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Reactivity of Urinary Bladder Smooth Muscle in Guinea Pigs to Acetylcholine and Carbachol: Participation of Acetylcholinesterase

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Summary

The authors examined the influence of acetylcholinesterase inhibitor (neostigmine) on the *in vitro* reactivity of urinary bladder smooth muscle (UBSM) in guinea pigs. The aim of the present study was to determine the participation of pharmacokinetic properties of acetylcholine and carbachol in different UBSM reactivity to these mediators. *In vitro* method of organ baths was used and reactivity of UBSM strips to cumulative doses of acetylcholine and carbachol was tested before and after the incubation with neostigmine (10^{-4} mol.l^{-1}). Neostigmine caused a significant increase of UBSM reactivity to acetylcholine. The UBSM reactivity to acetylcholine was significantly higher at concentrations of 10^{-5} and 10^{-4} mol.l^{-1} compared to carbachol at the same concentrations. These findings indicate that in addition to different mediator affinity to muscarinic receptors and to their different intrinsic activity, the pharmacokinetic properties of acetylcholine and carbachol also participate in UBSM reactivity.

Key words

Urinary bladder • Smooth muscle • Neostigmine • Acetylcholine • Carbachol.

Introduction

Urinary bladder smooth muscle fulfils an important role in morphological as well as functional state of the organism. The performance of urinary bladder and behavior of an individual is conditioned by its properties, as the ability to accumulate urine followed by its release belongs to basic physiological functions. Only a normal and coordinated urinary bladder activity is able to assign trouble-free social adaptation of the individual. Any changes in this basic need impair its integrity and social state leading to significant lowering of the quality of life. Therefore, it is absolutely necessary to know all the mechanisms participating in this physiological functions and in the case of failure to be able to adjust them. However, without good understanding the role and the position of smooth muscle in these processes, the causal therapy would be limited, if not unrealistic.

From the clinical point of view, „stability" disorders of the urinary bladder are relatively common. A recent study by Švihra *et al.* (2001) showed that the incidence of overactive (unstable) bladder in the Slovak population increases with age. The major symptoms of an overactive bladder include frequent voiding, full bladder feeling, incomplete voiding feeling as well as incontinence in the final stages. Especially incontinence is a symptom which significantly decreases the patient´s quality of life and makes an active participation in common daily and social activities more difficult.

The research objectives in this field are focused on elucidating the mechanisms participating in the pathogenesis and symptoms of urinary bladder functional disorders. Moreover, one of the major aims is to find new possibilities of its therapeutic modulation.

The urinary bladder wall consists of complex smooth muscles fascicules – musculus detrusor – innervated *via* intramural ganglia from three neural groups: 1) cholinergic (corpus vesicae) with major mediator acetylcholine, 2) adrenergic (basis vesicae) with major mediators epinephrine and norepinephrine, and 3) non-adrenergic non-cholinergic innervation with mediators ATP (purinergic), substance P and vasoactive intestinal peptides (predominantly sensory function).

By introducing the organ bath *in vitro* method at our department we found that acetylcholine and carbachol cause dose-dependent contraction of urinary bladder smooth muscle strips in guinea pigs. Furthermore, carbachol had a significantly stronger effect on smooth muscles compared to acetylcholine, predominantly at higher concentrations (Mokrý *et al.* 2002).

It is generally known that the acetylcholine molecule contains a quaternary ammonium group with a partial positive charge and an esteric group with a partial negative charge. Especially the latter part of the molecule is very susceptible to rapid hydrolysis by acetylcholinesterase (AChE). In the esteric part of the carbachol molecule, ammonium group replaces the methyl group. This substitution causes decreased susceptibility of carbachol to AChE hydrolytic activity leading to prolongation of the effect on smooth muscles (Rang *et al.* 1999). The aim of this study was to examine the participation of pharmacokinetic properties of contractile mediators acetylcholine and carbachol in the difference of urinary bladder smooth muscle reactivity.

Neostigmine – as a representative of indirect parasympathomimetics – is a reversible inhibitor of AChE. Its administration prolongs the action of acetylcholine in the synaptic cleft leading to longer contractions. Neostigmine is also used in clinical practice for the pharmacological treatment of myasthenia gravis and post-operative ileus. We were interested to know how can neostigmine modulate the reactivity of urinary bladder smooth muscle reactivity to acetylcholine and carbachol.

Material and Methods

The *in vitro* organ bath method was used in our experiments (Fraňová 2001). There were eight guinea pigs (male, weight 250-350 g) in the group. The animals were killed by interrupting the cervical spinal cord and thereafter the urinary bladder was cut into thin strips of about 2x2x15 mm. All the experiments were conducted in accordance with basic ethical norms and the Helsinki Declaration of 1975, revised in 1983.

Four strips were obtained from each animal. All of them were placed into 30 ml organ bath chambers. The chambers were filled with Krebs-Henseleit´s solution (in $mmol.l^{-1}$: NaCl 110, KCl 4.80, $CaCl_2$ 2.35, $MgSO_4$ 1.20, $KHPO_4$ 1.20, $NaHCO_3$ 25, glucose 10 in glass-distilled water) saturated by pneumoxide (95 % O_2 + 5 % CO_2) maintained at pH 7.5 ± 0.1. The temperature was held at 36 ± 0.5 ºC. Single strips were fixed onto the sliding arm and the other end was bound with a thin thread to a hook of a tensometer (TSR 10G, Vývoj Martin, Slovakia). The tissue strips were initially set to 4 g tension (30 min loading phase). After this period, the tension in each strip was readjusted to a baseline of 2 g (30 min adaptation phase). The tension was transmitted through an amplifier (M1101 SUPR, Mikrotechna Prague, Czech Republic) to a linear recorder TZ 4620 (Laboratorní přístroje, Prague, Czech Republic), which recorded the intensity of contractile responses.

For appropriate nutrition of the strips and sufficient clearance of metabolic products, regular scour was done every 10 min with a prewarmed solution. After one-our lasting adaptation and equilibration, cumulative doses of contractile mediator acetylcholine and carbachol (both Sigma Aldrich) (at concentrations 10^{-8}-10^{-3} $mol.l^{-1}$, respectively) were added into the chambers. When maximal contraction was reached, all the strips were scoured four times (immediately, after 5 min and later twice after 10 min) for complete removal of contractile mediators and recovery of the strips. Thereafter neostigmine (Sigma Aldrich) was added to the samples to reach the concentration of 10^{-4} $mol.l^{-1}$ in the chambers.

After 15 min of „incubation", both contractile mediators in increasing concentrations were administered to the chambers in a cumulative manner and the changes of smooth muscle contractility were recorded.

During the whole experiment continual graphical recordings of contractile responses were made. These recordings were evaluated for single concentrations of acetylcholine and carbachol. The force of contraction was calculated in g/100 mg of tissue. Non-parametric ANOVA test was used for the statistical analysis and for comparison of the „control" recordings (strip contractility after stimulation by only cumulative doses of acetylcholine). The probability level of $p<0.05$ was accepted as statistically significant.

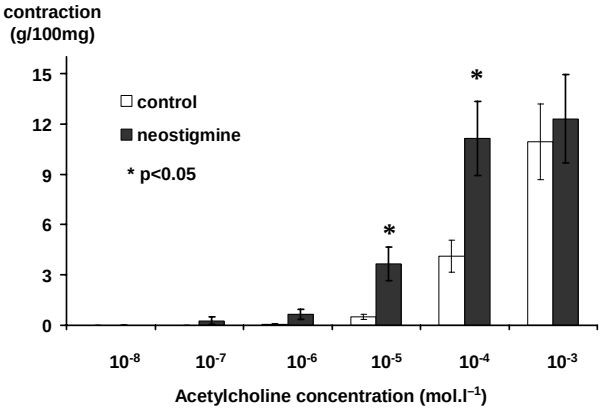

Fig. 1. The comparison of urinary bladder smooth muscle reactivity to acetylcholine before (Control) and after adding neostigmine at a concentration of 10^{-4} mol.l^{-1}. The columns represent the mean contraction (g/100 mg) and the standard error of the mean (S.E.M.). An asterisk represents significance of difference with $p<0.05$.

Results

The incubation with neostigmine at concentration of 10^{-4} mol.l^{-1} resulted in significantly stronger contractile responses of urinary bladder smooth muscle to acetylcholine (Fig. 1) at concentrations of 10^{-5} – 10^{-4} mol.l^{-1}. The presence of neostigmine did not influence the reactivity of urinary bladder smooth muscle to cumulative doses of carbachol in guinea pigs (Fig. 2). By comparison of urinary bladder smooth muscle contractility to acetylcholine and carbachol after incubation with neostigmine at concentration of 10^{-4} mol.l^{-1} we found that at concentrations of 10^{-5} and 10^{-4} mol.l^{-1} the contractile responses to acetylcholine were significantly stronger compared to carbachol (Fig. 3).

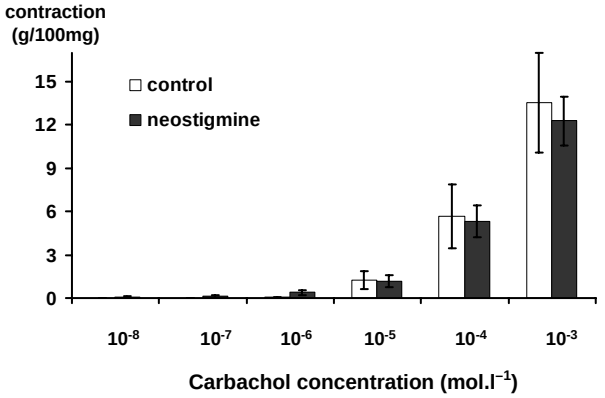

Fig. 2. The comparison of urinary bladder smooth muscle reactivity to carbachol before (Control) and after adding of neostigmine at concentration of 10^{-4} mol.l^{-1}. For description see Fig. 1.

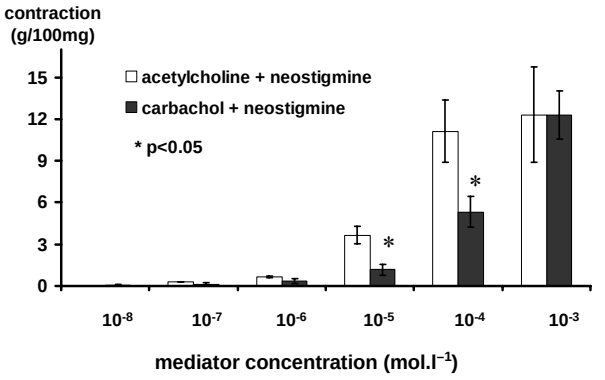

Fig. 3. The comparison of urinary bladder smooth muscle reactivity to acetylcholine and carbachol after adding neostigmine at a concentration of 10^{-4} mol.l^{-1}. For description see Fig. 1.

Discussion

The urinary bladder smooth muscle, similarly to other organ systems, needs coordinated innervations by autonomic nervous system for its physiological function (Brozmanová *et al.* 2002). In the detrusor, the postganglionic parasympathetic innervation is the most important and predominant. Acetylcholine, which can bind to muscarinic receptors, is considered to be the major contractile mediator in smooth muscles. At present, five different subtypes of muscarinic receptors (M_1-M_5) are distinguished. There are predominantly M_2 and M_3 receptors in the urinary bladder (approximately 80 % of all muscarinic receptors in the detrusor are represented by M_2 subtype). However, the M_3 receptor was confirmed as the major receptor subtype for the mediation of contractions. M_3 receptors are responsible for a direct

contraction of smooth muscle.

This process begins with binding the acetylcholine to the receptor and following activation of Gq/p protein, leading to activation of phospholipase C (PLC) (Rhee and Bae 1997). PLC catalyses the dissociation of phosphatidylinositol bisphosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG; Fig. 4). IP_3, as the second messenger, induces a release of intracellular calcium stores into the cytoplasm and contraction itself (Hall 2000).

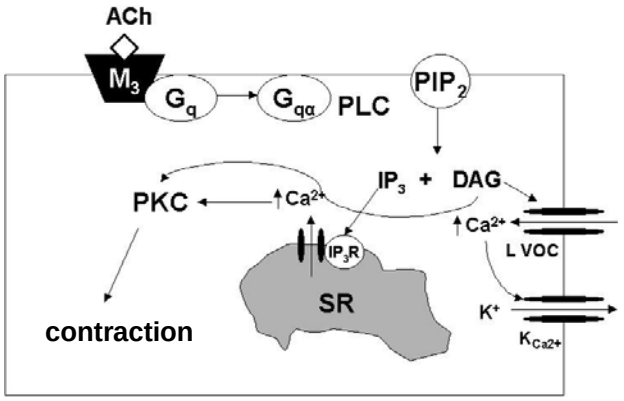

Fig. 4. Influx of calcium into the cell after activation of M_3 receptor. (ACh – acetylcholine, G_q – G-protein activating phospholipase C (PLC), PIP_2 – phosphatidyl inositol bisphosphate, IP_3 – inositol triphosphate, DAG – diacylglycerol, L VOC – voltage-operated calcium channel – type L, K_{Ca2+} - calcium activated potassium channel, SR – sarcoplasmatic reticulum, IP_3R – receptor for IP_3, PKC – proteinkinase C activated by calcium.

M_2 receptors prevent smooth muscle relaxation induced by the sympathetic nervous system and thus prolong the contraction (Hedge and Eglen 1999). The acetylcholine binding to these receptors activates protein G_i followed by inhibition of enzyme adenylylcyclase. This leads to decreased transformation of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). Cyclic AMP (the second messenger) is decomposed by enzyme phosphodiesterase to inactive AMP (Hudec *et al.* 2003, Fig. 5). Lower levels of intracellular cAMP are sufficient for keeping increased concentrations of intracellular calcium and prolonging the contraction.

Acetylcholine is synthesized in presynaptic nerve endings from acetylcoenzyme A and choline. This synthesis is mediated by enzyme cholineacetyl-transferase. Another enzyme – acetylcholinesterase (AChE) – is responsible for decomposition of acetylcholine in the synaptic cleft. Carbachol also binds directly to muscarinic receptors, but it is not decomposed by AChE and its effect is long-lasting.

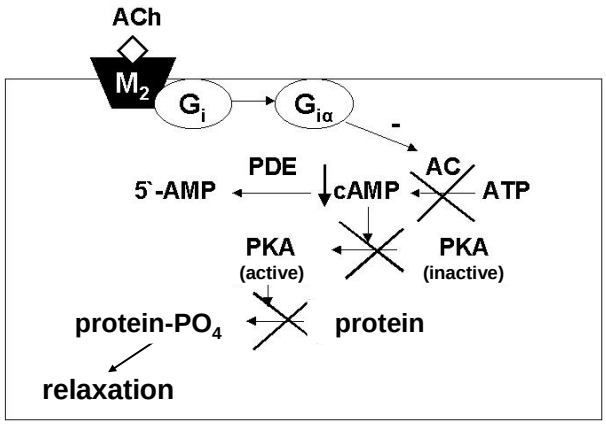

Fig. 5. Inhibition of relaxation after activation of postsynaptic M_2 receptor. ACh – acetylcholine, G_i – inhibitory G-protein, PDE – phosphodiesterase, AC – adenylyl cyclase, PKA – protein kinase A

Schneider *et al.* (2004) found that carbachol-induced contractions of human urinary bladder are mediated by M_3 receptors and largely dependent on calcium entry through nifedipine-sensitive channels and activation of Rho kinase. Phospholipase C or protein kinase C do not seem to be considerably involved in this pathway.

Jankovic *et al.* (1998) found in their study on smooth muscle strips isolated from the stomach that the affinity of agonists to muscarinic receptors decreases in the order carbachol > betanechol > metacholine > acetylcholine. They showed that the smooth muscle response to cholinergic agonists depends on intrinsic activity of these agents and not on their affinity to muscarinic receptors.

Similar results were obtained by Maize *et al.* (1998) on airways smooth muscle in ferrets. Tracheal and bronchial smooth muscles were more susceptible to administration of carbachol and metacholine as compared to acetylcholine.

Rhee and Bae (1997) found that carbachol stimulates phospholipase C 90-fold more effectively than acetylcholine. This could be considered as a reason for the observed difference in urinary bladder smooth muscle reactivity to cumulative doses of acetylcholine and carbachol (Mokrý *et al.* 2002).

Nakahara *et al.* (2003) compared in their experiments the action of neostigmine (AChE inhibitor) and tetraisopropylpyrophosphoramide (butyrylcholin-esterase inhibitor) on contractile responses of isolated rat urinary bladder smooth muscle to acetylcholine. They showed that both inhibitors caused a significant leftward shift of these contractile responses with predominant potentiation attributable to the inhibition of AChE. Thus,

AChE plays a critical role in controlling acetylcholine-induced contractions and in the regulation of resting tension of rat urinary bladder.

It has been proposed that in airway smooth muscle and pulmonary blood vessels a dual enzymatic process controls acetylcholine hydrolysis (Norel *et al.* 1993, Altiere *et al.* 1994). It means that AChE is mainly responsible for the local neuronal synaptic regulation of acetylcholine and butyrylcholinesterase protects against an increased release of acetlycholine or against an extra-neuronal appearance of acetylcholine. We found that in the guinea pig urinary bladder, even contractile responses to exogenous acetylcholine were significantly potentiated by AChE inhibition.

Furthermore, neostigmine elevated the resting tension in a concentration-dependent manner. It is therefore supposed that inhibition of AChE may not only prevent the endogenous acetylcholine degradation, but it can also stimulate the release of endogenous acetylcholine from nerve terminals (Nakahara *et al.* 2003).

Similar findings were described by Somogyi and de Groat (1992) – both neostigmine and physostigmine were shown to increase the amount of acetylcholine released by cholinergic nerves. It was suggested that they prevent breakdown of acetylcholine, which in turn, activates facilitatory M_1 receptors on the cholinergic nerves. This action requires an initial acetylcholine release. No direct stimulation of muscarinic receptors by neostigmine was observed (Andersson 1999).

In our experiments, the AChE inhibitor neostigmine increased urinary bladder smooth muscle reactivity to acetylcholine in guinea pigs. The reactivity was increased significantly more after cumulative doses of acetylcholine at concentrations of 10^{-5} and 10^{-4} mol.l^{-1} compared to carbachol at the same concentrations. These findings show that in addition to diverse mediator affinity to muscarinic receptors, different pharmacokinetic properties participate in changes of urinary bladder smooth muscle reactivity.

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