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5-Hydroxytryptamine and atropine inhibit nicotinic receptors in submucosal neurons

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Abstract

The whole-cell recording technique was used to investigate the pharmacological properties of acetylcholine-activated ion channels of cultured submucosal neurons from guinea-pig small intestine. Acetylcholine induced whole-cell membrane currents (I_{ACh}) in a concentration-dependent manner ($EC_{50} = 79$ μM). I_{ACh} exhibited strong inward rectification, had a reversal potential of $+19 \pm 2$ mV (Na^+ outside, Cs^+ inside), was reversibly inhibited in a concentration-dependent manner by hexamethonium ($EC_{50} = 5$ μM) and atropine ($EC_{50} = 1.6$ μM), and was unaffected by α-bungarotoxin (30 nM). Atropine was less potent in inhibiting the currents induced by 30 μM acetylcholine than those induced by 1 mM acetylcholine. I_{ACh} was mimicked by the current induced by nicotine (I_{Nic}; $EC_{50} = 52$ μM). I_{Nic} was also blocked by atropine ($EC_{50} = 1.7$ μM) and hexamethonium ($EC_{50} = 3.6$ μM). 5-Hydroxytryptamine (5-HT) also inhibited I_{ACh} in a concentration-dependent manner ($EC_{50} = 180$ μM) in the experiments carried out in the presence of a 5-HT_3 receptor antagonist. 5-HT had a similar inhibitory effect after the desensitization of 5-HT_3 receptors or in neurons with relative small 5-HT_3-mediated currents. The inhibitory actions of hexamethonium, atropine, and 5-HT on I_{ACh} were voltage-dependent. Thus, inhibition was significantly smaller for outward currents (recorded at $+40$ mV) than for inward currents (recorded at -60 mV). Our observations indicate that the I_{ACh} of submucosal neurons are mediated by activation of nicotinic channels, which are blocked by atropine, 5-HT, and hexamethonium. The possibility that one of the 5-HT roles in the gastrointestinal tract might be to directly modulate nicotinic channels is discussed.

Keywords: Enteric neuron; Submucosal neuron; Nicotinic channel; Acetylcholine; 5-HT (5-hydroxytryptamine, serotonin); 5-HT_3 receptor; Ligand-gated channel; Autonomic neuron; Ion channel; Nicotinic receptor; Atropine; Electrophysiology

1. Introduction

Acetylcholine is a major neurotransmitter in the sympathetic, parasympathetic, and enteric ganglia, where it is responsible for fast excitatory postsynaptic potentials (fast EPSPs) and slow EPSPs (Nishi and North, 1973; Hirst et al., 1974; Surprenant, 1984; North et al., 1985; Tack and Wood, 1992; Nishimura, 1997). These synaptic potentials are mediated by nicotinic and muscarinic receptors, respectively. Nicotinic acetylcholine receptors are non-selective, ligand-gated cation channels; whereas muscarinic receptors are part of the G-protein-linked receptor superfamily. Nicotinic acetylcholine receptors are also found in the central nervous system neurons and at the end plate of the neuromuscular junction.

Nicotinic acetylcholine receptors are thought to be made up of a pentamer of subunits (see reviews by Mcgehee and Role, 1995; Sargent, 1993; Corringer et al., 2000). Several neuronal nicotinic receptor subunits have been isolated and cloned. Using low-stringency DNA probing, 12 genes have been found in various animals (rat, chicken, Drosophila, cockroach, goldfish, and human) that share homology with the muscle α-subunit. These genes encode for the protein neuronal subunits α2–α9 and β2–β5. The function and some pharmacological properties of nicotinic acetylcholine receptors have been shown to be dependent on the subunits that they are composed of. By expressing different combinations of these subunits in Xenopus oocytes, various authors have shown that α7 subunits can form functional homomeric channels. Similar results were obtained with α8 and α9 subunits. On the other hand, α2–α6 subunits

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need β subunits to form functional channels. In enteric neurons, a recent study (Kirchgessner and Liu, 1998) showed immunostaining for α3, α5, α7 and β4 in both submucosal and myenteric plexuses. Immunostaining was present in the neuronal soma, dendrites, and axons.

In general, nicotinic acetylcholine receptors can be distinguished from muscarinic receptors by their specific agonism to nicotine and specific blockage by hexamethonium (Mcgehee and Role, 1995; Sargent, 1993). However, this is not always the case. Nicotine, for example, has been shown to inhibit the currents induced by acetylcholine (I_{ACh}) for the homomeric receptor α9 (Elgoyhen et al., 1994). Bungarotoxins from snakes have also widely been used to characterize nicotinic acetylcholine receptors. One such toxin, α-bungarotoxin, binds the muscle nicotinic acetylcholine receptors with high affinity but is generally ineffective in blocking nicotinic receptors that underlie synaptic transmission in autonomic ganglia (Fiordalisi et al., 1991). Furthermore, some nicotinic acetylcholine receptors present in non-ganglionic neurons (e.g., rat hippocampus; Alkondon and Albuquerque, 1993) are sensitive to α-bungarotoxin. Neuronal nicotinic acetylcholine receptors have also been shown to be sensitive to other antagonists including atropine (Zwart and Vijverberg, 1997; Minota and Kuba, 1999), curare (Peng et al., 1994; Elgoyhen et al., 1994), strychnine (Peng et al., 1994; Elgoyhen et al., 1994), and some endogenous substances like 5-hydroxytryptamine (5-HT; Akasu and Koketsu, 1986; Garcia-Colunga and Miledi, 1995).

In a previous study, we found that melatonin (Barajas-López et al., 1996b), a hormone synthesized by 5-HT acetylation, blocks nicotinic receptors in submucosal neurons of guinea-pig small intestine in a noncompetitive manner. This led to the hypothesis that 5-HT might have a similar effect as melatonin in submucosal neurons. In the present study, we test this hypothesis and describe other important pharmacological and electrophysiological properties of the whole-cell inward currents induced by acetylcholine of submucosal neurons from the guinea-pig small intestine.

2. Materials and methods

2.1. Tissue preparation

Young male and female guinea-pigs (150–300 g) were stunned and immediately exsanguinated by severing major

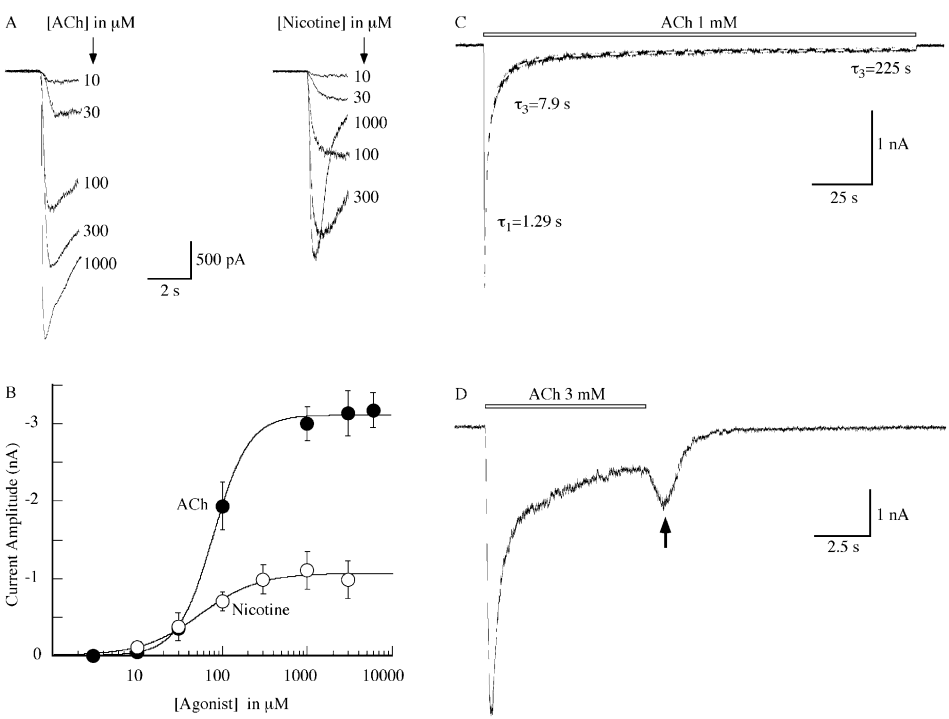

Fig. 1. Whole-cell inward currents induced by various concentrations of acetylcholine and nicotine in submucosal neurons. (A) Whole-cell inward currents induced by various concentrations of acetylcholine and nicotine in two different submucosal neurons. (B) Acetylcholine and nicotine induced a fast whole-cell current with similar concentration dependency. Symbols are the mean ± S.E.M. values of the currents induced by acetylcholine ($n = 9–18$) or nicotine ($n = 3–9$). Sigmoidal lines were fitted with a three-parameter logistic function. (C) Desensitization kinetics of the current induced by acetylcholine application. This kinetics was better fitted by the sum of three exponential functions (dashed lighter). The values of the three exponentials are as indicated. (D) As shown by the arrow, a transitory current increase was observed when acetylcholine was removed and when high concentrations (≥ 3 mM) of acetylcholine were used. Similar current transients were recorded with concentrations of nicotine ≥ 1 mM (not shown). Currents were measured at a holding potential of −60 mV.

neck blood vessels. A segment of small intestine (jejunum; about 5 cm in length) was removed, placed in modified Krebs solution (in mM: NaCl 126, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5, KCl 5, $NaHCO_3$ 25, glucose 11; gassed with 95% O_2 and 5% CO_2) and opened longitudinally. The mucosa was removed and the submucosal layer (submucosal preparation) was dissected from the underlying layers of smooth muscle.

2.2. Dissociation of submucosal neurons

Methods for dissociating and culturing submucosal neurons have been described elsewhere (Barajas-López et al., 1996a). Briefly, the submucosal preparation was dissociated using sequential enzymatic treatments, first with a papain solution (0.01 ml/ml; activated with 0.4 mg/ml of L-cysteine) and later with a collagenase (1 mg/ml) and dispase (4 mg/ml) solution. After washing out these enzymes, submucosal neurons were plated on round coverslips coated with sterile rat tail collagen. Culture medium was minimum essential medium 97.5%, containing 2.5% guinea-pig serum, L-glutamine 2 mM, penicillin 10 U/ml, streptomycin 10μ g/ml and glucose 15 mM.

2.3. Patch-clamp recordings

Whole-cell currents were recorded from short-term (2–72 h) primary cultures of submucosal neurons. Membrane currents were recorded using an Axopatch 1D amplifier. Patch pipettes were made as previously described (Barajas-López et al., 1996a) and had resistances between 1 and 3 MΩ. Except when otherwise noted, the holding potential was −60 mV. The standard solutions used, unless otherwise mentioned, had the following millimolar composition. Inside the pipette: Cs^+-glutamate 160, EGTA 10, HEPES 5, NaCl 10, ATPMg 3, and GTP 0.1. External solution: NaCl 160, $CaCl_2$ 2, glucose 11, HEPES 5 and CsCl 3. The pH of these solutions was adjusted to 7.3–7.4 with either CsOH (pipette solutions) or NaOH (external solutions). With these standard solutions, the usual input resistance of the neurons ranged from 1 to 10 GΩ. Whole-cell currents were recorded on a PC using Axotape software (Axon Instruments). Analysis of these currents was performed on a Macintosh computer using Axograph software (Axon Instruments). Membrane potentials were corrected for the liquid junction potential (pipette 11 mV negative). The recording chamber was continuously superfused with external solution at approximately 2 ml/min. Rapid changes in the external solution were made by using an eight-barrelled device (Barajas-López et al., 1994). The external application of experimental substances was achieved by abruptly changing the tube delivering the external solution in front of the cell being recorded for a tube delivering the same solution plus the drug(s). Substances were washed out by switching back to the delivery tube containing only the external solution and by flushing of the bath. External solutions were delivered by gravity. The response to the same agonist was measured with a minimal interval of 5 min. Whole-cell experiments were performed at room temperature (~23°C) and, at least, 5 min after going into the whole-cell configuration. Experiments reported here are from cells in which no significant run down of the currents was noticed during the recording time.

2.4. Drugs used

Acetylcholine, 5-HT, and 3-tropanyl-indole-3-carboxylate methiodide (ICS 205–930) were purchased from Research Biomedical (Natick, MA, USA). All other substances (atropine, α-bungarotoxin, nicotine, hexamethonium) were purchased from Sigma (St. Louis, MO, USA). Stock solutions of 0.1–100 mM were made and diluted in the physiological saline. Stock solutions were maintained at −4°C.

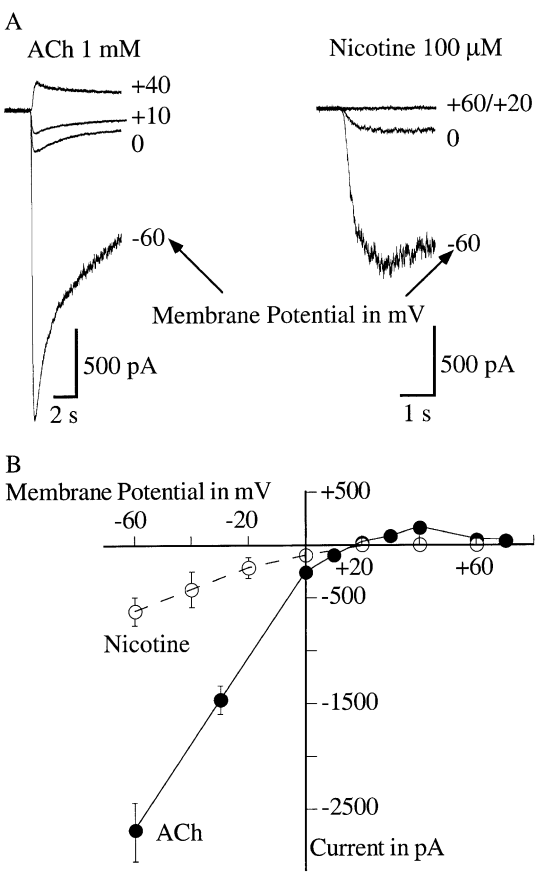

Fig. 2. Currents induced by acetylcholine and nicotine shows a similar voltage-dependent inward rectification. (A) Whole-cell inward currents induced by acetylcholine (1 mM) or nicotine (300 μM) at different holding potentials from two different submucosal neurons. (B) Current/voltage relationships for acetylcholine ($n = 6–15$) or nicotine ($n = 3–6$). Very small currents were recorded at positive potentials with acetylcholine and no current was detected at similar potentials with nicotine.

2.5. Data analysis

Results were expressed as means ± S.E.M. and the number of cells used as *n*. The paired Student's *t*-test was used to evaluate the differences between mean values obtained from the same cells and the unpaired Student's *t*-test was used for data obtained from different groups of cells. Two-tailed *P* values of 0.05 or less were considered statistically significant. Data of Figs. 1B, 3B,C, and 4A) were fitted to a three-parameter logistic function as previously reported (Kenakin, 1993).

3. Results

3.1. General properties of the acetylcholine activated currents in guinea-pig submucosal neurons

Acetylcholine application induced a fast inward current (I_{ACh}) in 90% of a total of 152 neurons. The amplitude of this current is plotted as a function of acetylcholine concentration in Fig. 1. These data were analyzed using a logistic model and the EC_{50} value for acetylcholine was 79μ M, the Hill coefficient value was 2.1 ± 0.2, and the maximal calculated current was −3110 pA (Fig. 1B).

The amplitude of I_{ACh} increased by hyperpolarizing the membrane, it was inward below +20 mV and outward above +20 mV. The amplitude of these outward currents increased very little by further depolarization of the membrane and, in fact, tended to decrease above +40 mV (Fig. 2). The calculated reversal potential of I_{ACh} was +19 ± 2 mV ($n = 9$).

At −60 mV holding potential, the I_{ACh} induced by 1 mM acetylcholine reached the half-maximal values in 150 ± 10 ms and reached its peak in the next second. At concentrations exceeding 100 M, after reaching maximal amplitude, the currents decreased despite the continuous presence of the transmitters—indicating tachyphylaxis (Fig. 1A and C). The kinetics of this desensitization was analyzed with 1 mM acetylcholine applications for ~3 min. This desensitization was modeled by the sum of three exponential functions. The tau values of these exponentials were $\tau_1 = 1.6 \pm 0.3$, $\tau_2 = 8.7 \pm 1.2$, $\tau_3 = 135 \pm 28$ s ($n =$ 12, Fig. 1C). After acetylcholine was removed, at concentrations lower or equal than 1 mM I_{ACh} decayed rapidly, and can be modeled by a single exponential function with an τ value of 299 ± 25 ms ($n = 20$; 1 mM acetylcholine). At concentrations usually equal or larger than 3 mM and sometimes even with 1 mM, I_{ACh} increased transitorily before decaying (Fig. 1D). It is, therefore, possible that at

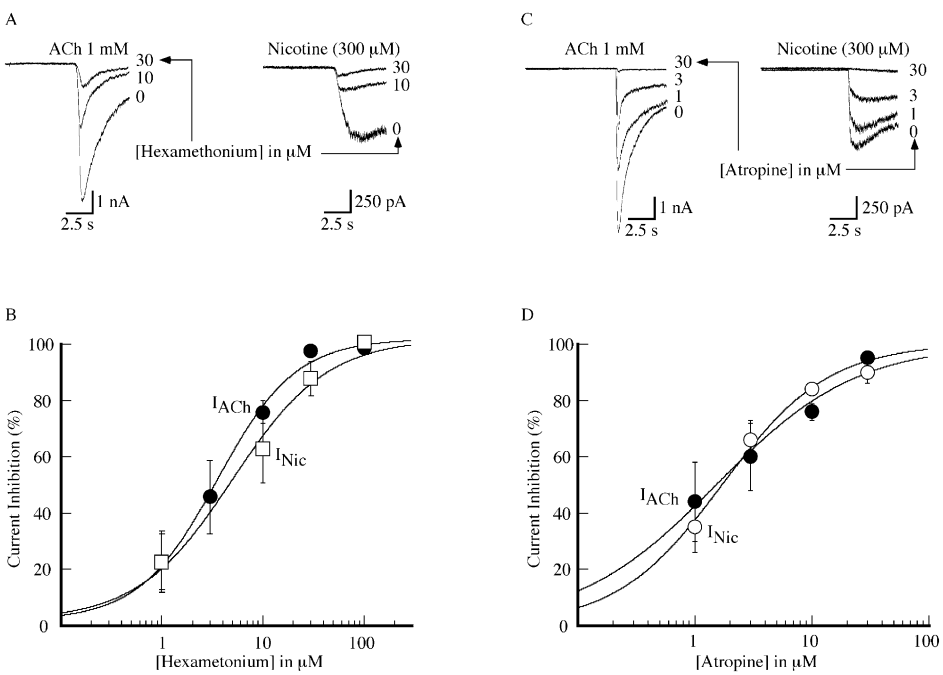

Fig. 3. Hexamethonium and atropine inhibit both acetylcholine (I_{ACh}) and nicotine (I_{Nic}) evoked. (A) Whole-cell inward currents induced by 1 mM acetylcholine or by 300 μM nicotine in the presence of 10 and 30 μM hexamethonium, in two different submucosal neurons. (B) Symbols are means ± S.E.M. of currents induced by 1 mM acetylcholine ($n = 4$–11) and 300 μM nicotine ($n = 3$–5) in various concentrations of hexamethonium. The hexamethonium IC_{50} values were 5 and 3.6 μM for I_{ACh} and I_{Nic}, respectively. (C) Whole-cell inward currents induced by 1 mM acetylcholine or by 300 μM nicotine in the presence of 1, 3 and 30 μM atropine, in two different submucosal neurons. (D) Symbols are the means ± S.E.M. of currents induced by 1 mM acetylcholine ($n = 3$–6) or 300 μM nicotine ($n = 3$–9) in the presence of various concentrations of atropine. The atropine IC_{50} values were 1.6 and 1.7 μM for I_{ACh} and I_{Nic}, respectively. Sigmoidal lines of (C) and (D) were fitted with a three-parameter logistic function. Experiments were carried out at a holding potential of −60 mV.

these high concentrations, acetylcholine might be blocking I_{ACh}, as has been proposed by others (Zwart and Vijverberg, 1997).

3.2. Pharmacological properties of submucosal I_{ACh}

Nicotine application also induced an inward current (I_{Nic}) in 85% of a total of 40 submucosal neurons (Figs. 1A, 2A, 3A, and 4A). At concentrations equal to or larger than 1 mM and sometimes as low as 300 μM, I_{Nic} increased transitorily before decaying (not shown) in a similar manner as observed for I_{ACh} in Fig. 1D. The amplitude of I_{Nic} is plotted as a function of the nicotine concentration in Fig. 1B. From this concentration–response curve, we calculated an EC_{50} value of 52 μM, a Hill coefficient of 1.3 ± 0.3 and a maximal current of -1065 pA. I_{Nic} was more reproducible and tended to recover faster when nicotine concentrations were equal to or lower than 300 μM. As a result, we avoided higher concentrations of this drug in most of the experiments reported below.

I_{Nic} had similar voltage dependency as I_{ACh}, as shown in Fig. 2. Thus, a similar inward rectification is observed in the I/V plots (Fig. 2B). An important difference, however, was that no current was recorded by application of nicotine (100 μM) when the membrane was held at potentials greater than or equal to $+20$ mV (Fig. 2). This was observed even in two neurons that display a relatively large I_{Nic} (about -1 nA) when held at -60 mV. Conversely, outward I_{ACh} currents were observed when the membrane potential was held at $+30$ mV in the presence of as little as 50 μM. In these experiments, the average outward I_{ACh} was $+26 \pm 5$ pA at $+30$ mV compared to an average inward current of -621 ± 83 at a membrane potential of -60 mV ($n = 3$). This inward current was similar to that observed with 100 μM nicotine at this potential (Fig. 2B).

3.3. I_{Ach} and I_{Nic} are blocked by hexamethonium and atropine

Inward currents induced by acetylcholine (1 mM) and nicotine (300 μM) were inhibited by hexamethonium in a concentration-dependent manner, as shown in Fig. 3A and B. The IC_{50} value for this hexamethonium effect was 5 and 3.6 μM for I_{ACh} and I_{Nic}, respectively.

I_{ACh} (induced by 1 mM acetylcholine) and I_{Nic} (induced by 0.3 mM nicotine) were also inhibited by micromolar concentrations of atropine, a well-known muscarinic receptor antagonist. This atropine effect was concentration-dependent (Fig. 3C and D) and the IC_{50} value for this atropine action was 1.6 μM (I_{ACh}) and 1.7 μM (I_{Nic}). The data shown in Fig. 3D were collected from experiments in which atropine was present 3–5 min before applying acetylcholine or nicotine. Atropine also has a similar inhibitory effect when it is applied simultaneously with the agonists (data not shown), indicating that this inhibitory effect occurs with very rapid kinetics. Removing atropine from the external solution could wash out this effect. The potency of atropine to inhibit I_{ACh} was decreased when the current was induced by a lower concentration of acetylcholine (30 μM). Thus, in three experiments the average control responses were -402 ± 280, and -435 ± 362 and -211 ± 350 pA in the presence of 1 and 10 μM of atropine, respectively. Atropine by itself did not affect the holding current in any of the tested neurons.

3.4. α-Bungarotoxin effects on I_{ACh}

The snake venom toxin, α-bungarotoxin, has been used to specifically block certain subtypes of nicotinic recep-

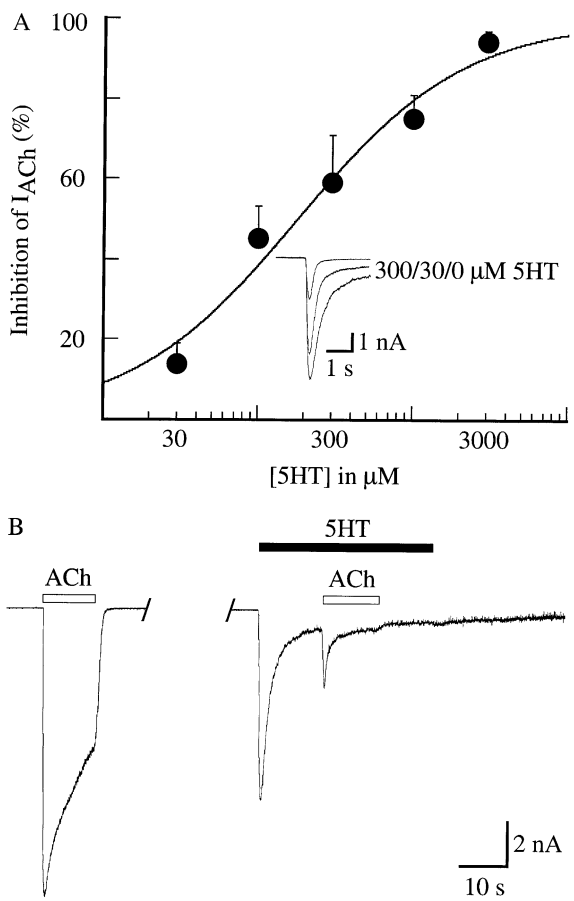

Fig. 4. 5-Hydroxytryptamine (5-HT) inhibited the current induced by acetylcholine (I_{ACh}). (A) Symbols are the means ± S.E.M. of currents induced by 1 mM acetylcholine ($n = 3$–8) in the presence of various concentrations of 5-HT. Activation of 5-HT_3 receptors was prevented by 300 nM ICS 205-930 in these experiments. Sigmoidal line were fitted with a three-parameter logistic function. The 5-HT IC_{50} values was 196 μM. *Inset* are whole-cell inward currents recorded in response to 1 mM acetylcholine in various concentrations of 5-HT plus 300 nM ICS 205-930. (B) Whole-cell inward currents induced by 1 mM acetylcholine before (left) and after partial desensitization of the 5-HT (1 mM) response. Substances were added as indicated by lines. Similar results were observed in six different neurons. All these currents were measured at a holding potential of -60 mV.

tors. In our preparations, α-bungarotoxin did not block I_{ACh} at a concentration of 30 nM. The mean control value for I_{ACh} was -426 ± 93 pA and the mean current was -366 ± 85 pA after 5–10 min of α-bungarotoxin superfusion ($n = 9$).

3.5. 5-HT effects on I_{ACh}

In order to investigate the effects of 5-HT on I_{ACh}, we blocked 5-HT_3 receptors using ICS 205-930, a potent antagonist of these receptors ($IC_{50} = 12$ nM; Vanner and Surprenant, 1990). The pharmacological and electrophysiological properties of submucosal 5-HT_3 receptors have been previously characterized and their activation is known to mediate fast cationic currents (Derkach et al., 1989) with similar kinetics as those recorded here (see Fig. 5A). These fast currents induced by 5-HT ($I_{5\text{-HT}}$) were totally blocked by fast 300 nM ICS 205-930 ($n = 6$), indicating that they are mediated by 5-HT_3 receptors. ICS 205,930 has also been reported to inhibit nicotinic mediated depolarizations in submucosal neurons (Vanner and Surprenant, 1990). In our experiments, 1 or 10 μM ICS 205-930 inhibited $44 \pm 8\%$ and $73 \pm 8\%$ I_{ACh} (induced by 1 mM acetylcholine), respectively ($n = 5$; $P = 0.01$). At 300 nM, however, ICS 205-930 had no effect I_{ACh} ($n = 6$) and therefore, the following experiments were carried out in presence of the 300 nM ICS 205-930.

Under these conditions, we found that 5-HT inhibits I_{ACh} in a concentration-dependent manner, with an IC_{50} of 180 μM (Fig. 4A). This effect was completely reversible

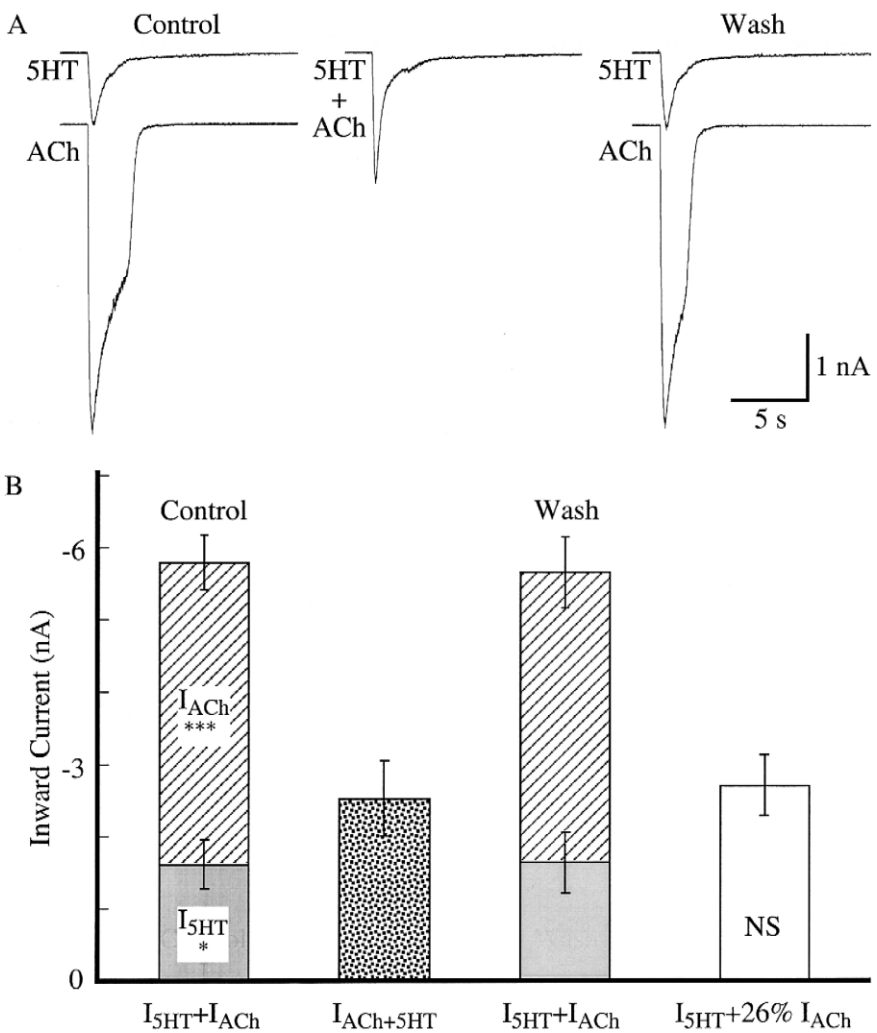

Fig. 5. 5-Hydroxytryptamine (5-HT) blocks the current induced by acetylcholine (I_{ACh}) very rapidly. Panel (A) shows recordings from one neuron of a typical experiment (left) and panel (B) the average values of four similar experiments. Currents were induced by application of either acetylcholine (1 mM) or 5-HT ($I_{5\text{-HT}}$; 1 mM) and by the simultaneous application of both agonists ($I_{ACh+5\text{-HT}}$). I_{ACh} and $I_{5\text{-HT}}$ were recorded 5 min before (Control) and 5 min after (Wash) $I_{ACh+5\text{-HT}}$. Error bars show the S.E.M.. The mean $I_{ACh+5\text{-HT}}$ was significantly lower ($^{***}P < 0.005$) than I_{ACh} and significantly larger ($^{*}P < 0.05$) than $I_{5\text{-HT}}$. According to the concentration–response curve of panel (A), about 26% of I_{ACh} should still be recorded in the presence of 1 mM 5-HT. In agreement with this interpretation, we found that the mean value of $I_{5\text{-HT}} + 26\%$ of I_{ACh} is represented by the open bar and was found to be non-significantly different (NS) than $I_{ACh+5\text{-HT}}$. Whole-cell currents were measured at a holding potential of -60 mV.

only few seconds after the removal of 5-HT (see Fig. 6B). This effect was determined not to be a result of an unknown interaction between ICS 205-930 and 5-HT because a similar inhibitory effect of 5-HT on I_{ACh} was observed in experiments ($n = 6$) in which we desensitized most of the response to 5-HT (Fig. 4B).

In addition, a similar inhibition of I_{ACh} by 5-HT was observed when both agonists were applied together. In experiments performed in neurons ($n = 4$) in which $I_{5\text{-HT}}$ amplitude was less than 50% of the I_{ACh} amplitude, we measured the peak I_{ACh} and $I_{5\text{-HT}}$, as well as the currents induced by simultaneous application of both agonists ($I_{ACh+5\text{-HT}}$). The prediction was that if 5-HT is blocking I_{ACh}, then $I_{ACh+5\text{-HT}}$ should be smaller than I_{ACh}. We found that $I_{ACh+5\text{-HT}}$ was significantly lower than I_{ACh} alone ($P < 0.005$) but significantly larger than $I_{5\text{-HT}}$ alone ($P < 0.05$; Fig. 5). Another important observation was that the $I_{ACh+5\text{-HT}}$ kinetics better resembles that of $I_{5\text{-HT}}$ than I_{ACh} (Fig. 5). 5-HT only blocks about 74% of the control I_{ACh} in the presence of ICS 205-930 (Fig. 4A), suggesting that $I_{ACh+5\text{-HT}}$ was composed by $I_{5\text{-HT}}$ and a smaller I_{ACh} component resistant to 1 mM 5-HT. In agreement with this interpretation, the addition of $I_{5\text{-HT}}$ plus 26% of I_{ACh} was no different than $I_{ACh+5\text{-HT}}$ (Fig. 5B). These experiments indicate that 5-HT effects are as fast as the activation of nicotinic acetylcholine receptors and that they cannot be mediated by the activation of second messenger cascades.

In order to demonstrate that 5-HT's inhibitory effect on I_{ACh} was not due to cross-desensitization between nicotinic and 5-HT_3 receptors, we measured the amplitude of I_{ACh} and $I_{5\text{-HT}}$ before and immediately after (~ 5 s) a relatively long application of acetylcholine or 5-HT (1 mM each). This long application lasted for at least 15 s or until the induced current had desensitized approximately 80% (usually within 30 s). Some typical recordings and the average data from such experiments are shown in Fig. 6. We observed that nicotinic receptor desensitization with acetylcholine decreased I_{ACh} significantly (from -2687 ± 468

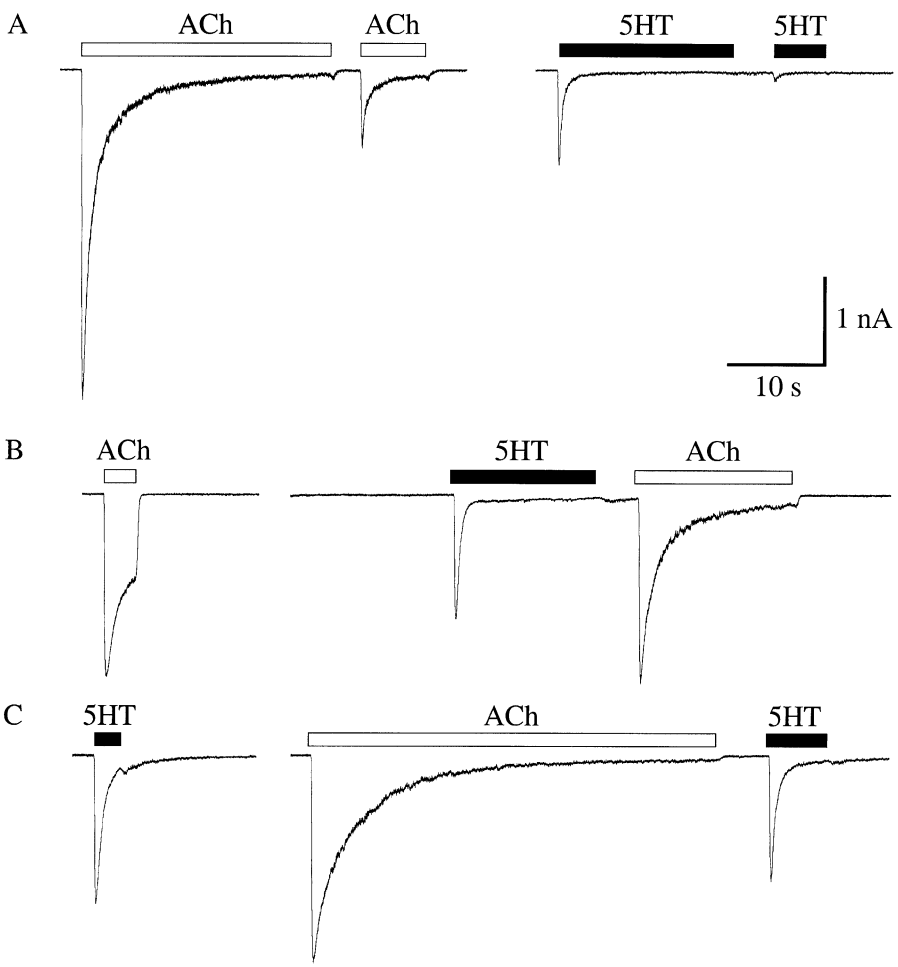

Fig. 6. No cross-desensitization was observed between nicotinic and 5-HT_3 receptors. (A) Currents induced by acetylcholine (I_{ACh}) and 5-HT ($I_{5\text{-HT}}$) were desensitized by relatively long applications of these agonists. Control I_{ACh} (B) and $I_{5\text{-HT}}$ (C) were recorded 5 min before (left recordings in B and C) and immediately after (~ 5 s) a prolonged application of the other agonist. Similar observations were performed in other four experiments. The holding potential was -60 mV.

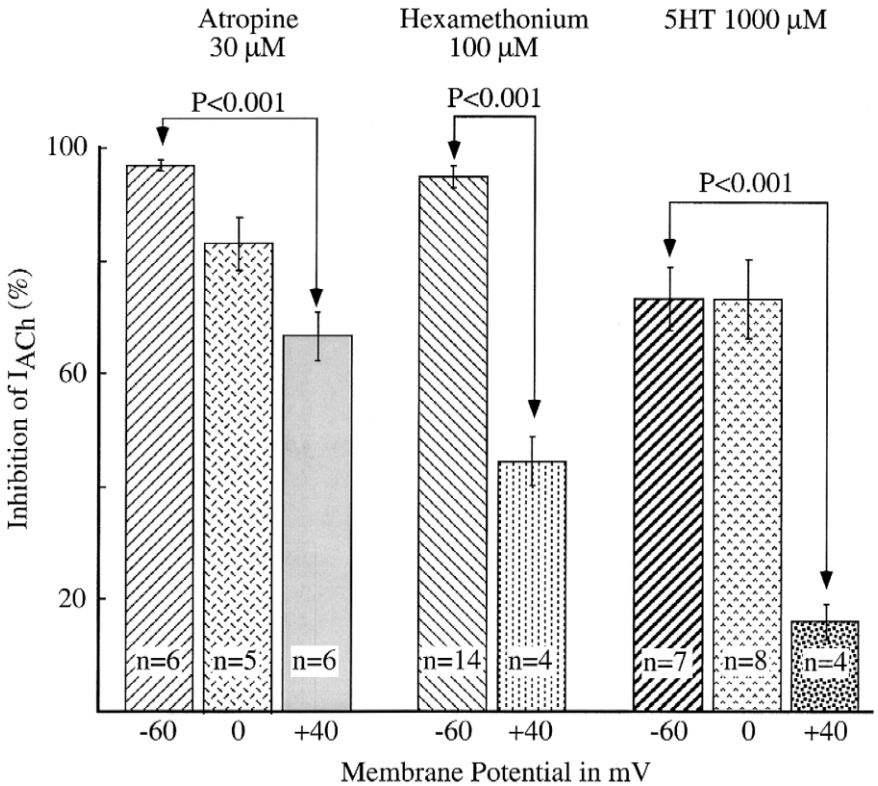

Fig. 7. The inhibition of the acetylcholine-activated current (I_{ACh}) by atropine, hexamethonium, and 5-hydroxytryptamine (5-HT) was voltage-dependent. Bars represent the average current inhibition measured at −60, 0, or +40 mV membrane potentials. The number of cells (n) and P values are indicated.

to -1061 ± 400 pA; $P < 0.05$; $n = 4$; left recording of Fig. 6A), whereas it did not affect $I_{5\text{-HT}}$ (Fig. 6C; $n = 4$). Similarly, 5-HT_3 receptor desensitization with 5-HT decreased $I_{5\text{-HT}}$ significantly (from -2047 ± 315 to 172 ± 29; $P < 0.05$; $n = 3$; right recording of Fig. 6A), whereas it did not affect I_{ACh} (Fig. 6B; $n = 4$). In other words, no cross-desensitization was observed between nicotinic and 5-HT_3 receptors.

3.6. Atropine, hexamethonium, and 5-HT effects on I_{ACh} at +40 mV

The mean inhibitory effect induced by atropine, hexamethonium, and 5-HT on I_{ACh} was lower at +40 mV than at −60 mV when outward and inward currents were recorded, respectively (Fig. 7). Cells were clamped at these potentials for at least 5 min before these experiments were performed.

4. Discussion

We found that nicotinic receptors expressed by submucosal neurons are inhibited by atropine and 5-HT. The later substance is highly concentrated in the gut (see Gershon, 1999). It is therefore possible that this 5-HT inhibitory effect might play a neuromodulatory role in the gastrointestinal tract. Atropine, on the other hand, is a potent antagonist of muscarinic receptors and it has been used to prevent the activation of these receptors in many gastrointestinal studies, at concentrations equal to or larger than 1 μM. We found that, at these concentrations, atropine is also able to inhibit submucosal nicotinic acetylcholine receptor.

4.1. Whole cell inward currents induced by acetylcholine in submucosal neurons are mediated by nicotinic receptors

The currents induced by acetylcholine could also be mimicked by the application of nicotine. Furthermore, both I_{ACh} and I_{Nic} were blocked by hexamethonium, indicating that they are mediated by nicotinic receptors. The maximal currents induced by nicotine are smaller than those induced by acetylcholine. An easy explanation for this is that the nicotine is activating only a subset of the receptors activated by acetylcholine. Further support for this interpretation comes from the fact that the Hill's coefficient for the concentration–response curves of these two agonists is different and no outward current was evoked by nicotine. This interpretation is in agreement with a recent report by Kirchgessner and Liu (1998), who found immunoreactivity for α3, α5, α7, and β4 subunits in submucosal neurons.

Each nicotinic receptor is thought to contain at least two alpha subunits. Therefore, it is possible that if two or three α-subunits are expressed in the same submucosal neuron, they might get combined differently to form various nicotinic acetylcholine receptor subtypes.

The α7 subunits are able to form homomeric receptors that are known to be atropine- (Peng et al., 1994) and α-bungarotoxin (IC_{50} = 2–3 nM)-sensitive (Alkondon and Albuquerque, 1993; Couturier et al., 1990). However, it is unlikely that the submucosal nicotinic acetylcholine receptors observed are α7 homomeric receptors because α7 desensitizing kinetics, as described by Peng et al. (1994), is far more rapid than those observed here and submucosal nicotinic acetylcholine receptors were not affected by 30 nM α-bungarotoxin.

Experiments carried out in *Xenopus laevis* oocytes (Zwart and Vijverberg, 1997) showed that atropine (1 μM) inhibits by 23 ± 5% and 51 ± 3% the currents induced by 1 mM acetylcholine of channels formed by the combination of α3β4 and α2β4 subunits. Currents through other nicotinic acetylcholine receptor (e.g., α4β2 and α4β4) were also inhibited by atropine (~ 50%) when 1 mM acetylcholine was used to evoke the current but the same concentration of atropine (1 μM) potentiates (~ 60%) the currents induced by a much lower concentration of acetylcholine (1 μM). Here, we found an inhibitory effect of 44 ± 14% (for 1 mM acetylcholine) and 35 ± 9% (for 300 μM nicotine) with the same concentration of atropine and no potentiatory effect was observed when lower concentrations of acetylcholine (30 μM) were used. Thus, the pharmacological properties of submucosal native nicotinic acetylcholine receptor resemble better those of α3β4 and α2β4. The fact that immunoreactivity for α3 and β4 subunits is present in submucosal neurons (Kirchgessner and Liu, 1998) supports the hypothesis that these subunits could form the native nicotinic acetylcholine receptors of submucosal neurons. In agreement with this interpretation, other ganglionic nicotinic receptors are believed to be formed by the last two subunits (Wong et al., 1995).

Native nicotinic acetylcholine receptors expressed in bullfrog sympathetic ganglion cells (Minota and Kuba, 1999; Connor et al., 1983) and in guinea-pig chromaffin cells (Inoue and Kuriyama, 1991) are also atropine-sensitive in contrast to those expressed in solitary rat retinal ganglion cells (Lipton et al., 1987), which are not affected by even a relatively large concentration of this substance (10 μM).

4.2. 5-HT blocks submucosal nicotinic receptors

This is the first study reporting the blocking effect of 5-HT on I_{ACh} in enteric neurons and we propose that this might be just another mechanism by which 5-HT modulates the enteric nervous system. The presence of serotoninergic neurons and the existence of a complex population of 5-HT receptors in the enteric nervous system has been interpreted in favour of the hypothesis that 5-HT is a neurotransmitter and neuromodulator in the enteric nervous system (see reviews by Talley, 1992; Gershon, 1999). In the present study, we found that concentrations as low as 30 μM of 5-HT can inhibit the submucosal nicotinic acetylcholine receptor. We speculate that similar or even higher concentrations of 5-HT might be reached in putative serotoninergic enteric synapses. Indeed, 5-HT_3 channels present in submucosal neurons are activated at 5-HT concentrations (Derkach et al., 1989) similar to those required to inhibit nicotinic channels (present study).

A large portion of the 5-HT present at a given time in the body is produced in the gastrointestinal tract, mainly by enterochromaffin cells (see Gershon, 1999). It is also possible that similar micromolar concentrations are reachable in the vicinity of enterochromaffin cells during 5-HT release and therefore, the blockage of nicotinic channels in nerve terminals could be one of the postulated paracrine effects of 5-HT. In agreement with this hypothesis, some axons are known to incorporate nicotinic receptors into their membrane (Buckley and Caulfield, 1992). The function of these receptors is unknown but it has been proposed that they might modulate transmitter release (Carneiro and Markus, 1994; McMahon et al., 1994). In the gut, the mucosa is known to be richly innervated (Javed and Cooke, 1992) including enterochromaffin cells (Ahlman et al., 1996). Some of these mucosal nerves might contain nicotinic receptors and they might run close enough to enterochromaffin cells so as to be affected by their secretory products, e.g. 5-HT. In agreement with this hypothesis, 5-HT stimulates mesenteric afferents by a direct action on $5HT_3$ receptors, which are likely to be present on vagal mucosal afferent terminals (Hillsley et al., 1998). Altogether, these observations indicate that the inhibitory effect of 5-HT on nicotinic channels might be involved in a neuromodulatory action of this substance in the gut mucosa.

Other serotoninergic drugs also inhibit submucosal nicotinic acetylcholine receptors. Thus, melatonin, a hormone synthesized by 5-HT acetylation, also inhibits I_{ACh} in submucosal neurons of guinea-pig small intestine (Barajas-López et al., 1996b). In agreement with a previous report (Vanner and Surprenant, 1990), we found that ICS 205-930, a well-known 5-HT_3 receptor antagonist, also blocks these nicotinic acetylcholine receptors. These observations are also in agreement with previous studies that have shown an inhibitory effect of various serotoninergic substances on nicotinic acetylcholine receptor in other preparations (Nakazawa et al., 1995; Grassi et al., 1993).

The inhibitory effect of atropine, hexamethonium, and 5-HT on the submucosal I_{ACh} was voltage-dependent, which suggests that these drugs block nicotinic channels by binding to a site close to the channel mouth or by entering into the open pores. This interpretation is in agreement with previous results discussed by others regarding the mechanisms by which atropine (Zwart and

Vijverberg, 1997), hexamethonium (Bertrand et al., 1990; Ascher et al., 1979), melatonin (Barajas-López et al., 1996b), and 5-HT (Nakazawa et al., 1995; Grassi et al., 1993) inhibit other neuronal nicotinic channels. Indeed, atropine was more potent to inhibit I_{ACh} induced by larger concentrations of acetylcholine (1 mM vs. 30 μM).

In conclusion, 5-HT and atropine appear to directly inhibit the nicotinic submucosal ion channels. This effect of atropine is seen at concentrations of as low as 1 μM, which are often used to block muscarinic receptors in various gut preparations. Our results indicate that 5-HT might be a local modulator of nicotinic channels in the gastrointestinal tract.

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References

Ahlman, H., Nilsson, O., Wangberg, B., Dahlstrom, A., 1996. Neuroendocrine insights from the laboratory to the clinic. Am. J. Surg. 172, 61–67.

Akasu, T., Koketsu, K., 1986. 5-Hydroxytryptamine decreases the sensitivity of nicotinic acetylcholine receptor in bull-frog sympathetic ganglion cells. J. Physiol. (London) 380, 93–109.

Alkondon, M., Albuquerque, E.X., 1993. Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons: I. Pharmacological and functional evidence for distinct structural subtypes. J. Pharmacol. Exp. Ther. 265, 1455–1473.

Ascher, P., Large, W.A., Rang, H.P., 1979. Studies on the mechanism of action of acetylcholine antagonists on rat parasympathetic ganglion cells. J. Physiol. (London) 295, 139–170.

Barajas-López, C., Espinosa-Luna, R., Gerzanich, V., 1994. ATP closes a potassium and opens a cationic conductance through different receptors in neurons of guinea-pig submucous plexus. J. Pharmacol. Exp. Ther. 268, 1396–1402.

Barajas-López, C., Huizinga, J.D., Collins, S.M., Gerzanich, V., Espinosa-Luna, R., Peres, A.L., 1996a. P2x-purinoceptors of myenteric neurones from the guinea-pig ileum and their unusual pharmacological properties. Br. J. Pharmacol. 119, 1541–1548.

Barajas-López, C., Peres, A.L., Espinosa-Luna, R., Reyes-Vazquez, C., Prieto-Gomez, B., 1996b. Melatonin modulates cholinergic transmission by blocking nicotinic channels in the guinea-pig submucous plexus. Eur. J. Pharmacol. 312, 319–325.

Bertrand, D., Ballivet, M., Rungger, D., 1990. Activation and blocking of neuronal nicotinic acetylcholine receptor reconstituted in Xenopus oocytes. Proc. Natl. Acad. Sci. U. S. A. 87, 1993–1997.

Buckley, N.J., Caulfield, M., 1992. Transmission: acetylcholine. In: Burnstock, G., Hoyle, C.H.V. (Eds.), Autonomic Neuroeffector Mechanisms. Hardwood Academic Publishers, Chur, Switzerland, pp. 277–322.

Carneiro, R.C., Markus, R.P., 1994. Presynaptic nicotinic receptors involved in release of noradrenaline and ATP from the prostatic portion of the rat vas deferens. J. Pharmacol. Exp. Ther. 255, 95–100.

Connor, E.A., Levy, S.M., Parsons, R.L., 1983. Kinetic analysis of atropine-induced alterations in bullfrog ganglionic fast synaptic currents. J. Physiol. (London) 337, 137–158.

Corringer, P.J., Le Novere, N., Changeux, J.P., 2000. Nicotinic receptors at the amino acid level. Annu. Rev. Pharmacol. Toxicol. 40, 431–458.

Couturier, S., Bertrand, D., Matter, J.M., Hernandez, M.C., Bertrand, S., Millar, N., Valera, S., Barkas, T., Ballivet, M., 1990. A neuronal nicotinic acetylcholine receptor subunit (alpha 7) is developmentally regulated and forms a homo-oligomeric channel blocked by alpha-BTX. Neuron 5, 847–856.

Derkach, V., Surprenant, A., North, R.A., 1989. 5-HT_3 receptors are membrane ion channels. Nature 339, 706–709.

Elgoyhen, A.B., Johnson, D.S., Boulter, J., Vetter, D.E., Heinemann, S., 1994. Alpha 9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. Cell 79, 705–715.

Fiordalisi, J.J., Fetter, C.H., TenHarmsel, A., Gigowski, R., Chiappinelli, V.A., Grant, G.A., 1991. Synthesis and expression in *Escherichia coli* of a gene for kappa-bungarotoxin. Biochemistry 30, 10337–10343.

Garcia-Colunga, J., Miledi, R., 1995. Effects of serotonergic agents on neuronal nicotinic acetylcholine receptors. Proc. Natl. Acad. Sci. U. S. A. 92, 2919–2923.

Gershon, M.D., 1999. Review article: roles played by 5-hydroxytryptamine in the physiology of the bowel. Aliment. Pharmacol. Ther. 13 (Suppl. 2), 15–30.

Grassi, F., Polenzani, L., Mileo, A.M., Caratsch, C.G., Eusebi, F., Miledi, R., 1993. Blockage of nicotinic acetylcholine receptors by 5-hydroxytryptamine. J. Neurosci. Res. 34, 562–570.

Hirst, G.D.S., Holman, M.E., Spence, I., 1974. Two type of neurones in the myenteric plexus of duodenum in the guinea-pig. J. Physiol. (London) 236, 303–326.

Hillsley, K., Kirkup, A.J., Grundy, D., 1998. Direct and indirect actions of 5-hydroxytryptamine on the discharge of mesenteric afferent fibres innervating the rat jejunum. J. Physiol. (London) 506, 551–561.

Inoue, M., Kuriyama, H., 1991. Properties of the nicotinic-receptor-activated current in adrenal chromaffin cells of the guinea-pig. Pfluegers Arch. 419, 13–20.

Javed, N.H., Cooke, H.J., 1992. Acetylcholine release from colonic submucous neurons associated with chloride secretion in the guinea pig. Am. J. Physiol. 262, G131–G136.

Kenakin, R., 1993. Pharmacologic analysis of drug–receptor interaction. 2nd edn. Raven Press, New York, NY.

Kirchgessner, A.L., Liu, M.T., 1998. Immunohistochemical localization of nicotinic acetylcholine receptors in the guinea pig bowel and pancreas. J. Comp. Neurol. 390, 497–514.

Lipton, S.A., Aizenman, E., Loring, R.H., 1987. Neural nicotinic acetylcholine responses in solitary mammalian retinal ganglion cells. Pfluegers Arch. 410, 37–43.

Mcgehee, D.S., Role, L.W., 1995. Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. Annu. Rev. Physiol. 57, 521–546.

McMahon, L.L., Yoon, K.W., Chiappinelli, V.A., 1994. Electrophysiological evidence for presynaptic nicotinic receptors in the avian ventral lateral geniculate nucleus. J. Neurophysiol. 71, 826–829.

Minota, S., Kuba, K., 1999. Restoration of the nicotinic receptor-channel activity from the blockade by atropine in bullfrog sympathetic ganglia. Br. Res. J. Cell Biol. 146, 203–218.

Nakazawa, K., Akiyama, T., Inoue, K., 1995. Block by 5-hydroxytryptamine of neuronal acetylcholine receptor channels expressed in Xenopus oocytes. Cell. Mol. Neurobiol. 15, 495–500.

Nishi, S., North, R.A., 1973. Intracellular recording from the myenteric plexus of the guinea-pig ileum. J. Physiol. (London) 231, 471–491.

Nishimura, T., 1997. Effects of adenosine on cholinergic transmission in mammalian vesical parasympathetic ganglia. Tokai J. Exp. Clin. Med. 22, 1–5.

North, R.A., Slack, B.E., Surprenant, A., 1985. Muscarinic M1 and M2 receptors mediate depolarization and presynaptic inhibition in guinea-pig enteric nervous system. J. Physiol. (London) 368, 435–452.

Peng, X., Katz, M., Gerzanich, V., Anand, R., Lindstrom, J., 1994.

Human alpha 7 acetylcholine receptor: cloning of the alpha 7 subunit from the SH-SY5Y cell line and determination of pharmacological properties of native receptors and functional alpha 7 homomers expressed in Xenopus oocytes. Mol. Pharmacol. 45, 546–554.

Sargent, P.B., 1993. The diversity of neuronal nicotinic acetylcholine receptors. Annu. Rev. Neurosci. 16, 403–443.

Surprenant, A., 1984. Slow excitatory synaptic potentials recorded from neurones of guinea-pig submucous plexus. J. Physiol. (London) 351, 343–361.

Tack, J.F., Wood, J.D., 1992. Synaptic behaviour in the myenteric plexus of the guinea-pig gastric antrum. J. Physiol. (London) 445, 389–406.

Talley, N.J., 1992. Review article: 5-hydroxytryptamine agonists and antagonists in the modulation of gastrointestinal motility and sensation: clinical implications. Aliment. Pharmacol. Ther. 6, 273–289.

Vanner, S., Surprenant, A., 1990. Effects of 5-HT_3 receptor antagonists on 5-HT and nicotinic depolarizations in guinea-pig submucosal neurones. Br. J. Pharmacol. 99, 840–844.

Wong, E.T., Holstad, S.G., Mennerick, S.J., Hong, S.E., Zorumski, C.F., Isenberg, K.E., 1995. Pharmacological and physiological properties of a putative ganglionic nicotinic receptor, alpha 3 beta 4, expressed in transfected eucaryotic cells. Brain Res. Mol. Brain, 101–109.

Zwart, R., Vijverberg, H.P., 1997. Potentiation and inhibition of neuronal nicotinic receptors by atropine: competitive and noncompetitive effects. Mol. Pharmacol. 52, 886–895.