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Activation of cholinergic receptors blocks non-adrenergic non-cholinergic contractions in the rat urinary bladder

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Abstract

In the present study, the plasticity of the non-adrenergic non-cholinergic (NANC) response was investigated. Isolated rat bladder strips were electrically stimulated and the evoked contractions were isometrically recorded. The NANC part of the contractions were unmasked by applying 500 nM 4-DAMP, a potent muscarinic antagonist. Treatment of the bladder strips with 10 μ M carbachol (a cholinergic agonist) increased the muscle tone but did not alter the neurally evoked contractions. However, carbachol decreased: (1) the NANC response from 74.6% to 33.3% of control and (2) the purinergic contractile response to α,β methylene ATP (α,β mATP) (10 μ M) from 97.0% to 43.4% ($p < 0.05$). Treatment with the cholinesterase inhibitor eserine (10 μ M) also significantly decreased the NANC response to 21.1% ($p < 0.0001$). The purinergic receptor antagonist suramin (100 μ M) did not affect the neurally evoked contractions, however; subsequent addition of 4-DAMP decreased the contractions to 31%. Activation of the smooth muscle cholinergic receptors (with carbachol or eserine) and purinergic receptors (with α,β mATP) decreased the NANC contractions and the direct contractile response to α,β mATP. When the electrically evoked contractions were facilitated by the L-type Ca^{2+} channel activator, Bay-K 8644 the subsequent application of 4-DAMP did not unmask inhibited NANC contractions. We conclude that activation of muscarinic receptors by cholinergic agonist, carbachol or by endogenous acetylcholine (ACh) induce a cascade of events that leads to diminished purinergic response and consequently an inhibition of the bladder NANC response.

Keywords

nicotinic acetylcholine receptors; muscarinic acetylcholine receptors; P2X purinergic receptors; non-adrenergic, non-cholinergic (NANC) contractions; receptor interaction; bladder smooth muscle; bladder contractility

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1. Introduction

During electrical stimulation of the rat bladder, acetylcholine (ACh) and ATP are released from parasympathetic nerve terminals and activate M₃ muscarinic and P2X₁ purinergic receptors, respectively, to elicit a bladder contraction. Consequently, muscarinic antagonists such as atropine or 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine methiodide) are not fully effective in inhibiting neurally evoked bladder contractions [1,17,24] due to a non-adrenergic, non-cholinergic (NANC) contractile component. In addition, P2X purinergic antagonists inhibit most of the remaining electrically evoked contraction after atropine treatment indicating that the NANC contraction in rat bladders is regulated, in part, by purinergic pathways [8,9, 20]. The relationship between the cholinergic and the NANC response is influenced by the frequency of nerve stimulation since at higher frequencies (10-40Hz) more ACh was released and the bladder contractions became more cholinergic [14,17]. Although the participation of the NANC response is fairly constant at a given frequency of stimulation in normal bladders, it can be altered during pathology. For instance, spinal cord injury or bladder injury in rats induces bladder contractions which become more responsive to the cholinergic transmitter ACh, and less responsive to the purinergic transmitter ATP. In concert with these findings, the muscarinic blocker atropine has a greater inhibitory effect in these pathological models [17, 18].

Several bodies of evidence demonstrate that ATP can be released from postjunctional sites (i.e. smooth muscle) unlike traditional nerve transmitters such as ACh or norepinephrine (NE). For example, ACh and NE binding to smooth muscle induce post-junctional release of ATP that may overcome its neuronal release. This kind of ATP release in the adrenergic system of the vas deferens was described as cascade transmission [22,23]. Similar postjunctional release of ATP has been also demonstrated in the cholinergic system of the guinea pig ileum [10] where released ACh or exogenously applied cholinergic agonist induced the release of considerable amounts of ATP.

In this paper, we investigated the NANC response in the rat bladder in the presence of the cholinergic agonist carbachol, or in the presence of the cholinesterase inhibitor eserine, conditions associated with high extracellular levels of ACh. We also measured changes in the purinergic response to the P2X agonist α,β methylene ATP (α,β m-ATP) or the P2X antagonist suramin following muscarinic receptor activation. Finally, we determined the change in NANC response to carbachol induced by pretreatment of the preparation with a muscarinic antagonist.

2. Materials and Methods

2.1 Surgical procedures, electrical stimulations, and pharmacologic agents

Experiments were performed on female Sprague-Dawley rats weighing 250-300g. After euthanization of the rats the urinary bladder was removed and 4 longitudinal strips were prepared. The strips were mounted in 5 mL double jacketed organ baths containing oxygenated Krebs (NaCl 113, KCl 4.7, CaCl₂ 1.25, MgSO₄ 1.2, NaHCO₃ 25, KH₂PO₄ 1.2, and D-glucose 11.5 mM) at 37°C. Electrical field stimulation was applied with a Grass 88 stimulator (Astro-Med, W. Warwick, RI) via platinum wire electrodes. A pretension of 10 mN was applied to all strips prior to starting the experiments and isometric contractions were measured with a force transducer (World Precision Instruments, Sarasota, FL), connected to a bridge amplifier (Transbridge 4M, World Precision Instruments, Sarasota, FL). The data were collected in real-time using the WINDAQ data acquisition program (DataQ Instruments, Akron, OH) at a sampling rate of 20 Hz. Trains of square wave impulses (0.25 ms, 20 Hz, 200 shocks) were applied at a voltage that produced maximal contractions every 100 s. Drugs were added to the organ bath following the experimental paradigms described in Fig. 1. All animal experiments

were carried out in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals, and were approved by the institutional review board.

Experimental protocol—The experimental protocol is summarized in Fig 1. The α,β mATP was added at the start and the end of the experiments as shown in Fig 1a through 1c. The time difference between the two applications was approximately 60 min. The amplitudes of the electrically evoked contractions were measured and the average of three contractions before the first drug addition was considered as 100% (A1). All the changes were expressed as percent of control. In Fig. 1a, b and c the change in the amplitude of α,β mATP induced contractions to treatment was expressed as percent of the contraction amplitude taken at the beginning of the experiment (A1). At the end of all experiments the viability of tissue contractions was tested by adding 100mM K^+ to the tissue bath.

2.2 Statistics

The experimental results were expressed as mean $\pm$ S.E.M. Paired or unpaired t-tests were performed as appropriate using the statistical function of PRISM graphical program (GraphPad Software Inc, San Diego, CA). Statistical significance was considered at the level of $P \leq 0.05$.

2.3. Drugs

The pharmacologic agents carbachol, eserine, atropine, 4-DAMP, α,β mATP, suramin and BAY-K 8644 and all constituents of Krebs solution were obtained from Sigma-Aldrich, Inc (St Louis, MO).

3. Results

3.1 Effect of carbachol on the amplitude of NANC contractions

In rat bladder strips, 4-DAMP decreased the amplitude of neurally evoked stimulations by 25.4% ($n=14$, Fig. 3). In other words, the NANC response accounted for $74.6 \pm 5.0\%$ of the neurally evoked contractions in the controls. Treatment with 10 μ M carbachol prior to 4-DAMP application (paradigm 'b', Fig. 1) caused a robust contraction of the bladder strips that persisted until 4-DAMP was applied subsequently (Fig. 2A). In the presence of carbachol the neurally evoked contractions were superposed on the increased baseline but the contraction amplitude did not change significantly as compared to the control. The contraction amplitude was significantly decreased after 0.5 μ M 4-DAMP was added to the solution to $33.3 \pm 3.5\%$ of controls ($n=13$, $p<0.0001$, Figs. 2A and 3). However, when bladder strips were treated with a lower concentration of carbachol (1.0 μ M) that also caused contractions of the bladder strips, the subsequent application of 4-DAMP did not significantly decrease the contraction amplitude over the inhibitory effect of 4-DAMP alone ($69.1 \pm 14.0\%$ of controls, $n=4$, $p>0.1$). Adding 4-DAMP before 10 μ M carbachol to preemptively block the muscarinic receptors (paradigm 'c') did not significantly alter the NANC response ($72.0 \pm 12.7\%$ of controls, $n=5$, $p=0.82$) as compared to the inhibition produced by 4-DAMP alone (Fig. 1, paradigm "a")

3.2 Effect of the cholinesterase inhibitor eserine on the amplitude of NANC contractions

In this series of experiments, 10 μ M of eserine was added to the organ bath. After 30 min incubation time, the amplitude of the electrically evoked contractions was initially increased then slightly decreased while the baseline tone became elevated. When 4-DAMP (0.5 μ M) was added to the bath, the elevated baseline tone returned to the control level and at the same time the amplitude of the electrically evoked contractions was abruptly reduced ($21.1 \pm 8.0\%$ of controls; $p<0.0001$; $n=4$, Figs. 2B and 3).

3.3 Effect of Ca^{2+} channel activation on the amplitude of NANC contractions

To test whether facilitation of the electrically evoked contractions induced downstream from the muscarinic receptors contributes to the enhanced inhibitory effect of 4-DAMP, the L-type Ca^{2+} channels were activated by BAY-K-8644 (BAY-K) (10 μM) [16]. BAY-K enhanced the neurally evoked contractions and increased the basal tone of the bladder strips (Fig. 4) similar to what happened in the presence of eserine, but subsequent application of 500 nM 4-DAMP only slightly inhibited the neurally evoked contractions and had no greater inhibitory effect than when 4-DAMP was applied alone.

3.4 Effect of P2X_1 receptor activation on the amplitude of NANC contractions

After inhibiting the muscarinic receptor-evoked contractions with 4-DAMP (500 μM), 10 μM α,β mATP was added to the tissue bath. The activation of the P2X_1 receptors by α,β mATP caused a transitory contraction and then further decreased the neurally evoked contractions to 31.4% of controls ($n=6$, $p<0.001$) (Fig. 5).

3.5. Effect of P2X_1 receptor inhibition on the amplitude of NANC contractions

In this series of experiments, the P2X receptor antagonist suramin was applied in a concentration of 100 and 500 μM [8]. Suramin did not affect the neurally evoked contractions in either concentration; however, subsequent application of 4-DAMP caused a robust inhibition similar to that observed when 4-DAMP was applied after carbachol (Fig. 6). When suramin was applied after carbachol it did not affect the neurally evoked contractions; however, 4-DAMP exerted an enhanced inhibitory effect similar to that produced without suramin. (Fig. 6).

3.6 Effect of carbachol and 4-DAMP on the contractile response to α,β mATP

In paradigm a, b, and c, 10 μM α,β mATP was applied at the beginning and the end of each experiment (A1 and A2) approximately 60 min apart. The change in amplitude of the purinergic receptor-activated contractions was calculated as the percent decrease in amplitude of A2 compared to A1. In paradigm 'a' when only 4 DAMP (500 nM) was applied between A1 and A2, the amplitude of A2 was almost identical to A1 indicating that under this experimental condition no desensitization of purinergic receptors occurred ($97.0 \pm 18.7\%$ compared to A1, Fig.7). Using paradigm 'b', carbachol and subsequent 4-DAMP treatment caused a significant decrease in amplitude of A2 ($43.4 \pm 7.1\%$ compared to A1, $p<0.05$, $n=6$). However, when muscarinic receptors were inhibited by 4-DAMP before carbachol (paradigm 'c'), the amplitude of A2 was not significantly different from that of A1 ($83.1 \pm 5.3\%$ compared to A1, $p>0.10$, $n=4$ Fig.7).

4. Discussion

In this paper, we demonstrate a reduction of non-adrenergic non-cholinergic (NANC) bladder contractions after activating the smooth muscle cholinergic receptors by carbachol or by increased levels of endogenous ACh. The NANC contraction is defined as the neurally evoked contractile response remaining after cholinergic and adrenergic blockade. Although the adrenergic transmitter NE is released in response to electrical stimulation [12], it does not significantly contribute to the rat bladder contraction and therefore, we chose not to address the adrenergic system in this study. Our results show that carbachol and subsequent application of a muscarinic antagonist reduce the contractile effects of the stable ATP analogue, α,β mATP on the bladder smooth muscle. Since ATP is the main component of the NANC contraction [4,8] we concentrated our attention on the role of cholinergic and purinergic receptors in the down-regulation of the NANC response.

4.1. Effect of cholinergic receptor activation

High frequency (10-20Hz) stimulation evokes facilitation of ACh release in the bladder parasympathetic nerve terminals that is mediated via M1 muscarinic receptors [14]. This facilitation is further augmented by increasing the extracellular concentration of ACh by the cholinesterase blocker eserine [13] and can be inhibited by muscarinic inhibitors [14,17]. In contrast, in other peripheral tissues like ileum or heart, carbachol or eserine inhibits ACh release by activating presynaptic inhibitory M2 or M4 receptors in the parasympathetic nerve terminals. Consequently, in these tissues muscarinic antagonists significantly increase ACh release by blocking these presynaptic inhibitory receptors [14,15,21]

One would expect a marked increase in the amplitude of neurally evoked contractions through enhancing ACh release with carbachol or eserine application. The fact is that the increase in contractile amplitude varied after carbachol or eserine application; however, we did not experience any major increase in the contractile amplitude that would approach the vast increase in ACh release. In other words, the facilitated release of ACh does not translate to a major enhancement in the contractile amplitude [5]. On the other hand, the muscarinic antagonist 4-DAMP evoked a more significant inhibition of the contractile amplitude after carbachol application as compared to the inhibition produced by 4-DAMP alone. This finding suggests that the cholinergic contractile component becomes more prominent under these experimental conditions. In other words, carbachol reduced the sensitivity of the purinergic receptors to released ATP while at the same time enhanced the muscarinic response. The outcome of these bidirectional changes is that the amplitude of the electrically evoked contractions does not change considerably following massive activation of the cholinergic receptors.

One might question whether a smaller degree of muscarinic receptor activation would result in a smaller shift towards a more prominent cholinergic contractile response. It seems that the diminished NANC response depends on the degree of cholinergic activation, as smaller concentrations of carbachol (0.5-1 μ M) did not induce an increased inhibition of NANC by muscarinic antagonists. In addition, when muscarinic receptors were blocked before addition of carbachol the reduction in the amplitude of NANC did not occur. Thus the above results suggest that: 1) Muscarinic receptors should be activated to a certain degree to evoke the increased NANC inhibition and 2) functional muscarinic receptors should be present to elicit the carbachol-evoked enhanced NANC inhibition.

4.2. Effect of purinergic receptor activation

When the cholinergic part of the neurally evoked contractions was eliminated by 4-DAMP (i.e. the NANC response was unmasked), we found that addition of α,β mATP further inhibited the NANC response indicating that additional activation of purinergic receptors makes them less responsive to the released ATP. This can be explained in either of two ways: 1) The release of ATP is inhibited by α,β mATP or, 2) P2X₁ receptors are desensitized by high concentrations of α,β mATP or ATP. The first possibility should be abandoned because it has been demonstrated that α,β mATP or ATP increases ATP release from peripheral smooth muscle tissues [22] and from urothelial cells [19]. However, the second possibility is a more plausible explanation. When α,β mATP was applied to the bath we observed that it caused a transitory increase in smooth muscle tone that rapidly returned to the baseline [25] which can be explained by complete desensitization of the P2X₁ receptors. When we applied 10 μ M α,β mATP at the beginning and at the end of the experiments the amplitude of the smooth muscle tone increase was not significantly different. Since the two α,β mATP induced contractions were applied at least 60 min apart, we concluded that if there was any short term desensitization of the P2X₁ receptors after the first application of α,β mATP, it was reversed by the time of the second application occurred. However, when carbachol was applied before the second α,β mATP

induced contraction, the α,β mATP-evoked contraction amplitude became significantly lower, suggesting that carbachol exerted an inhibitory effect on smooth muscle P2X₁ receptors. Since it has been shown that ATP release is enhanced by carbachol [10] in other smooth muscle preparations, it is plausible to conclude that the carbachol induced release of ATP may desensitize purinergic receptors in the bladder smooth muscle. The question arises as to why this inhibition is only unmasked after the smooth muscle muscarinic receptors are blocked. If the purinergic and cholinergic signals were additive in the smooth muscle, the change in amplitude should occur immediately after purinergic desensitization by carbachol. The idea of the non-additive nature of the purinergic and cholinergic contractions observed in these experiments is further supported by the fact that blocking the purinergic receptors with the non-specific blocker suramin after carbachol did not induce any inhibition in the contraction amplitude. In this case the purinergic receptors were already inhibited and the cholinergic system already compensated for the missing purinergic response. Subsequent administration of a muscarinic blocker unmasked the increased inhibition of NANC.

In another set of experiments we tried to address whether suramin itself can affect the amplitude of the electrically evoked contractions. Suramin has been shown to exert a moderate inhibitory effect on the electrically evoked contractions at 4Hz stimulation and an intense inhibition on the NANC at the same frequency [8]. In our experimental paradigm we used 20 Hz stimulation with 100 shocks that by itself induces a significant facilitation of ACh release [14] while lower frequencies (5 Hz) have only a modest facilitation [17]. At this stimulation paradigm a moderate concentration of suramin (100 μ M) that is 8 times higher than its IC₅₀ [8] failed to inhibit the neurally-evoked contractions but subsequent application of a muscarinic antagonist produced a significant inhibition. We think that while the purinergic receptors were inhibited by suramin the cholinergic receptors compensated for the loss in purinergic contractile response so no change in amplitude was observed. The enhanced cholinergic receptor activity was completely blocked by muscarinic receptor inhibition and not a reduction in the NANC response was unmasked.

4.3. Putative receptor interactions

What is the mechanism for the simultaneous down-regulation of the purinergic response and up-regulation of the cholinergic response in the presence of carbachol or eserine? It is clear that facilitation of ACh release and smooth muscle contractions downstream from the muscarinic receptors with the Ca²⁺ channel agonist BAY-K 8644 [10] does not evoke an intense inhibition of the NANC contraction. Thus, muscarinic receptors may have a central role in the enhanced inhibition of the NANC. This is supported by the fact that when the muscarinic receptors were inhibited by 4-DAMP before carbachol application, we did not observe the NANC inhibition. If we accept the hypothesis that the purinergic inhibition is the consequence of a short term desensitization by carbachol induced ATP release we can speculate that muscarinic receptor activation may enhance the purinergic receptor desensitization. This kind of desensitization observed with muscarinic receptor activation has been described regarding nicotinic receptors in the enteric ganglia [6]. Whether muscarinic receptors can couple to other types of ionotropic receptors like P2X₁ receptors needs further investigation and these experiments will be performed in the near future. Operation of other possible receptor interactions should also be taken into account. It is tempting to consider that after inhibition of the muscarinic receptors with 4-DAMP the remaining site of action for carbachol would be with nicotinic receptors. Recently, several subtypes of nicotinic receptors have been demonstrated in vascular smooth muscle [7]. In addition, investigators have described that nicotinic and purinergic receptors can negatively interact with each other in the neural cells of sympathetic or enteric ganglia [2,11,26].

In summary, muscarinic antagonists exert a higher inhibitory effect on the neurally evoked NANC contraction when given in the presence of a cholinergic agonist. Since the muscarinic blockade unmasks the NANC part of the contraction, we conclude that when we sequentially activate and then inhibit the muscarinic receptors with carbachol and 4-DAMP, respectively, the NANC response is diminished. This does not occur when the drug application sequence is reversed, emphasizing the importance of functional muscarinic receptors in this process. Our results suggest that following muscarinic receptor activation, the cholinergic component of the electrically evoked contractions is increased while, at the same time, the purinergic contractions are decreased. Thus, the neurally evoked amplitude does not or only slightly changes. The same phenomenon was observed when the purinergic blocker suramin was applied; the amplitude of the contractions was unaffected but muscarinic inhibition unmasked the NANC contraction. These results give important insight into the NANC mechanism of bladder function, since the NANC response decreases with pathology (spinal cord injury, bladder injury) in the rat model [17,18] and increases following pathology in human bladders [3].

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Fig. 1.

Experimental paradigms of bladder strips using various sequences of agonists and antagonists to study the plasticity of the NANC response. Paradigm 'a' served as control experiment where only the muscarinic antagonist 4-DAMP was applied to unmask the NANC response; while paradigm 'b' examined the effects of cholinergic stimulation (carbachol) on the NANC response. In paradigm 'c', 500 nM 4-DAMP was administered before carbachol to preemptively block the muscarinic receptors. Paradigm 'd' investigated the effects of increasing synaptic acetylcholine concentration by applying the cholinesterase inhibitor eserine. Paradigm 'e' studied the effects of P2X₁ purinergic receptor activation by α,β -mATP. For the experiments with BAY-K 8644 and suramin basically the paradigm 'b' was applied

but the drugs were added to the bath in lieu of carbachol. Symbols (Δ +W) washout; (Δ +C) control for the calculations; (Δ +R) reading the drug effect (result); ($\blacktriangle$) addition of drug; A1 α,β mATP at the beginning and A2 at the end of the experiments.

Fig. 2. Representative tracing showing the effect of the cholinergic agonist carbachol (10 μ M) (panel A) and the cholinesterase inhibitor eserine (10 μ M) (panel B) on the neurally evoked contractions and the action of subsequent application of the muscarinic antagonist, 4-DAMP (500 nM). Stimulation parameters: 20 Hz 200 shocks 0.25 ms every 100 s.

Fig. 3. Effects of the cholinergic agonist carbachol (CCh, 1 μ M, 10 μ M) and the cholinesterase inhibitor eserine (10 μ M) on NANC response of bladder strips. The data were expressed as percent of control. The NANC response was elicited by 4-DAMP (0.5 μ M). *** $p < 0.0001$.

Fig. 4. Effect of BAY-K 8644 (10 μ M) on the neurally evoked contractions and the effect of the subsequently applied atropine (4-DAMP, 0.5 μ M). Left panel: tracing from one individual experiment; right panel: summarized effect of 5 experiments. *** $P < 0.001$ (paired t test). The amplitude of neurally evoked contractions before addition of BAY-K served as control.

Fig. 5. Effects of the stable ATP analogue α,β mATP (10 μ M) on the contractile amplitude of the NANC response. α,β mATP was added to the bath after 4-DAMP application (paradigm 'e', *** $p < 0.001$). The amplitude of the neurally evoked contractions before 4-DAMP was used as control for these experiments.

Fig. 6.

Effect of the purinergic receptor antagonist suramin on neurally evoked contractions. Upper panel shows the individual tracing with suramin (500 μ M) and subsequently applied 4-DAMP (0.5 μ M). Lower left panel shows the sequential application of carbachol (10 μ M) suramin (100 μ M) and 4-DAMP (n=6). Suramin did not inhibit the neurally evoked contractions when applied after carbachol, while 4-DAMP (0.5 μ M) exerted an enhanced inhibitory effect. Lower right panel demonstrate that 4-DAMP induced an enhanced inhibitory effect after suramin. Baseline means $\Delta=C$ as it was defined in Fig 1. (n=6)

Fig. 7.

Effects of carbachol (CCh, 10 μ M) on the amplitude of the direct contractile response evoked by α,β mATP. Purinergic contractions were evoked by 10 μ M α,β mATP at the beginning (A1) and the end (A2) of the experiments. The data are expressed as percent of the first contraction (A1). When only 4-DAMP was applied between the A1 and A2 the contraction amplitudes were not significantly different ($p > 0.2$ paired t-test; column 'A' $n=6$). When carbachol (10 μ M) was applied before 4-DAMP (500 nM) the A2 contractions were significantly reduced ($p < 0.05$ $n=6$; column 'B'). However, the sequence of application of CCh and 4-DAMP is crucial; when carbachol was applied against inhibited muscarinic receptors the direct contractile effect of α,β mATP was not reduced at A2 ($p > 0.1$ $n=4$; column 'C')