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POSGRADO EN CIENCIAS EN BIOLOGIA MOLECULAR

Interacciones Funcionales entre los Receptores Canal GABAA y 5-HT3 en neuronas del Plexo Mientérico.
(Functional interactions between GABAA and 5-HT3 channels in myenteric neurons)

Tesis que presenta

Luz María García Hernández

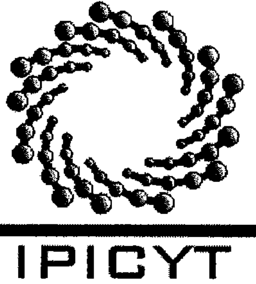
Para obtener el grado de

Maestra en Ciencias en Biología Molecular

Director de la Tesis:

Dr. Carlos Barajas López

San Luis Potosí, S.L.P., Julio de 2004.



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Luz María García Hernández

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Interacciones Funcionales entre los Receptores Canal GABAA y 5-HT3 en neuronas del Plexo Mientérico. (Functional interactions between GABAA and 5-HT3 channels in myenteric neurons)

que se desarrolló bajo la dirección de

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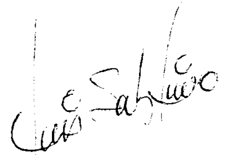
La presente tesis de maestría en ciencias en la especialidad de biología molecular fue elaborada por Luz María García Hernández y aprobada el 25 de Junio de 2004 por los suscritos, designados por el Colegio de Profesores del Departamento de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C.

Dr. Carlos Barajas López

Director de la tesis

Dr. Rubén López Revilla

Miembro del comité tutoral



Dr. Luis Salazar Olivo

Miembro del comité tutoral

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Abstract

Ionotropic receptors play an important role in fast transmission in many neuroneuronal synapses. Recently it has been shown that the *cys loop* superfamily of these receptors maintain inhibitory interactions with P2X receptors. However, it is not known whether or not members of the *cys loop* superfamily establish similar interactions among themselves; the aim of this study was to investigate and to functionally characterize the putative inhibitory interactions between native $GABA_A$ and 5-HT_3 receptors in myenteric neurons from the guinea-pig small intestine. We hypothesized that $GABA_A$ and 5-HT_3 receptors, being structurally similar to nACh receptors, could also maintain a similar interaction as the one observed between these three channels and the P2X receptors. Whole-cell currents were recorded in myenteric neurons induced by maximal concentrations of γ-aminobutyric acid (GABA), serotonin (5-HT), and by both agonists. We found that GABA and 5-HT open two different channel populations that which are functionally dependent as ionic currents carried through them are occluded when they are simultaneously activated. This occlusion is observed as soon as channel activation and the currents induced by simultaneous application of both agonists are carried through both $GABA_A$ and 5-HT_3 channels. Altogether, these observations indicate that the current occlusion is mediated by cross-inhibition and a direct interaction between $GABA_A$ and 5-HT_3 receptors. These interactions play an important role in synaptic integration.

Key words: Ion channels; $GABA_A$ receptors; γ-aminobutyric acid; 5-HT_3 receptors; serotonin; ionotropic receptors; ligand-gated channel; enteric neurons; myenteric neurons; patch clamp recordings; electrophysiology.

Resumen

Los receptores ionotrópicos juegan un papel importante en la transmisión sináptica rápida entre neuronas. Recientemente, se mostró que los receptores de la superfamilia *cys loop* mantienen interacciones inhibidoras con los receptores P2X, aunque se desconoce si miembros de la superfamilia *cys loop* interaccionan entre ellos. El propósito de este estudio fue investigar y caracterizar funcionalmente las posibles interacciones inhibidoras entre los receptores canal nativos $GABA_A$ y los 5-HT_3 presentes en neuronas mientéricas del intestino delgado del cobayo. Los receptores canal nACh, $GABA_A$ y los 5-HT_3 pertenecientes estructuralmente a la familia *cys loop*, podrían mantener interacciones similares a las observadas entre estos y los P2X. Las corrientes de célula completa fueron registradas en neuronas mientéricas inducidas por la aplicación del ácido γ-aminobutírico (GABA), serotonina (5-HT) o por la aplicación de ambos agonistas. Encontramos que el GABA y la 5-HT abren dos diferentes poblaciones de receptores canal. Sin embargo, estos no son funcionalmente independientes ya que sus corrientes iónicas son ocluidas cuando son activados simultáneamente. Esta oclusión es observada tan pronto como las corrientes son activadas. Las corrientes inducidas por la aplicación simultánea de ambos agonistas es conducida a través de ambos canales $GABA_A$ y 5-HT_3. En resumen, estas observaciones sugieren que la oclusión de las corrientes es mediada por la inhibición cruzada y por la interacción directa entre los canales $GABA_A$ y 5-HT_3. La importancia de tales interacciones en la integración sináptica es discutida.

Palabras clave: Canales iónicos; receptores $GABA_A$; ácido γ-aminobutírico; receptores 5-HT_3; serotonina; receptores ionotrópicos; receptores activados por ligando; neuronas entéricas; neuronas mientéricas; registros de patch clamp; electrofisiología.

Introduction

Fast synaptic communication in the nervous system is critical for information processing and synaptic plasticity and is achieved through the activation of neurotransmitter-gated channels also known as ionotropic receptors [Sakmann 1992]. Classically, it has been considered that ionotropic receptors are functionally and physically independent. The present study provides further evidence that this is not the case.

Serotonin (5-HT) and γ-aminoburytic acid (GABA) are both known to play a role as fast neurotransmitters. 5-HT_3 receptors are ligand-gated ion channels present in some neuronal-derived cell lines, and in peripheral and central neurons [Kilpatrick et al. 1987; Kilpatrick et al. 1988; Barnes et al. 1990; Jones et al. 1992; Krantis 2000]. The cloning of a functional 5-HT_3 receptor subunit (5-HT_{3A}) from mice, rat, and human brain tissue confirmed that this receptor is a member of the *Cys-loop* superfamily of ionotropic receptors [Maricq et al. 1991; Hope et al. 1993], which mediate fast excitatory transmission in the central nervous system [Sugita et al. 1992]. 5-HT_3 channels are likely constituted by the combination of five subunits. Homomeric channels can be formed from the 5-HT_{3A} subunits and heteromeric channels can also be obtained by the combination of the 5-HT_{3A} with a second known subunit of this subfamily (5-HT_{3B}). Nonfunctional channels result from the expression of only 5-HT_{3B} subunits [Maricq et al. 1991; Hope et al. 1993].

$GABA_A$ receptors are also part of the *Cys-loop* ligand-gated channel superfamily and are also heterooligomers composed of the α-, β-, δ-, γ-, and ε-subunits. A great variety of receptors can be formed by the combination of five of these subunits, which correlates with the many different physiological and pharmacological profiles reported for the GABA-mediated neurotransmission [DeFeudis 1990]. Krantis' group [Poulter et al. 1999], shows evidence that rat enteric neurons express various types of ∝1-6,[1] β2-3, and γ1,3 subunits. Yet, there was no evidence for γ2 subunits. Moreover, while the β3 and γ3 subunits are

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[1] Subscribed digits indicate the number of a certain subunit within a receptor, whereas regular ones describe the subunit type.

expressed in some neurons of the myenteric plexus, these were not found in submucosal neurons.

Recent experimental evidence indicates that the channels activated by acetylcholine (nACh) and ATP (P2X) are not independent and that they can inhibit each other when they are simultaneously activated [Barajas-Lopez et al. 1998; Zhou and Galligan 1998]. This inhibitory interaction is very fast and might be mediated by an allosteric interaction between nACh and P2X channels. A similar cross-inhibition between the $P2X_2$ and nicotinic $\propto 3\beta 4$ receptor subtypes was observed when these were co-expressed in *Xenopus* oocytes [Khakh et al. 2000]. Analogous interactions have been shown between P2X and the $GABA_A$ receptors in dorsal root ganglia [Sokolova et al. 2001], and between P2X and 5-HT_3 receptors in enteric neurons [Barajas-Lopez et al. 2002; Boue-Grabot et al. 2003]. In apparent contradiction with the latter studies, in myenteric neurons P2X channels were reported to interact specifically with nACh channels and not with other members of the *Cys-loop* superfamily (e.g. $GABA_A$ and 5-HT_3 receptors) present in these neurons [Zhou and Galligan 1998]. This discrepancy might indicate that these interactions are tissue specific and could be explained by receptor heterogeneity. Adding to this complexity, other types of pharmacological interactions appear to exist between the serotoninergic and cholinergic systems. It has been reported that 5-HT can block nACh channels at similar concentrations than those required to activate 5-HT_3 receptors in various cell types including submucosal neurons [García-Colunga and Miledi 1995; Barajas-Lopez et al. 2001].

Our aim was to investigate and to functionally characterize the putative inhibitory interactions between $GABA_A$ and 5-HT_3 native receptors present in myenteric neurons [Barajas-Lopez et al. 1998; Zhou and Galligan 1998; Barajas-Lopez et al. 2001]. Our hypothesis was that these receptors, being structurally similar to nACh receptors, maintain a similar interaction as that observed between the *Cys-loop* superfamily receptors and the P2X channels.

Our findings indicate that activation of $GABA_A$ and 5-HT_3 receptors opens two different channel populations. These two channels, however, negatively

modulate each other when they are simultaneously activated. This inhibitory interaction occurs concomitantly with current activation, implying that they are mediated by direct interaction between these receptors. These interactions are important in synaptic integration.

Methods

Tissue preparation. Young guinea pigs (150-300 g), either male or female, were killed by decapitation and five cm of proximal jejunum were 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 of this intestinal segment was removed and the thin submucosal layer was dissected before removing most of the circular muscle layer leaving behind the longitudinal layer with the myenteric plexus embedded with it.

Dissociation of enteric neurons. The myenteric preparation was incubated for 10 min at 37°C in Ca^{2+} and Mg^{2+} Hanks' balanced salt solution, which contained 0.1 ml/ml papain activated with 0.4 mg/ml L-cysteine. The papain solution was removed, and the preparation was washed with modified L-15 medium, containing 90% of Leibovitz's L-15 medium, 10% of fetal calf serum, 2 mM L-glutamine, 15 mM glucose, 10 U/ml penicillin, and 10 μg/ml streptomycin. Preparation was incubated for another 10 min in Ca^{2+} and Mg^{2+} Hanks' balanced salt solution containing 1 mg/ml collagenase and 4 mg/ml dispase instead of papain. After 10-15 min, the preparation was triturated by using a long-neck pasteur pipette until single neurons were observed. The suspension containing the dispersed neurons and large clumps of undissociated cells was allowed to settle. After 5 min, the fluid containing the suspended cells was carefully removed (leaving the large clumps behind) and layered with modified L-15 medium. This fluid was centrifuged, the supernatant was discarded, and cells were resuspended in growth medium. Cells were plated into culture wells with small rounded coverslips in their bottom. The coverslips were previously coated with sterile rat-tail collagen. Growth medium was constituted by: 97.5% of minimum essential medium, 2.5% of guinea pig serum, 2mM L-glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin, and 15 mM glucose. The source for growth medium components was GIBCO Lab (Life Technologies, Grand Island, NY) except for the guinea pig serum, which was from our own laboratory.

Patch-clamp recordings. 5-HT and GABA are known to modulate the potassium membrane conductance of enteric neurons via G-protein linked receptors [Pan et al. 1997; Krantis 2000]. In order to isolate the ionic membrane currents mediated by activation of ligand-gated channels, the experiments were carried out in the presence of Cs^+ (a potassium channel blocker) and currents were measured by the whole-cell patch clamp configuration, which is also known to prevent various effects mediated by second messengers [Gillis et al. 1991]. Membrane currents induced by 5-HT and GABA were recorded using short-term (2-80 hours) primary cultures of myenteric neurons and a Axopatch 1D amplifier. Patch pipettes were made as previously described [Barajas-Lopez et al. 1996] and had resistances between 1-3 MΩ. Except when otherwise mentioned, the holding potential was -60 mV. The standard solutions used, unless otherwise mentioned, had the following composition (in mM). Inside the pipette: CsCl 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 all 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) and analyzed on a Macintosh computer using Axograph software (Axon Instruments). Membrane potentials were corrected for the liquid junction potential (pipette -11 mV). 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-Lopez et al. 1994]. The external application of experimental substances was achieved by abruptly changing the tube delivering control external solution in front of the cell being recorded, for a tube delivering the same solution plus the drug(s). Experimental substances were removed by returning back to the tube containing the control external solution. External solutions were delivered by gravity. Experiments were performed at room temperature (~23°C).

GABA, picrotoxin, and tropisetron were purchased from Research Biomedical Inc. (Natick, MA, USA). All other substances were purchased from Sigma (St. Louis, MO., USA).

Data analysis. Results are expressed as means ± S.E.M. and the number of cells used as *n*. The paired Student's *t*-test was used to evaluate 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.

Results

Pharmacological and electrophysiological properties of the whole-cell currents induced by 5-HT and GABA

Whole-cell currents or conductances activated by GABA (I_{GABA}) and 5-HT ($I_{5\text{-}HT}$) in enteric neurons have been previously characterized [Cherubini and North 1984; Derkach et al. 1989; Zhou and Galligan 2000; Barajas-Lopez et al. 2001]. $I_{5\text{-}HT}$ is mediated by activation of nonspecific cationic channels, whereas I_{GABA} is mediated by activation of Cl^- channels.

5-HT induced an inward current in 88% of a total of 97 neurons, whereas GABA caused an inward current in only 76% for the neurons tested. Concentration-response curves (Fig. 1A) were obtained for these transmitters in myenteric neurons and analyzed as previously [Barajas-Lopez et al. 1996]. The mean current amplitudes induced by maximal concentrations of 5-HT (1 mM) and GABA (300 μM) were -1289 ± 183 pA and -1888 ± 536 pA, respectively, but had a variable amplitude in different cells ranging from only a few pA up to -7.4 nA. The amplitude of I_{GABA} was larger than that of $I_{5\text{-}HT}$.

As shown in Figure 1B, $I_{5\text{-}HT}$ (1 mM) and I_{GABA} (300 μM) were almost totally inhibited by tropisetron (0.3 μM) and picrotoxin (1 mM), respectively. Similar results were observed in three additional experiments when the specific 5-HT_3 receptor antagonist ondasetron (0.3 μM) was used instead of tropisetron, a 5-HT_3 and 5-HT_4 receptor antagonist [Hoyer et al. 1994].

The $I_{5\text{-}HT}$ and I_{GABA} onset were virtually the same (Fig. 2A). Thus, the time required to reach the half-maximal of these currents was not significantly different in the eight cells analyzed, which had average values of 191 ± 35.5 ms and 195 ± 32 ms for $I_{5\text{-}HT}$ and I_{GABA}, respectively. Both currents usually reached their peak within the following second.

After currents had reached their maximal amplitude, they decreased despite the continuous presence of the transmitters, indicating tachyphylaxis. $I_{5\text{-}HT}$ desensitized faster that I_{GABA} (Fig. 2B). Furthermore, $I_{5\text{-}HT}$ desensitization was better fitted by the sum of three exponential functions, whereas I_{GABA}

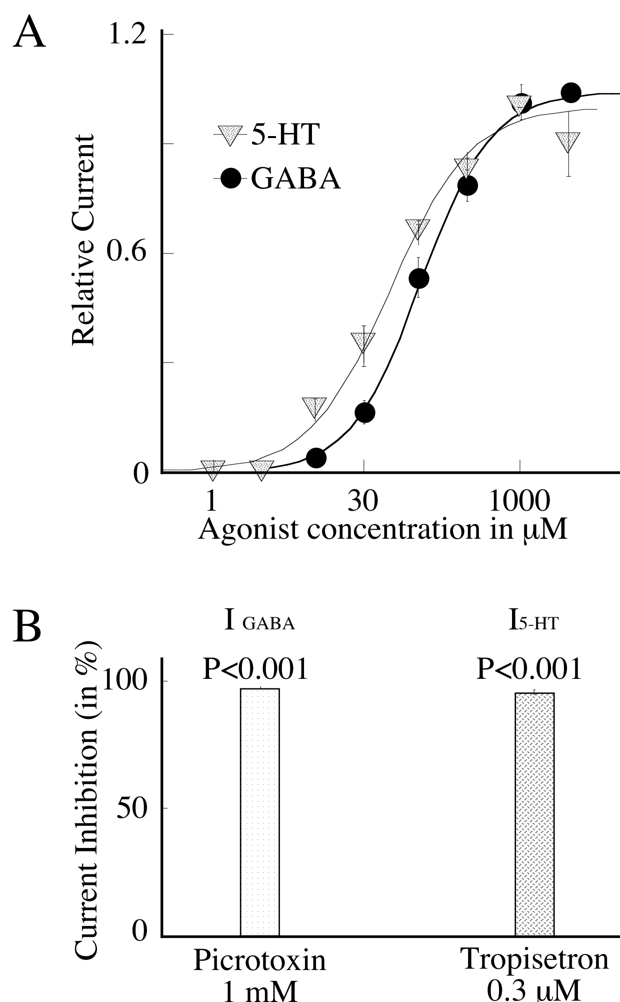

Figure 1. Whole-cell inward currents induced by GABA (I_{GABA}) and serotonin ($I_{5\text{-}HT}$) are mediated by two different receptors. A: Concentration-response curves showing the currents (relative values) induced by GABA and serotonin (5-HT). The concentrations required to induced fifty percent of the maximal response (EC_{50}) were 105 and 55 mM for GABA and 5-HT, respectively, while the Hill coefficients were 1.27 ± 0.1 and 1.0+0.1. Symbols are mean ± S.E.M. of six to eighteen experiments. Sigmoidal lines were fitted with 2-parameter logistic model. Relative currents were calculated assuming the effect of 1 mM as maximal. B: Picrotoxin (1 mM; a $GABA_A$ receptor blocker) blocks I_{GABA}. Tropisetron (0.3 µM; a 5-HT_3 receptor antagonist) blocks $I_{5\text{-}HT}$; similar observations were obtained with ondasetron, a more specific 5-HT_3 receptor antagonist (not shown). Whole-cell inward currents were measured from the same myenteric neurons, at a holding potential of -60 mV. In B, currents were induced with 1 mM (5-HT) and 300 µM (GABA) and were recorded before (control) and 5 min after starting the superfusion of picrotoxin or tropisetron. Bars represent the average current inhibition expressed in percentage whereas S.E.M. is indicated by line at top of the bars.

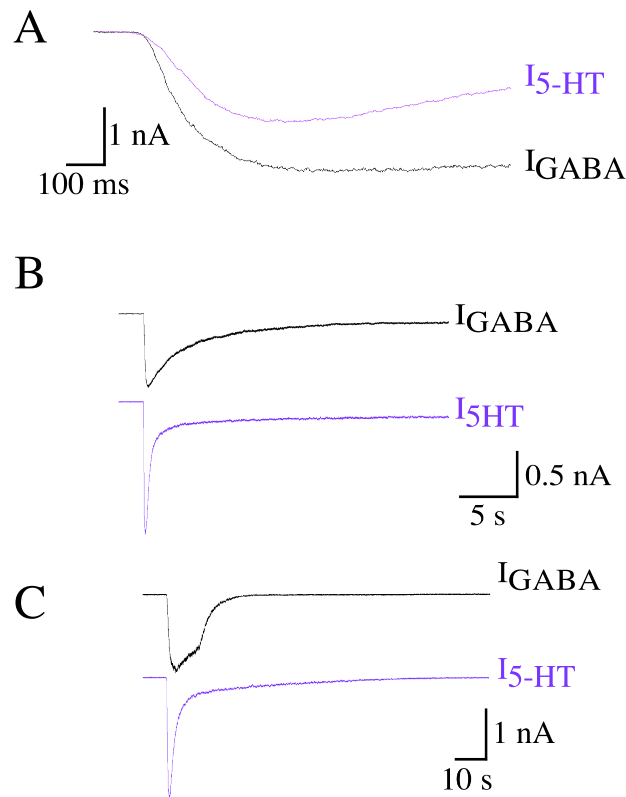

Figure 2. Electrophysiological properties of the whole-cell currents induced by 5-HT ($I_{5\text{-}HT}$) and GABA (I_{GABA}). Representative recordings of I_{GABA} and $I_{5\text{-}HT}$ recorded from three myenteric neurons. A: Onset of the currents showing that activation of I_{GABA} has similar kinetics than $I_{5\text{-}HT}$. B: After these two currents reached their maximal amplitude, quickly decreased despite the continuous presence of the neurotransmitters. Notice that $I_{5\text{-}HT}$ desensitized faster than I_{GABA}. C: After agonist removal from the external solution, both currents decay to reach their control values. $I_{5\text{-}HT}$ decay is far slower than that of I_{GABA}. All these experiments were measured at a holding potential of -60 mV.

desensitization was better fitted by the sum of two exponential functions (see below).

After agonist removal from the external solution, $I_{5\text{-HT}}$ decayed much more slowly than I_{GABA} (Fig. 2C). This decay was well fitted by a single exponential function for both currents, and their average τ values were significantly different ($P < 0.05$; $n = 9$), 8.3 ± 1.4 and 0.8 ± 0.1 s for I_{GABA} and $I_{5\text{-HT}}$, respectively.

Ionic currents induced by 5-HT and GABA are not additive

The experiments described above demonstrated that $I_{5\text{-HT}}$ and I_{GABA} have completely different kinetics and that they are mediated by activation of pharmacologically distinct receptors. In the following experiments, it was investigated whether or not these currents are mediated by two independent channel populations. If this is the case, then the currents induced by *quasi* maximal concentrations (i.e. receptor occupancy is expected to be close to 100%) of GABA and 5-HT should be additive. We therefore measured the peak whole-cell currents induced by maximal concentrations (1 mM 5-HT, 300 μM GABA) of independent or simultaneous application of both agonists ($I_{5\text{-HT+GABA}}$) in the same neuron. We found that the addition of individual currents ($I_{5\text{-HT}}$ + I_{GABA} = expected current [$I_{expected}$]) was significantly larger ($P < 0.01$) than $I_{5\text{-HT+GABA}}$, revealing an occlusion between $I_{5\text{-HT}}$ and I_{GABA} (Fig. 3A and 3B). In these experiments, $I_{5\text{-HT+GABA}}$ (-2546.5 ± 487 pA) was also different than the current induced by the most effective of these transmitters (-2254 ± 438). This indicates that there was partial additivity of the currents.

Kinetics of the whole-cell currents induced by simultaneous application of both agonists

Figure 3A shows the mean onset of $I_{5\text{-HT}}$, I_{GABA}, $I_{5\text{-HT+GABA}}$, and $I_{expected}$ from eight neurons. As it is shown in Figure 4B, the average time required to reach the half-maximal amplitude of these currents. Notice that the half onset time of $I_{5\text{-HT+GABA}}$ is not different than that of $I_{5\text{-HT}}$ or I_{GABA}.

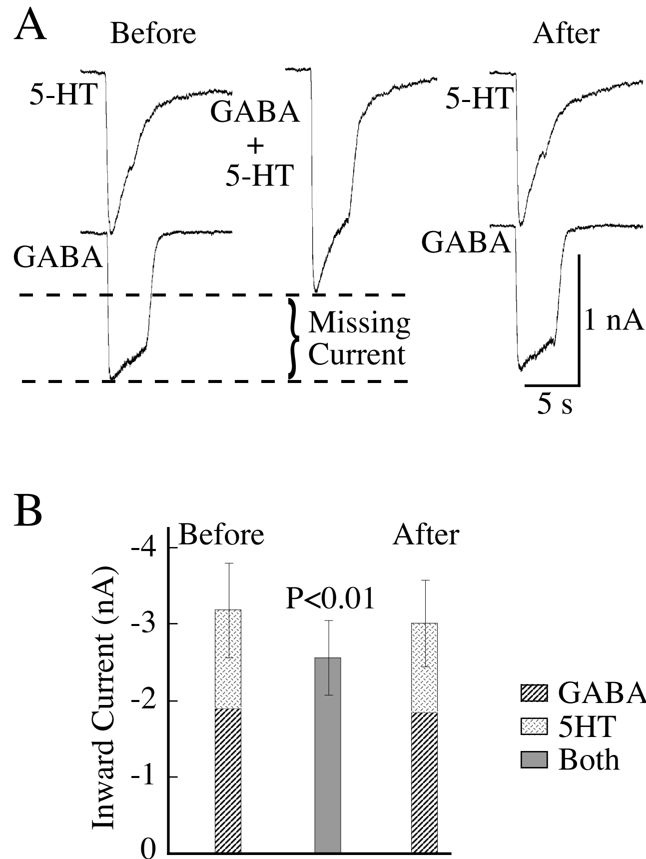

Figure 3. $I_{5\text{-}HT}$ and I_{GABA} in myenteric neurons are not additive revealing a current occlusion. A: whole-cell recordings from a neuron of a typical experiment. Currents were induced by application of either 5-HT (1 mM), GABA (300 μM) or by the simultaneous application of both agonist ($I_{5\text{-}HT+GABA}$). $I_{5\text{-}HT}$ and I_{GABA} were recorded five minutes before and five minutes after $I_{5\text{-}HT+GABA}$. B: the average (bars) values of seventeen experiments. The first and third bars show $I_{5\text{-}HT}$ and I_{GABA}; the sum of these individual currents represents expected current ($I_{expected} = I_{5\text{-}HT} + I_{GABA}$). S.E.M. are the lines on the top of the bars for $I_{expected}$ (first and last bars) and $I_{5\text{-}HT+GABA}$ (second bar). $I_{5\text{-}HT+GABA}$ is significantly lower than $I_{expected}$.

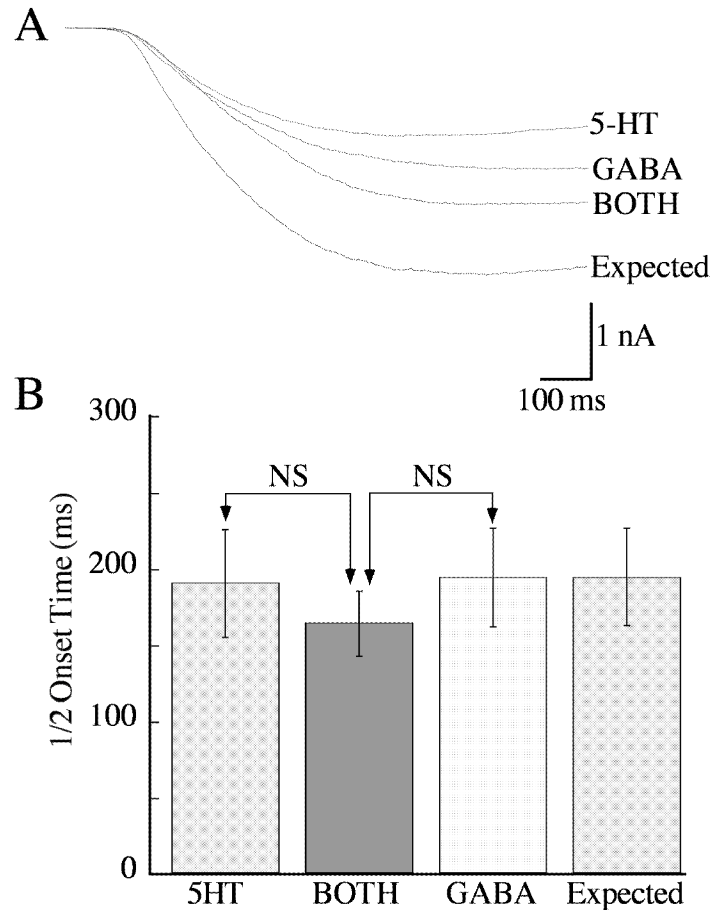

Figure 4. Average onset of the currents from myenteric neurons ($I_{5\text{-HT+GABA}}$) is smaller than $I_{expected}$. A: average of the onset of the currents recorded from myenteric neurons showing that $I_{5\text{-HT+GABA}}$ is smaller than $I_{expected}$ since the onset of these currents. The expected current shown in A is a graph representing the addition of $I_{5\text{-HT}}$ and I_{GABA}. B: the mean half-onset time of $I_{5\text{-HT+GABA}}$ was significantly different than that of $I_{5\text{-HT}}$ and I_{GABA}. Whole-cell currents were measured at a holding potential of –60 mV.

The amplitude of $I_{5\text{-}HT+GABA}$ was, since the beginning of the current, smaller than $I_{expected}$. This would indicate that the current occlusion occurs as soon as $GABA_A$ and 5-HT_3 receptors are activated.

Visual inspection of the currents induced by short lasting applications (see Fig. 3A) revealed that $I_{5\text{-}HT+GABA}$ desensitized faster than I_{GABA} but slightly slower than $I_{5\text{-}HT}$. To further investigate this, agonists were applied for long periods (90 s; *n*=7) and exponential function fits were performed using the data from the current peak to the "steady-state" (end of the agonist application; Fig. 5A). These fits were carried out using the Axograph software (Axon Instruments). As with $I_{5\text{-}HT}$, desensitization of $I_{5\text{-}HT+GABA}$ was better fitted by the sum of three exponential functions and the correspondent τ values of these two current were not different (Fig. 5B). However, the correspondent τ values of $I_{5\text{-}HT+GABA}$ and I_{GABA} were significantly different.

As mentioned before, currents decay to their control values after agonist removal from the external solution. Figure 6A depicts that $I_{5\text{-}HT+GABA}$ decay is quite different than those of either $I_{5\text{-}HT}$ or I_{GABA}. Indeed, the decay of $I_{5\text{-}HT+GABA}$ was best fitted by the sum of two exponential functions (n = 9) whereas the decay of individual currents was best fitted by a single exponential function. We tested the hypothesis that the first and the second exponential functions of $I_{5\text{-}HT+GABA}$ were indeed the exponentials of $I_{5\text{-}HT}$ and I_{GABA}. Thus, the average τ value of the first exponential (τ_1) was virtually the same as the τ_1 of I_{GABA} decay. The average τ value of the second exponential (τ_2) of $I_{5\text{-}HT+GABA}$ decay was also not different than τ_1 of $I_{5\text{-}HT}$ decay (Fig. 6B). Altogether, these results indicate that $I_{5\text{-}HT+GABA}$ are mediated by the opening of both 5-HT_3 and $GABA_A$ channels.

Application of both agonists desensitizes both 5-HT_3 and $GABA_A$ receptors

In the following experiments we measured the amplitude of both $I_{5\text{-}HT}$ and I_{GABA} before and immediately after (about 5 s) a long application (90 s) of 5-HT, GABA, or 5-HT+GABA. Typical recordings and the average data from such experiments are shown in Figures 7A-D. We observed that $GABA_A$ receptor desensitization with GABA significantly decrease I_{GABA} whereas this did not affect $I_{5\text{-}HT}$ (Fig. 7C).

Similarly, desensitization of 5-HT_3 receptors with 5-HT significantly decreased $I_{5\text{-}HT}$ yet did not affect I_{GABA} (Fig. 7D). In others words, no cross-desensitization was observed between 5-HT_3 and $GABA_A$ receptors. However, when receptors were desensitized by simultaneous application of 5-HT + GABA, both $I_{5\text{-}HT}$ and I_{GABA} were significantly decreased (Fig. 7A, 7B, and 7E). These observations couple with the fact that $I_{5\text{-}HT+GABA}$ kinetics is different than the kinetics of $I_{5\text{-}HT}$ or I_{GABA} (Fig. 5 and 6) support the hypothesis that $I_{5\text{-}HT+GABA}$ is carried through both 5-HT_3 and GABA channels.

Prereceptor mechanisms and technical artifacts as the origin for the current occlusion The following experiments rule out that current occlusion is due to prereceptor mechanisms. In four analyzed cells with no initial response to GABA (Fig. 8A and C), $I_{5\text{-}HT+GABA}$ (-1482 ± 779 pA) had the same amplitude and similar kinetics as $I_{5\text{-}HT}$ alone (-1275 ± 709 pA). Similarly, in another three cells with no initial response to 5-HT (Fig. 8 B and 8 C), $I_{5\text{-}HT+GABA}$ (-3291 ± 774 pA) had the same amplitude and similar kinetics as I_{GABA} alone (-3031 ± 907 pA).

Several observations rule out the possibility that current occlusion was due to a technical artifact of our recording system. First, current occlusion also occurred in seven neurons in which $I_{expected}$ was equal to or lower than –2646 pA (average amplitude of –1609 ± 260 pA). This average value is smaller than that of I_{GABA} alone in all tested neurons (-1888 ± 536 pA). In those seven neurons, $I_{GABA+5\text{-}HT}$ (-1346 ± 217 pA) was still significantly ($P < 0.05$; $n = 7$) lower than $I_{expected}$ (–1609 ± 260 pA). Second, our amplifier was capable of recording large and fast ionic currents in the same neurons and under exactly the same recording conditions. We were able to record voltage-activated Ca^{2+} and Na^+ inward currents as large as -8000 pA (unpublished observations; see also [Barajas-Lopez et al. 1996], which, in most of the observed cases, is larger than $I_{expected}$ (range -566 to –8459 pA). It is also important to highlight that the onset of voltage-activated Ca^{2+} and Na^+ currents is more rapid (few milliseconds) than that of I_{GABA} and $I_{5\text{-}HT}$ (hundreds of milliseconds). Finally, experiments in which lack of voltage clamp was noticed were disregarded.

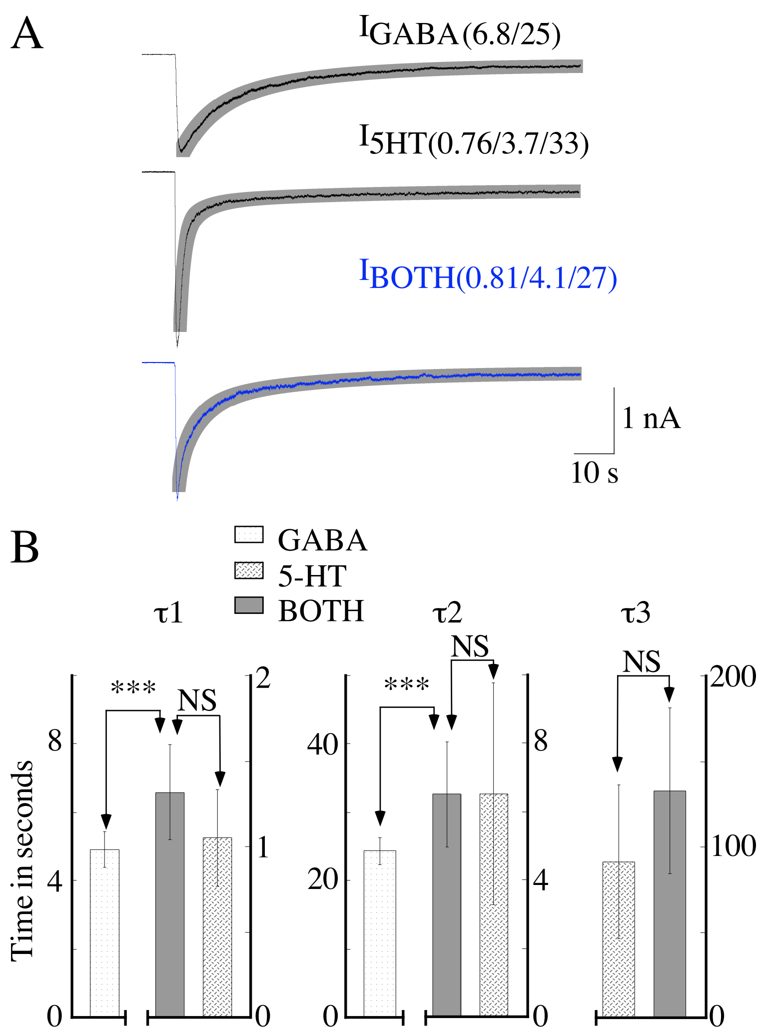

Figure 5. Desensitization kinetics of $I_{5\text{-}HT+GABA}$ cannot be explained by the desensitization kinetics of $I_{5\text{-}HT}$ or I_{GABA} alone. A: representative recordings from a myenteric neuron of $I_{5\text{-}HT}$, I_{GABA} and $I_{5\text{-}HT+GABA}$. The desensitization of $I_{5\text{-}HT+GABA}$ and $I_{5\text{-}HT}$ were best fitted by the sum of three exponential functions (thick gray lines), whereas I_{GABA} desensitization was best fitted by the sum of two exponential functions. Notice that $I_{5\text{-}HT+GABA}$ desensitizes faster that I_{GABA} but similarly than $I_{5\text{-}HT}$. B: bars in the lower graphs represent the mean and S.E.M. of the τ values of these exponential functions. The τ values of I_{GABA} exponentials were significantly (***, $P<0.001$) smaller than the correspondent τ values of $I_{5\text{-}HT+GABA}$The τ values of the three exponentials of $I_{5\text{-}HT}$ were not different (NS, not significant) than the correspondent τ values of $I_{5\text{-}HT+GABA}$ exponentials. Exponential fits were performed using the data from current peak to the "steady-state" component. In these experiments, the agonists were applied for approximately 90 seconds, and the holding potential was –60 mV.

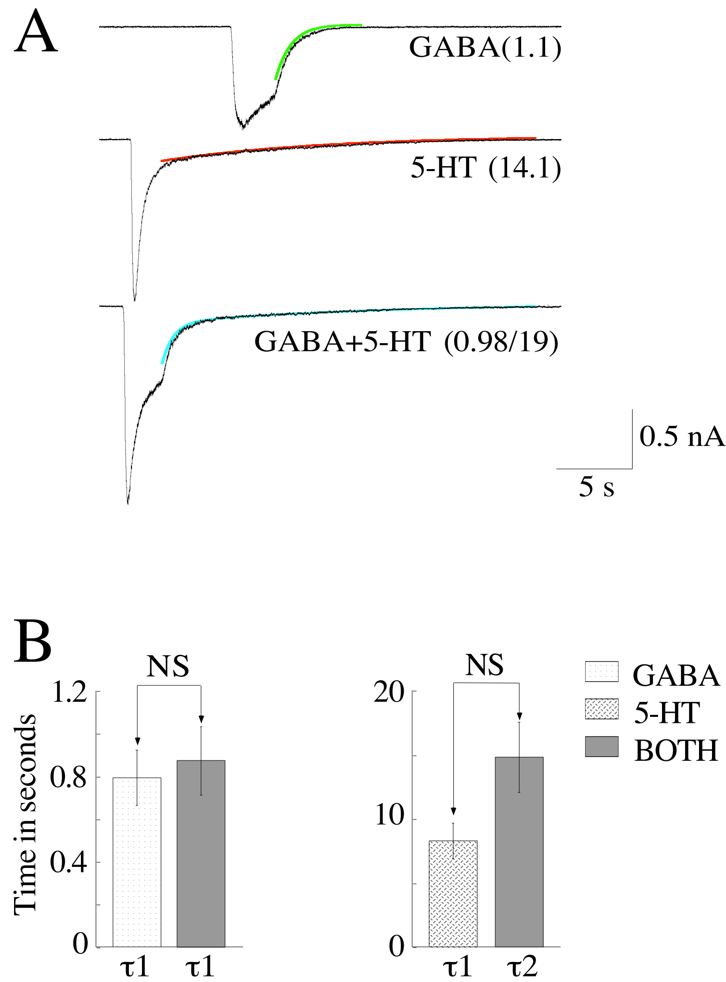

Figure 6. Decay kinetics of $I_{5\text{-}HT+GABA}$ is neither explained by the decay kinetics of $I_{5\text{-}HT}$ nor I_{GABA} alone. A: representative recordings from a myenteric neuron of $I_{5\text{-}HT}$, I_{GABA} and $I_{5\text{-}HT+GABA}$. The decay of $I_{5\text{-}HT+GABA}$ was best fitted by the sum of 2 exponential functions (thick grey line), whereas $I_{5\text{-}HT}$ and I_{GABA} desensitization were best fitted by a single exponential function. B: bars in these graphs represent the mean and S.E.M. of the τ value of the decay exponential functions. The τ value of I_{GABA} exponential was not significantly (NS) different than τ_1 of $I_{5\text{-}HT+GABA}$. The τ value of $I_{5\text{-}HT}$ exponential was also not significantly different that τ_2 of $I_{5\text{-}HT+GABA}$. Exponential fits were performed using the data from a couple hundred milliseconds after removing the agonists to the steady-state component. These experiments were carried out at a holding potential of –60 mV.

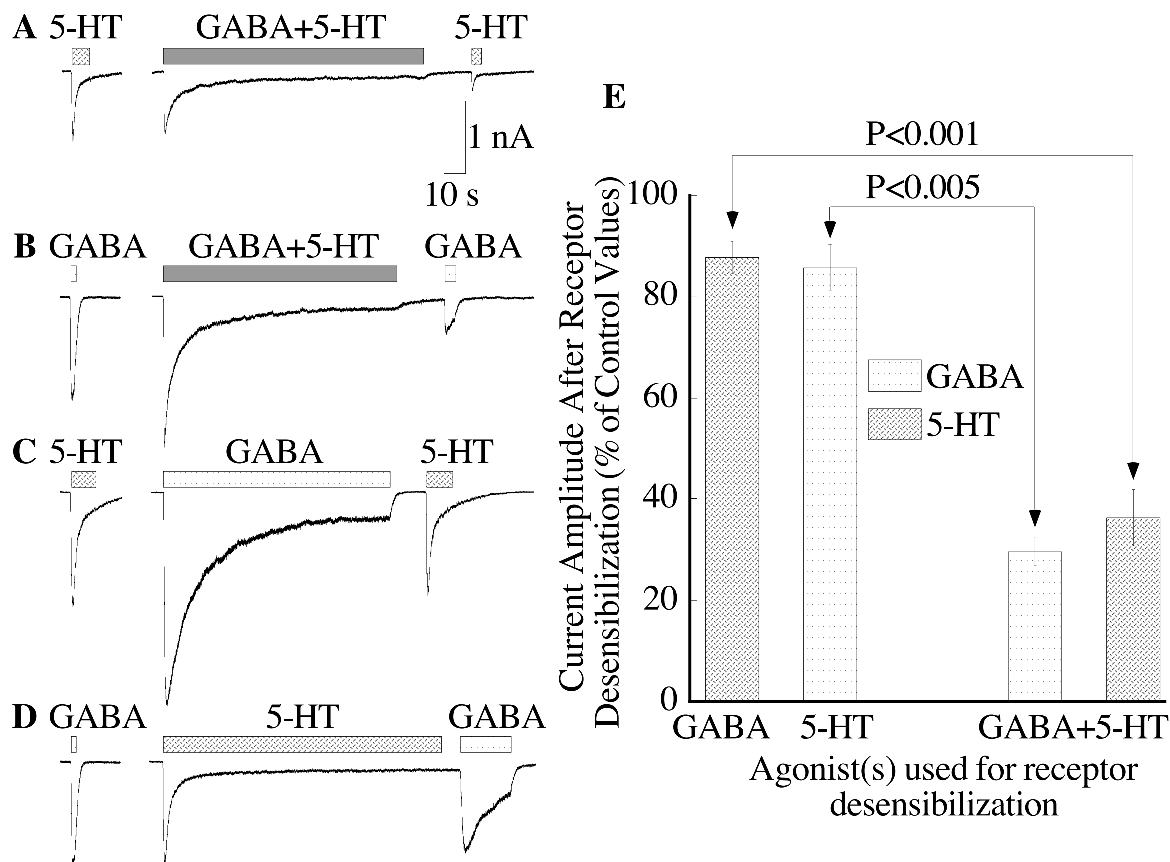

Figure 7. Simultaneous application of GABA and 5-TH desensitized both receptors, through a mechanism that does not involve cross-desensitization. A and B: simultaneous application of both agonists desensitized both receptor populations. C: $GABA_A$ receptor desensitization did not modify $I_{5\text{-}HT}$. D: similarly, desensitization of 5-HT_3 receptors did not modify I_{GABA}. Control $I_{5\text{-}HT}$ (C) and I_{GABA} (D) were recorded 5 minutes before (left) and approximately 5 s after a prolonged application of the other agonist. E: average amplitude of $I_{5\text{-}HT}$ and I_{GABA} recorded after the prolonged application of GABA, 5-HT, or 5-HT+GABA as a percentage of control response. Lines on top of the bars are S.E.M. Recordings were taken at the holding potential of –60 mV.

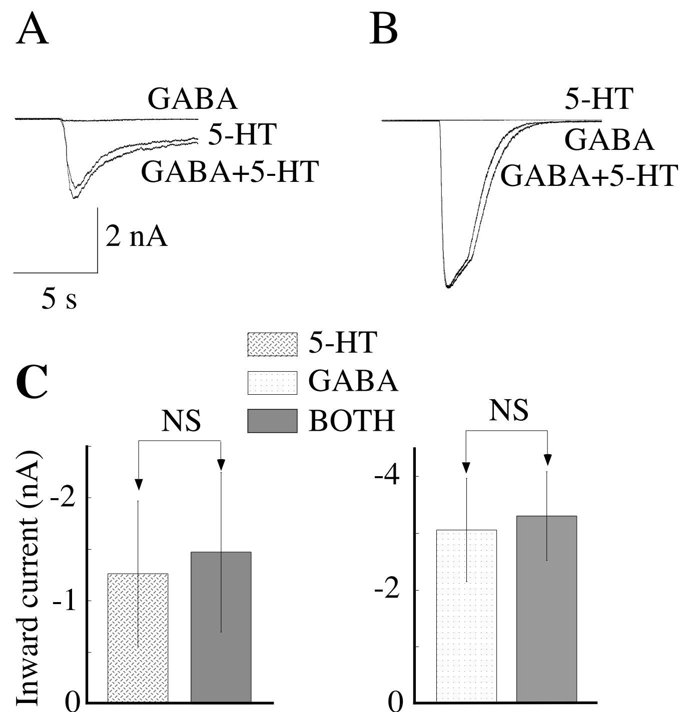

Figure 8. Inhibitory interactions between $5\text{-}HT_3$ and $GABA_A$ receptors require the presence of functional channels. A: recording from a neuron with a prominent $I_{5\text{-}HT}$ but with no response to GABA. Notice that GABA did not modify the $I_{5\text{-}HT}$ amplitude when both agonists were applied. B: similarly, 5-HT did not alter the I_{GABA} amplitude in neurons with no $I_{5\text{-}HT}$. C: mean and S.E.M. of four (left graph) and three (right graph) similar experiments.

Discussion

Our findings demonstrate that activation of $GABA_A$ and 5-HT receptors/channels is not functionally independent and that the ionic currents carried through them are occluded when both channel types are simultaneously activated. This current occlusion is observed as soon as currents are activated. Currents induced by the simultaneous application of both agonists are carried through both $GABA_A$ and 5-HT channels. Altogether, these observations suggests that this current occlusion is mediated by cross-inhibition and by a direct interaction between $GABA_A$ and 5-HT_3 receptors/channels. In general, similar interactions have been shown in the dorsal root ganglia neurons between: $GABA_A$ and P2X receptors [Sokolova et al. 2001], nACh and P2X receptors [Barajas-Lopez et al. 1998; Zhou and Galligan 1998; Khakh et al. 2000], 5-HT_3 and P2X receptors [Barajas-Lopez et al. 2002], and between dopamine (D2) and somatostatin (SSTR5) receptors [Rocheville et al. 2000], and between dopamine (D5) and $GABA_A$ channels [Liu et al. 2000]. Altogether, these findings indicate that receptor-protein interactions play an important role in neuronal signaling.

Activation of $GABA_A$ and 5-HT_3 receptors/channels is not independent

Despite these relatively high concentrations, the whole-cell currents induced by either GABA (1 mM or 300 μM) or 5-HT (1 mM) are mediated by pharmacologically distinct receptors. This is demonstrated by the specific inhibitory effect of picrotoxin (a $GABA_A$ channel blocker; [Bowery et al. 2002]) on I_{GABA}, and tropisetron (a 5-HT_3 channel blocker [Bowery et al. 2002]) on $I_{5\text{-}HT}$. The permeability of these channels in myenteric neurons is also different; $GABA_A$ channels are permeable to Cl^- [Cherubini and North 1985; Zhou and Galligan 2000] and 5-HT_3 channels are permeable to cations [Derkach et al. 1989]. Furthermore, the fact that the amplitudes of these two currents are independent from each other in the recorded neurons indicates that these ligand-gated channels are expressed independently in these neurons. These results agree with

the fact that $GABA_A$ and 5-HT_3 channels have different molecular structures [Hoyer et al. 1994; Bowery et al. 2002].

Activation of these channels is, however, not independent when they are simultaneously activated as shown by the fact that inward currents carried through $GABA_A$ and 5-HT_3 channels were not additive, revealing current occlusion. The unlikely possibility that current occlusion was due to interactions between 5-HT and GABA molecules, is ruled out by the following observations. First, in neurons with no initial response to 5-HT, $I_{GABA+5\text{-}HT}$ had virtually the same amplitude and kinetics as the current induced by GABA alone. Second, in cells with no initial response to GABA, $I_{GABA+5\text{-}HT}$ had virtually the same amplitude and kinetics as $I_{5\text{-}HT}$. These observations also rule out the possibility that GABA blocks 5-HT_3 channels or that 5-HT blocks $GABA_A$ channels, an effect that was demonstrated before for serotonin on nACh channels of submucosal neurons [Barajas-Lopez et al. 2001].

Currents induced by the simultaneous application of both agonists are carried through both $GABA_A$ and 5-HT_3 channels

At least four different observations indicate that $I_{GABA+5\text{-}HT}$ are carried through both $GABA_A$ and 5-HT_3 channels and not only through one population of these channels. **1**) $I_{GABA+5\text{-}HT}$ kinetics are clearly different than the kinetics of I_{GABA} or $I_{5\text{-}HT}$ alone. Thus, $I_{GABA+5\text{-}HT}$ desensitizes faster than I_{GABA} but similarly to $I_{5\text{-}HT}$. Furthermore, the decay of $I_{GABA+5\text{-}HT}$ resembles the decay of both, I_{GABA} and $I_{5\text{-}HT}$. **2**) When 5-HT + GABA are simultaneously applied, both $GABA_A$ and 5-HT_3 receptors are desensitized, whereas no cross-desensitization is observed when $GABA_A$ and 5-HT_3 receptors are individually desensitized. **4**) In experiments in which one of the currents was marginal as compared with the response induced by the other transmitter, $I_{GABA+5\text{-}HT}$ amplitude was the same as that of the current induced by the most effective neurotransmitter, and was significantly larger than the current induced by the weaker transmitter. These observations imply that $I_{GABA+5\text{-}HT}$ is carried through both $GABA_A$ and 5-HT_3 channels and that inhibition between these channels is reciprocal.

Cross-inhibition between $GABA_A$ and 5-HT_3 channels is mediated by a direct interaction between these receptors

Enteric neurons also express metabotropic 5-HT and GABA receptors, which are linked to G-proteins, second messengers and protein phosphorylation [Cherubini and North 1984; Pan et al. 1997; Barajas-Lopez et al. 2000]. At least one of our observations strongly indicates that activation of these receptors is not required for current occlusion. This occlusion occurs as soon as $GABA_A$ and 5-HT_3 channels are activated, indicating that it occurs as fast as the activation of these ligand-gated channels. However, in future experiments, we will further investigate the possibility that G-proteins, second messengers or protein phosphorylation might be involved in these channel interactions by testing the effects of G-protein (NEM or GDP-β-S) and protein kinase (with staurosporine+K-252+genistein) inhibitors on these interactions.

Functional implications for $GABA_A$ and 5-HT_3 channel interactions

Current experimental information suggests that inhibitory interactions between ligand-gated channels might be a widely used mechanism to limit the ionic currents through the cellular membrane. For example, functional interactions such as the one demonstrated here have been shown to exist between P2X and nicotinic channels in enteric neurons [Barajas-Lopez et al. 1998; Zhou and Galligan 1998], between P2X and 5-HT_3 receptors in enteric neurons [Barajas-Lopez et al. 2002] and between P2X and $GABA_A$ receptors in dorsal root ganglion neurons [Sokolova et al. 2001]. This does not appear to always be the case since, according to Zhou and Galligan [Zhou and Galligan 1998], P2X receptors do not interact with $GABA_A$ or 5-HT_3 receptors in myenteric neurons.

The important role of 5-HT and GABA as neurotransmitters [Sugita et al. 1992; Hoyer et al. 1994; Krantis 2000; Pan and Gershon 2000; Bowery et al. 2002] leads the hypothesis that the inhibitory interactions between their ionotropic receptors, shown here, might play an important modulator role in synaptic transmission. In the myenteric plexus, fast synaptic potentials mediated

by GABA and 5-HT have also been reported [Galligan et al. 2000] and therefore it is possible that inhibitory interactions between these channels might be important for the synaptic integration of this plexus.

In conclusion, our results indicate that there is a very fast inhibitory interaction between $GABA_A$ and 5-HT_3. This interaction occurs as fast as the activation of $GABA_A$ and 5-HT_3 channels, supporting the hypothesis that these receptors are located very close to each other in the neuronal membrane, perhaps forming functional units constituted by at least one channel of each type. The putative role of these interactions in synaptic transmission requires direct demonstration.

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