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Bladder contractility is modulated by K_v7 channels in pig detrusor

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ABSTRACT

K_v7 channels are involved in smooth muscle relaxation, and accordingly we believe that they constitute potential targets for the treatment of overactive bladder syndrome. We have therefore used myography to examine the function of K_v7 channels in detrusor, i.e. pig bladder, with a view to determining the effects of the following potassium channel activators: ML213 ($K_v7.2/K_v7.4$ channels) and retigabine ($K_v7.2–7.5$ channels). Retigabine produced a concentration-dependent relaxation of carbachol- and electric field-induced contractions. The potency was similar in magnitude to that of ML213-induced relaxation, suggesting that $K_v7.2$ and/or $K_v7.4$ channels constitute the subtypes that are relevant to bladder contractility. The effects of retigabine and ML213 were attenuated by pre-incubation with 10 μM XE991 ($K_v7.1–7.5$ channel blocker) ($P < 0.05$), which in turn confirmed K_v7 channel selectivity. Subtype-selective effects were further investigated by incubating the detrusor with 10 μM chromanol 293B ($K_v7.1$ channel blocker). Regardless of the experimental protocol, this did not cause a further increase in the evoked contraction. In contrast, the addition of XE991 potentiated the KCl-induced contractions, but not those induced by carbachol or electric field, indicating the presence of a phosphatidyl-inositol-4, 5-biphosphate-dependent mechanism amongst the K_v7 channels in detrusor. qRT-PCR studies of the mRNA transcript level of $K_v7.3–7.5$ channels displayed a higher level of $K_v7.4$ transcript in detrusor compared to that present in brain cortex and heart tissues. Thus, we have shown that $K_v7.4$ channels are expressed and functionally active in pig detrusor, and that the use of selective $K_v7.4$ channel modulators in the treatment of detrusor overactivity seems promising.

1. Introduction

The innervation of the urinary bladder is complex and therefore there is a large risk amongst the population at large of developing disorders related to its function. One such disorder is the overactive bladder syndrome (OAB) which is a symptom-based disease. OAB patients suffer from one or more of the following symptoms: urgency, usually in combination with frequency and nocturia and/or urge incontinence (Abrams, 2003). The disease has a tremendous impact on the patients' health-related quality-of-life (Stewart et al., 2003). In fact, it has been estimated that 17% men and 30% women suffer from the overactive bladder syndrome and that its prevalence increases with age (Coyne et al., 2012; Irwin et al., 2006). The use of muscarinic receptor antagonists is the main pharmacological treatment employed to control the symptoms of OAB. However, the development of new drugs is urgently required, as the effect of muscarinic antagonists is modest at best, and their side effects are many (Andersson et al., 2009; Athanasopoulos and Giannitsas, 2011; Chapple et al., 2008).

Retigabine (D23129, N-(2-amino-4–51 (4-flurobenzylamino)-phenyl) carbamic acid ethyl ester) is a selective and positive $K_v7.2–7.5$ channel modulator that has been approved by the U.S. Food and Drug Administration for the treatment of epilepsy. Interestingly, patients treated with retigabine experienced side effects associated with the bladder, i.e. an increased incidence of urinary hesitation, retention, residual urine volume and decreased urine flow compared to placebo (Brickel et al., 2012). These findings have been further confirmed in rats and guinea pigs (Anderson et al., 2009; Rode et al., 2010; Streng et al., 2004; Svalø et al., 2011). The studies revealed that K_v7 channels are indeed expressed in the urinary bladder of rodents and that the pharmacological modulation of these channels affects detrusor contractility, both in vivo and in vitro.

As a result, scientists have argued that K_v7 channels could be a potential target in the treatment of diseases linked to smooth

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muscle cells, since K_v7 channels are expressed in the peripheral organs (Jepps et al., 2012) while also being present in the central nervous system (Brown and Passmore, 2009).

K_v7 channels are encoded by the KCNQ gene. Currently 5 different subtypes are known to exist, of which only KCNQ3–5 have been described in pigs. Since most of our knowledge regarding the properties of the K_v7 channels is based on work carried out on small animals, i.e. guinea-pigs, rats and mice, we lack information on K_v7 channel properties in the detrusor of larger animals and humans. Since human bladder tissue is scarce, good animal models of urinary bladder function are sorely needed. The urodynamic and functional characteristics of the pig bladder is similar and hence comparable to that of humans. Therefore the use of pig bladders, rather than those of small laboratory animals, is more appropriate when studying clinically relevant effects. This is the reason why we, in our present study, aimed to study the expression and functional characteristics of modulating K_v7 channels in pig detrusor. The role of K_v7 channels in isolated pig detrusor strips was evaluated using retigabine and ML213 (N-Mesitylbicyclo[2.2.1]heptane-2-carboxamide). Retigabine and ML213 are selective and positive modulators of $K_v7.2$–7.5 and $K_v7.2$ and $K_v7.4$ channels, respectively (Yu et al., 2011). K_v7 channel selectivity was confirmed by applying the following negative modulators: XE991 (10,10-bis(4-pyridinyl-55 methyl)-9(10H)-anthracenone), which is a $K_v7.1$–7.5 channel blocker, and chromanol 293B (trans-N-[6-Cyano-3,4-dihydro-3-hydroxy-2,2-dimethyl-2H-1-benzopyran-4-yl]-N-methyl-ethanesulfonamide), which is a $K_v7.1$ channel blocker. The functional experiments were validated by employing qRT-PCR to analyse the relative levels of $K_v7.3$–7.5 mRNA.

2. Materials and methods

2.1. Tissue collection

Danish landrace pigs ($n=33$, 6–7 month-old, 105 kg) (The Danish Meat Trade College, Roskilde) were euthanised in accordance with the regulations and guidelines from the Danish Ministry of Food, Agriculture and Fisheries. Then, the urinary bladders were isolated and placed in cold Tyrode's salt solution (Sigma) with 20 mM HEPES, transported on ice and used for the myograph experiments (DK-10-3-oth-036) within two hours in the Smooth Muscle Research Center.

Detrusor tissue ($n=14$) for the qRT-PCR experiments was collected from pigs at the Department of Basic Animal and Veterinary Sciences, Faculty of Science, University of Copenhagen. The tissue was snap frozen in liquid nitrogen and stored at −80 °C until further processing. All experiments were carried out at the Smooth Muscle Research Center.

2.2. Drugs

Retigabine and chromanol 293B were purchased from Santa Cruz (California, USA) and Sigma-Aldrich (St. Louis, MO, USA) whereas XE991 was kindly provided by NeuroSearch (Ballerup, Denmark) and ML213 by Vanderbilt University (Nashville, TN, USA (MLCPN probes)). The stock solutions (10^{-2} M) were prepared in 100% DMSO (Merck, VWR international, Herlev, Denmark) and further diluted in physiological saline solution (PSS) just before experiments. Carbachol, papaverine and tetrodotoxin (TTX) were purchased from Tocris (VWR international, Herlev, Denmark), Sigma-Aldrich (St. Louis, MO, USA) and Ascent Scientific (Cambridge, UK), respectively. The stock solutions were prepared in distilled H_2O and further diluted in standard PSS (NaCl 118.99 mM, KCl 4.69 mM, $MgSO_4 \cdot 7H_2O$ 2.40 mM, KH_2PO_4 1.18 mM, glucose 6.06 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 1.6 mM) just before experiments.

2.3. RNA extraction and cDNA synthesis

RNA extraction, cDNA synthesis and qRT-PCR were performed as described by Rosenbaum et al. (2012). Tissue biopsies were grinded in liquid nitrogen in a mortar, and homogenised in 1–1.5 ml TriZol (Invitrogen, Denmark) using a rotor-stator homogeniser. Total RNA was extracted from (32–160 mg) grinded tissue using the Trizol method (manufacturer's instruction), including bromochloropropane (VWR, Denmark) for phase-separation. The quantity of extracted RNA was checked spectrophotometrically with a nanophotometer (Implen, VWR, Denmark). To ensure RNA integrity, only samples with an A260/A280 optical density (OD) ratio of 1.9–2.0 were used. 1 μg was loaded on a 1% agarose gel containing ethidium bromide for electrophoresis and visualised by a UV-transilluminator (Uvitec, Kem-en-tec, Taastrup, Denmark). RNA (10 μg) samples were purified by DNase treatment in order to remove contaminating genomic DNA. This was done using an RNase-free DNase kit according to the manufacturer's instructions for rigorous DNA removal (50 μl reaction) (Ambion, Applied Biosystems, Denmark). The quantity and purity of DNase treated RNA was checked spectrophotometrically and with the help of gel electrophoresis.

RNA was reversely transcribed into complementary DNA, (cDNA) with 4.5 μl oligo(dT) primers (100 ng/μl), and reverse transcriptase (1.5 μl), in a 30 μl reaction volume from 1200 ng DNase treated RNA. This was done using the Affinity Script qPCR cDNA synthesis kit (Stratagene, AH Diagnostics, Denmark) as per the manufacturer's instructions. Cycling parameters were 5 min at 25 °C; 15 min at 42 °C; 5 min at 95 °C; and 30 min at 25 °C using a Mx3000P QPCR system (Stratagene, AH Diagnostics, Aarhus, Denmark). cDNA was stored at −20 °C until further processing. Pig cDNA for the positive control tissues (heart and brain cortex) was purchased from Zyagen (Immunodiagnostic, Finland).

2.4. Primers

Primers for the genes of interest, KCNQ3–5 were designed using Primer3 (NCBI) (Table 1), primer properties were determined by Oligo Analyzer (IDT) software. The primers were ordered and purified by MALDI-TOF from TAG-Copenhagen (Denmark). The primers were designed to match all known splice variants of the given gene. To ensure primer specificity a BLAST search against the NCBI database was performed, and a melting curve analysis ensured that only one product was amplified. Primers for the reference genes ACTB, ATP5G, B2M, GAPDH, GPI, GPX1, GSR, PPIA, SDHA, SHAS2, UBC and eEF-1 were included in a geNorm Pig REF gene kit (PrimerDesign Ltd., UK).

Table 1
Primer sequences and efficiencies.

Gene	Primer sequence	Amplicon bp	Region spanned	Efficiency %	R^2
KCNQ3	F-5′-AAGACGGGACCCTACTGCTG-3′ R-5′-CGGTACTTGGCGTTGTTCCT-3′	127	347–366, 454–473	92.3	0.90
KCNQ4	F-5′-GACACCACCTCCGACTACCA-3′ R-5′-AAGCGCCTCAGTCCATGTTG-3′	109	1855–1874, 1944–1963	105.7	0.89
KCNQ5	F-5′-CCCTGTGGACAGCAAAGACC-3′ R-5′-GTCTGGGCGCTGAACTCATT-3′	126	2076–2095, 2182–2201	98.9	0.99