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Human urinary bladder smooth muscle is dependent on membrane cholesterol for cholinergic activation

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ABSTRACT

Voiding is mediated by muscarinic receptors in urinary bladder smooth muscle cells. Lipid rafts and caveolae are cholesterol enriched membrane domains that modulate the activity of G protein-coupled receptors and second messenger systems. Conflicting findings regarding sensitivity of muscarinic signalling to cholesterol desorption, which perturbs lipid rafts and caveolae, have been reported, and no study has used human urinary bladder. Here, the dependence of human bladder muscarinic receptor signalling on plasma membrane cholesterol was examined. Nerve-mediated contraction, elicited by electrical field stimulation of human bladder strips, was impaired by desorption of cholesterol using methyl-β-cyclodextrin, and the concentration–response curve for the muscarinic agonist carbachol was right-shifted. No effect of cholesterol desorption was observed in rat, and in mouse increased maximum contraction was seen. Expression of caveolin-1, $PLC_{\beta 1}$ and M_3 muscarinic receptors did not differ between species in a manner that would explain the differential sensitivity to cholesterol desorption. In human bladder, threshold depolarisation eliminated the difference between cyclodextrin-treated and control preparations. Contraction elicited by depolarisation *per se* was not affected. M_3 muscarinic receptors appeared clustered along plasma membrane profiles as shown by immunohistochemical staining of human bladder, but no redistribution in association with cholesterol reduction was seen. Thus, muscarinic receptor-induced contraction of the urinary bladder exhibits species-specific differences in its sensitivity to cholesterol desorption suggesting differential roles of lipid rafts/caveolae in muscarinic receptor signalling between species.

1. Introduction

Lower urinary tract dysfunction (incontinence, overactive bladder) is a major pathology affecting the quality of life of millions of people, and with considerable economic impact (Levy and Muller, 2006; Milsom et al., 2000). The cholinergic nervous system plays a critical role in voiding through activation of muscarinic receptors in the bladder (Hegde, 2006). Muscarinic receptors in the urinary bladder are mainly represented by the muscarinic M_2 and M_3 receptor subtypes (Wang et al., 1995). Studies using knock-out mice have established a major role for muscarinic M_3 receptors (Matsui et al., 2002), which couple to Gq causing bladder contraction and voiding. Muscarinic M_2 receptors may prevent β-adrenergic formation of cyclic AMP, indirectly promoting bladder contraction (Ehlert et al., 2005), and this receptor subtype may assume a more prominent role in bladder pathology (Braverman et al., 1998).

Lipid rafts are dynamic aggregates of cholesterol and sphingolipids in the plasmalemma that are considered to play a role in signalling from G protein-coupled receptors (Simons and Toomre, 2000). Caveolae, a subcategory of lipid rafts, are 50 to 100 nm flask-shaped invaginations in the membrane (Cohen et al., 2004) and these organelles have been proposed to organize receptors and signalling intermediaries central to smooth muscle contraction (Bergdahl and Swärd, 2004). Caveolae are abundant in the detrusor (Gabella and Uvelius, 1990). Mice lacking caveolin-1 also lack caveolae in the bladder and exhibit several urological defects including decreased contractility on stimulation with carbachol (Lai et al., 2004, 2007; Woodman et al., 2004). Genetic ablation of caveolae was found to be associated with a 70% decrease in acetylcholine release from bladder nerve terminals (Lai et al., 2004).

Desorption of cholesterol from the cell membrane using cyclodextrins (Kilsdonk et al., 1995) causes reversible disassembly of caveolae (Dreja et al., 2002; Rothberg et al., 1992). Cholesterol lowering is not specific for caveolae and also affects lipid rafts and membrane fluidity. An advantage, however, over the genetically caveolae-ablated mice, is that compensation developing in response to life-long loss of caveolins is avoided. Moreover, cyclodextrins allow probing of the role of lipid rafts/caveolae in species other than the mouse, including humans.

A few studies on the role of caveolae for the function and localization of muscarinic receptors have accumulated. Feron et al. (1997) demonstrated that muscarinic M_2 receptors dynamically target rat cardiomyocyte

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caveolae. Moreover, in guinea pig intestinal smooth muscle muscarinic M_2 receptors were demonstrated to co-localize with caveolae (Iino and Nojyo, 2006), but the consequences for signalling are uncertain as discrepant findings were reported in mouse and rat (El-Yazbi et al., 2008; Shakirova et al., 2006; Somara et al., 2007). M_3 muscarinic receptors were recently colocalized with caveolin-1 in canine airway smooth muscle, and disruption of caveolae impaired cholinergic Ca^{2+} signalling in both canine and human airway muscle cells (Gosens et al., 2007). Only one study has probed the role of caveolae in cholinergic bladder contraction in a species other than the mouse. Cristofaro et al. (2007) disrupted caveolae in rat bladder using cyclodextrin and found that cholinergic contractions were unchanged.

In view of the apparent controversy regarding the dependence of cholinergic signalling on rafts/caveolae we hypothesized that differences may exist between species. In the present study the sensitivity of human bladder cholinergic contraction to cholesterol desorption was examined and compared with that of rat and mouse bladders.

2. Materials and methods

2.1. Patients and animals

Human bladder tissue was obtained from patients undergoing radical cystectomy for treatment of localized bladder cancer. 25 patients, 20 male and 5 female, with ages ranging from 39 to 82 years (median 67) were selected on the basis of localization, size and spread of the bladder cancer. 16–22 week old C57Bl6/J mice were obtained from Taconic (Ejby, Denmark). Female Sprague–Dawley rats weighing 250 g were obtained from the same vendor. Human experiments were approved by the regional human ethics committee and all patients gave their written informed consent. The local animal ethics committee in Lund/Malmö approved all experiments involving animals.

2.2. Tissue preparation

Human bladder specimens were dissected from the ventral midportion of the excised bladder, and rapidly transported to the lab in ice-cold HEPES buffered Krebs (composition in mM: NaCl 135.5, KCl 5.9, $MgCl_2$ 1.2, glucose 11.6, HEPES 11.6, pH 7.4). Only healthy tissue far from the tumor was used for experimentation. Mice and rats were killed by CO_2 asphyxiation and the bladder was rapidly removed. Bladders were immediately placed in cold nominally Ca^{2+}-free HEPES buffered Krebs solution. Homogenous bundles of urinary bladder smooth muscle were carefully dissected free of fat and connective tissue and the urothelium was removed. Strips measuring 1 mm (width) × 0.2 mm (thickness) × 3 mm (length, mouse dimensions), 1 mm × 0.3 mm × 3 mm (rat), and 1 mm × 0.8 mm × 3 mm (human) were mounted horizontally using silk sutures.

2.3. Electrical field stimulation

Using silk sutures, strips were mounted on steel pins connected to a force transducer in 5 ml open organ baths with Krebs solution (composition in mM: NaCl 119, KCl 4.6, NaH_2PO_4 1.2, $NaHCO_3$ 15, $MgCl_2$ 1.2, glucose 5.5 and $CaCl_2$ 1.5), gassed with 95% O_2 and 5% CO_2 at 37 °C (pH 7.4). The passive tension was adjusted to 3 mN and preparations were allowed to equilibrate for 45 min. Strips were first activated with high K^+ solution (obtained by exchanging NaCl for KCl) twice (5 min each). The mean value of the initial peak responses was used as reference for normalization of subsequent force responses. Platinum electrodes were then mounted on both sides of the preparation. Strips were activated at two-minute intervals for 5-s periods at 20 Hz (pulse duration 0.5 ms) with increasing voltage to find the optimal stimulation voltage. This voltage was used during the remainder of the experiment. Cholesterol was removed by incubation for 40 min in 10 mM methyl-β-cyclodextrin (mβcd), (Dreja et al., 2002; Kilsdonk et al., 1995). Treatment with mβcd depletes roughly 20% of tissue cholesterol and leads to reversible disruption of caveolae (Dreja et al., 2002). Control and cyclodextrin-treated preparations were always run in parallel. Frequency–response curves were then determined by increasing the frequency in steps from 1 to 50 Hz (5 s stimuli at two-minute intervals). The experiments were terminated by high K^+ activation to ensure that force was not lost during the course of the experiments.

2.4. Myograph experiments

Smooth muscle strips were mounted in myographs (610 M, Danish MyoTechnology). Baths contained aerated HEPES buffered Krebs solution with 2.5 mM Ca^{2+}. A basal tension of 3 mN was applied as above. After equilibration for 30 min at 37 °C, the solution was changed to high K^+ solution, obtained by replacing 60 mM NaCl for KCl, for 7 min. Following relaxation cholesterol was extracted with cyclodextrin as above. Carbachol was added in a cumulative manner to final concentrations ranging between 10^{-8} and 3×10^{-5} M. Each concentration was maintained for 7 min. Force responses to agonists were expressed relative to mean high K^+ induced contraction. In Fig. 3D preparations were partially contracted using 25 mM K^+ following cholesterol desorption and washing. Carbachol was then added cumulatively during sustained contraction as above. The force caused by 25 mM K^+ did not differ between groups and was not subtracted from the carbachol-induced force in 3A.

2.5. Western blotting

Western blotting was performed as described (Shakirova et al., 2006), with antibody dilutions as recommended by the manufacturers. Antibodies against caveolin-1 (clone 2297), PTRF-cavin (Polymerase and Transcript Release Factor-cavin, a.k.a. cavin-1), and phospholipase $C_{\beta1}$ ($PLC_{\beta1}$) were from BD Biosciences Pharmingen. Anti-M_3 (H-210) was from Santa Cruz Biotechnology (Santa Cruz, CA). Protein concentration was determined using EZQ protein assay (R-33200, Molecular Probes, CA). Equal amounts of protein were loaded in all lanes. After transfer of proteins to nitrocellulose membranes, proteins remaining on the gel were stained with Coomassie brilliant blue. Blots and gels were analyzed in the Fluor-S™ MultiImager (BIO-RAD) using general background subtraction. Optical densities times the area (mm^2) of the bands of interest was normalized to total protein in the same lane on the gels. Sections of these gels, centered over actin, are shown below blots as controls for protein loading.

2.6. Immunohistochemistry

Following relaxation in Ca^{2+}-free solution for 30 min at room temperature, human urinary bladder preparations were fixed in Histochoice (Amresco, Solon, Ohio, USA) overnight and embedded in paraffin. M_2 and M_3 muscarinic receptors were visualized on 3 µm serial paraffin sections using an automated immunohistochemistry robot (Autostainer, DakoCytomation, Glostrup, Denmark) and the EnVision™ Detection System Peroxidase/DAB (K5007, Dako, Glostrup, Denmark). In preliminary tests two distinct antibodies against the M_3 receptor were evaluated (AB9217, Chemicon International, Inc., Millipore, Temecula, CA, USA, and GTX13063, GeneTex, Inc., Irvine, CA, USA). Both tested antibodies produced similar M_3 staining patterns but the GeneTex antibody was selected for the remainder of the study.

Briefly, sections were deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval using a low pH citrate buffer (pH 6; only M_3) and a pressure cooker (2100 Retriever, Prestige Medical Ltd., Blackburn, England). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide before incubation with rabbit polyclonal

antibodies against M_2 (1:300, AB9452, Chemicon International) or M_3 muscarinic receptors (1:100, GTX13063, GeneTex). The primary antibodies were detected with polymer-bound anti-rabbit secondary antibodies and horseradish peroxidase (HRP) (EnVision™, Dako). After counter staining with Mayer's haematoxylin cell nuclei appear blue whereas M_2 or M_3 immunoreactivity is identified by the brown HRP product. All slides were examined by bright field microscopy (Nikon E80i, Nikon, Tokyo, Japan). Negative controls were performed by omitting the primary antibody or using matched control sera.

2.7. Chemicals

All chemicals not specified above were of analytical grade or better and obtained from Sigma (Sigma Aldrich, Sweden).

2.8. Statistics

In the typical experiment an even number of preparations ranging from 4 to 12 from each patient or animal was mounted. Half of the preparations were treated with mβcd and the other half with vehicle. The means of the contractile responses of all control- and all mβcd-treated strips from each individual were then entered into graphs and statistical tests and considered to represent one observation. Means based on all subjects (±S.E.M.) are shown in the figures. The number of animals/human subjects are denoted by *h* (number of human subjects), *r* (number of rats), and *m* (number of mice). Student's *t*-test for paired or unpaired data, as appropriate, was used to test for differences between groups. $P<0.05$ was considered significant. One-way ANOVA followed by Bonferroni's multiple comparisons test was used for multiple comparisons. Significance is indicated by $^{*}P<0.05$, and $^{**}P<0.01$.

3. Results

3.1. EFS-induced contraction of human bladder strips following desorption of cholesterol

Electrical field stimulation (EFS), at frequencies ranging from 1 to 50 Hz, caused a frequency-dependent increase of contraction in human bladder strips. At frequencies exceeding 10 Hz EFS-induced contraction was impaired following reduction of cholesterol using methyl-β-cyclodextrin (mβcd, Fig. 1A). Threshold depolarisation with 25 mM K^+ eliminated the effect of cholesterol lowering and reduced the amplitude of EFS-induced contractions (Fig. 1B). Stimulation with K^+-high solution following either EFS protocol caused indistinguishable contraction in control and depleted preparations (Fig. 1C).

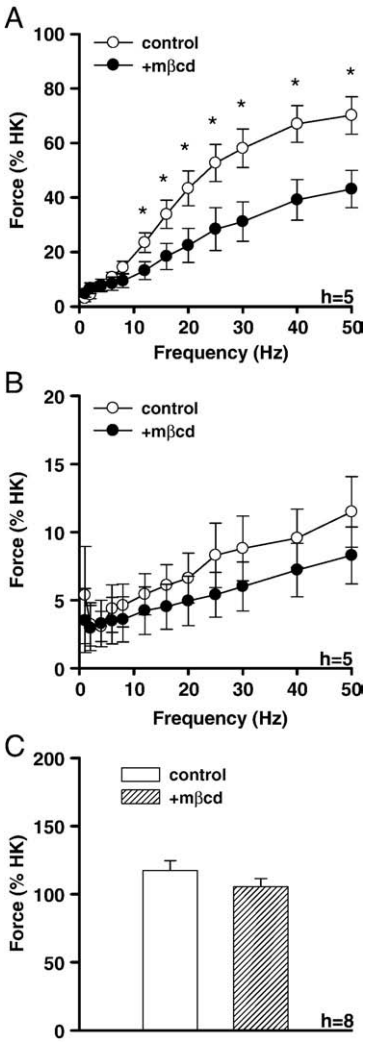

Fig. 1. (A) shows force elicited by electrical field stimulation (EFS), normalized to the mean of two reference high K^+ contractions (125 mM). Open symbols represent vehicle-treated control strips from human urinary bladder and filled symbols represent methyl-β-cyclodextrin-treated (mβcd, 10 mM, 40 min) strips from the same patients run in parallel. At frequencies between 10 and 50 Hz EFS-induced contraction was impaired following depletion of cholesterol ($P<0.05$). (B) shows an identical experiment to that in (A) except that all preparations were pre-contracted with 25 mM K^+ prior to EFS. (C) shows force elicited by 125 mM K^+ following vehicle/mβcd and EFS. Force is normalized to the mean of two reference high K^+ contractions (125 mM) prior to the depletion protocol. In this and the following figure *h* denotes the number of human subjects.

3.2. Effect of cholesterol desorption on carbachol contraction in urinary bladder from different species

Full concentration–response curves for the muscarinic receptor agonist carbachol were generated in human (Fig. 2A), rat (Fig. 2B) and mouse (Fig. 2C) bladder strips. In human bladder strips, contractile responses to carbachol were significantly suppressed between 0.03 and 3 μM following cholesterol reduction. Contractility at saturating concentrations of carbachol was however unaffected. Cholesterol reduction had no effect in rat bladder (Fig. 2B). In the mouse, cholesterol desorption increased force at the two highest carbachol concentrations (Fig. 2C), but did not right- or left-shift the curve.

3.3. Effect of depolarisation on cholinergic contraction following cholesterol desorption from human urinary bladder

Depolarisation with 25 mM K^+ completely eliminated the effect of cholesterol desorption on the concentration–response curve for carbachol in human urinary bladder (Fig. 3A), similar to the effect of threshold depolarisation on EFS-induced contraction (cf. Fig 1B). Moreover, the difference in the concentration–response relationships between control and cholesterol-depleted preparations appeared less pronounced in the presence of nifedipine (1 μM, Fig. 3B), but a significant difference was still seen at 0.3 μM. Contraction induced by depolarisation was not significantly affected by cholesterol desorption at any concentration of K^+ (Fig. 3C).

3.4. Effect of cholesterol desorption on purinergic contraction in human bladder

The peak response of human bladder strips on application of a single concentration of the purinergic agonist ATP (1 mM) was not affected by cholesterol desorption (26 ± 13% of HK for control vs. 25 ± 10% for mβcd, $h=4$).

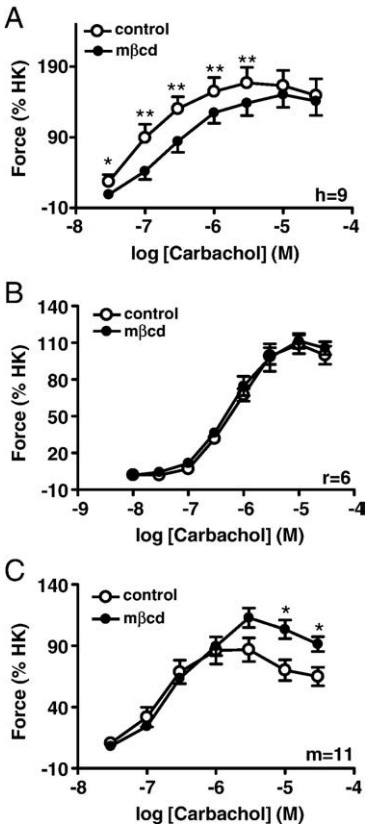

Fig. 2. Concentration–response relationships for the muscarinic receptor agonist carbachol in human (A), rat (B), and mouse (C) control (white symbols) and cholesterol-depleted (black symbols) urinary bladder strips. Force was normalized to the depolarisation-induced contraction (60 mM K^+, HK) elicited before the depletion protocol. *h*, *r*, and *m* refer to the number of humans, rats, and mice, respectively.

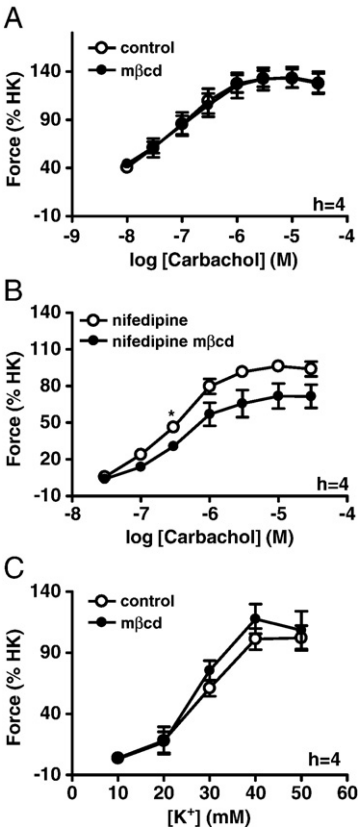

Fig. 3. (A) shows concentration–response relationships for carbachol in control (white circles) and mβcd-treated (black circles) human bladder strips following pre-treatment with 25 mM K^+. (B) shows concentration–response relationships for carbachol in human bladder in the presence of the L-type Ca^{2+}-channel blocker nifedipine (1 μM). (C) shows concentration–response relationships for K^+-high solution.

3.5. Expression of M_3, phospholipase $C_{\beta 1}$, caveolin-1 and PTRF-cavin

The expression of proteins involved in caveolae-formation and muscarinic receptor signalling was next compared between species using Western blotting. The expression of caveolin-1, PTRF-cavin, M_3, and phospholipase $C_{\beta 1}$ did not differ between human and rat bladder (Fig. 4A–D). Caveolin-1 appeared higher in rat than in mouse bladder, but this difference did not reach significance. $PLC_{\beta 1}$ was higher in mouse bladder compared to both human and rat bladders. Mouse urinary bladder also expressed more M_3 receptor protein in comparison to both human and rat bladders. PTRF-cavin levels did not differ between human, mouse, and rat urinary bladders.

3.6. Immunohistochemical demonstrations of M_2 and M_3 muscarinic receptors in human bladder tissue

Immunoreactivity for M_2 and M_3 muscarinic receptors was observed in the detrusor smooth muscle of human urinary bladder as demonstrated in Fig. 5A (M_2) and 5C (M_3). Desorption of cholesterol did not affect the pattern or intensity of the M_2 or M_3 immunoreactivity (compare 5B vs. 5A for M_2 and 5D vs. 5C for M_3). The staining for M_2 was homogenously distributed over the smooth muscle cells. In contrast, the M_3 immunoreactivity was clustered to distinct areas at or near the membrane suggesting compartmentalization of M_3 receptors on smooth muscle cells (Fig. 5E and F). A handful of such clusters per cell were seen in cross-sections at high magnification (Fig. 5F). Apart from positive staining in the detrusor smooth muscle, M_2 (Fig. 5G) and M_3 (Fig. 5H) muscarinic receptor immunoreactivity was also localized to the endothelium and vascular smooth muscle of blood vessels in the urinary bladder.

4. Discussion

In the present study species-specific differences in the sensitivity of muscarinic receptor signalling to cholesterol desorption are unveiled. It is well established that the reduction of plasma membrane cholesterol leads to disruption of lipid rafts and caveolae. This is found here to cause sizeable inhibition of cholinergic contraction in human, but not in mouse or rat urinary bladder. Differences in the ability of cyclodextrins to remove cholesterol and to ablate caveolae between species are possible. In rat bladder, a desorption-protocol similar to that used here was however found to affect serotonin, angiotensin II, and bradykinin contraction as well as the ultra structure of caveolae, despite leaving carbachol responses unaltered (Cristofaro et al., 2007). Thus, rat bladder muscarinic contraction does not resist cholesterol desorption because caveolae are insensitive to the effects of cyclodextrins. The effect of cholesterol desorption in human bladder does not represent a general impairment of contractility because cholesterol reduction did not affect purinergic contraction or contraction induced by membrane depolarisation. Moreover, threshold depolarisation eliminated the effect of cholesterol lowering, suggesting that the signalling mechanism targeted may be located downstream of receptor activation and involving membrane depolarisation.

This work is not the first to document differences between human and rodent bladder function. Recent work has demonstrated that relaxation by cathecholamines is dominated by β_3-adrenergic receptors in human bladder whereas β_2-adrenergic receptors dominate in the mouse (Wuest et al., 2009). Moreover, Ca^{2+} influx mechanisms were found to differ between mouse, pig, and human detrusor, with a smaller contribution of L-type Ca^{2+} channels in

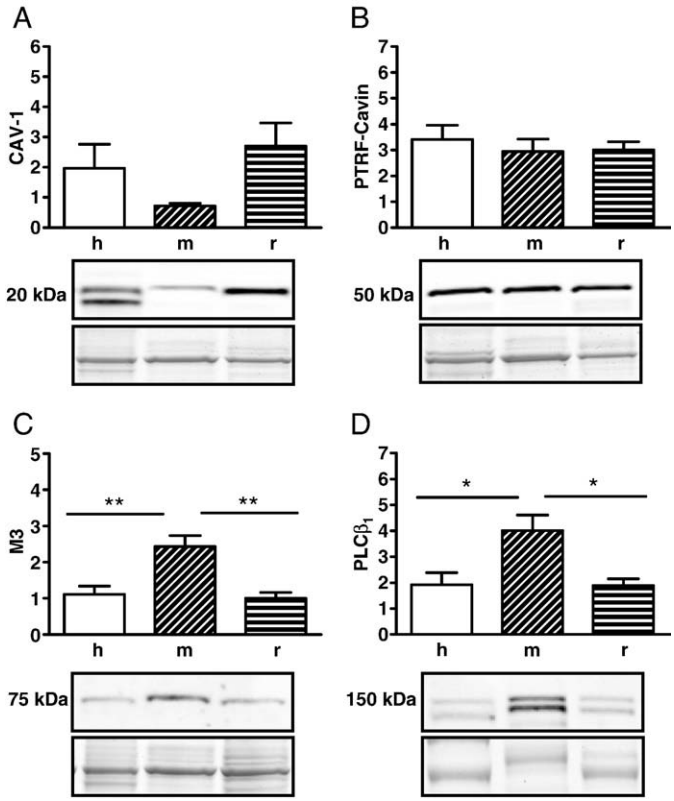

Fig. 4. The results of Western blotting for caveolin-1 (A), PTRF-cavin (B), M_3 (C), and phospholipase $C_{\beta 1}$ (D) are summarized as bar graphs for human, mouse, and rat urinary bladders. Expression was normalized to total protein on the Coomassie brilliant blue stained gel. Representative Western blots and excerpts from the stained gel, centered over actin, are shown below each bar graph. Positions of molecular weight markers are indicated to the left of the blots. *h*, *m*, and $r = 4$.

human as compared to pig and mouse (Wuest et al., 2007). The atropine resistant component of contractions elicited by electrical field stimulation is also considerably larger in mouse (50–75%) than in human (25%) bladder (Wuest et al., 2005). A different dependence of muscarinic receptors on membrane cholesterol, and thus presumably on rafts/caveolae, may now be added to this list of discrepancies between man and rodent detrusor function.

It is necessary to discuss what desorption of cholesterol vis à vis lifetime genetic ablation of caveolin tells us about the physiological function of caveolae in the bladder. While lowering of cholesterol causes clear-cut disassembly of caveolae (Dreja et al., 2002; Cristofaro et al., 2007; Rothberg et al., 1992) it also affects lipid rafts, which are planar assemblies of glycosphingolipids, cholesterol, and glycosylphosphatidylinositol-anchored proteins. Moreover, cyclodextrin treatment may affect rather specific interactions between e.g. receptors and cholesterol molecules as well as the fluidity of the membrane. Thus, cholesterol reduction cannot be equated with disruption of the caveolin-1 gene, and these interventions do indeed often result in opposite effects. For example, the activity of endothelial nitric oxide synthase (eNOS), which is a prototypical caveolae-associated enzyme, is inhibited by desorption of cholesterol (Darblade et al., 2001), but increased in caveolin-1-deficient mice (Drab et al., 2001; Razani et al., 2001; Zhao et al., 2002).

Findings in the mouse bladder seemingly replicate the situation with eNOS with regard to the opposing functions of cholesterol and caveolin-1. It has been reported that cholinergic contraction is impaired in caveolin-1-deficient mouse bladder (Lai et al., 2004; Woodman et al., 2004; Wuest et al., 2009), and that the gravity of this phenotype increases with age (Lai et al., 2007). As shown here, acute disruption of caveolae and lipid rafts actually has the opposite, albeit modest, effect in mouse bladder, i.e. it increases the efficacy of carbachol.

In order to better understand the differences in cholinergic sensitivity to reduction of cholesterol, the expression of proteins involved in formation of caveolae and in muscarinic signalling was examined. Differences in the expression of M_3 muscarinic receptors, and phospholipase $C_{\beta 1}$ were noted between mouse and rat, and occasionally human, bladder. It was recently discovered that PTRF-cavin (also known as cav-p60, PTRF, and cavin-1) plays a key role in formation of caveolae (Hill et al., 2008), and similar amounts of this protein were detected in all species. Importantly, all of the proteins examined appeared to be equally expressed in human and rat bladders. Thus different densities of caveolin-1, muscarinic M_3 receptors, or phospholipase $C_{\beta 1}$ are not likely to explain the differences in cholinergic sensitivity to cholesterol lowering between man and rat.

Our immunohistochemical staining supported subcellular compartmentalization of muscarinic M_3 but not M_2 receptors in human urinary bladder smooth muscle. The sarcolemma in most gastrointestinal and urogenital smooth muscle cells has a bipartite organization where longitudinal strands of membrane studded with caveolae are separated by so called dense bands. We have previously found that 7–15 such "caveolae domains" can be distinguished in cross-sectioned intestinal smooth muscle cells (Shakirova et al., 2006). This agrees roughly with the number of cross-sectional clusters of muscarinic M_3 receptors found here ($\approx$7). Our findings thus appear to be in accordance with the results of Gosens et al. (2007) who found muscarinic M_3 receptors to be clustered and to co-localize with caveolae domains in airway smooth muscle. The staining for muscarinic M_2 receptors, on the other hand, may at first glance appear to be at variance with the reported targeting of muscarinic M_2 receptors to caveolae in cardiomyocytes and intestinal smooth muscle (Feron et al., 1997; Iino and Nojyo, 2006). However, our tissues were fixed under non-stimulated and fully relaxed conditions precluding us from capturing any dynamic changes in localization.

Difficulties in raising specific antibodies against the family of G protein-coupled receptors have previously been recognized (Michel et al., 2009). Indeed, recent publications have raised concerns regarding the specificity of several commercially available anti-muscarinic receptor antibodies (Jositsch et al., 2009; Pradidarcheep et al., 2008, 2009). A number of hard criteria for acceptable specificity were set forth: i) absent staining in knock-out animals or after knock down, ii) increased staining in over-expressing cells lacking related receptor subtypes, and iii) a similar staining pattern using multiple antibodies against different receptor epitopes. Immunization peptides for pre-absorption control experiments were not available for the antibodies used in this study. One of the hard criteria above was however fulfilled, namely that the same pattern of immunoreactivity was produced by distinct antibodies against the same receptor.

In the human bladder our analysis allows us to generally localize the defect in muscarinic signalling that is caused by lowering of cholesterol. Because the response to depolarisation by K^+-high solution was unchanged, an effect on voltage gated Ca^{2+}-channels can be ruled out. It was found that threshold depolarisation eliminated the effect of mβcd treatment on both EFS-induced contractions and the concentration–response curve for carbachol. This would not occur if cholesterol reduction was acting at the receptor level and therefore favours a site of action located downstream of receptor activation and involving processes of membrane depolarisation. In support of this reasoning, introduction of the L-type Ca^{2+}-channel blocker nifidipine similarly appeared to reduce the effect of cholesterol desorption. Moreover, the cellular distribution of M_3 receptors was apparently not altered by cholesterol extraction. Thus, our findings appear to place the defect incurred by cholesterol desorption in human bladder at the level of

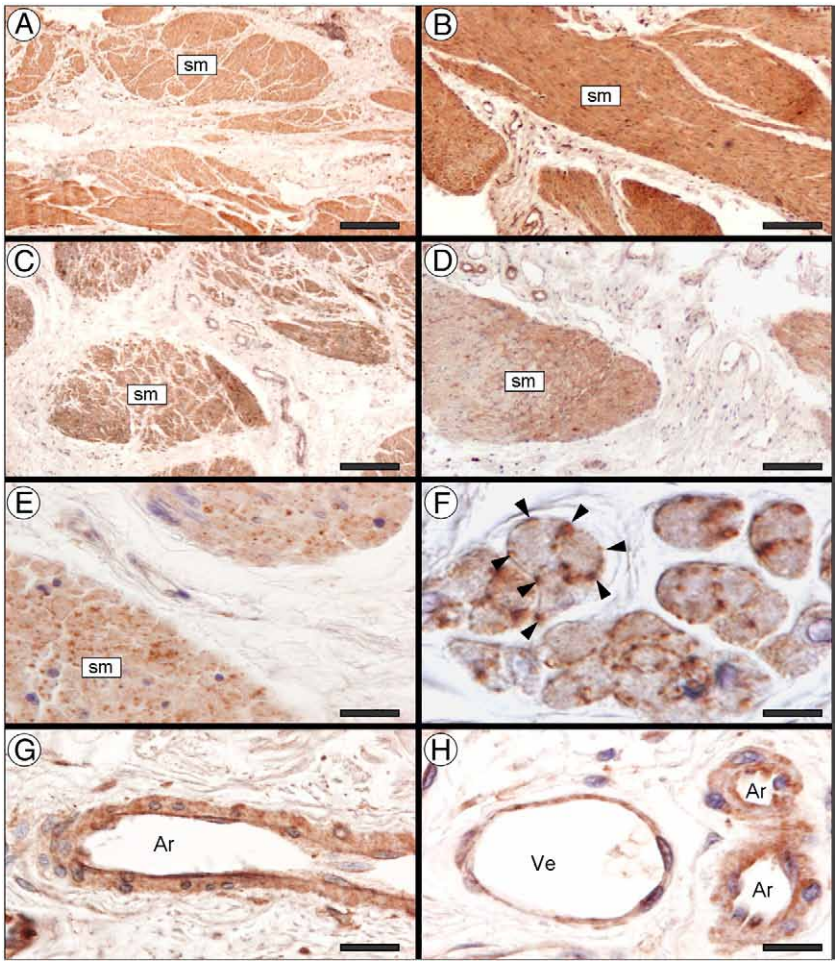

Fig. 5. Immunohistochemical labelling of muscarinic receptors in human bladder. The peroxidase activity of the secondary antibodies results in brown pigments and nuclei are stained blue. M_2 receptor in the human detrusor muscle (sm) (A) and in cholesterol-depleted detrusor (B). M_3 immunoreactivity in human detrusor (C) and in cholesterol-depleted urinary bladder (D). The distribution of M_3 was punctuated (E and F). A handful of such clusters per cell (arrowheads) were evident at higher magnification (F). Immunolocalization of M_2 (G) and M_3 (H) in arteries (Ar) and venules (Ve) of the urinary bladder. Scale bar: A, C = 100 μm; B, D = 50 μm; E, G, and H = 10 μm; F = 2.5 μm.

membrane depolarisation. This is consistent with the findings by Gosens et al. (2007). These authors depleted cholesterol from human airway smooth muscle cells and found that this impaired acetylcholine-mediated Ca^{2+} mobilization at intermediate agonist concentrations whereas neither the K_d nor B_{max} for muscarinic receptors was affected. Clearly, however, a contribution of effects involving e.g. transmitter release cannot be ruled out in our EFS experiments.

Taken together, the present study reveals that cholinergic contraction of the urinary bladder exhibits species-specific differences in its sensitivity to cholesterol desorption. Pinpointing the exact mechanism of action in human bladder will require further study, but the present data do suggest differential or even opposing roles of lipid rafts/caveolae in muscarinic signalling between species. Thus, attempts to target raft/caveolae-associated signalling mechanism in the urinary bladder for therapeutic benefit in man on the basis of results obtained in rodents may be unwarranted.

Acknowledgements

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