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Pharmacological Characterization of Ionotropic Receptor Antagonists on Guinea-Pig Myenteric Neurons

Tesis que presenta

Esri Hazael Juárez

Para obtener el grado de

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Esta tesis fue elaborada en el Laboratorio de Neurobiología de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C., bajo la dirección y codirección del Dr. Carlos Barajas López y la Dra. Marcela Miranda Morales, respectivamente.

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MAESTRO EN CIENCIAS EN BIOLOGÍA MOLECULAR

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Esri Hazael Juárez

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Este trabajo esta dedicado a todas las personas que me ayudaron a concluirlo, desde aquellas que me ayudaron en el Instituto, hasta aquellas que desde siempre me apoyaron sin importar la distancia, estado o salud.

GRACIAS MAMÁ, ABUELA Y FAMILIA, SIN USTEDES NUNCA LO HUBIERA LOGRADO, POR ESO LES DEDICO ESTE TRABAJO.

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Contenido

Resumen

El sistema nervioso enterico (ENS) esta formado por una serie de redes neuronales complejas capaces de controlar y modular la mayoría de las funciones gastrointestinales. Los somas de las neuronas entéricas se localizan en dos principales plexos ganglionados, el mientérico y el submucoso. Estas expresan al menos dos familias de canales iónicos activados por ligando (LGIC), los *Cys-loop* y los P2X. Los receptors *Cys-loop* más comumente expresados por las neuronas entéricas son los nicotínicos, (activados por acetilcolina; nACh), $5HT_3$ (activados por serotonina; 5HT) y $GABA_A$ (activados por ácido–γ-amino-butírico; GABA). Para estudiar los efectos y funciones de estos receptores, los investigadores utilizan antagonistas como hexametonio (para receptores nACh), ondansetron (para receptores $5HT_3$) y para los receptores $GABA_A$, picrotoxina y bicuculina. En general, estos antagonistas son considerados específicos sobre los receptores mencionados, aunque hay evidencia experimental de que algunas de estas substancias inhiben a más de un LGIC. El objetivo de este trabajo fue investigar la especificidad de estos antagonistas sobre LGIC en neuronas mientéricas del intestino delgado de cobayo. Nosotros usamos la técnica de Patch Clamp en su configuración de célula completa para medir las corrientes entrantes inducidas por los receptores nACh, $5HT_3$, $GABA_A$ y P2X, usando virtualmente concentraciones máximas de ACh (1 mM), 5HT (1 mM), GABA (0.3 mM) y ATP (1 mM). Fue caracterizado el efecto de cada antagonista sobre los receptores P2X y *Cys-loop* mencionados. Para ello se obtuvieron curvas concentración-respuesta. Por una parte, encontramos que hexametonio inhibió solo las corrientes inducidas por ACh y por otra parte picrotoxina y bucuculina inhibieron las corrientes inducidas por ACh, 5HT y GABA, mientras que no afectaron aquellas inducidas por ATP. Sin embargo, picrotoxina fue menos potente que ondansetron y que el hexametonio para inhibir las corrientes inducidas por 5HTo ACh, respectively. Nuestros datos muestran que hexametonio es específico sobre los receptores nACh, picrotoxina y bicuculina pueden inhibir todos los receptores *Cys-loop* estudiados en este trabajo. Ondansetron parece ser específico sobre los receptores $5HT_3$.

Palabras clave: *picrotoxina, bicuculina, hexametonio, ondansetrón, antagonismo competitivo, antagonismo no competitivo, cobayo y neuronas mietéricas.*

Abstract

The enteric nervous system (ENS) consists in a series of complex neuronal networks able to control and modulate many of the gastrointestinal functions. Enteric neurons form two major ganglionated plexuses, the myenteric and the submucosal, and express at least two families of ligand-activated ion channels (LGIC), the *Cys-loop* and P2X. The *Cys-loop* receptors most commonly present in enteric neurons are the nicotinic (activated by acetylcholine; nACh), $5HT_3$ (activated by serotonin; 5-HT) and $GABA_A$ (activated by γ-amino-butyric acid; GABA). To study the effects and functions of these receptors researchers have been using antagonists such as hexamethonium (for nACh receptors), ondansetron (for $5HT_3$ receptors), and, for $GABA_A$ receptors, picrotoxin and bicuculine. In general, these antagonists had been considered as specific on the mentioned receptors, although there is experimental evidence indicating that at least some of these substances might also inhibit more than one LGIC. The aim of this work was to investigate the specificity of these antagonists on LGIC in myenteric neurons from the guinea-pig small intestine. We used the Whole-Cell configuration of the Patch Clamp techniques to measured inward currents mediated by nACh, $5HT_3$ and $GABA_A$ receptors using virtually maximal concentration of ACh (1 mM), 5HT (1 mM), GABA (0.3 mM) and ATP (1 mM). The effect of each receptor antagonists was tested and concentration-response curves were obtained for the mentioned *Cys-loop* and P2X receptors. On one hand, we found that hexamethonium inhibited only the currents induced by ACh. On the other hand, picrotoxin and bicuculline inhibited the currents induced by ACh, 5HT and GABA whereas did not affect those induced by ATP. However, picrotoxin was less potent than ondansetron or hexamethonium to inhibit currents induced by 5HT or ACh, respectively. Ondansetron did not inhibit the GABA-evoked currents. Our data shows that hexamethonium is specific on nACh receptors and picrotoxin and bicuculline can inhibit all *Cys-loop* receptors tested in this study. Ondansetron appears to be specific on $5HT_3$ receptors.

Key words: *picrotoxin, bicuculline, hexamethonium, ondansetron, competitive antagonism, non-competitive antagonism, guinea pig, and myenteric neuron*

INTRODUCTION

Enteric Nervous System

The enteric nervous system (ENS) is located into the gastrointestinal wall, and contains motor neurons, interneurons and sensory neurons (Bertrand & Thomas, 2004) and altogether can integrate sensory information and provide organized reflex responses, which control important gastrointestinal functions (Wood, 2004). The ENS is constantly under the control of the central nervous system (CNS) but can also function as an independent system. Thus, the ENS controls and coordinates motility, blood flow and secretions, to meet, the digestion needs of the individual (Grundy & Schemann, 2005). The ENS consists of the myenteric and submucosal plexuses. The absorptive and secretory functions of the gastrointestinal epithelium, neuro-inmune function and local blood flow are controlled by submucosal plexus (Galligan, 2002b) whereas, the myenteric plexus controls the relaxation and contraction of the gastrointestinal smooth muscle and coordinates the contractions with the epithelium functions (absorption and secretion).

There are many cell surface receptors express by enteric neurons, which play and essential role in cell communication. These receptors respond to synaptically released neurotransmitters, circulating hormones and locally released substances. Besides their physiological role, cell surface receptors are also targets for many therapeutically used drugs.

Ligand-gated ion channels (LGIC), are one of various types of receptors expressed by enteric neurons. LGIC are proteins that incorporate both the receptor and an ion channel. Binding of a specific ligand to the receptor increase the open probability

of the channel, which is usually selective for cations or anions. Ion movement through these channels will generate a membrane current that can be recorded using a voltage clamp technique. The direction of the resulting current is determined by the charge of the ion and their electrochemical gradient across the membrane (Barry & Lynch, 2005). The magnitude of these currents depends on the single channel current, their mean open probability and the number of activated channels.

LGIC expressed by enteric neurons are those activated by acetylcholine (nACh), 5-Hydroxytryptamine ($5HT_3$), γ-aminobutyric acid ($GABA_A$), N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), glycine and adenosine 5'-triphosphate (ATP; P2X) (Galligan, 2002a). P2X, 5-HT_3 and nACh receptors mediate fast synaptic potentials recorded in enteric neurons (Galligan, 2002a). The present study will focus on P2X and three *Cys-loop* channels ($GABA_A$, 5-HT_3, and nACh receptors).

P2X receptors constitute the most recently cloned family of LGIC. These receptors, which are gated by ATP, are cation channels that mostly have substantial Ca^{2+} permeability. They form a new family of transmitter-gated ion channels with only two transmembrane domains (Robertson *et al.*, 2001).

$GABA_A$ receptors mediate fast synaptic transmission. They are assembled in a heteropentameric arrangement of individual subunits from seven families (α, β, γ, δ, ε, π and θ). Most native $GABA_A$ subtypes are believe to consist of α, β and γ subunits. The expression pattern off individual subunits in a space-temporal dependent manner generates a high diversity of $GABA_A$ receptors with different functional properties (Kneussel, 2002). In order to study Gabaergic synapsis, researchers had been used the

no-competitive antagonist Picrotoxin and Bicuculline to study them (Ong & Kerr, 1984) (Fargeas *et al.*, 1988) (Galligan, 2002b).

5-HT_3 channels are activated by serotonine (5-hydroxytriptamine) these receptors are responsible for rapid chemical transmission where binding the neutransmitter to their receptor results in the opening of an integral cation-selective pore. The aminoacid sequences of three classes of 5-HT_3 receptor subunits are currently known A, B and C. The 5-HT_{3A} receptor subunit can form functional homopentameric receptors (Reeves & Lummis, 2002). One of the inhibitors used to characterize these ligand-gated ion channels is ondansetron a competitive antagonist (Butler *et al.*, 1988).

nAChR's are ligand-gated cation channels activated by acetilcoline (ACh), composed of pentameric combinations of 11 subunits (α and β). The specific subunit composition yields receptors with pharmacological and electrophysiological properties that are unique to that subunit combination (Zhou *et al.*, 2002). Competitive antagonists like Hexamethonium have been used to characterize the nAChR's.

Antagonists

The use of antagonist like hexamethonium, picrotoxin, bicuculline, and ondansetron as tools to study the effect and functions of LGIC had been wide (Galligan, 2002a, b; Hamrouni *et al.*, 2006). In general these antagonists have been considered specific on a kind of LGIC (Fargeas *et al.*, 1988; Chebib & Johnston, 2000). However, experimental evidence indicates that some of them might affect more than one LGIC. For instance, on neurons from the stomatogastric ganglion of the crab, picrotoxin blocks a

postsynaptic depolarizing ACh response (Marder & Paupardin-Tritsch, 1980), Furthermore, ondansetron reversibly inhibited 5-HT_{3A} receptors and nACh muscle receptors expressed in Xenopus laevis oocytes in a concentration-dependent manner (Paul *et al.*, 2005). The depolarizing actions of 5-HT on sensory and autonomic ganglia and on afferent nerve endings are sensitive to the antagonism of picrotoxin (Simonds & DeGroat, 1980).

Despite their importance, the specificity of LGIC antagonists on native receptors from myenteric neurons has not been studied. Our aim in this study was to characterize the antogonistic effect of hexamethonium, picrotoxin, ondansetron and bicuculline on *Cys-loop* and P2X receptors from myenteric neurons of the guinea-pig small intestine. We found that hexamethonium specifically inhibited the currents induced by ACh. Picrotoxin and bicuculline were not specific on $GABA_A$ receptors but did not affect ATP-induced currents. Ondansetron inhibit the 5HT responses but did not affect the GABA induced currents.

MATERIALS AND METHODS

Guinea-Pig Myenteric Plexus Dissection

Young guinea pigs (150-200), either female or male, were killed by decapitation, and a segment of five centimeters of proximal jejunum was removed, and placed in modified Krebs solution (in mM): NaCl 126, NaH_2PO_4 1.2. $MgCl_2$ 1.2, $CaCl_2$ 2.5, KCl 5, $NaHCO_3$, Glucose, 11; gassed with 95% O_2 and 5% CO_2, after, opened longitudinally. The mucosa and submucosal layer of this intestinal segment were dissected, before removing most of the circular muscle layer leaving only the longitudinal layer with the myenteric plexus embedded with it.

Enzymatic and Mechanical Dissociation

The myenteric preparation was dissociated using a sequential treatment with two enzymatic solutions, described before by Barajas-Lopez and coworkers (Barajas-Lopez *et al.*, 1996), the first contained papain (0.01 ml/ml; activated with 0.4 mg/ml of L-cysteine) and the second, collagenase (1mg/ml) and dispase (4 mg/ml). The enzymes were removed by washing with L15 and the neurons were plated on rounded coverslips coated sterile rat tail collagen. Culture medium was minimun essential medium 97.5%, containing 2.5% guinea pig serum, L-glutamine 2 mM, penicillin 10 U/ml, streptomycin 10 µg/ml and glucose 15 mM.

2.3 Electrophysiology

The currents were measured by Axopatch 200B amplifier (Axon Instruments), recorded and analyzed with Axoscope 9.0 (PC) and Axograph 4.0 (Macintosh software), respectively; to measured the responses we used two techniques: *Patch clamp* in whole cell configuration and *voltage clamp*. The cultures were short-term (24-72 hours) primary cultures to prevent space clamp problems due to neurite growth, plus the membrane extension is bigger and makes difficult de voltage clamp. Patch pipettes were made as previously describe Barajas-Lopez and coworkers (Barajas-Lopez *et al.*, 1996) and the pipettes access resistance was 1-5 MΩ. All the measures were made at holding potential -60 mV and ninety to ninety-five percent of the series resistance was compensated.

Solutions

ACh, ATP, GABA and 5HT are known to modulate membrane potassium channels of enteric neurons via G-protein coupled receptors (Christofi *et al.*, 1997; Barajas-Lopez *et al.*, 2000; Krantis, 2000). In order to decrease these responses G-protein coupled receptors, the experiments were carried out of Cs^+ (a potassium channels blocker). The external solution composition used to make the experiments is below (in mM): $CaCl_2 \cdot 2H_2O$ 2, CsCl 3, glucose 11, HEPES 4.8, NaCl 160 and pH was adjusted to 7.3-7.4 with NaOH; the pipette solution was composed by (in mM):, CsCl 150, EGTA 10, , HEPES 5, NaCl 10, ATPMg 4.5, GTP 0.1and pH was adjusted too at 7.3-7.4 with CsOH. The recording chamber was continuously superfused with external solution at approximately 2 ml/min.

Drug Application

Experimental solutions were applied by using an eight-tubes device to allow rapid changes between control and experimental solution. Each tube is connected to a syringe, every syringe contain control solution with or without experimental drug. The experimental drug application mechanism was made placing the control solution in front the cell and being recorded and the external application of experimental substances were achieved by rapid change for a tube containing the control solution plus the drug(s) and change again to the tube with control solution. Every application was at approximately for 5 s, and flows by gravity adjusting every change level to avoid any flow variation. Experiments were performed at room temperature (~23° C). To study the antagonism effect, first we applied the agonist and antagonist separately by ~5 s (Controls), then we applied the agonist and antagonist (at increasing concentration) together by ~5 s and finally we repeat controls; between experiments we wait at least 5 s.

Data Acquisition

Hexamethonium was purchased from Research Biomedical Inc. (Natick, MA, USA). All other substances were purchased from Sigma (St. Louis, MO., USA). pH of the external solution containing ATP, used to induce the ATP-current, was always readjusted with NaOH to 7.3-7.4. Addition of other substances did not alter the pH of the external solution.

2.7 Data Analyses

Results were expressed as the mean ± S.E.M. Antagonist concentration-response curves obtained from neurons were fit using the Hill equation as follows.

$$y = I_{max}(x^n / I_{50}{}^n + x^n)$$

where I_{max} is the maximum inhibition, I_{50} is the half-maximal effective concentration, n is the slope factor, and x is the antagonist concentration. Each point of every concentration-response curve has at least 3 or more cells measured. The amount of inhibited current was obtained subtracting the peak current at a given concentration from the control current (without antagonist). Data was plotted as percentage inhibition that was the inhibited current expressed as a percentage of the control value for each experiment.

RESULTS

All experiments were carried out on guinea-pig myenteric neurons from the proximal jejunum. To evoke the inward currents we used agonist concentrations to activate at least 80% of receptors. The inhibitory effect for all antagonists was concentration-dependent and reversible.

Hexamethonium

Hexamethonium, even at the highest concentration used (100 μM), did not change the holding current or the currents induced by ATP, GABA, and 5HT (Figs 1B, 1C, and 1D). However, the current induced by ACh was inhibited in a concentration-dependent manner by this substance. The concentration that produced half of the maximal inhibitory effect (IC_{50}) was 11.1 ± 2.1 μM and the Hill coefficient was 0.78 ± 0.1 (Figs 1A and 2).

Picrotoxin

Picrotoxin had an inhibitory effect on all *Cys-loop* family receptors tested (Figs 3, 4A and 4B) but did not affect ATP-induced currents (Fig 4C). The inhibitory effect of PTX, on *Cys-loop* family receptors, was concentration-dependent. The IC_{50} for picrotoxin on $GABA_A$ and nACh receptors 9.6 ± 1.5 μM and 55.16 ± 8.4 μM (Fig 5, Table 1) respectively, indicating similar potency to inhibit both, $GABA_A$ and nACh receptors; in contrast, picrotoxin was less potent to inhibit $5HT_3$ receptors (IC_{50} 443.9 μM), than $GABA_A$ and nACh receptors as shown in table 1. Hill coefficient values for $GABA_A$, nACh, and $5HT_3$ receptors were 0.75, 0.86, and 1.2 respectively (Table 1). All Hill

coefficients are not different than unity, which indicates the lack of cooperativity of the picrotoxin effects. The picrotoxin order of potency on *Cys-loop* family receptors tested was $GABA_A$ > nACh >> $5HT_3$. With these results, we show that picrotoxin is not a specific antagonist in myenteric neurons.

PTX vs	*IC_{50} (μM)*	*Hill Coeficiente*
GABA	9.6 ± 1.5	0.75
ACh	55.2 ± 8.4	0.86
5HT	443.9 ± 52.8	1.2
ATP	-	-

Table 1. Picrotoxin IC_{50} and Hill coefficient were calculated from concentration-response curves shown in Fig 5. There are inhibitory effects of picrotoxin on ACh, GABA, and 5HT induced currents. This antagonist did not affect P2X receptors.

Ondansetron

Ondansetron had inhibitory effect only on $5HT_3$ receptors. This effect was concentration-dependent (Figs 6 and 7). The IC_{50} tell us that this substance is a potent $5HT_3$ receptor antagonist and the Hill coefficient value was not different than unity (Fig 7) indicates lack of cooperativity. GABA-induced inward currents were not affected by ondansetron.

Bicuculline

The whole-cell inward currents evoked by ACh, GABA, and 5HT were inhibited by bicuculline. These effects appear to be concentration-dependent. However, we need to increase the number of experiments to build concentration-response curves for these effects. For the time being our data indicates that bicuculine is not a specific antagonist to $GABA_A$ receptors.

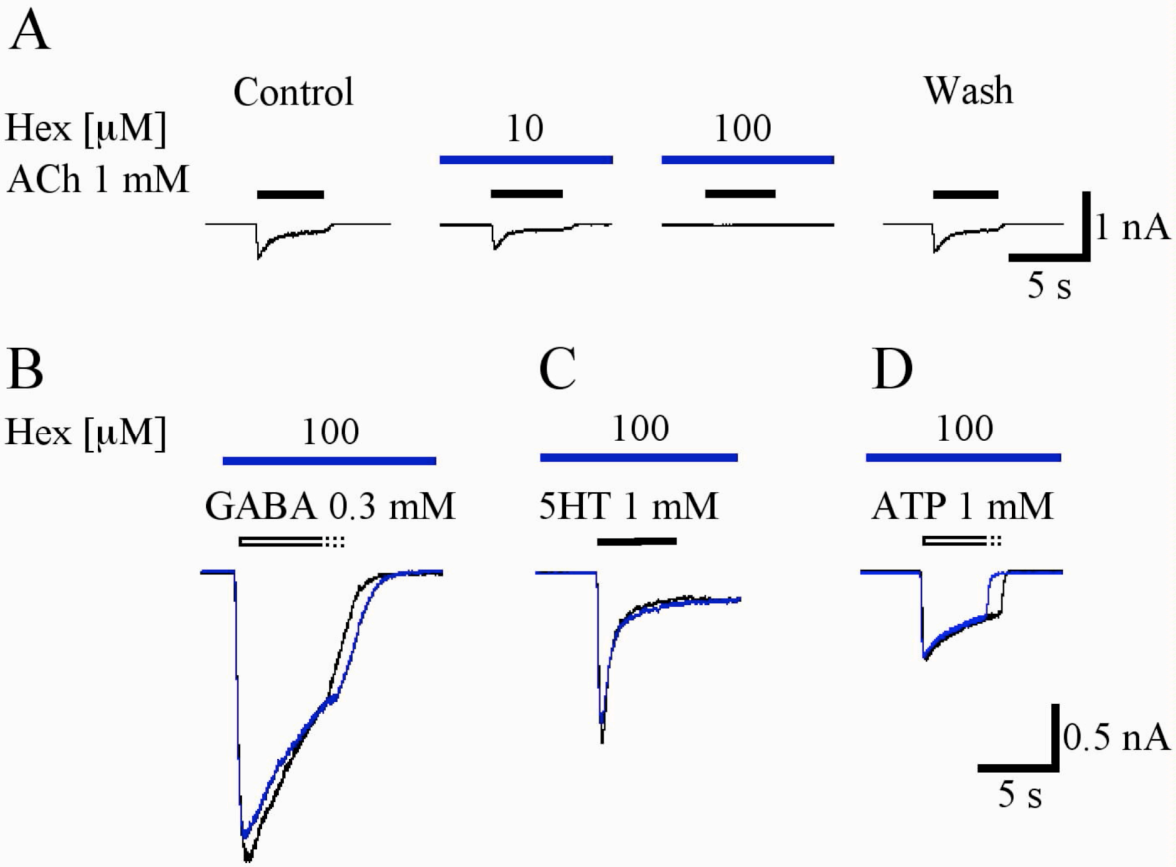

Figure 1. Hexamethonium (Hex) blocks specifically the nicotinic receptors. **A-D**. Whole-cell inward currents induced by: acetylcholine (ACh), adenosine 5'-triphosphate (ATP), γ-aminobutyric acid (GABA), and 5-hydroxytryptamine (5HT) in presence or absence of hexamethonium (Hex). **A**, The ACh-induced current decrease in presence of hexamethonium in a concentration-dependence manner, the black bars indicate ACh application, the blue bars indicate hexamethonium application. Notice that at 100 μM completely blocks the ACh response. **B-D**, GABA-, 5HT-, and ATP-induced currents (black trace) did not change in presence of hexamethonium 100 μM (blue trace). Recordings shown in all panels were obtained from four different myenteric neurons and at holding potential of -60 mV; Recordings were carried out every 5 min to avoid receptor desensitization.

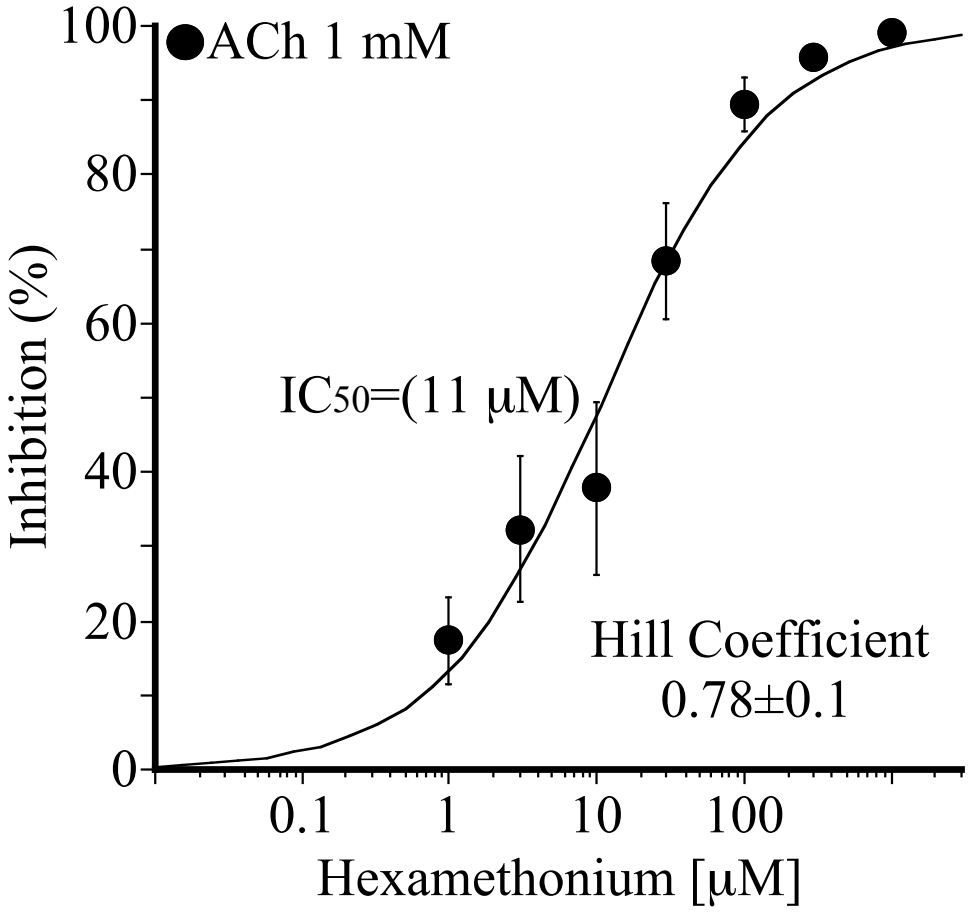

Figure 2. Hexamethonium (Hex) inhibits ACh-induced inward currents in a concentration-dependent manner. Whole-Cell currents were induced by 1 mM ACh and each point represents the average data of three different neurons. The response is expressed as percentage of inhibition of the ACh-induced current. Lines on the symbols are the S. E. M.

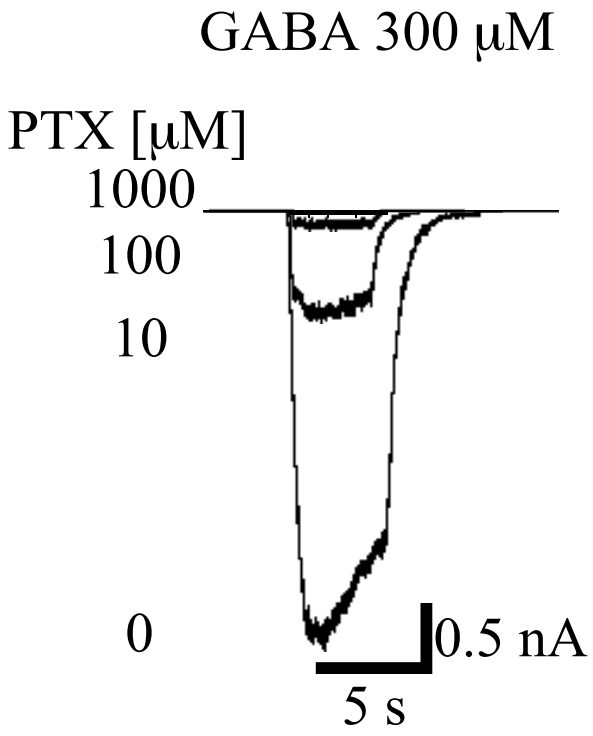

Figure 3. Picrotoxin (PTX) inhibits GABA-induced inward current in a concentration-dependant manner. Notice that at a concentration of 100 μM picrotoxin blocks most of the GABA (300 μM) response. Recordings are from the same myenteric neuron and were carried out at holding potential of -60 mV and every 5 min to prevent receptor desensitization.

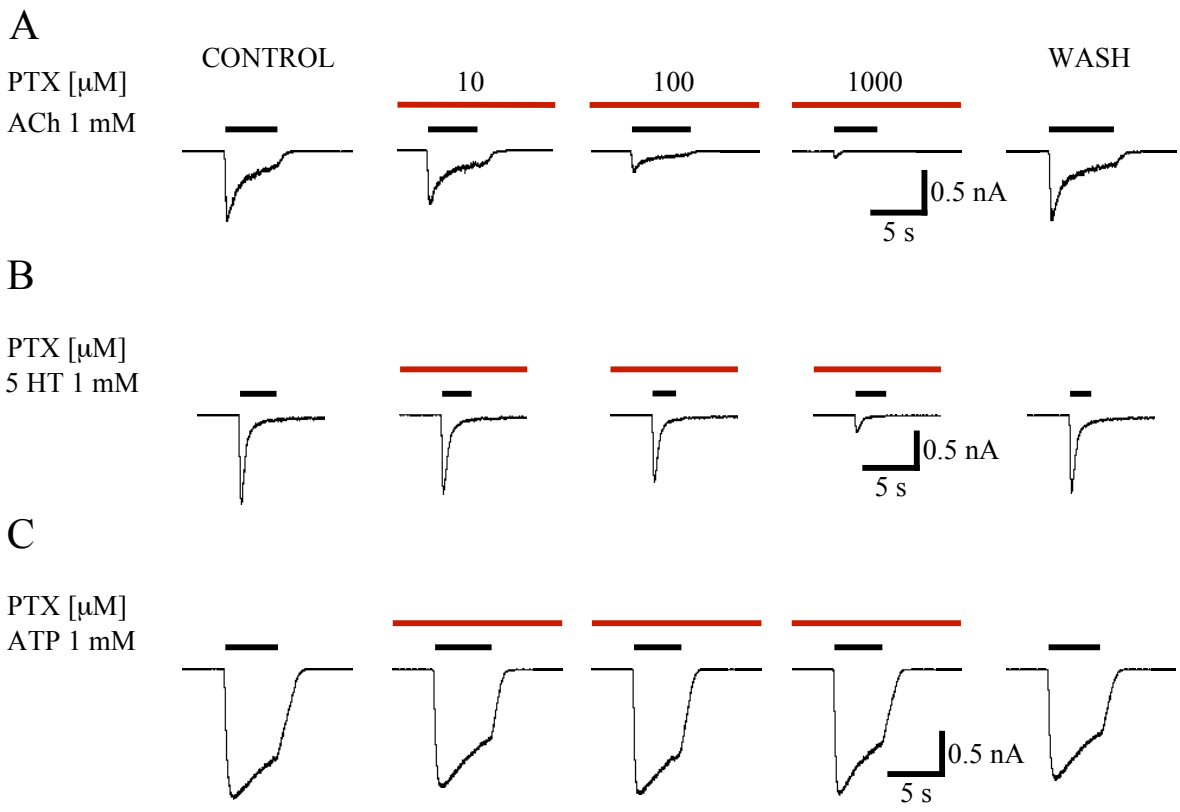

Figure 4. Picrotoxin (PTX) also inhibits the currents induced by ACh and 5HT but not those induced by ATP. Whole-cell inwards currents induced by 1 mM concentration of ACh, 5HT or ATP before and in the presence of the indicated picrotoxin concentrations. Currents shown in each panel were measured from the same myenteric neuron at holding potential of -60 mV.

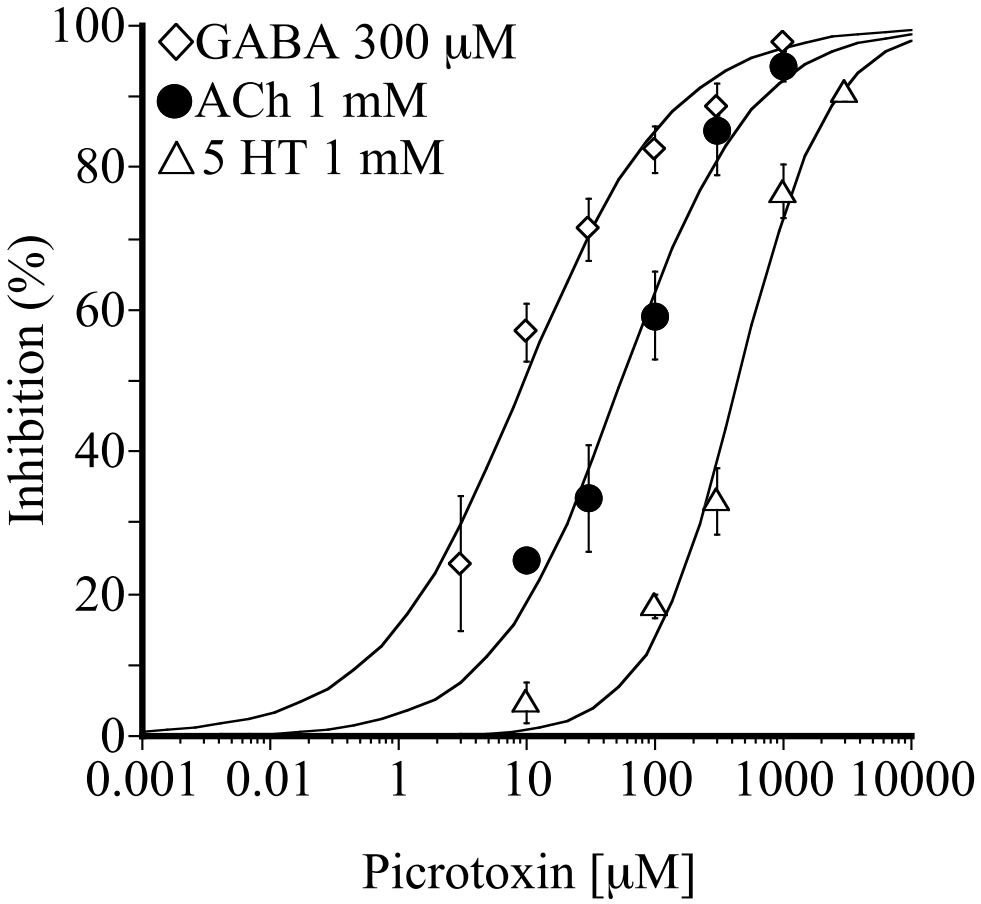

Figure 5. Picrotoxin inhibits ACh-, 5HT-, and GABA-induced inward currents in a concentration-dependent manner. The order of potency for this picrotoxin effects on the different receptors is $GABA_A$>nACh>>$5HT_A$. Each point represents the average obtained from at least three neurons. The response is expressed as percentage of inhibition of the agonist-induced current. Lines on the symbols are the S. E. M.

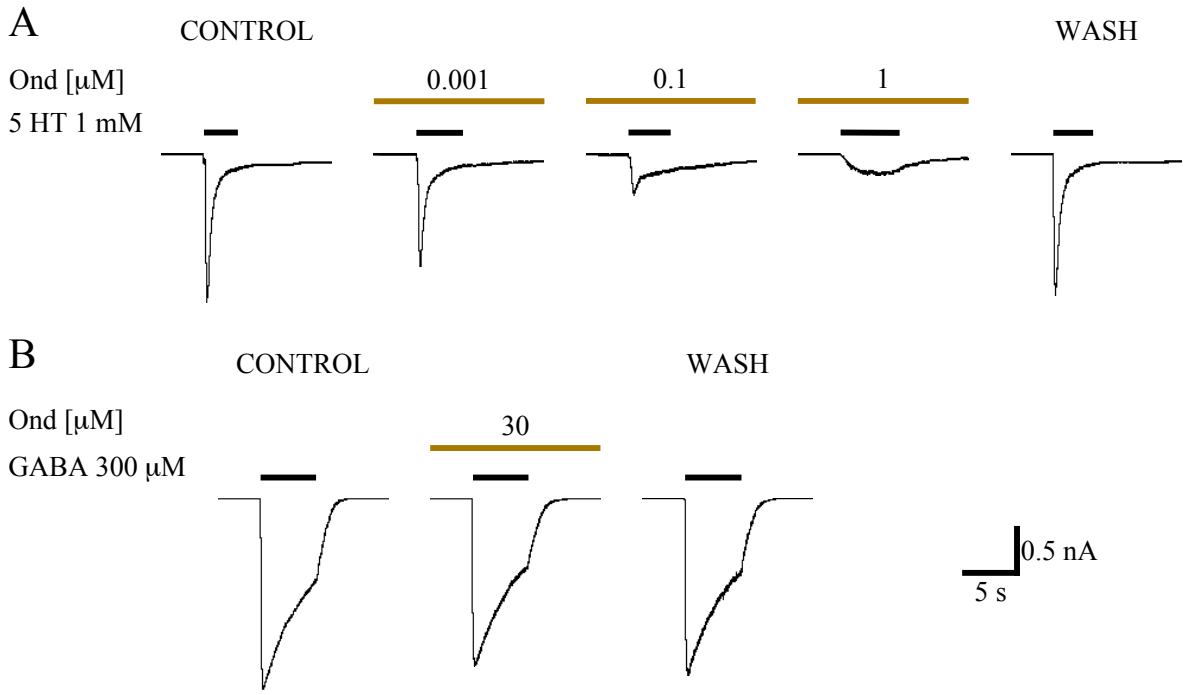

Figure 6. Ondansetron (Ond) inhibits 5HT-induced current but not those induced by GABA. Whole-cell inwards currents induced by 5HT (1 mM) or GABA (300μM), before and in the presence of the indicated picrotoxin concentrations. Currents shown in each panel were measured from the same myenteric neuron at holding potential of -60 mV.

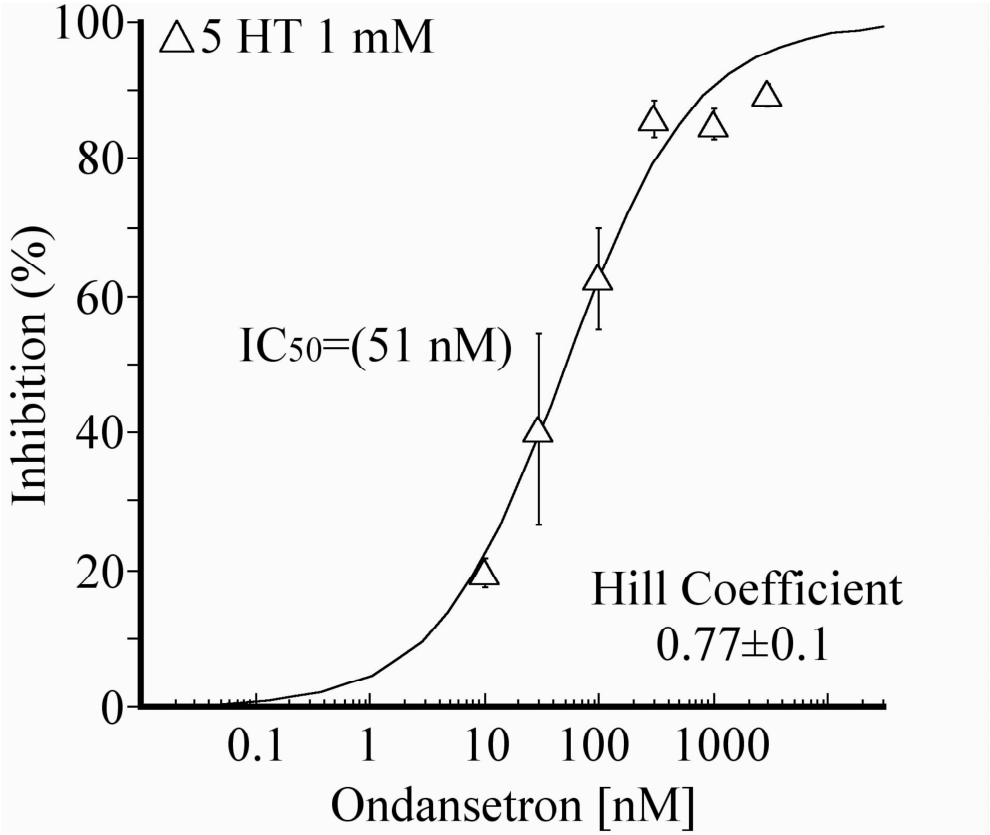

Figure 7. Ondansetron is a potent antagonist of the $5HT_3$ receptors. This effect is concentration-dependent. Each point represents the average obtained from at least three neurons. The response is expressed as percentage of inhibition of the 5HT-induced current. Lines on the symbols are the S. E. M.

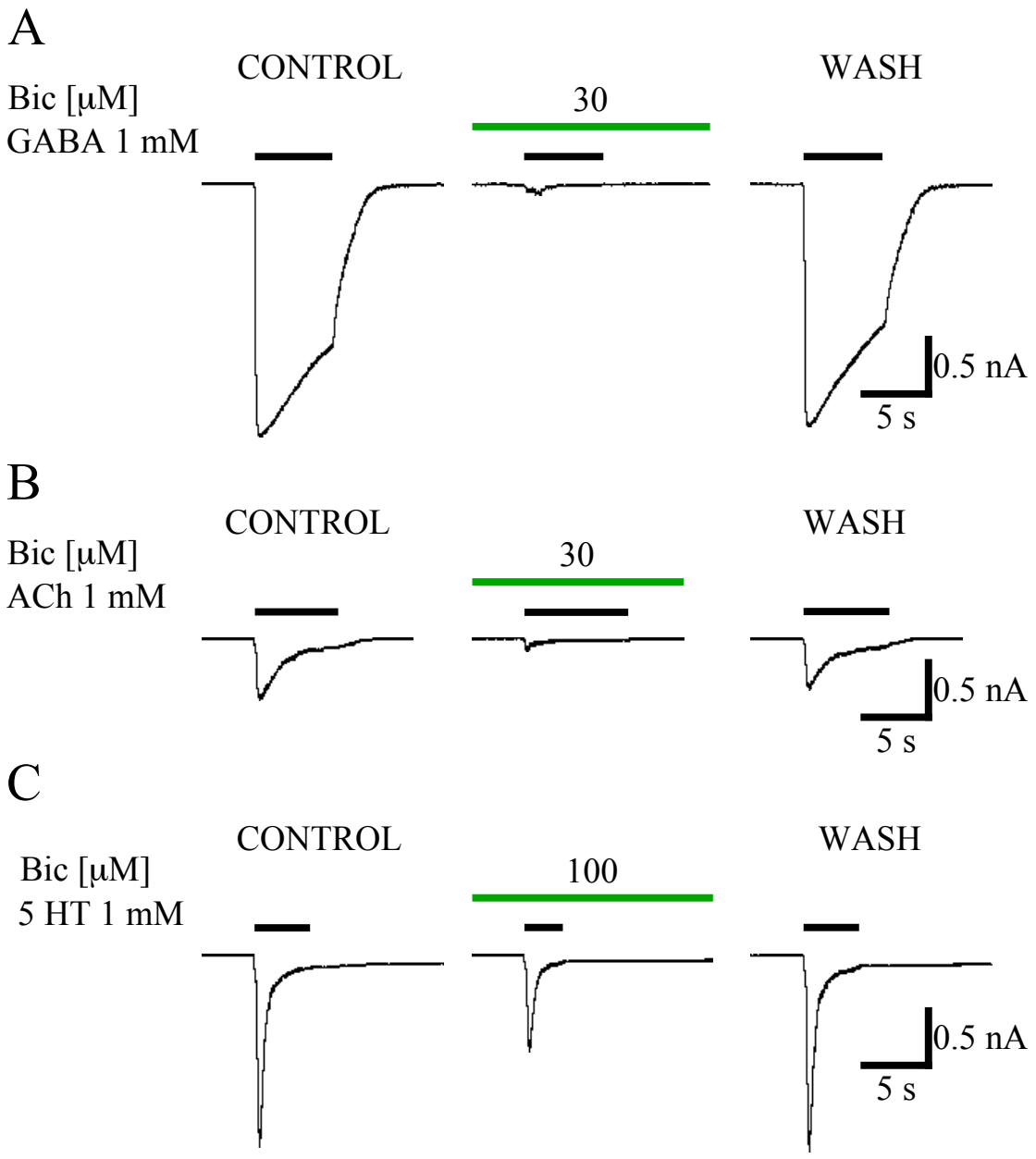

Figure 8. Bicuculine (Bic) inhibits the GABA-, ACh-, and 5HT-induced currents. Currents induced by GABA, ACh or 5HT were recorded before and in the presence of the indicated bicuculline concentration. Currents of each panel were measured from the same myenteric neuron at holding potential of -60 mV.

DISCUSION

The present study shows that the well-known $GABA_A$ antagonists, picrotoxin and bicuculline, are not specific and they also inhibit other *Cys-loop* receptors present in myenteric neurons. Furthermore, we found that hexamethonium and ondansetron inhibit specifically nACh and $5HT_3$, respectively. P2X receptors where not affected by any of the mentioned antagonists.

Picrotoxin on nACh receptors

Our data shows clearly that picrotoxin inhibits the nACh native receptors present in myenteric neurons, which is in agreement with previous studies carried out with different animal models and tissues. For instance, picrotoxin has been shown to inhibit nACh receptors from crab gastric mill (Marder & Paupardin-Tritsch, 1980), from Kenyon cells of the honey bee (Wustenberg & Grunewald, 2004), and specific receptors ($\alpha_3\beta_4$ and α_7) expressed in oocytes (Erkkila *et al.*, 2004).

Picrotoxin on 5-HT_3 receptors

Picrotoxin also inhibited the 5-HT_3 native receptors of myenteric neurons with a relatively low potency (IC_{50} = 444 μM), which might indicate that they might be formed with $5HT_{3A}$ and $5HT_{3B}$ subunits. This hypothesis is based in the fact that picrotoxin inhibits $5HT_{3A}$ homomeric channels, expressed in HEK-293, with a lower potency (IC_{50} = 30 μM) (Das *et al.*, 2003). Using the same expression technique, it has also been shown that heteromeric $5HT_{3A}$-$5HT_{3B}$ channels are less sensitive to picrotoxin (IC_{50} = 1135 μM) than $5HT_{3A}$ homomeric channels, which clearly indicates that picrotoxin

sensitivity is subunit-dependent (Das & Dillon, 2005). $5HT_{3A}$ and $5HT_{3As}$ receptors are known to be present in guinea pig intestine (Lankiewicz *et al.*, 1998). As far as we know, our electrophysiological evidence is the first reported data suggesting the presence of $5HT_{3B}$ subunit in the guinea-pig small intestine.

Bicuculline on nACh and $5HT_3$ receptors

Our findings indicate that bicuculline, besides blocking $GABA_A$ receptors, also blocks nACh and $5HT_3$ receptors in myenteric neurons of the guinea pig small intestine. A similar effect has previously been reported on $5HT_3$ receptors in rat cerebral cortical neurons (Mayer & Straughan, 1981) and feline autonomic and sensory ganglia reviewed by (Mayer & Straughan, 1981). Similarly, it has been reported that bicuculline inhibits nACh receptors of honeybee Kenyon cells (Wustenberg & Grunewald, 2004).

Hexamethonium was Specific on nACh Receptors

As it has previously been shown (Zhou *et al.*, 2002), we found that hexamethonium inhibited nACh receptors in myenteric neurons of the guinea pig small intestine. However, we found that this effect was less potent (IC_{50} = 11.13 μM) than the one described by Zhou *et al.* (IC_{50} = 1.6 μM). This discrepancy might be explained by the fact that they used newborn whereas we used young guinea pigs. It possible that the subunit composition of nACh receptors change during animal development, as previously reported in neurons of the CNS (Keiger *et al.*, 2003; Azam *et al.*, 2007).

Ondansetron Acts Specifically on $5HT_3$ Receptors

Our preliminary data indicate that ondansetron does not modify GABA induced currents but totally blocks $5HT_3$ receptors. The relatively low potency of ondansentron to inhibit $5HT_3$ native receptors of myenteric neurons (IC_{50} = 51.3 nM) suggests that they are heteromeric. Thus, ondansentron is more potent (IC_{50} = 6.4 nM) on mammalian $5HT_{3A}$ homomeric receptors expressed in Xenopus oocytes (Paul *et al.*, 2005).

Mechanism of Action of Picrotoxin *Versus* Hexamethonium and Ondansentron

Picrotoxin effect on 5-HT_3 (Das *et al.*, 2003) and nACh (Erkkila *et al.*, 2004) receptors is a non-competitive antagonism. The high homology between *Cys-loop* family receptors could be the reason why picrotoxin blocks $GABA_A$, nACh, and $5HT_3$ channels (Erkkila *et al.*, 2004). Our observation that picrotoxin did not affect P2X receptors in the present study is in agreement with such an interpretation. The lack of effect of hexamethonium and ondansetron on all tested *Cys loop* receptors indicates that these drug binds on a site that is only present in nACh and $5HT_3$ receptors, respectively.

In summary, our data shows that hexamethonium and ondansentron might be useful tools to study the functions of ACh and 5HT and their respective LGIC in myenteric neurons of the guinea pig small intestine. Picrotoxin and bicuculline affect all LGIC studied here and therefore, they are non-specific antagonists of $GABA_A$ receptors. These substances are not recommended for physiological studies aimed to investigate the functions of GABA in these neurons.

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