values reported previously for native erg channels (1, 28). Cisapride, dofetilide, and haloperidol, which have all been reported to block HERG (32), mimicked the contractile effects of E-4031, pointing to erg channels as a direct target and effector molecule of the drugs. The E-4031-induced concentration-dependent increase in epididymal SC amplitude may reflect an increase in action potentials per “slow wave.” E-4031 also increased the amplitude of spontaneous contractions in the guinea pig urinary bladder (14) and the rabbit small intestine (18), whereas in the human jejunum, high concentrations of the compound (1 μM) replaced phasic contractions with small tonic contractures due to the elimination of slow waves (8). The mechanism of the E-4031-evoked reduction in epididymal SC frequency is unknown, but the phenomenon equals the findings in the portal vein. Here, a block of erg channels is suggested to delay action potential repolarization and to decrease the activity of hyperpolarization-activated pacemaker channels, thereby prolonging intercontraction intervals (28).

E-4031 induced transient contraction in epithelium-denuded corpus segments, which lacked spontaneous phasic activity. The latter concurs with previous findings supporting a crucial role of epididymal epithelial factors for the generation of SC in the proximal duct (23). Moreover, E-4031 evoked monophasic and amplified OT-induced contraction in normally quiescent cauda (m) segments. In conjunction with the immunohistochemical localization of erg1 protein to the epididymal muscle
wall, it seems likely that E-4031 depolarizes the resting potential in epididymal SMC, thereby increasing their excitability. All spontaneous and OT-/E-4031-induced rhythmic activity in the epididymal duct was totally suppressed by nifedipine, pointing to an essential role of L-type Ca\(^{2+}\)/H\(\text{11001}\) channels in the generation of phasic contractions. An E-4031-dependent induction of SC was also observed in the quiescent esophageal circular smooth muscle, presumably due to an increase in intracellular Ca\(^{2+}\)/H\(\text{11001}\) concentration following the opening of voltage-dependent Ca\(^{2+}\)/H\(\text{11001}\) channels (1). By contrast, inhibition of SERCA pumps with thapsigargin showed to have no effect on OT-/E-4031-induced rhythmic contractions and only slightly reduced the SC amplitude, indicating that SR Ca\(^{2+}\)/H\(\text{11001}\) release is not obligatory for the induction and maintenance of epididymal rhythmic activity. Because the SR Ca\(^{2+}\)/H\(\text{11001}\) uptake inhibitor induced a loss of tone in the proximal duct and reduced the OT-induced increase in tone in the cauda (m), a tone-relevant role of SR Ca\(^{2+}\)/H\(\text{11001}\) release can be assumed. However, it remains to be elucidated why E-4031 affected basal tone only in the proximal duct and why the blocker produced only monophasic contraction in the cauda (m) when administered alone. Possibly, these data reflect regionally different actions of E-4031 on SMC Ca\(^{2+}\)/H\(\text{11001}\) handling and a higher threshold of excitability in the cauda region.

The SC-suppressive effects of NS-1643 confirmed the presence and functional role of erg channels in the epididymal duct. The EC\(_{50}\) value (8.3 \(\mu M\)) determined for the reduction in SC amplitude in the corpus was very similar to that found for HERG channel activation in oocytes (10.5 \(\mu M\); see Ref. 11). At a concentration of 30 \(\mu M\), the erg channel activator totally suppressed spontaneous rhythmic activity in the proximal duct regions as well as the OT-induced contraction in cauda (m) segments. This effect was unexpected in view of the fact that NS-1643 increases HERG currents by shifting the steady-state inactivation curve to more depolarized potentials without altering the activation threshold (4).

**Epididymal expression of erg channel subunits.** The results of the Western blots suggest the presence of the erg1 splice variants erg1a and erg1b in all regions of the bovine epididymal duct. The partially varying molecular weights of the erg1 subunits found in the rat and CHO preparations could indicate differences with respect to protein glycosylation or splicing of the **erg1** gene product. In addition, nonspecific binding of the used anti-erg1 antibody as well as posttranslational protein modifications other than glycosylation may account for a subset of the additional bands seen in the bovine and rat native tissue compared with transfected CHO cells. The higher expression level of the putative erg1b isoform compared with that of erg1a in the epididymal proximal duct regions may point to a relatively higher portion of homomultimeric erg1b channels or a higher percentage of erg1b subunits in heteromultimeric channels. A marked predominance of erg1b over erg1a tran...
scripts was also detected in portal vein myocytes (28). Erg1a and erg1b have been shown to be coexpressed in several tissues (9, 12, 19, 21, 28) and to form heteromultimeric channels in the heart (15). Heteromeric erg1 channels significantly differ from homomeric erg1 channels in their response to hormonal stimuli (16) and biophysical properties, such as the deactivation kinetics and steady-state inactivation (19, 21).

RT-PCR analyses by the use of specific erg primers, allowed amplification of mRNA transcripts for b-erg1, however, not for the two other Kv.11 family members b-erg2 and b-erg3 in the epididymis. The presence of erg1 mRNA has also been reported for other smooth muscles such as the esophagus, stomach, duodenum and urinary bladder (27), the portal vein (28), and the gallbladder (29). In accordance with the present findings, erg3 gene transcripts were absent from all of these tissues. Likewise, expression of erg2, which is also suggested to be widely brain-specific (35), was only detected in the gastric antrum (27). Interestingly, no RT-PCR signals of all erg subtypes could be detected in preparations from the rat vas deferens (27).

NS-1643 effects on heterologously expressed erg1b channels. NS-1643 has been described to enhance HERG (erg1a) currents by shifting the steady-state inactivation curve to more positive potentials without affecting the voltage dependence of activation (4, 11). Because our present results indicate a considerable epididymal expression of erg1b subunits and because erg1b channels exhibit less inactivation than erg1a (16), we examined the effects of NS-1643 on erg1b channels using HEK-293 cells as a mammalian heterologous expression system. Surprisingly, the effects of NS-1643 on the erg1b isofrom were much more pronounced than those exerted on HERG channels. In addition, the activator-induced changes in the biophysical properties turned out to be completely different, since NS-1643 increased erg1b currents by a marked leftward shift in the voltage dependence of channel activation. A qualitatively similar NS-1643 effect was recently reported for erg2 channels, where 30 µM NS-1643 shifted the voltage for half-maximal channel activation by ~6 mV to the left (7). In contrast, the drug-induced leftward shift in erg1b channel activation found in the present study amounted to ~60 mV, resulting in an activation threshold even below ~80 mV in the presence of 30 µM NS-1643. This finding can readily explain the inhibitory effects of the drug on epididymal myogenic activity. A NS-1643-induced hyperpolarization due to increased erg conductance would inhibit the activation of voltage-dependent Ca2+ channels obligatory for the induction of epididymal phasic activity (23). The same mechanism of action could account for the total suppression of OT-induced contraction in the cauda produced by the drug. However, a direct antagonistic effect of NS-1643 on voltage-gated Ca2+ channels cannot totally be excluded, although neither L-type nor N-type calcium channel currents were found to be affected by 10 µM NS-1643 in guinea pig ventricular myocytes (11).

Putative functional role of erg channels. The data of the present study indicate a functional role for erg1 channels in the regulation of epididymal motility patterns and therefore sperm transport. The presence and functional role of inwardly rectifying, E-4031-sensitive erg K+ currents has already been shown in various tissues of the gastrointestinal tract (1, 8, 20), the gallbladder (29), and the portal vein (28). It is generally accepted that the concentration-dependent modulation of electrical and mechanical activity by E-4031 in smooth muscle is connected with the fundamental importance of erg currents for the control of the resting membrane potential. In interstitial cells of Cajal, erg channels are supposed to be the most important K+ channels regulating cellular excitability (41). Pacemaker-like cells are also suspected to be present in those epididymal duct regions that exhibit spontaneous activity (23), and they may form a target of the E-4031 effects observed in the present study. Ohya et al. (28) suggest that erg channels are crucial for smooth muscles that frequently discharge action potentials, whereas in quiescent blood vessels other K+ currents are more important. In fact, in gallbladder myocytes, erg1 channels seem to serve a similar function as the rapidly activating inward-rectifying IKr in cardiac muscle (31, 40) by contributing to the repolarization of the plateau phase of action potentials (29).

Perspectives and Significance

Inhibition and activation of erg channels resulted in marked changes in epididymal duct motility. Controlled passage of sperm through the duct depends on its phasic activity and is of critical importance for sperm maturation (22, 26, 37). Because erg channels are targets for many pharmaceutics and hormonal regulation, mostly resulting in decreased channel activity (2, 3, 33), it is tempting to speculate that erg channel modulation by drug intake interferes with sperm quality and male fertility. Especially, the ability of erg channel activation to suppress duct peristalsis raises the question about a potential contraceptive significance of erg channel modulators. However, because erg1 channels are crucial for normal cardiac function (32), an epididymis-restricted effect of a given erg channel modulator must be a prerequisite for its potential therapeutic use in male contraception.

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GRANTS

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REFERENCES


