Muscarinic receptors couple to modulation of nicotinic acetylcholine receptor desensitization in myenteric neurons

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Running head: nAChR desensitization in myenteric neurons

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Abstract

Signaling mechanisms coupled to activation of different neurotransmitter receptors interact in the enteric nervous system. Acetylcholine (ACh) excites myenteric neurons by activating nicotinic acetylcholine receptors (nAChRs) and muscarinic receptors expressed by the same neurons. These studies tested the hypothesis that muscarinic receptor activation alters the functional properties of nAChRs in guinea pig small intestinal myenteric neurons maintained in primary culture. Whole-cell patch clamp techniques were used to measure inward currents caused by ACh (1 mM) or nicotine (1 mM). Currents caused by ACh and nicotine were blocked by hexamethonium (100 μM) and showed complete cross-desensitization. The rate and extent of nAChR desensitization was greater when recordings were obtained with ATP/GTP-containing compared to ATP/GTP-free pipette solutions. These data suggest that ATP/GTP-dependent mechanisms increase nAChR desensitization. The muscarinic receptor antagonist, scopolamine (1 μM) decreased desensitization caused by ACh but not by nicotine, which does not activate muscarinic receptors. Phorbol 12, 13 dibutyrate (10 - 100 nM), an activator of protein kinase C (PKC), but not 4-α-phorbol 12-myristate 13-acetate (a PKC inactive phorbol ester) increased nAChR desensitization caused by ACh and nicotine. Forskolin (1 μM), an activator of adenylate cyclase, increased nAChR desensitization but this effect was mimicked by dideoxyforskolin, an adenylate cyclase inactive forskolin analog. These data indicate that simultaneous activation of nAChRs and muscarinic receptors increases nAChR desensitization. This effect may involve activation of a PKC-dependent pathway. These data also suggest that nAChRs and muscarinic receptors are coupled functionally through an intracellular signaling pathway in myenteric neurons.

Key words: enteric nervous system, electrophysiology, intracellular signaling
Introduction

It has been known for some time that “cross-talk” between intracellular signaling pathways activated by different classes of cell surface receptors is a mechanism for integration of information coming in to individual cells, including neurons (12, 24). Typically, these interactions involve G-protein-linked receptors and two or more intracellular signaling pathways composed of multiple enzymatic processes and diffusible signaling molecules (12, 23). Recently, it has been shown that there are interactions between different ligand-gated ion channels that result in inhibition of signals mediated by each receptor type. This interaction does not involve diffusible second messenger molecules but instead may involve direct protein-protein interaction. For example, in myenteric neurons nicotinic acetylcholine receptors (nAChRs) and P2X receptors exhibit cross inhibition when these receptors are activated simultaneously (9, 26). In the submucosal plexus, a similar direct inhibitory interaction occurs between P2X receptors and 5-HT₃ receptors (1).

Acetylcholine, acting at nAChRs and at M₁ muscarinic receptors, is an important excitatory neurotransmitter in the enteric nervous system (ENS)(6, 7, 15, 16). M₁ Muscarinic receptors are G-protein coupled receptors that link to activation of phospholipase C (PLC) and generation of inositol triphosphate and diacylglycerol (DAG)(19). DAG activates protein kinase C (PKC) and PKC can phosphorylate a variety of intracellular targets including ion channels (21). M₁ muscarinic receptors mediate some slow excitatory postsynaptic potentials (sEPSPs) in the ENS (17, 18). Furthermore, drugs which inhibit the PLC-PKC signaling pathways can inhibit sEPSPs while drugs which activate this system can mimic sEPSPs (2). The sEPSPs are associated with a long-lasting increase in excitability. ACh acting at nAChRs mediates most fast excitatory postsynaptic potentials (fEPSPs)
in the ENS. There are neurons in the ENS which receive dual excitatory synaptic input mediated by ACh acting at nAChRs and muscarinic receptors (17,18). The long-lasting increase in excitability subsequent to muscarinic receptor activation can potentiate subsequent fEPSPs mediated by nAChRs on the same neuron. This is a mechanism by which two different receptor mechanisms can interact in the ENS. However, it is also known that nAChRs are targets for phosphorylation which alters desensitization and other functional properties of the nAChR (4, 5, 8, 20). It is not known if the functional properties of nAChRs in the ENS can be modified following activation of intracellular signaling pathways. In the present study, we tested the hypothesis that simultaneous activation of nAChRs and muscarinic receptors is associated with an alteration in desensitization of nAChRs expressed by the same myenteric neurons. Data from these studies would provide evidence that different types of receptor for the same transmitter can interact functionally and therefore modify interneuronal signaling.
MATERIALS AND METHODS

Primary culture of myenteric neurons. The procedures used in these studies are similar to previously published methods using similar preparations (9, 26, 27). Newborn (1 to 2 days old) guinea pigs were anesthetized by halothane inhalation, stunned, and exsanguinated by severing major neck blood vessels. The entire small intestine was placed in 4°C Krebs bicarbonate buffer of the following composition (millimolar): NaCl, 117; KCl, 4.7; CaCl₂, 2.5; MgCl₂, 1.2; NaH₂PO₄, 1.2; NaHCO₃, 25; glucose, 11. The longitudinal muscle myenteric plexus was stripped free using a moist cotton swab and then cut into 5 mm pieces. Dissected tissues were divided into two equal aliquots and each aliquot was transferred to 1 mL of sterile-filtered Krebs’ solution containing 1600 U of trypsin (Sigma Chemical Co., St. Louis, MO) for 30 minutes at 37°C. Following trypsin incubation, tissues were triturated 30 times through a fire-polished Pasteur pipette and centrifuged at 900 g for 10 minutes using a bench-top centrifuge. The supernatant was discarded and the pellet was resuspended in 1 mL Krebs’ solution containing 4000 U crab hepatopancreas collagenase (Calbiochem-Novabiochem, Corp., La Jolla, CA). The suspension was triturated using fire-polished Pasteur pipette and then centrifuged for 10 minutes. The pellet was resuspended in Eagle’s minimum essential medium (MEM) containing 10% fetal bovine serum, gentamycin (10 μM), penicillin (100 U/mL) and streptomycin (50 mg/mL) (all from Sigma). Aliquots (200 μL) was plated onto sterile, poly-L-lysine (MW 30-70K, Sigma) - coated glass cover slips placed in 35 mm plastic culture dishes containing 3 mL of MEM. After two days of incubation, cytosine arabinoside (10 μM) was added to the MEM to limit smooth muscle and fibroblast proliferation. Cultures were maintained at 37 °C in a tissue culture incubator containing a 5% CO₂ atmosphere. Cultures were maintained up to 2 weeks after plating with medium replacement every 2 days.
Whole-cell patch clamp recordings. The glass cover slips containing neurons were removed from the culture dishes and placed in a plexiglass recording chamber (3 mL volume) with a glass bottom. The recording chamber was placed on the stage of an inverted microscope and neurons were viewed using Hoffman modulation contrast optics. The extracellular solution was the standard Krebs’ solution described above. The Krebs’ solution was superfused through the recording chamber at a flow rate of 4 mL/min. Fire polished patch clamp pipettes were fabricated from borosilicate glass capillary tubes (World Precision Instruments, Sarasota FL). The fire polished pipettes had a tip resistance of 3-7 MΩ. Electrode liquid junction potentials and series resistance was compensated. The standard intracellular (pipette) solution for patch clamp recording is as follows (millimolar): CsCl, 160; MgCl₂, 2.9; EGTA, 10; HEPES, 10; ATP, 0.5; GTP, 0.25; pH was adjusted to 7.4 using CsOH. The CaCl₂/EGTA ratio yielded a resting level of free [Ca²⁺] of less than 100 nM. Experiments were performed using an Axopatch 200B patch clamp amplifier, a Digidata 1200 A/D converter and pCLAMP 6.01 programs for acquisition, storage and analysis of data (Axon Instruments, Burlingame, CA). Data was filtered at 2 kHz using a 4 pole Bessel filter (Warner Instruments, New Haven, CT) and were digitized at a rate of 5 kHz and stored on the computer hard drive. Unless otherwise indicated the holding potential was -60 mV.

Drug application. Drugs were applied via flow tubes gated by computer-controlled solenoid valves. Four of these flow through tubes were glued together at the tip and each was connected via polyethylene tubing to a reservoir syringe (10 mL) containing a known concentration of drug. The reservoir platform was positioned above the recording chamber so that drugs were gravity fed through the flow tubes. Platform height was adjusted to provide a flow rate of 0.1 mL/min. The flow tube array was mounted on a micromanipulator and drugs were applied by positioning the flow
tubes over the neuron. This method permitted rapid adjustments between control and drug-containing solution (equilibrium < 100 ms).

**Drugs.** All drugs were obtained from Sigma Chemical Company (St. Louis, MO). Acetylcholine and nicotine were prepared as 1 M stock solutions in deionized water. Phorbol 12,13 dibutyrate (PDBu), 4-α-Phorbol 12-myristate 13-acetate (4-α-PMA), forskolin and dideoxyforskolin (DDF) were prepared as stock solutions in 50% (v/v) ethanol/deionized and were then diluted to working concentrations in Krebs’ buffer. The final ethanol concentration in the buffer did not exceed 0.05%.

**Statistics.** All data were expressed as mean ± S.E.M. and “n” values refer to the number of neurons from which data were obtained. Differences between treatment groups were established using Student’s t-test for paired and unpaired data, and one-way analysis of variance and Student Newman Keul’s test for multiple comparisons. A P value < 0.05 was taken as the level of statistical significance.
RESULTS

Acetylcholine (ACh) and nicotine activate nAChRs in myenteric neurons. ACh (1 mM) caused rapidly developing and desensitizing inward currents in >90% of neurons tested (Fig. 1A). ACh caused inward currents that did not decline in amplitude over 20 minutes, the time course of most experiments done here. In 8 neurons, the initial ACh response was 1.3 \( \pm \) 0.3 nA while at 20 minutes the response amplitude was 1.5 \( \pm \) 0.3 nA (P \( > \) 0.05). These data are similar to those published previously (27). ACh-induced currents were due to activation of nAChRs as they were mimicked by nicotine (1 mM)(Fig. 1A). In addition, ACh- and nicotine-induced currents were blocked completely and reversibly by the nAChR antagonist hexamethonium (Fig. 1B). ACh and nicotine also activated the same receptors as responses caused by nicotine and ACh exhibited cross desensitization. In these experiments ACh (1 mM) was applied for 2 seconds and after a 5 minute recovery, nicotine was applied for 2 s. After an additional 5 minute recovery ACh was applied again for 7 seconds and the inward current decayed to a steady state level during this time (see below). Nicotine was applied again at this point and the nicotine-induced current was reduced significantly(Fig. 1C). After a 5 minute recovery period the ACh response returned to near its initial amplitude (Fig. 1C)

Modulation of nAChR desensitization during muscarinic receptor activation. ACh-induced currents decayed in the continued presence of agonist and the ACh-induced current reached a steady state level within 7 s (Fig. 2A and 2B). The time course of desensitization was quantified as the time to half decay (\( T_{1/2} \)) of the ACh current in each neuron. The average time course for the decay of the ACh response under different recording conditions is presented in Fig. 2B. The ACh
current amplitude was measured at the peak response ($I_{peak}$) and this was set as time 0. The amplitude of the ACh current was then measured at 1 s intervals ($I_{time}$) until the current reached a steady state level ($I_{steady\ state}$) (Fig. 2A). The amount of nAChR desensitization was quantified by calculating the ratio of $I_{steady\ state}$ vs. $I_{peak}$ in the same neuron (Fig. 2A). ACh-induced currents declined by half in less than 3 s and reached a steady state level of about 77% of the initial peak current (Fig. 2B, Table 1).

Desensitization of nAChRs can be modulated by a variety of intracellular signaling pathways and receptor phosphorylation (20). Therefore we tested the effects of removing ATP and GTP from the pipette solution on the rate and extent of nAChR desensitization in myenteric neurons. The peak amplitude of the ACh-induced current recorded using an ATP/GTP-free pipette solution was not different from the peak amplitude obtained using ATP/GTP containing pipette solutions (Table 1). Furthermore, the ACh-induced current recorded without ATP/GTP in the recording pipette was stable in amplitude over a 20 minute recording period. When using an ATP/GTP-free pipette solution, the initial ACh-induced current was $1.5 \pm 0.5$ nA while 20 minutes after establishing the whole-cell recording the ACh response amplitude was $1.4 \pm 0.5$ nA ($n = 6$, $P > 0.05$). When recordings were obtained using an ATP/GTP-free pipette solution, the $T_{1/2}$ for decay of the ACh-induced current increased to 4 s while the steady state current was approximately 60% of the initial peak current (Fig. 2B, Table 1).

Myenteric neurons express muscarinic cholinergic receptors which can couple to activation one or more intracellular signaling pathways leading to excitation of myenteric neurons. It is possible that simultaneous activation of muscarinic receptors by ACh could modulate nAChR function. Therefore, we tested the effects of scopolamine (1 µM), a muscarinic receptor antagonist,
on the rate and extent of nAChR desensitization recorded using an ATP/GTP-containing pipette solution. The peak amplitude of the ACh-induced current recorded in the presence of scopolamine was not different from that recorded under control conditions (Table 1). Furthermore, it was found that scopolamine decreased both the rate and extent of nAChR desensitization (Fig. 2B, Table 1).

Scopolamine also increased the rate of recovery from desensitization of nAChRs caused by ACh. This conclusion is based on studies in which ACh (1 mM) was applied for 7 s to cause nAChR desensitization and ACh was re-applied at several time points after the initial response to determine the time course of recovery from desensitization (Fig. 3A). Under control recording conditions the ACh response had a time to half recovery of 0.1 ± .04 min (n = 7) while in the presence of scopolamine (1 µM) the time to half recovery was reduced to 0.03 ± 0.01 min (n = 6, P < 0.05)(Fig. 3B).

*Scopolamine does not alter nAChR desensitization caused by nicotine.* It is possible that scopolamine could alter the rate and extent of nAChR desensitization through mechanisms other than blockade of muscarinic receptors. To rule out this possibility, we tested the effects of scopolamine on currents caused by nicotine which will not activate muscarinic receptors. It was found that both the time course and extent of nAChR desensitization caused by nicotine were unaffected by scopolamine (Fig. 4A, Table 1). Scopolamine also did not alter the time course of recovery from desensitization caused by nicotine (Fig. 4B). The time to half recovery from nicotine desensitization under control conditions was 0.67 ± 0.1 min while in the presence of scopolamine this value was 0.51 ± 0.1 min (P > 0.05).

*Bethanechol (BeCh) increases nAChR desensitization.* The data presented above indicate that muscarinic receptor activation can increase nAChR desensitization. This issue was further
investigated by using nicotine to selectively activate and desensitize nAChRs in the absence and presence of the muscarinic receptor agonist BeCh (100 μM). It was found that under control conditions, the amount of desensitization in the same neuron was stable on successive nicotine applications (Fig. 5 left). However, when neurons were treated with BeCh, the amount of nAChR desensitization caused by nicotine increased significantly (Fig. 5 right). BeCh did not change the amplitude of the peak nicotine induced current. The control peak nicotine current was 1.7 ± 0.5 nA while in the presence of BeCh this value was 1.8 ± 0.6 nA (P > 0.05, n=5).

Phorbol ester-induced modulation of nAChR desensitization. Myenteric neurons express M₁ type muscarinic receptors which couple via G_q to the phosphatidyl inositol (PI)-dependent signaling pathway and activation of PKC (19). Therefore, PDBu was used to activate PKC in order to investigate a role for this enzyme in modulating nAChR desensitization. ACh (1 mM) (in the presence of 1 μM scopolamine) or nicotine (1 mM) were applied after pretreating neurons with vehicle (0.05% EtOH) or PDBu (10 - 300 nM). It was found that PDBu increased the maximum nAChR desensitization occurring during a 7 s application of ACh (Fig. 6A) or nicotine (Fig. 6B). However, PDBu did not change the amplitude of the peak ACh- or nicotine-induced current (Table 2). To test that PDBu was not acting via an PKC-independent mechanism to alter nAChR desensitization, a PKC inactive phorbol ester analog, 4-α-PMA was tested. 4-α-PMA (10 - 300 nM) did not alter the amount of nAChR desensitization caused by either ACh (Fig. 6A) or nicotine (Fig. 6B). Furthermore, 4-α-PMA did not change the peak ACh- or nicotine-induced currents (Table 2).

Effects of forskolin on nAChR desensitization. The next set of experiments tested the possibility that adenylate cyclase-dependent mechanisms could also modulate nAChR desensitization. These studies used forskolin (1 μM) to stimulate adenylate cyclase. Forskolin did
not alter the peak current amplitude caused by either ACh or nicotine (Table 2). Forskolin increased
the maximum nAChR desensitization caused by both ACh (scopolamine present) and nicotine (Fig.
7A and 7B). However, it was also found that dideoxyforskolin (DDF), a forskolin analog that does
not activate adenylate cyclase, also caused an apparent increase in nAChR desensitization caused
by ACh (Fig. 7A) and nicotine (Fig. 7B). These data suggest that forskolin decreased the steady
state nAChR current at least partly through an adenylate cyclase-independent mechanism.
ACh is the principal excitatory neurotransmitter in the ENS and ACh acts at nAChRs and M₁ muscarinic receptors expressed by the same neurons to cause synaptic excitation (6, 7, 17, 18). The different time course and signaling pathways coupled to nAChRs and M₁ muscarinic receptors provide a basis for integration of synaptic input and output of the neuron receiving the mixed cholinergic input. In addition, the data from the present study indicate that activation of muscarinic receptors can alter the time course of responses mediated by nAChRs on the same neurons.

*nAChR-mediated inward currents in myenteric neurons.* Previous studies have shown that ACh acts at nAChRs to cause an inward current in myenteric neurons maintained in primary culture (24). These results were confirmed in the present study where it was shown that ACh caused a rapidly developing and desensitizing inward current that was blocked completely by the nAChR antagonist, hexamethonium. The ACh-induced current was mimicked by the selective nAChR agonist, nicotine and the nicotine-induced current was also blocked by hexamethonium. In addition, the ACh- and nicotine-induced currents cross-desensitized indicating that these responses were mediated at the same population of nAChRs. The amplitude of the ACh-induced current is stable over the period of at least 20 minutes (see also 27) and omitting ATP and GTP from the recording pipette solution did not alter the peak ACh current over this time period. However, it was found that the time course and extent of nAChR desensitization were altered by omitting ATP and GTP from the pipette solution. This result suggests that ATP- and or GTP-dependent processes modify nAChR desensitization. As ATP and GTP are substrates for kinase-dependent reactions, phosphorylation of the nAChR is a mechanism by which desensitization can be modulated (8, 15).
Muscarinic receptor activation increases nAChR desensitization. Myenteric neurons express both nAChRs and M₁ muscarinic receptors (6, 7, 17, 18). M₁ muscarinic receptors are G-protein coupled receptors that are linked to activation of phospholipase C and polyphosphoinositide (PI) hydrolysis (19). PI hydrolysis can lead to release of diacylglycerol and PKC activation (21). The data from the present study indicate that simultaneous activation of nAChRs and muscarinic receptors increases the amount of nAChR desensitization occurring during a 7 s application of ACh. This conclusion is based on the result showing that the addition of scopolamine, a muscarinic receptor antagonist, to the extracellular solution decreased the rate and extent of nAChR desensitization caused by ACh. Therefore, simultaneous activation of muscarinic receptors modulates the function of nAChRs expressed by the same neuron. M₁ muscarinic receptors mediate sEPSPs in the myenteric plexus (17, 18). The M₁ mediated sEPSP is due to an inhibition of potassium channels that are open near the resting membrane potential (18). The pipette solution used in the present study contained cesium ions to block all potassium channels. Therefore, changes in resting conductance of the neurons would not contribute to the muscarinic receptor-mediated change in nAChR desensitization.

Scopolamine could have effects on myenteric neurons that are independent of muscarinic receptor antagonism. For example, scopolamine could interact directly with the nAChR ion channel to alter its functional properties (14). To test the possibility that scopolamine altered nAChR desensitization by a muscarinic receptor-independent mechanism, nicotine was used as a nAChR agonist. As nicotine does not activate muscarinic receptors, scopolamine should not alter nAChR desensitization if it acts only at muscarinic receptors. It was found that the rate and extent of nicotine-induced nAChR desensitization was unaffected by scopolamine and therefore, scopolamine
did not interact with the nAChR via a muscarinic receptor-independent mechanism.

The data discussed above suggest that simultaneous activation of muscarinic receptors alters nAChR desensitization. To test this hypothesis more directly, nicotine and the muscarinic receptor agonist, BeCh, were applied simultaneously to individual neurons. When BeCh was co-applied with nicotine, the amount of nAChR desensitization increased compared to the level occurring during the control recording. Taken together, these data indicate that activation of muscarinic receptors increases nAChR desensitization during simultaneous activation of the two cholinergic receptors.

**PKC activation modulates nAChR desensitization.** As M₁ muscarinic receptors couple to generation of DAG (19) and DAG activates PKC (21), we tested the effects of the PKC activator, PDPu, on nAChR desensitization. PDBu caused a concentration-dependent increase in the amount of nAChR desensitization caused by ACh (in the presence of scopolamine to block muscarinic receptors) and by nicotine. However, PDBu can alter nAChR desensitization by an action that is independent of PKC activation (16). To test this possibility, we used 4-α-PMA, a phorbol ester that does not activate PKC, in our desensitization protocol. 4-α-PMA, in a concentration range identical to that used for PDBu, did not alter nAChR desensitization caused by either ACh or nicotine. These data suggest that PDBu-induced changes in nAChR desensitization were mediated by PKC activation. Although PDBu increased nAChR desensitization, the phorbol ester did not alter the peak currents caused by either ACh or nicotine. This result indicates that PKC activation does not alter the affinity of the nAChR for agonist. In rat cardiac parasympathetic neurons, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating peptide (PACAP) both cause an increase in peak nAChR mediated currents and this increase in response has been attributed to an increase in affinity of the nAChR for ACh (11). VIP and PACAP receptors modify nAChR function
through a mechanism that is independent of a diffusible second messenger but may use a membrane delimited but G-protein dependent pathway (11). Also, in rat cardiac parasympathetic neurons, receptors for substance P couple to an inhibition of a subset of nAChRs. This mechanism also does not involve a diffusible, cytosolic second messenger and it may involve a change in desensitization or the conducting properties of the nAChR ion channel (3). Both of these mechanisms are different from the pathway activated by muscarinic receptors in myenteric neurons where the increase in PKC activity may alter the transition of the nAChR from the open and activated state to one or more desensitized states (20). As peak currents are not altered by muscarinic receptor activation or treatments that mimic downstream signaling, the change in function of the nAChR is likely to be independent of changes in ACh binding.

Regulation of nAChR desensitization by PKC is not unique to myenteric neurons. Earlier studies done on desensitization of nAChRs at the neuromuscular junction and in sympathetic ganglia demonstrated that PKC activation increases nAChR desensitization (4, 5, 10, 15). Therefore, regulation of desensitization by PKC is a general mechanism by which the function of nAChRs can be modulated. Protein kinase A (PKA) has also been shown to phosphorylate nAChRs and alter desensitization of these receptors (15, 20). Based on these previous data, we tested the effects of forskolin, an activator of adenylate cyclase which can to lead stimulation of PKA. Forskolin increased nAChR desensitization to a similar degree as PDBu, however forskolin can act as a nAChR ion channel blocker (25). Therefore, we tested the adenylate cyclase inactive forskolin analog, DDF, for its effects on nAChR desensitization. It was found that forskolin and DDF caused similar increases in nAChR desensitization leading to the conclusion that the effects of forskolin on desensitization are likely to be independent of adenylate cyclase and PKA activation.
Functional Significance. There are many examples of co-transmitters released simultaneously by autonomic nerves producing responses in synaptic targets that differ from responses produced by the individual transmitters acting alone (13). These effects can be mediated presynaptically resulting in either a facilitation or inhibition of transmitter release or transmitter interactions can be postsynaptic where one transmitter may sensitize the target cell to the actions of the second transmitter (13). In addition, it has been shown previously that nicotinic and P2X receptors and P2X and 5-HT3 receptors exhibit cross inhibition when these receptors are activated simultaneously (1, 9, 27). The data from the present paper indicate that there is a potential for postsynaptic modulation of responses to single transmitter (ACh) mediated by different classes (nicotinic and muscarinic) of receptor for that transmitter. Muscarinic receptor-mediated acceleration of the desensitization rate of nAChRs could function to limit postsynaptic excitation during periods of high cholinergic nerve activity. The delay in recovery from desensitization of the nAChR would serve a similar function.
References


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Table 1. *Properties of nAChR desensitization under different recording conditions.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peak current (nA)</th>
<th>T(_{1/2}) (s)</th>
<th>I(<em>{\text{steady state}})/I(</em>{\text{peak}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh (1 mM)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control (n = 10)</td>
<td>1.4 ± 0.2</td>
<td>2.5 ± 0.3</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>without ATP/GTP (n = 10)</td>
<td>1.8 ± 0.4</td>
<td>3.9 ± 0.4*</td>
<td>0.44 ± 0.04*</td>
</tr>
<tr>
<td>Scopolamine (1 μM)(n=10)</td>
<td>1.2 ± 0.2</td>
<td>5.0 ± 0.4*†</td>
<td>0.55 ± 0.04*</td>
</tr>
<tr>
<td>Nicotine (1 mM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n = 7)</td>
<td>1.3 ± 0.2</td>
<td>3.8 ± 0.4</td>
<td>0.4 ± 0.04</td>
</tr>
<tr>
<td>Scopolamine (1 μM)(n = 6)</td>
<td>1.3 ± 0.3</td>
<td>4.1 ± 0.8</td>
<td>0.39 ± 0.03</td>
</tr>
</tbody>
</table>

Data are the mean ± S.E.M. of measurements obtained from “n” neurons. *indicates significantly different from the Control ACh groups (P < 0.05). †Indicates significantly different from the without ATP/GTP group. Data analyzed using analysis of variance and Student-Newman Keuls multiple comparisons test.
Table 2. *Peak ACh and nicotine currents (nA) before and after treatment with forskolin, DDF, PDBu or 4-α-PMA.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ACh (1 mM)</th>
<th>Nicotine (1 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Forskolin 1 μM</td>
<td>2.1 ± 0.7 (n = 9)</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>DDF 1 μM</td>
<td>1.2 ± 0.2 (n = 8)</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>PDBu 10 nM</td>
<td>1.0 ± 0.1 (n = 6)</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>PDBu 30 nM</td>
<td>1.4 ± 0.3 (n = 9)</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>PDBu 100 nM</td>
<td>1.2 ± 0.2 (n = 10)</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>4-α-PMA 10 nM</td>
<td>1.0 ± 0.2 (n = 5)</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>4-α-PMA 30 nM</td>
<td>1.3 ± 0.2 (n = 5)</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>4-α-PMA 100 nM</td>
<td>1.7 ± 0.2 (n = 5)</td>
<td>1.8 ± 0.3</td>
</tr>
</tbody>
</table>

Each concentration of drug was tested in separate groups of neurons and each neuron served as its own control. Data are the mean ± S.E.M. of measurements obtained from “n” neurons. Data were analyzed by Student’s t-test for paired data. None of the treatments altered peak currents.
Figure Legends

Fig. 1. Pharmacological properties of inward currents caused by ACh and nicotine. A, Representative traces of currents caused by a 7 s application of ACh (1 mM) and nicotine (1 mM) in the same neuron. The inward current desensitizes during continued application of each agonist. B, Responses caused by ACh and nicotine are blocked reversibly by the nAChR antagonist, hexamethonium (C6, 100 μM). *indicates significantly different from ACh (P < 0.05). † indicates significantly different from control and wash responses (P < 0.05). C, Responses caused by ACh (1 mM) and nicotine (1 mM) cross-desensitize. *indicates significantly smaller than ACh responses (P < 0.05). † indicates significantly different from ACh and nicotine alone (P < 0.05). # indicates significantly different from ACh + nicotine (P < 0.05). Data analyzed by analysis of variance and Student-Newman Keuls multiple comparisons test.

Fig. 2. A, Representative recording of an inward current caused by ACh. ACh-induced responses desensitized to a steady state level (I_{steady state}) within 7 s. ACh was applied during the period indicated by the bar above the trace. B, Time course of desensitization of ACh responses under control recording conditions (drug free extracellular solution, with ATP 1 mM and GTP, 0.25 mM in the whole cell-pipette solution), when ATP and GTP were omitted from the pipette solution (without ATP/GTP) and when scopolamine (1 μM) was added to the extracellular solution (with ATP and GTP in the pipette solution). The rate and extent of desensitization were reduced either by omitting ATP and GTP from the pipette solution or by adding scopolamine to the extracellular solution (see Table 1 for statistical analysis).
**Fig. 3.** Blockade of muscarinic receptors increases the rate of recovery from desensitization caused by ACh. A, representative recordings, under control conditions, of inward currents caused by a 7 s application of ACh at time 0 ($I_{initial}$) and at several time points after the initial application ($I_{time}$). B, Time course of recovery of nAChRs from desensitization caused by a 7 s application of ACh in the absence (Control) and presence of scopolamine (1 $\mu$M). Data points are the mean $\pm$ s.e.m. of measurements made on 7 neurons for each group.

**Fig. 4.** Scopolamine does not alter desensitization or recovery from desensitization of nAChRs caused by nicotine. A, Time course or extent of nAChR desensitization caused by a 7 s application of nicotine is not changed by scopolamine (1 $\mu$M). Data are the mean $\pm$ s.e.m. of measurements made in 7 neurons in the control group and 9 neurons in the scopolamine group. B, Recovery from desensitization caused by nicotine is not altered by scopolamine. Data are the mean $\pm$ s.e.m. of measurements made in 8 neurons in the control group and 10 neurons in the scopolamine group.

**Fig. 5.** The muscarinic receptor agonist, bethanechol (BeCh) increases nAChR desensitization caused by nicotine. Nicotine caused similar amounts of nAChR desensitization during successive applications in the absence or presence of the BeCh vehicle ($H_2O$) ($n = 10$). In a separate group of neurons ($n = 5$), addition of BeCh to the extracellular medium increased desensitization caused by a second application of nicotine. *indicates significantly different from control ($P < 0.05$).

**Fig. 6.** Phorbol 12,13 dibutyrate (PDBu) increases nAChR desensitization. A, The amount of nAChR desensitization caused by ACh is increased after treatment with PDBu but not the PKC
inactive phorbol ester, 4-α-PMA. Scopolamine (1 μM) was present in the extracellular solution throughout these studies. B, The amount of nAChR desensitization caused by nicotine is increased after treatment with PDBu but not 4-α-PMA. For both figures the data are expressed as the mean ± s.e.m. percent change in the amount of desensitization caused by a 7 s application of ACh or nicotine before and after treatment with the phorbol esters. Each concentration of phorbol ester was tested in separate groups of neurons. *indicate significantly different from the amount of desensitization occurring before phorbol ester treatment.

**Fig. 7.** Forskolin and dideoxyforskolin (DDF) increase nAChR desensitization. A, The amount of nAChR desensitization caused by ACh is increased after treatment with forskolin and the adenylate cyclase inactive forskolin analog, DDF. Scopolamine (1 μM) was present in the extracellular solution throughout these studies. B, Nicotine-induced nAChR desensitization is also increased after treatment with forskolin and DDF. For both figures the data are expressed as the mean ± s.e.m. percent change in the amount of desensitization caused by a 7 s application of ACh or nicotine before and after treatment with vehicle (0.05% EtOH), forskolin or DDF. *indicates significantly different from the amount of desensitization occurring before forskolin or DDF treatment.
Figure 1

A

ACh

Nicotine

0.5 nA

5 s

B

Peak Current (nA)

2

1

0

Control

C6

Wash

ACh Nicotine

† †

0

1

2

ACh Nicotine ACh + Nicotine ACh recovery

C

Peak Current (nA)

2

1

0

ACh Nicotine ACh + Nicotine ACh recovery

*
Figure 2

A

ACh (1 mM)

I_steady state

2 s

0.5 nA

I_peak

B

\frac{I_{\text{time}}}{I_{\text{peak}}}

Time (s)

Control

without ATP/GTP

with Scopolamine
Figure 3

A

ACh (1 mM)

0 min 0.1 min 1 min 3 min

7 s 0.2 nA

B

I_{time} / I_{initial}

0 1 2 3 4 5

Time (min)

○ Control

□ Scopolamine
**Figure 4**

(A) Graph showing the decay of nicotine (1 mM) over time (s). The y-axis represents $I_{time}/I_{initial}$, and the x-axis represents time in seconds. The data points are labeled as 'Control' and 'with Scopolamine'.

(B) Graph showing the recovery over time (min) after exposure to nicotine (1 mM). The y-axis represents $I_{time}/I_{initial}$, and the x-axis represents time in minutes. The data points are labeled as 'Control' and 'with Scopolamine'.
Figure 5

- Control
- Treatment

Vehicle

BeCh (100 nM)

I_{steady state} / I_{peak}
Figure 6

A. ACh (1 mM)
- PDBu
- 4-α-PMA

B. Nicotine (1 mM)
- PDBu
- 4-α-PMA

Desensitization (% Change)

Levels: 10 nM, 30 nM, 100 nM

* Indicates significant changes.
Figure 7

ACh (1 mM)

- Vehicle
- Forskolin (1 μM)
- DDF (1 μM)

Nicotine (1 mM)

- Vehicle
- Forskolin (1 μM)
- DDF (1 μM)