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UROTHELIAL NICOTINIC RECEPTOR FUNCTION IN BOTH NORMAL AND PATHOLOGICAL RAT BLADDERS

Hypothesis / aims of study

Recent studies have demonstrated that the urinary bladder epithelium (known as the urothelium) plays a prominent role in bladder signalling, aside from its well-known barrier function. For example, it has been shown that the urothelium expresses receptors that can respond to mechanical and chemical stimuli by causing the release of transmitters that may then act on underlying afferent nerves to affect bladder sensations and voiding. One class of receptors expressed by the urothelium are the nicotinic acetylcholine receptors (nAChRs). It has been demonstrated that the urothelium expresses two distinct types of nAChRs, based on their composition and ability to modulate reflex bladder voiding in the rat when administered intravesically: 1) inhibitory α7 receptors and 2) excitatory α3-containing receptors (α3*). However, little is known about the distribution/function of the nAChRs in the urothelium or if these receptors could play a role in pathological bladder conditions such as interstitial cystitis or overactive bladder syndrome. Therefore, the aims of the present study were to 1) determine the distribution of nAChR subunits in the urothelium, 2) examine the role of nAChRs in modulating intracellular calcium levels in cultured rat urothelial cells and 3) determine if nAChR mediated expression/signalling is altered in cyclophosphamide treated rats, a model of bladder pathology.

Study design, materials and methods

Immunohisotchemistry: Bladders were removed from female Sprague Dawley rats (200-250g) and snap frozen in OCT compound using liquid nitrogen. These bladders were then sectioned at 10μm, placed on slides and fixed in 2% paraformaldehyde in phosphate buffered saline (PBS) for 30 minutes. Following washes in PBS, the slides were incubated in 0.3M glycine for 20 minutes to reduce auto-fluorescence. The slides were then permeabilized in 0.1% Triton X-100 and blocked for 30 minutes. The blocking solution contained 0.1% Triton X-100, 0.5% bovine serum albumin and 10% normal goat serum. The slides were then incubated with Alexa-488 α-bungarotoxin (for α7 receptors, 100nM) or rabbit anti-α3 polyclonal antibody (1:100 dilution) overnight at 4˚C. After primary incubation, α3 slides were washed with PBS and incubated with a goat anti-rabbit-FITC secondary antibody (1:250 in PBS) for 1 hour. Each slide was also incubated with TO-PRO3 (1:1,000 in PBS for 15 minutes) to provide a nuclear counterstain. Following final washes with PBS, the slides were mounted and viewed.

Cell Culture: Female SD rats (~250g) were euthanized, the bladders removed and pinned face up on Sylgaard coated dishes in MEM containing dispase. After incubation at room temperature for 5 hours, the mucosa was gently scraped off using a spatula and dissociated in trypsin for 5 minutes. Cells were washed and centrifuged twice in MEM and then resuspended and plated on collagen-coated coverslips in a small volume of keratinocyte media (cell concentration: 500,000 cells/ml).

Ca^{+2} Imaging: For cell imaging, cultured urothelial cells were used 1-2 days following plating. For Ca^{+2} imaging, coverslips were incubated in 5μM Fura-2AM in HBSS solution for 30 minutes at 37˚C. Following incubation, each coverslip was placed in a perfusion chamber and constantly infused with HBSS solution or a drug solution and imaged with a Dage-MTI cooled CCD camera. The coverslips were alternatively excited at 340 and 380nm using a monochromator attached to a xenon lamp and fluorescence recorded. A Dage-MTI Gen. II system image intensifier and software package from Compix Inc. was used to collect data.

CYP Experiments: Rats were chronically dosed with CYP (75mg/kg ip every 3 days for 8 days). The rats were then sacrificed and their bladders removed. The bladders were then placed in KREBS solution, cut open and the urothelium was gently teased away from the underlying tissue using fine forceps and scissors. Total RNA was then extracted from each urothelium using Trizol. The RT reaction was carried out in a GeneAMP 9700 thermocycler using Qiagen's Omniscript kit and 1μg of total RNA. qPCR was performed using a Bio-Rad MyiQ real-time thermocycler, utilizing 1μl of the RT product, subtype specific primers and Bio-Rad's SYBR Master Mix qPCR kit. Results were normalized to the expression of β-actin. For cystometrograms, chronically CYP-treated rats were anesthetized using urethane (1.2g/kg, s.c.) and a catheter was implanted into the dome of the bladder following a midline abdominal incision. The catheter was connected, via a three way stopcock, to a syringe pump and a pressure transducer connected to a computer data acquisition system (DATAQ). Bladders were continuously infused with either saline (control) or a drug solution at a constant rate of 0.04ml/min.

Results

Immunohistochemistry localizes nAChRs to umbrella cells: Staining of fixed bladder tissue with either a fluorescently tagged epitope of α-bungarotoxin (which specifically binds α7 receptors) or a polyclonal antibody against the α3 receptor subunit localizes nAChRs to the umbrella cells of the urothelium, with little staining elsewhere in the bladder. Additionally, staining of cultured cells reveals both α7 and α3 staining throughout all cells.

Stimulation of nAChRs elicits a functional effect – Use of Ca^{+2} imaging: Stimulation of cultured cells with 10μM or 100μM of either cytisine (a specific α3* agonist) or choline (a specific α7 agonist) resulted in significant increases in intracellular calcium. These increases were blocked by subtype specific antagonists (TMPH for α3*, and α-bungarotoxin for α7). Removal of extracellular calcium did not affect agonist induced intracellular calcium increases for either cytisine or choline.

In order to evaluate the involvement of intracellular stores in the Ca^{+2} signals observed following nicotinic agonist stimulation, we studied the effects of thapsigargin and ryanodine, which block the IP_3- and ryanodine-sensitive intracellular Ca^{+2} pathways, respectively. Pre-treatment of the cells with thapsigargin (10μM for 20 minutes) significantly inhibited cytosine (α3*) induced calcium increases, however it did not significantly affect choline (α7) induced signals. Conversely, pre-treatment with ryanodine (10μM, 20 minutes) blocked a significant portion of both signals.

Chronic CYP treatment decreases a7 message and functional response: Following chronic CYP treatment, only α7 mRNA was significantly decreased (83.5% decrease), with α3 mRNA remaining unchanged.

During cystometry, chronic treatment with CYP decreased the intercontraction interval (ICI, 62.4%) in anesthetized rats. Nicotine (50nM and 1μM), when instilled intravesically in normal rats significantly increased ICI, however, this inhibition of the bladder reflex was significantly attenuated in chronically CYP treated rats. Intravesical administration of choline (1, 10 and 100 μM), a specific α7 agonist, also inhibited bladder reflexes in normal rats, however choline failed to inhibit bladder reflexes in CYP treated rats.

Interpretation of results

Immunohistochemical staining for nAChR subunits localize nicotinic receptors to the umbrella cells of the urothelium, where they would be accessible to agents present in urine. This data corresponds well with the data presented here and in previous studies, which demonstrate intravesical administration of nicotinic agents can modulate bladder reflexes in the rat.

Increases in $[Ca^{+2}]_i$ induced by nAChR activation was due exclusively to mobilization from intracellular Ca^{+2} stores. And while stimulation of α3* and α7 receptors induce similar increases in $[Ca^{+2}]_i$, they do so by activating distinct signalling pathways. This separation of signals could be the basis for the opposing physiological effects *in vivo* previously demonstrated. It is unknown if Ca^{+2} release from two different internal stores is linked to differences in transmitter release, however, it is possible that this may contribute to the differences in α3 versus α7 mediated effects on bladder function.

Additionally, our results indicate that following cyclophosphamide treatment, an animal model of cystitis, nicotinic receptor mRNA expression is altered, with a significant decrease in the message of the inhibitory α7 receptor following chronic treatment. This decrease in mRNA correlates with a decrease in the expression of functional receptors, as stimulation of urothelial α7 receptors in CYP treated animals with choline or nicotine did not result in the inhibition of bladder reflexes as seen in control animals. These results suggest that at least part of the symptoms associated with pathological bladder disorders may be due to the loss of the inhibitory α7 receptor in the urothelium.

Concluding message

Taken together, these data further support the hypothesis that urothelial nAChRs can play an important role in the physiology of both the normal and pathological bladder. Therefore, urothelial nAChRs may represent another important target in the treatment of bladder disorders such as overactive bladder or interstitial cystitis.

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ANIMAL SUBJECTS: This study followed the guidelines for care and use of laboratory animals and was approved by University of Pittsburgh Institutional Animal Care and Use Committee