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La Apertura de los Canales Activados por Ligando no es Independiente

Tesis que presenta

Marcela Miranda Morales

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Director de la Tesis:

Dr. Carlos Barajas López

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A mi mamá Francis

A mi mamá Guille

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PÁGINA

Resumen

La apertura de los canales activados por ligando no es independiente

La interacciones inhibidoras entre los canales $GABA_A$ (activados por GABA) y P2X (activados por ATP) o 5-HT_3 (activados por serotonina) en neuronas mientéricas del intestino delgado del cobayo fueron caracterizadas utilizando registros de célula completa. Las corrientes inducidas por GABA (I_{GABA}) fueron inhibidas por picrotoxina o bicuculina, aquellas inducidas por ATP (I_{ATP}) fueron inhibidas por PPADS (un antagonista de los receptores P2X) y por una concentración alta de Ca^{2+}, mientras que, aquellas inducidas por serotonina ($I_{5\text{-}HT}$) fueron inhibidas por tropisetron. Las corrientes inducidas por GABA+ATP ($I_{GABA+ATP}$) o por GABA+5HT ($I_{GABA+5\text{-}HT}$) fueron menores que la suma de las corrientes individuales, revelando una oclusión de las corrientes. Esta oclusión requiere de la activación máxima de al menos uno de los receptores. Las características cinéticas y farmacológicas de $I_{GABA+ATP}$ o $I_{GABA+5\text{-}HT}$ indican que estas son mediadas por canales $GABA_A$ y P2X o por canales $GABA_A$ y 5-HT_3, respectivamente. El ATP (o la 5-HT) no afectó I_{GABA} en neuronas en las cuales: i) los canales P2X (o los 5-HT_3) no estaban presentes, ii) después de inhibir los canales P2X con Ca^{2+}, iii) en presencia del PPADS (o en presencia del tropisetron), iv) después de desensibilizar los receptores P2X (o los 5-HT_3), o v) en el potencial de inversión de la I_{ATP}. De manera similar, el GABA no afectó las corrientes mediadas por los canales P2X (o por los 5-HT_3) en neuronas en las cuales: i) los canales $GABA_A$ no estaban presentes, ii) en presencia de picrotoxina o bicuculina, antagonistas de los receptores $GABA_A$, iii) después de

desensibilizar los receptores $GABA_A$, o iv) en el potencial de inversión de la I_{GABA}. La oclusión de la corriente ocurrió tan rápido como la activación de los receptores. Estuvo presente en ausencia del Ca^{2+}, en experimentos a temperatura baja (11ºC), después de agregar a la solución de la pipeta varios inhibidores de las proteín-cinasas (estaurosporina, genisteina o K-252a), después de sustituir GTP por GDP-β-S en la pipeta, y después de tratar las células con *N*-etilmaleimida. Estos resultados son consistentes con un modelo de inhibición cruzada entre los receptores $GABA_A$ y P2X, y entre los receptores $GABA_A$ y 5-HT_3.

Palabras clave: sistema nervioso entérico, receptores $GABA_A$, cobayos, canales iónicos, receptores *Cys loop*, canales P2X, receptores 5-HT_3, ATP, serotonina, GABA, neurotransmisores, interacciones proteínicas, *patch clamp*, fijación de voltaje, cultivos neuronales, sinapsis

Abstract

Activation of ligand-gated channels is not independent

Inhibitory interactions between $GABA_A$ (activated by GABA) and P2X (activated by ATP) or 5-HT_3 (activated by serotonin) receptors of myenteric neurons from the guinea pig small intestine were characterized using whole-cell recordings. Currents induced by GABA (I_{GABA}) were inhibited by picrotoxin or bicuculline, those induced by ATP (I_{ATP}) were inhibited by PPADS (a P2X receptor antagonist) and a high concentration of Ca^{2+}, whereas those induced by serotonin ($I_{5\text{-}HT}$) were inhibited by tropisetron. Currents induced by GABA+ATP ($I_{GABA+ATP}$) or by GABA+5HT ($I_{GABA+5\text{-}HT}$) were smaller than the addition of the individual currents, revealing current occlusion. This occlusion requires maximal activation of at least one of these receptors. Kinetic and pharmacological properties of $I_{GABA+ATP}$ or $I_{GABA+5\text{-}HT}$ indicate that they are carried through both $GABA_A$ and P2X channels or $GABA_A$ and 5-HT channels, respectively. ATP (or 5-HT) did not affect I_{GABA} in neurons in which: i) P2X (or 5-HT_3) channels were not present, ii) after inhibiting P2X channels with Ca^{2+}, iii) in the presence of PPADS (or in the presence of tropisetron), iv) after P2X (or 5-HT_3) receptor desensitisation, or v) at I_{ATP} reversal potential. Similarly, GABA did not affect P2X (or 5-HT_3) mediated currents in neurons in which: i) $GABA_A$ channels were not present, ii) in the presence of picrotoxin or bicuculline, $GABA_A$ receptor antagonists, iii) after $GABA_A$ receptor desensitisation, or iv) at the I_{GABA} reversal potential. Current occlusion occurred as fast as current activation. It was still present in the absence of Ca^{2+}, when

experiments were performed at low temperature (11ºC), after adding to the pipette solution a cocktail of protein kinase inhibitors (staurosporine, genistein or K-252a), after substituting the GTP in the pipette with GDP-β-S, and after treating the cells with *N*-ethylmaleimide. These results are consistent with a model of cross-inhibition between $GABA_A$ and P2X and between $GABA_A$ and $5\text{-}HT_3$ receptors.

Keywords: enteric nervous system, $GABA_A$ channels, guinea pig, ion channels, *Cys loop* receptors, P2X channels, $5\text{-}HT_3$ receptors, ATP, serotonine, GABA, neurotransmitters, protein interactions, patch clamp, voltage clamp, neuronal cultures, synapse

Capítulo 1

Antecedentes generales

1.1 Introducción General

La habilidad de las neuronas de responder a cambios en el medio ambiente es llevada a cabo a través de la expresión en la superficie celular de un repertorio de receptores específicos. Estos receptores por lo tanto transducen las señales del medio ambiente y generan respuestas químicas o eléctricas apropiadas a través de rutas de señalización membranal e intracelular. La transmisión sináptica química depende de la conversión de una señal química a una eléctrica que se manifiesta por cambios en el potencial de membrana a nivel postsináptico. Este tipo de procesos es frecuentemente mediado por un tipo de proteínas receptoras presentes en la membrana neuronal las cuales contienen un canal iónico por lo que se les conoce como receptores ionotrópicos o receptores activados por ligando (LGIC; por sus siglas en inglés).

Los LGIC son transmembranales y la apertura de su poro iónico se realiza mediante un cambio conformacional cuando se une el ligando extracelular. La apertura de estos canales permite un cambio selectivo en la permeabilidad de membrana a iones específicos lo cual altera el potencial de membrana de una célula blanco. El flujo iónico a través de estos canales genera corrientes eléctricas que pueden ser registradas con precisión mediante las técnicas de fijación de voltaje y las de *Patch clamp*.

El concepto de interacciones directas entre LGICs es relativamente nuevo y es actualmente objeto de estudio de varios laboratorios. El hallazgo de Nakazawa (Nakazawa, 1994) de que las corrientes inducidas por ATP (mediadas por la

activación de receptores P2X) y por ACh (mediada por la activacion de receptores nACh) no se suman fue interpretada como evidencia de que dos receptores distintos comparten un mismo canal. El trabajo de Nakazawa fue fundamental, y a lo largo de estos años han surgido varios estudios que describen este fenómeno, incluyendo interacciones entre otros receptores y en diferentes tipos neuronales, inclusive entre miembros de la superfamilia de receptores *Cys-loop*. A continuación proporciono un resumen de las propiedades más importantes de las interacciones entre diversos LGICs. En el capítulos 2 y 3 se describe la evidencia experimental que demuestra la existencia de una interacción inhibitoria entre los canales P2X y $GABA_A$ y los entre canales 5-HT_3 y los canales $GABA_A$ respectivamente, en neuronas del plexo mientérico.

1.2 Revisión Bibliográfica

1.2.1 Comunicación sináptica

El sitio donde una neurona se comunica con otra célula se conoce como **sinapsis** (Kandel & Siegelbaum, 2000). Dos elementos pueden ser identificados en todas las sinapsis, el presináptico y el postsináptico. Las sinapsis se pueden clasificar en dos tipos, de acuerdo al mecanismo que se utiliza para establecer la comunicación. Las sinapsis químicas utilizan substancias llamadas neurotransmisores, las cuales son liberadas del elemento presináptico y activan proteínas receptoras en el postsináptico. En este tipo de sinapsis los dos elementos están separados por un pequeño espacio llamado hendidura sináptica. El segundo tipo de sinapsis se les conoce como eléctricas debido a que la

comunicación se establece a través de canales intercelulares (uniones comunicantes) que se caracterizan por una baja resistencia eléctrica (Fig 1.1).

Durante ambos tipos de comunicación sináptica se modulan el potencial de membrana en reposo del elemento postsináptico. En la transmisión química, estos cambios de potencial suceden por la activación de receptores que modifican el flujo iónico a través de la membrana plasmática. La modulación de estos flujos iónicos se realiza mediante receptores asociados a proteínas G o receptores directamente asociados a canales iónicos. En el primer ejemplo, las proteínas G activadas pueden modular directamente canales iónicos alterando el potencial de membrana o indirectamente a través de la activación de enzimas y segundos mensajeros (Kandel & Siegelbaum, 2000). En el segundo ejemplo, el receptor y el canal son parte de la misma proteína y el canal se abre cuando el neurotransmisor se une a su receptor específico. Estos receptores son conocidos como ionotrópicos o bien como canales activados por ligando (LGIC; por sus siglas en inglés) (Kandel & Siegelbaum, 2000).

1.2.2 Los canales activados por ligando

Los LGIC son proteínas constituídas por varias subunidades (unidas por uniones no-covalentes), las cuales forman un poro central acuoso. Los sitios receptores están presentes en los dominios extracelulares. Cuando el neurotrasmisor se une a su receptor, la proteína cambia su conformación y pasa a un estado en el que el poro acuoso es permeable a iones. Estos canales tienen una selectividad iónica específica, esto es, son permeables sólo a un tipo de iones. El movimiento iónico genera una

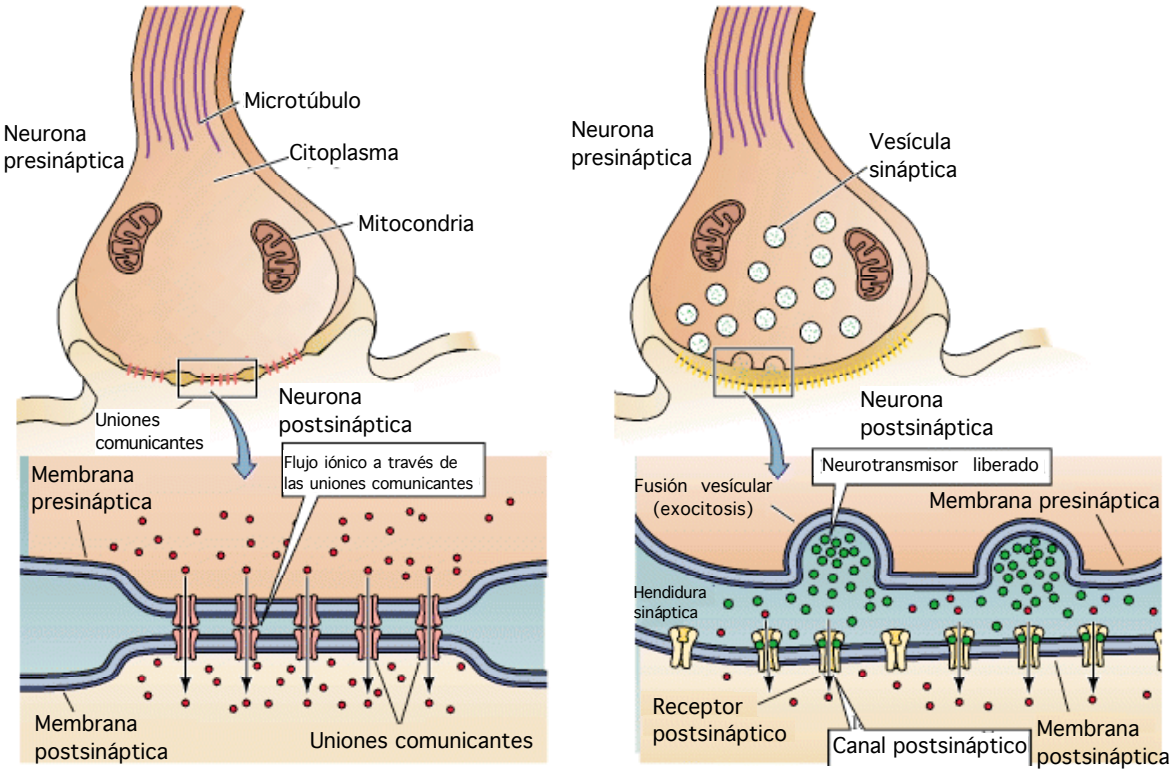


Figura 1.1 Las sinapsis eléctrica y química difieren en sus mecanismos de transmisión. (A) En las sinapsis eléctricas, las uniones comunicantes entre las membranas pre y postmembranal permiten el flujo pasivo a través de canales intercelulares. Este flujo de corriente cambia el potencial postsináptico membranal, iniciando (o en algunos casos inhibiendo) la generación de potenciales de acción a nivel postsináptico. (B) En las sinapsis químicas no hay continuidad intercelular, y por lo tanto, no hay flujo directo de corriente de la célula pre a la postsináptica. La corriente fluye a través de la membrana postsináptica sólo en respuesta a la secreción de neurotransmisores la cual abre o cierra canales iónicos postsinápticos después de la unión de las moléculas al receptor [modificada de (Purves *et al.*, 2001)].

corriente de una magnitud proporcional al número de canales abiertos y a la corriente unitaria de cada canal. La dirección de la corriente depende del tipo de iones y del sentido del flujo hacia el citoplasma o hacia el exterior de la membrana. Por convención, el movimiento neto de cargas positivas hacia el interior se le llama corriente entrante y el movimiento neto de cargas positivas hacia el exterior se conoce como corriente saliente. Los LGIC pueden existir en tres estados principales, normalmente están cerrados en ausencia de ligando, y cuando éste se une a su sitio receptor le tomará ~0.02 ms para pasar al estado abierto, y cuando el ligando se disocia el canal pasará al estado cerrado nuevamente. Otro estado de estos canales se observa en presencia contínua de concentraciones relativamente altas del ligando y se conoce como desensibilizado. En este estado los LGIC no se abren a pesar de que el ligando este unido a su sitio receptor. Un canal desensibilizado sólo puede ser reactivado hasta que el ligando se disocie de su sitio y regrese a su estado cerrado (Barry & Lynch, 2005).

Los LGIC median transimisión rápida en el sistema nervioso central, periférico y en la unión neuromuscular, su principal función es convertir una señal química (unión del neurotransmisor) a una señal eléctrica. Los LGIC pueden ser divididos en cuatro superfamilias, la superfamilia *Cys-loop*, los receptores a glutamato [(NMDA (N-metil-D-aspartato), AMPA (ácido α-amino-3-hidroy-5-metil-D-aspartato) y kainato], los canales TRP (receptores de potencial transitorio) y los canales (P2X) activados por ATP (trifosfato de adenosina).

La configuración de *Patch clamp* que se utiliza con mayor frecuencia para estudiar los LGIC es la de célula completa (*Whole cell*) y fue utilizada en los estudios reportados en los capítulos 2 y 3. Esta se logra después de obtener un gigasello (sello eléctrico de al menos un GΩ) entre la pipeta de cristal (electrodo) y los lípidos de la membrana celular (configuración de célula pegada). Después de obtener la configuración de célula pegada (*Cell attached*), el interior de la pipeta se conecta con el citoplasma de la célula rompiendo la membrana que inicialmente separa a estos dos componentes [veáse (Purves et al., 2001)]. Obteniendo así, la configuración de célula completa. La Figura 1.2 es un registro de una corriente típica obtenida con esta configuración. Se puede observar una corriente entrante inmediatamente después de la aplicación de GABA, la cual alcanza su máximo (pico) y se inactiva a pesar de la presencia del agonista. La corriente regresa a sus valores iniciales (Caída) después de retirar el GABA.

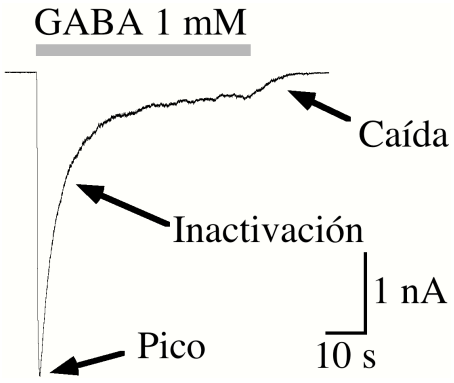


Figura 1.2 Registro típico de una corriente entrante inducida por GABA. La aplicación del GABA es indicada por la barra. El registro fue realizado utilizando la configuración de célula completa en una neurona mientérica del intestino de cobayo. El potencial fue fijado a -60 mV durante este registro.

1.2.2a Los receptores de la superfamilia *Cys-loop*

Los miembros de la superfamilia *Cys-loop* son diversos y su activación es responsable de potentiales sinápticos excitatorios e inhibitorios dependiendo de si su permeabilidad es a cationes o a aniones. Los receptores catiónicos [receptores activados por acetilcolina (nAChRs), y los 5-HT_3 (5-

hidroxitriptamina)] y aniónicos [GABA (Ácido γ-aminobutírico; que incluyen sólo los subtipos $GABA_A$ y $GABA_C$)], y los receptores a glicina (Connolly & Wafford, 2004).

Los receptores de la superfamilia *Cys-loop* poseen estructura similar. Los canales funcionales están formados por cinco subunidades. Cada subunidad está constituída por dominio amino terminal extracelular, cuatro dominios transmembranales (M1-M4) y un dominio carboxilo terminal extracelular. También tienen un dominio largo intracelular entre M3 y M4 (Jensen *et al.*, 2005), el cual ha sido implicado en una gran variedad de funciones del receptor, incluyendo facilitación de la interacción entre el receptor y la matriz intracelular.

1.2.2b Los receptores gabaérgicos

Los receptores $GABA_A$ miembros de la superfamilia *Cys-loop* son selectivamente permeables a iones cloruro, por lo que en la mayor parte de las situaciones celulares actúa inhibiendo la transmisión sináptica (Bormann, 2000; Kullmann *et al.*, 2005). A la fecha se conocen 19 subunidades del receptor $GABA_A$ (α_{1-6}, β_{1-3}, γ_{1-3}, δ y ε, π, θ, ρ_{1-3}). La mayoría de los receptores $GABA_A$ están compuestos por dos subunidades α, dos subunidades β y una γ, mientras que sólo la subunidad ρ puede formar canales homoméricos clasificados como receptores $GABA_C$ (Jensen *et al.*, 2005), encontrados principalmente en la retina del ojo (Bormann, 2000).

Cada uno de los receptores muestra diferentes afinidades de unión a neuromoduladores basados en la composición de sus subunidades. Por ejemplo la modulación de los canales $GABA_A$ por benzodiacepinas es dependiente de la subunidad γ y la ausencia de esta subunidad previene la potenciación de la

corriente gabaérgica inducida por diacepam (Jentsch *et al.*, 2002). Además, varias subunidades contienen sitios de fosforilación para proteín-cinasa A, proteín-cinasa-C y tirosín-cinasa y el alto grado de fosforilación es dependiente de la composición de subunidades del receptor. En general la fosforilación por PKA y PKC parece inhibir la función del receptor, mientras que la fosforilación por tirosin-cinasa la aumenta (Swope *et al.*, 1999).

Así como con otros miembros de la superfamilia *Cys-loop*, la localización específica de los receptores $GABA_A$ en la membrana celular es dependiente de la presencia de varios componentes citoesqueléticos. Ejemplos de estos componentes incluyen las proteínas asociadas al receptor $GABA_A$ (GABARAP) y las proteínas asociadas a microtúbulos (MAP) como la MAP1-B, la cual esta asociada con los receptores $GABA_A$ y $GABA_c$, respectivamente (Sheng & Pak, 2000).

1.2.2c Los receptores serotoninérgicos

Estos receptores fueron clasificados en base a las señales de transducción a las que se asocian y a su estructura (secuencia primaria de aminoácidos). A la fecha incluyen siete clases de receptores ($5\text{-}HT_1$ – $5\text{-}HT_7$), y cada una de ellas tiene una subclase (A, B, etc.). El receptor $5\text{-}HT_3$ pertenece a la superfamilia *Cys-loop*, por lo que es funcionalmente y estructuralmente distinto a las otras seis clases de receptores serotoninérgicos, los cuales están acoplados a proteínas G y median respuestas lentas a través de rutas de señalización por segundos mensajeros. Se han identificado cinco subunidades de los receptores $5\text{-}HT_3$ ($5\text{-}HT_{3A\text{-}E}$), aunque sólo se conocen las secuencia de aminoácidos de tres subunidades ($5\text{-}HT_{3A\text{-}c}$). Ha sido de mucho interés también la variante corta llamada $5\text{-}HT_{As}$, presente en

células NCB-20 (células híbridas de neuroblastoma de ratón y células embrionarias de cerebro de hámster chino), células HN9.10e (células hibridas de neuroblastoma de ratón e hipocampo), ganglios nodulares de rata e intestino delgado de ratón (Reeves & Lummis, 2002).

Ha sido demostrado que la subunidad 5-HT_{3A} forma canales homoméricos funcionales, mientras que la subunidad 5-HT_{3B}, necesita ser coexpresada con la subunidad 5-HT_{3A} para formar canales funcionales (Jensen *et al.*, 2005).

En secuencias descritas (cobayo y humano) de la subunidad 5-HT_{3A} se han encontrado secuencias consenso de glicosilación en el dominio extracelular, esta glicosilación podría jugar un papel en el ensamble del receptor. También existe un número de sitios consenso de fosforilación y por lo menos uno (Ser^{414}) parece ser fosforilado in vivo. La fosforilación del receptor parece tener un papel en los niveles de conductancia y desensibilización del receptor $5HT_3$ (Reeves & Lummis, 2002).

Los receptores $5HT_3$ son modulados por una gran variedad de sustancias incluyendo cationes divalentes, alcoholes, esteroides, anestésicos, etc. Sin embargo el conocimiento de su mecanismo de acción es limitado. Por ejemplo iones calcio actúan inhibiendo al receptor probablemente actúando en el sitio de unión al ligando. El receptor $5HT_3$ también es blanco principal de algunos anestésicos locales como halotano o isoflurano potenciando respuestas mediadas por este receptor (Reeves & Lummis, 2002).

1.2.2d Los receptores purinérgicos

Es la segunda familia de receptores importantes para este estudio. En la década de los 1970's fue propuesto el ATP como neurotransmisor y desde entonces muchos estudios han tratado de precisar el papel del ATP en la transmisión sináptica (Burnstock, 2006). Ha sido observado que el ATP y sus análogos activan receptores purinérgicos que son divididos en dos categorias: P1 y P2. Los receptores P1 son primariamente estimulados por adenosina, mientras que los P2 son activados por ATP y ADP (difosfato de adenosina). La categoria P2 es además subdividida en dos clases de receptores llamados P2X y P2Y (Abbracchio & Burnstock, 1994; Burnstock, 2006). La clase P2Y es metabotrópica, mientras que la P2X es ionotrópica (Burnstock, 2006) y es el receptor purinérgico involucrado en los estudios descritos en esta tesis.

Inicialmente se pensaba que el canal P2X tenia la misma estructura de la superfamilia de receptores *Cys-loop*, sin embargo después de un análisis estructural fue descubierto que hay diferencias sustanciales entre estas dos familias de receptores. Primero, las subunidades de la familia de receptores P2X tienen sólo dos dominios transmembranales (M1 y M2) con un largo dominio extracelular en forma de asa entre ellos, haciendo que ambos dominios, el amino y carboxilo terminal, sean intracelulares. El modelo más aceptado de la conformación de estos canales es el que considera que los canales funcionales están compuestos por tres subunidades que están organizadas como homotrímeros, y en algunos casos como heterotrímeros conteniendo dos subunidades diferentes también (Khakh, 2001; North, 2002).

A la fecha, han sido clonadas siete subunidades del receptor P2X ($P2X_{1-7}$). De éstas, todas excepto $P2X_6$ son capaces de formar canales homoméricos (North & Surprenant, 2000). También han sido descubiertos cuatro canales heteromericos que incluyen receptores $P2X_{2/3}$ $P2X_{4/6}$ $P2X_{1/5}$ y $P2X_{2/6}$ (Robertson *et al.*, 2001; North, 2002). En el sistema nervioso cada una de estas subunidades muestra caracteristicas cinéticas y de compartimentación distintas (Khakh, 2001; North, 2002). Por ejemplo, los receptores $P2X_3$ son dominantes en neuronas sensoriales incluyendo las del ganglio de la raiz dorsal (DRG) (Chen *et al.*, 1995; Lewis *et al.*, 1995), $P2X_2$, $P2X_4$ y $P2X_6$ están presentes en el cerebelo (Rubio & Soto, 2001) y $P2X_2$ y $P2X_3$ son las subunidades más abundantes en el sistema nervioso entérico (Xiang & Burnstock, 2004)(SNE).

Aunque los receptores P2X han sido encontrados en la membrana celular formando grupos, el mecanismo mediante el cual esto ocurre no ha sido todavía elucidado (Rubio & Soto, 2001). No han sido identificados dominios andamiaje en las secuencias del receptor P2X, haciendo poco probable que el receptor esté anclado a la célula por las ya identificadas MAP's (Robertson *et al.*, 2001). De tal manera que se requiere estudios adicionales acerca del anclaje de estos canales a la célula.

De los dos dominios transmembranales que constituyen el canal, el segundo dominio transmembranal de la subunidad P2X forma el poro del canal, el cual es permeable a diferentes cationes incluyendo sodio, potasio, y calcio (Khakh, 2001). La permeabilidad a calcio es importante porque permite a los receptores P2X modular diversas actividades intracelulares, por ejemplo la fosforilación. Diversos estudios han mostrado que existen sitios de fosforilación para PKA y

PKC presentes en el amino y carboxilo terminales de los receptores P2X que afectan su cinética de desensibilización. La fosforilación de los receptores P2X a través de PKC disminuye el nivel de desensibilización del receptor (Boue-Grabot *et al.*, 2000), mientras la fosforilación por PKA incrementa su velocidad de desensibilización (Chow & Wang, 1998). La modulación de los receptores P2X por fosforilación de proteínas puede utilizar los iones calcio que están presentes como resultado del flujo iónico a través de poro del canal, y podría ayudar a regular la actividad del canal (Burnashev, 1998).

1.2.3 Interacciones entre LGIC

El estudio de la interacción entre LGIC puede darnos herramientas para entender mejor el sistema nervioso. A pesar de que la neurotransmisión involucra la activación específica de receptores-canal por distintos neurotransmisores, diferentes clases de receptor pueden estar colocalizados en el mismo sitio sináptico y pueden ser activados por liberación de más de un tipo de neurotransmisor de la misma terminal sináptica, por ejemplo ATP y noradrenalina (Burnstock, 2004, 2006). Recientemente nuestro laboratorio y otros han demostrado que la activación simultánea de diferentes receptores postisinápticos por coaplicación de sus neurotransmisores específicos induce una modulación cruzada de sus propiedades de activación (Nakazawa, 1994; Barajas-López *et al.*, 1998; Zhou & Galligan, 1998).

Uno de los trabajos pioneros en las interacciones de los LGIC fué realizado por Nakazawa (Nakazawa, 1994), en el que demostró que la aplicación simultánea de ATP y ACh en neuronas simpáticas de rata generan una corriente de respuesta no-aditiva, en donde la corriente combinada es menor que la suma

de las dos corrientes componentes. Para explicar esta observación propuso la presencia de un solo canal que podría ser activado por ATP y ACh. Sin embargo nuestro laboratorio y otros han demostrado que la interacción entre los canales P2X y nAChRs no puede ser explicada por la presencia de un solo canal que es activado por dos agonistas. Así, ha sido demostrado que la respuesta combinada reducida es resultado de una modulación negativa de dos distintas poblaciones de canales en la membrana neuronal (Barajas-López *et al.*, 1998; Zhou & Galligan, 1998).

Registros generados de cultivos primarios de neuronas submucosas de cobayo verificaron los hallazgos de Nakazawa, en los que la aplicación simultánea de ACh y ATP genera una corriente menor a la suma de las dos corrientes componentes. Sin embargo en este estudio también se encontró que la adición de antagonistas llamados hexametonio y PPADS (ácido piridoxalfosfao-6-azofenil-2',4'-disulfonico) a las neuronas antes de administrar los agonistas, genera corrientes que corresponden a la administración de ATP o ACh respectivamente. Además estos estudios demuestran que no hay desensibilización cruzada entre estos dos canales. Estas observaciones sugieren la presencia de dos canales separados en la membrana neuronal. Además de un análisis cinético de las corrientes generadas por estos canales mostraron que la interacción entre estos dos canales ocurre rápidamente (en milisegundos) después de la aplicación simultánea de ambos transmisores. Fosforilación de proteínas o activación de proteínas G tampoco son requeridas para que esta interacción ocurra (Barajas-López *et al.*, 1998).

Todos estos resultados llevaron a formular un modelo para explicar la interacción, en el que estos dos canales están muy cercanos en la membrana formando grupos de por lo menos un canal de cada tipo (Fig 1.3). A favor de esta propuesta, un trabajo mediante fluorescencia por transferencia de energia por resonancia (FRET) y microscopía de fluorescencia por reflexión interna total (TIRF) encontró que los canales $P2X_2$ y nicotínicos $\alpha_4\beta_2$ parecen formar complejos a ~80 Å de distancia (Khakh *et al.*, 2005).

Conjuntamente estos hallazgos sugieren que la modulación negativa ocurre por una interacción directa entre los canales P2X y nAChRs. Dadas las similitudes funcionales entre los LGIC, surgieron otros trabajos que demostraron la existencia de este tipo de interacciones entre $5HT_3$ y P2X en neuronas mientéricas (Barajas-López *et al.*, 2002:Boue-Grabot, 2003 #28), $GABA_A$ y P2X en neuronas del ganglio de la raíz dorsal (Sokolova *et al.*, 2001). En un estudio más reciente llevado a cabo en ovocitos de rana fue encontrado que el dominio carboxilo terminal de los receptores $P2X_2$ y el dominio de las subunidades $GABA_A$ son requeridos para la interacción entre los receptores activados por ATP y GABA (Boue-Grabot *et al.*, 2004b).

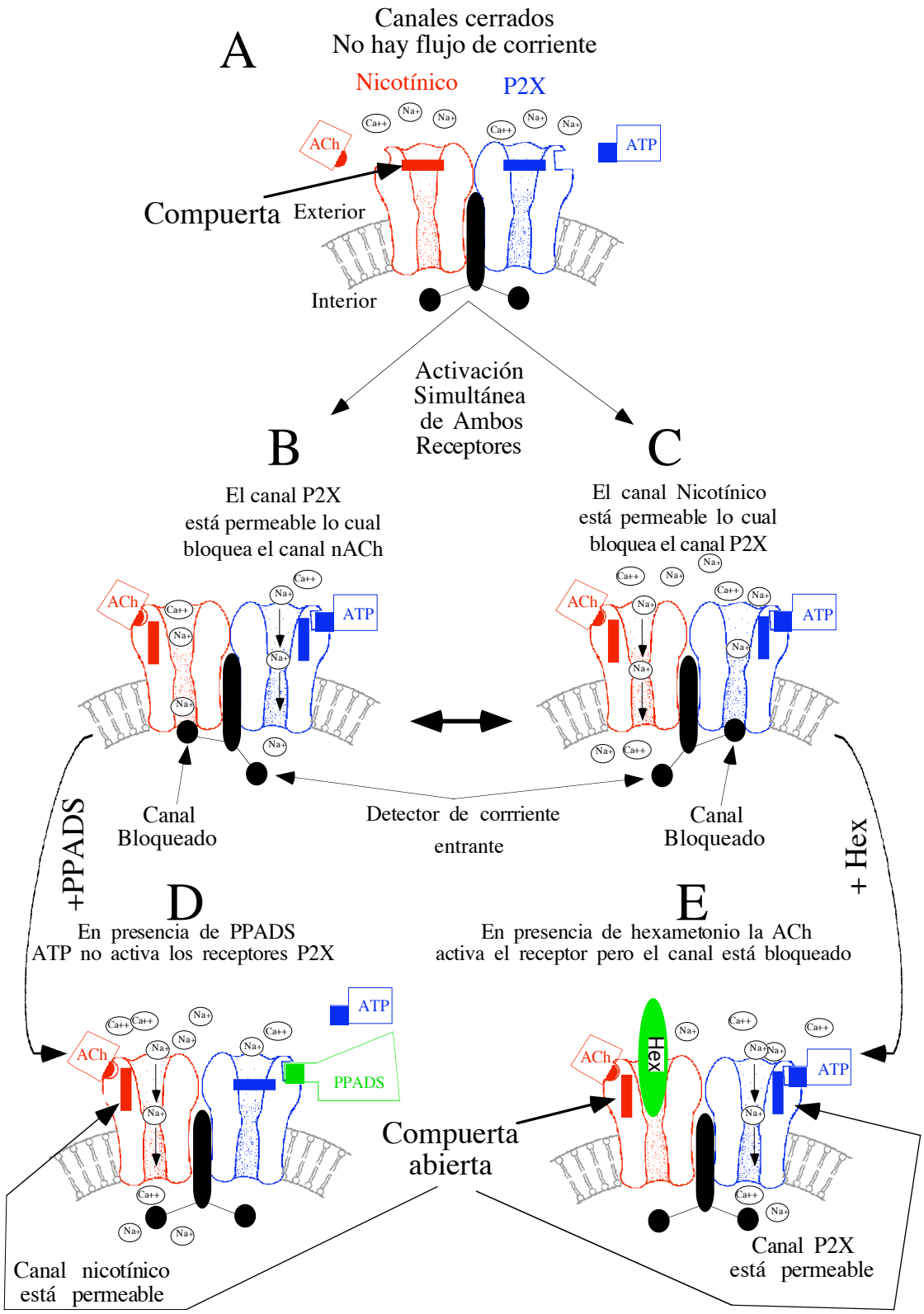
En aparente contradicción con estos estudios, en neuronas mientéricas los canales P2X interactuaban específicamente con los receptores nAChRs y no con otros miembros de la superfamilia *Cys-loop* presentes en estas neuronas (receptores $GABA_A$ y $5HT_3$; (Zhou & Galligan, 1998)). Sin embargo, un estudio reciente demostró la existencia de una interacción inhibitoria entre los receptores P2X y los $GABA_A$ en neuronas mientéricas de cobayo (Karanjia *et al.*, 2006).

Estas discrepancias podrían implicar que estas interacciones son tejido-específicas, dada la heterogeneidad de receptores.

Conjugando todos los hallazgos anteriores que demuestran interacciones entre los receptores P2X y miembros de la superfamilia *Cys-loop*, y dada la similtud funcional entre ellos, fue posible especular que miembros de la superfamilia de receptores *Cys-loop* puedan interactuar entre ellos. Se conoce a la fecha sólo un trabajo que demuestra una interacción inhibitoria asimétrica entre los receptores glicinérgicos y receptores $GABA_A$ en neuronas de la cresta dorsal en rata; esta interacción es independiente de la concentración intracelular de iones Ca^{2+}, potencial de membrana y cambios en el potencial de equilibrio del Cl^- (Li *et al.*, 2003). Se ha estudiado la interacción funcional entre los receptores $5HT_3$ y los nAChRs, y se encontró que esta inteacción es diferente, ya que moléculas de serotonina bloquean las corrientes nicotínicas en varios tipos celulares incluyendo neuronas submucosas (García-Colunga & Miledi, 1995; Barajas-López *et al.*, 2001).

Además de este tipo de interacciones entre miembros de la familia LGIC, también han sido reportadas interacciones entre estos y algunos receptores metabotropicos. Por ejemplo, entre receptores a dopamina (D2) y receptores a

Figura 1.3 Hipótesis de trabajo de las interacciones inhibitorias entre canales. **A**, los canales están organizados en unidades funcionales las cuales poseen al menos un receptor canal de cada tipo. Cuando los receptores son simultáneamente activados, únicamente uno de los dos canales de una unidad dada se abre en un tiempo dado, como se muestra en **B** y **C**. **B**, cuando el influjo iónico es detectado en el canal P2X, este induce el bloqueo del canal nicotínico, a través de un mecanismo que podría involucrar las terminales carboxílicas de los canales P2X. **C**, cuando el influjo iónico se detecta en el canal nicotínico esto bloquea los canales P2X. La unidad funcional podría cambiar del estado **A** al **B** o viceversa. Diferentes unidades funcionales de una célula dada podrían estar en el estado **A** o **B** de manera aleatoria. Este modelo está basado en el hecho de que la cinética de las corrientes inducidas por la aplicación simultánea de los dos agonistas no puede ser explicada por la cinética de las corrientes individuales, y porque en la presencia de PPADS (un antagonista de los receptores P2X) $I_{ACh+ATP}$ es muy similar a I_{ACh} y en la presencia de hexametonio (bloqueador de los canales nicotínicos) $I_{ACh+ATP}$ es muy similar a I_{ATP} sola (no mostrado).


A
Canales cerrados
No hay flujo de corriente
Nicotínico
P2X
ACh
ATP
Compuerta
Exterior
Interior
Activación
Simultánea
de Ambos
Receptores
B
El canal P2X
está permeable lo cual
bloquea el canal nACh
C
El canal Nicotínico
está permeable lo cual
bloquea el canal P2X
Canal
Bloqueado
Detector de corriente
entrante
Canal
Bloqueado
+PPADS
+ Hex
D
En presencia de PPADS
ATP no activa los receptores P2X
E
En presencia de hexametonio la ACh
activa el receptor pero el canal está bloqueado
PPADS
Hex
Compuerta
abierta
Canal nicotínico
está permeable
Canal P2X
está permeable

somatostatina (SSTR5) (Rocheville *et al.*, 2000), receptores a dopamina (D5) y receptores $GABA_A$ (Liu *et al.*, 2000). Todos estos estudios sugieren que las interacciones entre proteínas-receptor juegan un papel importante en la señalización neuronal.

1.2.4 Sistema nervioso entérico (SNE)

El SNE controla todas las funciones gastrointestinales y está a su vez bajo el control del sistema nervioso central y el sistema nervioso endócrino. El SNE lleva a cabo sus funciones mediante un complejo de reflejos neurales utlilizando varios neurotransmisores que aumentan y disminuyen la excitabilidad neuronal, muscular y de las células glandulares. Las neuronas del SNE se localizan fundamentalmente en dos plexos: el mientérico y el submucoso. El plexo mientérico controla la contracción y relajación del músculo liso intestinal. El plexo submucoso controla las funciones de absorción y secreción del epitelio gastrointestinal, flujo sanguíneo local y respuestas neuroinmunes (Galligan, 2002).

Hay muchos receptores de superficie expresados en las neuronas del SNE, estos receptores responden a neurotransmisores liberados en las sinapsis, hormonas circulantes y sustancias liberadas localmente. Los LGIC expresados en el neuronas del SNE son: nAChRs, P2X, $5HT_3$, $GABA_A$, NMDA, AMPA y receptores a glicina. P2X, $5HT_3$ y nAChRs participan en la transmisión sináptica rápida en neuronas tipo S en el SNE, mientras que las neuronas tipo AH expresan todos los LGIC (Galligan, 2002).

La motilidad intestinal del SNE es también regulada a través de neuronas sensoriales intrínsecas, varios tipos de interneuronas y neuronas motoras

excitatorias e inhibitorias. Neuronas excitatorias presentes en el músculo liso circular, contienen colina acetil-transferasa (ChAT) y takininas y se proyectan oralmente, transmitiendo via receptores muscarínicos activados por acetilcolina y takininas actúando sobre receptores NK1 y NK2. Las neuronas motoras inhibitorias presentes en el músculo circular contienen óxido nítrico sintetasa (NOS), péptido intestinal vasoactivo (VIP) y péptido activador de adenil-cliclasa pituitaria (PACAP); además ya ha sido caracterizada una transmisión proveniente de neuronas descendientes via ChAT/5-HT, ChAT/somatostatina (Bornstein *et al.*, 2004).

1.3 Planteamiento del problema

Pese a que la activación de receptores por su propio neurotransmisor se ha explicado como un proceso discreto e independiente, recientemente ha mostrado que es modulado por distintos receptores cuando son activados simultáneamente por su propio transmisor. Se han sido mostrado interacciones entre canales P2X con miembros de la superfamilia de receptores *Cys-loop* en distintos tipos neuronales.

Particularmente con receptores nicotínicos (Nakazawa, 1994; Barajas-López *et al.*, 1998; Zhou & Galligan, 1998), con canales 5-HT_3 (Barajas-López *et al.*, 2002; Boue-Grabot *et al.*, 2003) y con canales $GABA_A$. Sin embargo, el grupo de Galligan (Zhou & Galligan, 1998) reportó que no había interacción entre los canales P2X y $GABA_A$ en neuronas entéricas. Dada esta discrepancia, en la primera parte de esta tesis presento el trabajo realizado por nuestro grupo en el cual investigamos la interacción entre estos dos canales ya reportada por Sokolova (Sokolova *et al.*, 2001) en neuronas del ganglio de la raíz dorsal, lo cual

sugería que esta interacción era tejido específica. Por lo tanto, el primer objetivo de este reporte fue investigar las interacciones inhibitorias entre los canales P2X y $GABA_A$ en neuronas del plexo mientérico. A la fecha también ha sido reportada la existencia de interacciones entre dos miembros de la misma superfamila de receptores *Cys-loop* por Li *et al.* (2003). Estos autores han descrito la presencia de una interacción inhibitoria asimétrica entre los canales glicinérgicos y los $GABA_A$; esta interacción involucra dos canales aniónicos, por lo que la segunda parte de esta tesis describe el trabajo realizado para investigar y caracterizar la posible interacción inhibitoria entre dos miembros de la superfamilia *Cys-loop* uno catiónico 5-HT_3, y el otro aniónico los $GABA_A$ en neuronas del plexo mientérico.

Nuestros hallazgos indican que existe interacción inhibitoria entre canales P2X y $GABA_A$ y además también entre canales estructuralmente relacionados 5-HT_3 y $GABA_A$. En estas interacciones los canales se modulan uno al otro cuando son máximamente activados. Esta interacción inhibitoria ocurre tan pronto como la activación de los canales y no requiere Ca^{2+} ni fosforilación de proteínas. Conjuntamente, estos resultados implican que la inhibición es mediada por interacciones alostéricas entre esos pares de receptores.

1.4 Objetivos

1 Investigar y caracterizar las interacciones inhibitorias entre los canales P2X y $GABA_A$ en neuronas del plexo mientérico.

2 Estudiar y caracterizar las interacciones inhibitorias entre los canales 5-HT_3 y $GABA_A$ en neuronas del plexo mientérico.

Capítulo 2

Cross-Inhibitory Interactions Between $GABA_A$ and P2X Channels in Myenteric Neurons

2.1 Abstract

Inhibitory interactions between $GABA_A$ (induced by GABA) and P2X (activated by ATP) receptors of myenteric neurons from the guinea pig small intestine were characterized using whole-cell recordings. Currents induced by GABA (I_{GABA}) were inhibited by picrotoxin, whereas those induced by ATP (I_{ATP}) were inhibited by PPADS and a high [Ca^{2+}]. Currents induced by GABA+ATP ($I_{GABA+ATP}$), were only as large as the current induced by the most effective transmitter, revealing current occlusion. This occlusion requires maximal activation of at least one of these receptors. Kinetic and pharmacological properties of $I_{GABA+ATP}$ indicate that they are carried through both $GABA_A$ and P2X channels. ATP did not affect I_{GABA} in neurons in which: i) P2X channels were not present, ii) after inhibiting P2X channels with Ca^{2+}, iii) in the presence of PPADS, a P2X receptor antagonist, iv) after P2X receptor desensitisation, or v) at I_{ATP} reversal potential. Similarly, GABA did not affect P2X mediated currents in neurons in which: i) $GABA_A$ channels were not present, ii) in the presence of picrotoxin, a $GABA_A$ channel blocker, iii) after $GABA_A$ receptor desensitisation, or iv) at the I_{GABA} reversal potential. Current occlusion occurred as fast as current activation. It was still present in the absence of Ca^{2+}, when experiments were performed at low temperature (11ºC), after adding to the pipette solution a cocktail of protein kinase inhibitors (staurosporine+genistein+K-252a), after substituting the GTP in the pipette with GDP-β-S, and after treating the cells with *N*-ethylmaleimide. These results are consistent with a model of cross-inhibition between $GABA_A$ and P2X.

2.2 Introduction

γ-Aminobutyric acid (GABA) and ATP are known to play a role as neurotransmitters (DeFeudis, 1990; Evans *et al.*, 1992; Silinsky & Gerzanich, 1993; Zhou & Galligan, 1996) by directly activating $GABA_A$ chloride and P2X cationic channels on the postsynaptic membrane. $GABA_A$ receptors are part of the *Cys-loop* ligand-gated channel superfamily and twenty different subunits have been cloned. P2X receptors belong to a different ligand-gated channel superfamily and seven different P2X subunits have been cloned (Dunn *et al.*, 2001; Khakh *et al.*, 2001; North, 2002). Each of the $GABA_A$ subunits has four transmembrane domains (Cockcroft *et al.*, 1990a; Ortells & Lunt, 1995) whereas, P2X subunits appear to cross the membrane only twice (Dunn *et al.*, 2001; Khakh *et al.*, 2001; North, 2002). P2X subunits appear to form both homomeric and heteromeric channels while the $GABA_A$ channels are predominantly heteromeric pentomers.

Pioneer studies have shown that the nicotinic acetylcholine (nACh) and P2X channels are not independent and that they can inhibit each other when they are simultaneously activated (Nakazawa, 1994; Barajas-López *et al.*, 1998; Zhou & Galligan, 1998). This inhibitory interaction is very fast and might be mediated by an allosteric interaction between nACh and P2X channels. In favour of this hypothesis, $P2X_2$ and $\alpha_4\beta_2$ channels appear to form complexes, with channels lying ~80 Å apart (Khakh *et al.*, 2005). Analogous interactions have been shown between P2X and $5\text{-}HT_3$ receptors in enteric neurons (Barajas-López *et al.*, 2002; Boue-Grabot *et al.*, 2003) and P2X and the $GABA_A$ receptors in dorsal root

ganglia (Sokolova *et al.*, 2001). In apparent contradiction with these studies, in myenteric neurons, P2X channels were reported to interact specifically with nACh and not with other members of the *Cys-loop* superfamily present in these neurons (p. e. $GABA_A$ and 5-HT_3 receptors; (Zhou & Galligan, 1998)). This discrepancy might indicate that these interactions are tissue specific, probably arising from receptor heterogeneity and hence requiring further experimental analysis. Adding to this complexity, other types of pharmacological interactions appear to exist between the serotoninergic and cholinergic systems. Thus, it has been reported that serotonin (5-HT) molecules can themselves block nACh channels in various cell types including submucosal neurons and this occurs at similar concentrations from those required to activate $5HT_3$ receptors (García-Colunga & Miledi, 1995; Karanjia *et al.*, 2006).

Our aim, in the present study, was to investigate and to characterize the putative inhibitory interactions between $GABA_A$ and P2X native receptors of myenteric neurons. Contrary to what has been reported previously (Zhou & Galligan, 1998), it was found that currents induced by GABA and ATP are not additive, revealing current occlusion. This phenomenon is concentration-dependent (requires total saturation of at least one of these receptors), occurs simultaneously with activation of these ligand-gated channels, and does not require Ca^{2+}, G-proteins or protein phosphorylation, implying that it is mediated by allosteric interactions between these receptors.

2.3 Materials and Methods

Young guinea pigs (150-200 g), either male or female, were killed by decapitation and a segment of five cm of proximal jejunum (or ileum when indicated) 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 and submucosal layers of this intestinal segment were dissected, before removing most of the circular muscle layer leaving behind the longitudinal layer with the myenteric plexus embedded with it.

The myenteric preparation was dissociated using a sequential treatment with two enzymatic solutions, as described elsewhere (Barajas-López *et al.*, 1996b), the first contained papain (0.01 ml/ml; activated with 0.4 mg/ml of L-cysteine) and the second collagenase (1 mg/ml) and dispase (4 mg/ml). The enzymes were removed by washing with L15 and the neurons were plated on rounded 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 10 µg/ml and glucose 15 mM.

ATP and GABA are known to modulate the membrane potassium channels of enteric neurons via G-protein linked receptors (Christofi *et al.*, 1997; Barajas-López *et al.*, 2000; Krantis, 2000). In order to decrease the involvement of membrane currents other than those mediated by activation of ligand-gated channels, the experiments were carried out in the presence of Cs^+ (a potassium channel blocker). Furthermore, 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 ATP and GABA were recorded from myenteric neurons in short-term (2-80 hours) primary cultures using an Axopatch 1D amplifier. Patch pipettes were made as previously described (Barajas-López *et al.*, 1996b) and had resistances between 1-3 MΩ. Sixty to seventy percent of the series resistance was compensated in 10 neurons of the experiments reported here. Series resistance compensation, however, did not affect the lack of additivity of I_{ATP} and I_{GABA} (see Results), so in most cases no compensation was made for this factor. Except when otherwise mentioned the holding potential was -60 mV. The standard solutions used, unless otherwise mentioned, had the following compositions (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). 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-tubes device, each of them connected to syringes containing the control and experimental solutions. The tube containing the control solution was placed in front of the cell being recorded and the external application of experimental substances was

achieved by abruptly changing this tube for a tube delivering the same solution plus the drug(s). Experimental substances were removed by returning back to the control solution. External solutions were delivered by gravity and the level of the syringes was continuously adjusted to minimize changes in the flow rate. Experiments, unless otherwise stated, were performed at room temperature (~23°C).

Genistein, GABA, picrotoxin, and pyridoxalphosphate-6-azophenyl-2´,4´-disulfonic acid (PPADS) were purchased from Research Biomedical Inc. (Natick, MA, USA). Staurosporine and K-252a were supplied by Kamiya, (Thousand Oaks, CA, USA). All other substances were purchased from Sigma (St. Louis, MO., USA). The pH of the external solution containing ATP, used to induce the I_{ATP}, was always readjusted with NaOH. The addition of the other substances to the external solution did not alter its pH.

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 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.

2.4 Results

2.4.1 Pharmacological and electrophysiological properties of the whole-cell currents induced by GABA and ATP

Electrophysiological and pharmacological properties of the whole-cell currents or conductances activated by GABA (I_{GABA}) and ATP (I_{ATP}) in enteric neurons have been previously characterized (Cherubini & North, 1984, 1985; Barajas-López *et al.*, 1994; Barajas-López *et al.*, 1996a; Zhou & Galligan, 1996). These currents are mediated by activation of channels with different ion permeability, chloride and non-specific cationic channels, respectively.

Using the standard chloride internal solution, GABA and ATP induced an inward current in 87 (60%) and in 122 (84%) neurons out of 145 recorded neurons. Concentration-response curves (not shown) were obtained for these transmitters and analysed as previously reported (Barajas-López *et al.*, 1996a). The EC_{50} values for GABA and ATP were 104 and 64 μM, whereas the Hill coefficient values were 1.3±0.1 and 1.1±0.1 (non significantly different than unity), respectively. The current amplitude induced by maximal concentrations of GABA and ATP (1 mM) was variable in different cells but a typical value was between 1-3 nA (range from 0.05 up to -7.5 nA). The mean peak-amplitude of these currents was of -1.94±0.23, and -1.90±0.14 nA, respectively. A larger variability was noticed in the amplitude of I_{GABA} than in I_{ATP}. The amplitude of these currents was independent of each other and in fact some neurons only depicted I_{GABA} (4 out of 87) or I_{ATP} (31 out of 122 neurons). Indicating that these channels are expressed independently in these neurons.

As shown in Figure 2.1A and 2.1B, inward currents induced by GABA (0.3 mM) and ATP (1 mM) were totally inhibited by picrotoxin (1 mM; n=4) and PPADS (30 μM; n=5), respectively. However, neither picrotoxin affects I_{ATP} nor PPADS alters I_{GABA}. Similarly, I_{ATP} was almost totally blocked when the extracellular Ca^{2+} concentration was raised to 82 mM while this manoeuvre did not affect I_{GABA} (n=5; Fig. 2.1C).

The I_{GABA} onset clearly was slower than I_{ATP} onset. In agreement with this, it was found that the time required to reach the half-maximal current was significantly different ($P<0.01$) in ten analysed cells. This time had mean values of 131±14 and 84±9 ms for I_{GABA} and I_{ATP}, respectively. These currents usually reached their peak within the following second. After reaching its maximal amplitude, the currents decreased despite the continuous presence of the transmitters (current inactivation), indicating receptor tachyphylaxis. $GABA_A$ receptors desensitisation was just slightly faster than the one observed for P2X. In three out of five cells treated with long-term (1-2 min) applications of GABA, receptor desensitisation was better fitted by the sum of three exponential functions and by two exponential functions in the other two neurons. In all five cells treated with long-term (1-2 min) applications of ATP, current desensitisation was better fitted by the sum of two exponential functions. After the agonist removal from the external solution, currents rapidly decreased until they disappeared. This decay was well fitted by a single exponential function with mean τ values of 2.6±0.6 and 0.3±0.1 s for I_{GABA} and I_{ATP}, respectively (n=5). These values were significantly different ($P<0.05$).

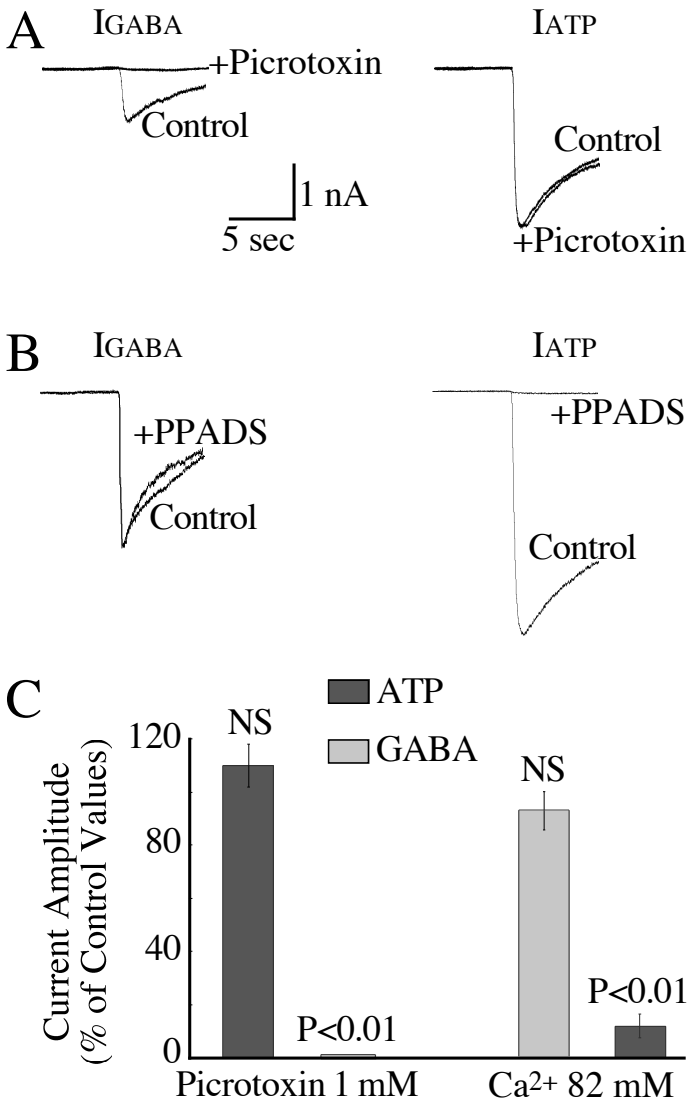


Figure 2.1 Whole-cell inward currents induced by ATP (I_{ATP}) and GABA (I_{GABA}) are mediated by two distinct receptors

A: Picrotoxin (1 mM; a $GABA_A$ receptor antagonist) blocks I_{GABA} without modifying I_{ATP}. **B**: PPADS (30 μM; a P2X receptor antagonist) blocks I_{ATP} without affecting I_{GABA}. **C**: Mean ± S.E.M. values (bars and lines on their top) obtained from five picrotoxin experiments and the average effects of elevating the extracellular concentration of Ca^{2+} up to 82 mM, on I_{ATP} and I_{GABA}. Whole-cell inward currents shown in **A** and **B** were measured from two different myenteric neurons, at a holding potential of –60 mV, and were induced by GABA (0.3 mM) and ATP (1 mM). Only the initial portion of the currents (~7 s) is shown, which were recorded before (Control) and five min after starting the receptor antagonist application. Notice that despite the relative high concentrations of the agonist (GABA and ATP) and inhibitors (PPADS, picrotoxin and Ca^{2+}) their effects are, as expected, on only one of the channels.

2.4.2 Currents induced by GABA and ATP, were not additive

The experiments described above demonstrated that I_{GABA} and I_{ATP} have different kinetics and that they are mediated by activation of pharmacologically distinct receptors. If the two currents are mediated by functionally independent ion channels then the currents induced by maximal concentrations of GABA and ATP (1 mM; when receptor occupancy would be expected to be close to 100%) should be additive. To investigate if this is the case, we measured the peak of I_{ATP}, I_{GABA}, and the current induced by the simultaneous application of a maximal concentration of both agonists ($I_{GABA+ATP}$) in the same neuron.

It was found that the addition of individual currents (I_{ATP}+ I_{GABA}=$I_{Expected}$; -4.57±0.79 nA) was significantly larger ($P<0.001$; n=6) than $I_{GABA+ATP}$; (-3.34±0.67 nA), revealing an occlusion between I_{GABA} and I_{ATP} (Fig. 2.2A). Virtually, the same results were obtained when 300 μM of GABA (n=13) were used instead of 1 mM. Therefore, we pulled together the results of all these nineteen experiments and their averages are shown in Fig. 2.2B. In all these experiments, $I_{GABA+ATP}$ (-2.65±0.28 nA) was not significantly different from the current induced by the more effective of these transmitters (-2.51±0.21 nA). For instance, if I_{ATP} was larger than I_{GABA}, then $I_{GABA+ATP}$ had about the same magnitude as I_{ATP} (p.e. Fig. 2.2A; see below). Indeed, in six analyzed experiments in which I_{ATP} (-2.22±0.21 nA) was significantly ($P<0.001$) larger than I_{GABA} (-0.78±0.16 nA), $I_{GABA+ATP}$ (-2.20±0.26 nA) was significantly ($P<0.001$) larger than I_{GABA} but was not different than I_{ATP}. Similarly, in five analyzed neurons in which I_{GABA} (-2.81±0.46 nA) was significantly

($P<0.05$) larger than I_{ATP} (-1.46±0.30 nA), $I_{GABA+ATP}$ (-3.06±0.45 nA) was also significantly ($P<0.05$) larger than I_{ATP} but was not different than I_{GABA}.

In a previous study (Zhou & Galligan, 1998), occlusion between I_{GABA} and I_{ATP} was not observed. This study was carried out in myenteric neurons from the whole small intestine whereas the results reported above are from jejunum neurons. We therefore, investigated if this current occlusion is present in myenteric neurons from the ileum. In eleven neurons that responded to both agonists, we found a significant current occlusion. Thus, $I_{GABA+ATP}$ (-2.75±0.52 nA) was significantly smaller ($P<0.01$) than the calculated $I_{expected}$ (-3.37±0.67 nA); being I_{ATP} and I_{GABA} -1.40±0.26 and -1.94±0.57 nA, respectively. This indicates that current occlusion also occurs in ileum neurons. A simple explanation for this discrepancy is the fact that Zhou & Galligan (Zhou & Galligan, 1998) used new born guinea pigs and myenteric neuronal cultures up to two weeks and different subtype of receptors might be expressed in such condition. The reason for this discrepancy is not clear and it is out of the scope of the present study.

2.4.3 Current kinetics of responses induced by simultaneous application of both agonists

Figure 2.3A shows the average onset of $I_{GABA,}$ I_{ATP}, $I_{GABA+ATP}$, and $I_{Expected}$ from six analyzed neurons and demonstrates the presence of current occlusion since the beginning of these currents. However, this occlusion was significant ($P<0.05$) 15 ms after the beginning of these currents (as indicated by arrow). At this point the average $I_{GABA+ATP}$ was 0.08±0.02 nA and $I_{Expected}$ 0.15±0.03 nA. In other words, current occlusion occurs rapidly and concomitantly with channel activation.

After the removal of the agonists from the external solution, $I_{GABA+ATP}$ decay appears to be different from that of either I_{GABA} or I_{ATP} (Fig 2.3B). Indeed, the decay of $I_{GABA+ATP}$ was well fitted by the sum of two exponential functions (Fig 2.3B; n=6). We tested the hypothesis that these two exponentials of $I_{GABA+ATP}$ are the single exponentials of the I_{ATP} and I_{GABA} decay. Thus, average τ value of the first exponential (τ_1) was virtually the same to τ_1 of I_{ATP} decay and the average τ value of the second exponential (τ_2) of $I_{GABA+ATP}$ did not differ from τ_1 of I_{GABA} decay (Fig 2.3C). These observations indicate that $I_{GABA+ATP}$ are mediated by the opening of both P2X and $GABA_A$ channels. In order to further investigate this interpretation, we test the hypothesis that the simultaneous application of both agonists desensitised both $GABA_A$ and P2X receptors.

2.4.4 Simultaneous application of both agonists desensitised $GABA_A$ and P2X receptors

In the following experiments, we measured the amplitude of both I_{GABA} and I_{ATP} before and immediately after (~5 s) a long application of GABA+ATP. This long application lasted for at least 45 s or until the induced current had desensitised more than 80% of its initial amplitude (usually within 90 seconds). Some typical recordings and the average data from such experiments are shown in Figs 2.4A-B. We observed that application of ATP+GABA, decreased both I_{GABA} and I_{ATP} to less than 20% of their control amplitude (Fig 2.4E). Such an inhibition was not due to cross-desensitisation since $GABA_A$ receptor desensitisation alone did not affect I_{ATP} (Fig 2.4C) and P2X receptor desensitisation alone did not affect I_{GABA} (Fig 2.4D). These observations, coupled with the fact that $I_{GABA+ATP}$ kinetics is different

from the kinetics of I_{GABA} or I_{ATP} alone (Fig 2.3), indicate that $I_{GABA+ATP}$ is carried through both $GABA_A$ and P2X channels. This would also imply that current occlusion is mediated by partial inhibition of both channels.

2.4.5 Sequential activation of $GABA_A$ an P2X receptors supports the hypothesis that channel inhibition is reciprocal

Figure 2.5 shows two panels with typical recordings from a series of experiments inwhich sequential applications of neurotransmitters were performed. As illustrated, GABA inhibited I_{ATP} (Fig. 2.5A; n=7) and ATP inhibited I_{GABA} (Fig. 2.5B; n=7) as I_{ATP} and I_{GABA} were significantly different from control currents recorded prior to the GABA or ATP application, respectively, compared with the control currents (first current tracing). The amount of missing current was normalized using the control currents as 100%, and the mean values were 63 ± 10% and 54 ± 12%, which were not significantly different.

2.4.6 Current occlusion requires channel activation

In a previous study, it was demonstrated that serotonin molecules directly block nACh channels of enteric neurons (Barajas-López *et al.*, 2001), suggesting that current occlusion might be mediated by a similar mechanism. This hypothesis and other pre-receptor mechanisms are ruled out by the following observations.

In three cells with a marginal or no initial response to ATP (-0.12±0.73 nA; Fig 2.6A), $I_{GABA+ATP}$ (-1.75±0.41 nA) had the same amplitude and similar kinetics to I_{GABA} alone (-1.63±0.45 nA).

Inhibition of I_{ATP} also prevented ATP actions on I_{GABA}. Thus, when I_{ATP} was inhibited to a value of -0.27±0.16 nA by addition of 82 mM Ca^{2+} to the external

solution (NaCl was substituted by an equimolar concentration of $CaCl_2$), $I_{GABA+ATP}$ (-2.59±0.134 nA) had similar amplitude and similar kinetics to I_{GABA} (-2.32±0.14 nA; n=5; Fig 2.6B). Similarly, in another three neurons, in which I_{ATP} (-17±17 pA) had previously been inhibited with PPADS (a P2X receptor antagonist; not shown), $I_{GABA+ATP}$ (-2.80±0.92 nA) had the same amplitude and similar kinetics to I_{GABA} (-2.77±0.93 nA).

In fourteen cells with a marginal or no response to GABA (-26±15 pA; Fig 2.5C) $I_{GABA+ATP}$ (-1.35±0.20 nA) had the same amplitude and kinetics to I_{ATP} (-1.53±0.23 nA). In another five cells in which I_{GABA} was totally blocked with picrotoxin (1 mM; Fig 2.5D), $I_{GABA+ATP}$ (-1.52±0.33 nA) had the same amplitude and similar kinetics to I_{ATP} (-1.39±0.33 nA).

Figure 2.6A shows a typical recording of I_{GABA} before (Control response; -2.69±1.22 nA) and during the continuous application of ATP, after desensitisation of >80% of I_{ATP}. This manoeuvre did not block I_{GABA}, which peak amplitude was -2.46±1.20 nA (n=4; Fig 2.6C). Similarly, the presence of GABA did not alter I_{ATP} (Fig 2.6B), which peak amplitude was -1.621±0.31 and -1.63±0.30 nA before and in presence of GABA, respectively (n=4; Fig 2.6C). These observations together with the ones described in the previous paragraph indicate that current occlusion is mediated by a post-receptor mechanism(s). These data also rule out the possibility that occlusion is mediated by ATP molecules acting on $GABA_A$ receptors or GABA molecules acting on P2X receptors.

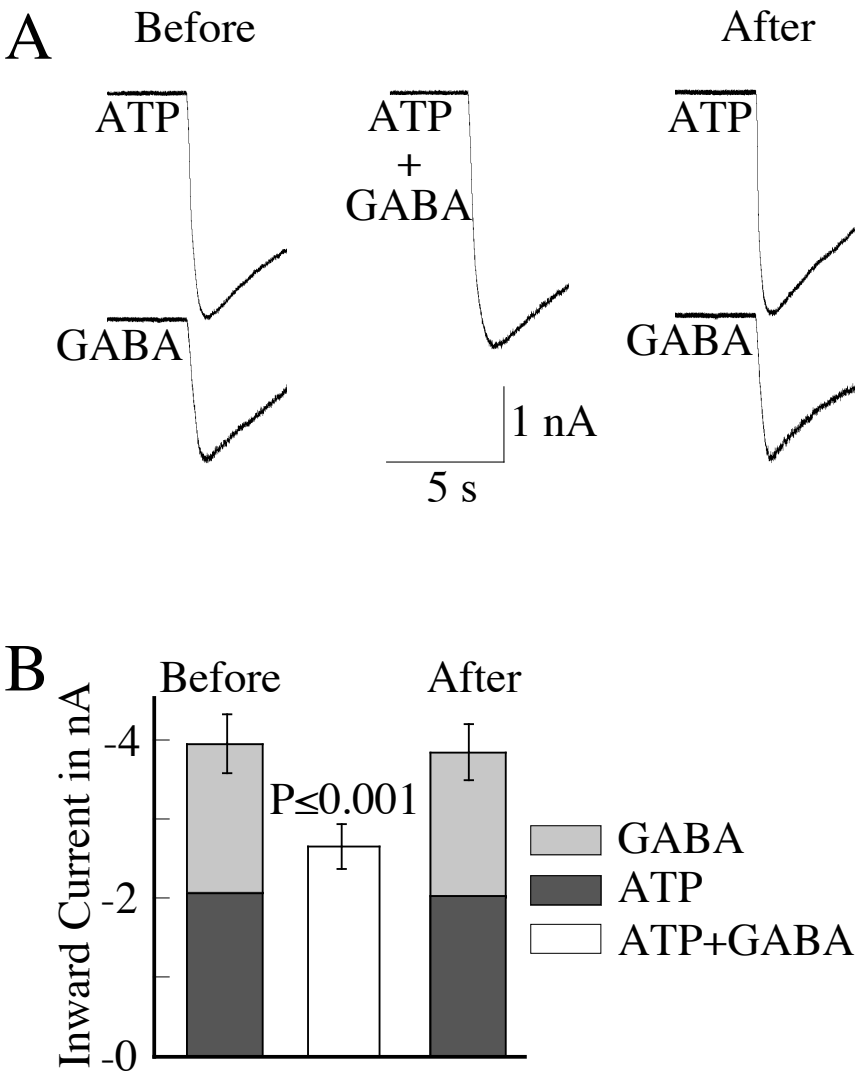


Figure 2.2 Whole-cell inward currents induced by GABA (I_{GABA}) and ATP (I_{ATP}) are not additive

A: shows recordings from one neuron of a typical experiment and **B**, the average (bars) values of nineteen experiments. Currents were induced by application of either GABA (0.3 or 1 mM) or ATP (1 mM) and by the simultaneous application of both agonists ($I_{GABA+ATP}$). I_{GABA} and I_{ATP} were recorded five minutes before and five minutes after $I_{GABA+ATP}$. Only the initial portion of the currents (~4 s) is depicted. **B**: the first and third bars show I_{GABA} and I_{ATP}, the addition of these currents represents expected current ($I_{Expected}$= I_{GABA}+I_{ATP}). S.E.M. are shown as lines in the top of the bars for $I_{Expected}$ and $I_{GABA+ATP}$. These two currents are significantly different ($P<0.001$), the latter one representing only 67% of $I_{Expected}$. $I_{GABA+ATP}$ (-2.65±0.28 nA), however, was not significantly different from the current induced by the most effective neurotransmitter (-2.51±0.21 nA, not shown).

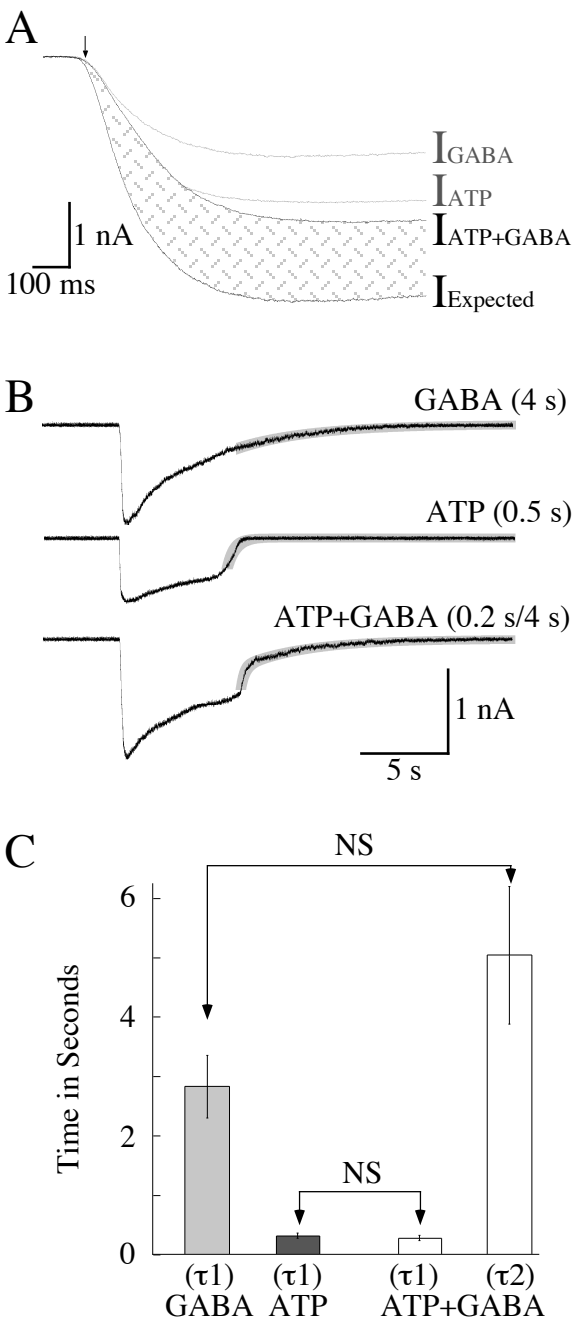


Figure 2.3 Onset and Decay kinetics of these currents indicate that occlusion is fast and bidirectional

A: Current occlusion is observed as soon as activation of the currents. All current tracings are mean currents from a total of six analysed myenteric neurons. The current induced by simultaneous application of both agonists ($I_{GABA+ATP}$) is smaller than the expected current ($I_{Expected}$= I_{GABA}+I_{ATP}) as soon as currents are detected. However, this difference (indicated by the shadow area between the last two currents) is significant ($P<0.05$) after the instant indicated by arrow. Whole-cell currents were measured at a holding potential of -60 mV. Only the initial portion of the currents (~1 s) is depicted. **B**: $I_{GABA+ATP}$ appear to be mediated by the opening of both channels, P2X and $GABA_A$. Currents induced by GABA (I_{GABA}), ATP (I_{ATP}) and $I_{GABA+ATP}$, are representative recordings from one myenteric neuron. Decay of $I_{GABA+ATP}$ was best fitted by the sum of two exponential functions (thick grey line), whereas I_{GABA} and I_{ATP} decay was best fitted by a single exponential function. Between brackets are the t of these exponential functions in seconds. **C**: bars in these graphs represent the mean ± S.E.M. values (n = 5) of the t of these exponential functions. The τ_1 of I_{ATP} was not significantly (**NS**) different from τ_1 of $I_{GABA+ATP}$. The τ_1 of I_{GABA} did not differ from τ_2 of $I_{GABA+ATP}$. Exponential fits were performed using the data from a couple hundred milliseconds after removing the agonists to the "steady-state" component. In these experiments the holding potential was -60 mV.

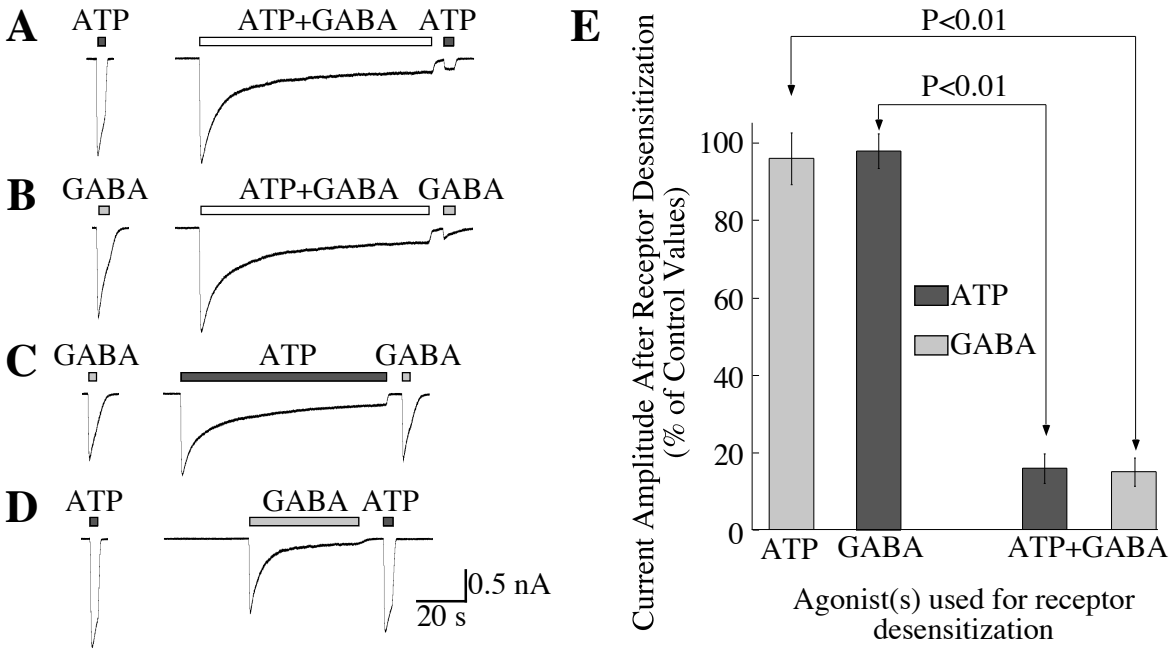


Figure 2.4 Simultaneous application of GABA and ATP induce desensitisation of both $GABA_A$ and P2X receptors, which was not due to cross-desensitisation between these receptors

Control I_{ATP} (**A** and **D**) and I_{GABA} (**B** and **C**) was recorded five minutes before (left recordings) and immediately after (~5 s), the prolonged application of both agonists (**A** and **B**), ATP (**C)** or GABA (**D**). **E**: average amplitude of I_{GABA} and I_{ATP} recorded after the prolonged application of ATP, GABA or GABA+ATP, as a percentage of control response (n=5). Line on top of the bars represents S.E.M. Recordings are from two neurons taken at the holding potential of -60 mV.

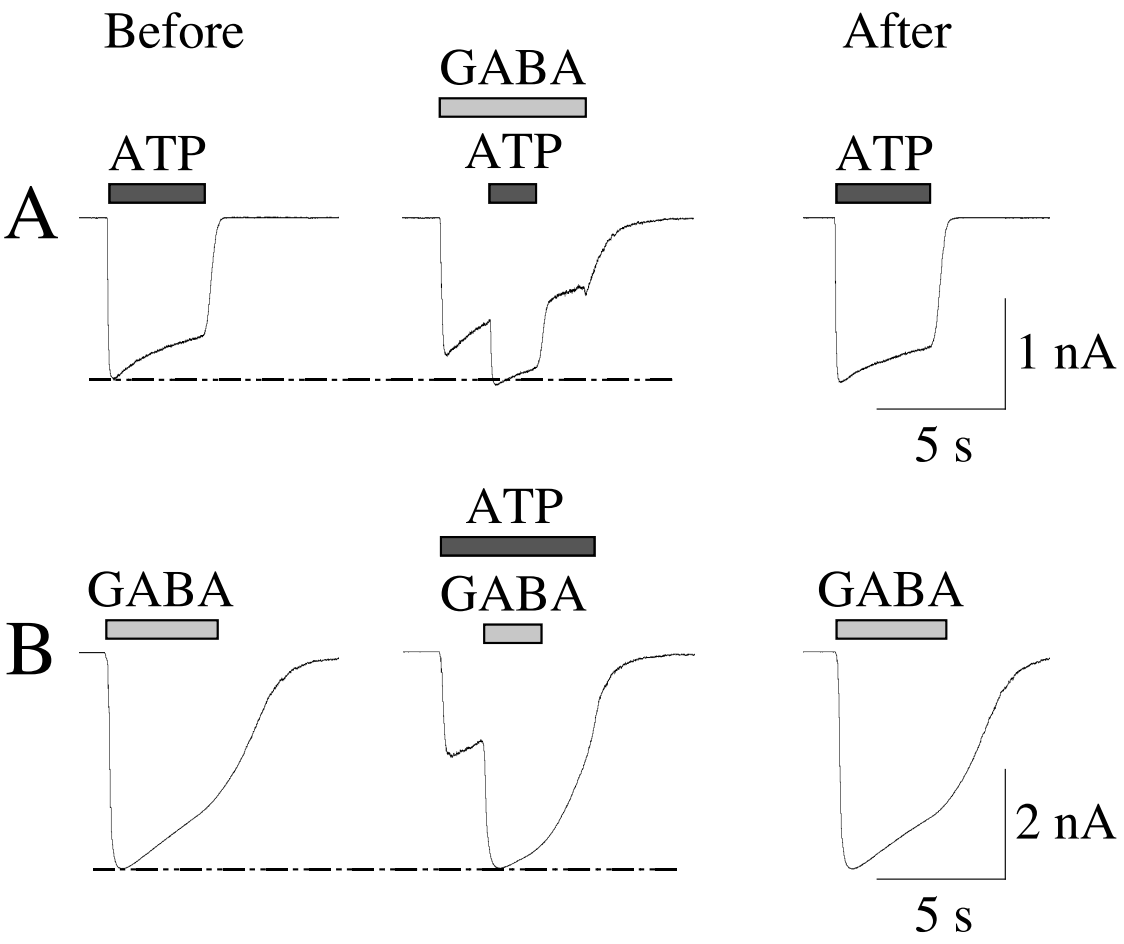


Figure 2.5. Sequential activation of these receptors suggests reciprocal channel inhibition

Current inhibition induced by sequential applications of neurotransmitters, GABA (0.3 mM) and ATP (1 mM) were applied as indicated by bars. **A**, activation of $GABA_A$ channels inhibited I_{ATP} up to 40% of its control amplitude, I_{ATP} was considered as the additional current observed after adding ATP. **B**, activation of P2X channels decreased I_{GABA} up to 59% of its control amplitude, I_{GABA} was considered as the additional current observed after adding GABA. Control recordings were taken 5 min before and after the middle recording. Each set of recordings is from the same myenteric neuron. Cell membrane potential was held at -60 mV.

In fourteen cells with a marginal or no response to GABA (-26±15 pA; Fig 2.5C) $I_{GABA+ATP}$ (-1.35±0.20 nA) had the same amplitude and kinetics to I_{ATP} (-1.53±0.23 nA). In another five cells in which I_{GABA} was totally blocked with picrotoxin (1 mM; Fig 2.5D), $I_{GABA+ATP}$ (-1.52±0.33 nA) had the same amplitude and similar kinetics to I_{ATP} (-1.39±0.33 nA).

Figure 2.6A shows a typical recording of I_{GABA} before (Control response; -2.69±1.22 nA) and during the continuous application of ATP, after desensitisation of >80% of I_{ATP}. This manoeuvre did not block I_{GABA}, which peak amplitude was -2.46±1.20 nA (n=4; Fig 2.6C). Similarly, the presence of GABA did not alter I_{ATP} (Fig 2.6B), which peak amplitude was -1.621±0.31 and -1.63±0.30 nA before and in presence of GABA, respectively (n=4; Fig 2.6C). These observations together with the ones described in the previous paragraph indicate that current occlusion is mediated by a post-receptor mechanism(s). These data also rule out the possibility that occlusion is mediated by ATP molecules acting on $GABA_A$ receptors or GABA molecules acting on P2X receptors.

2.4.7 Role of protein phosphorylation, G-protein activation and intracellular Ca^{2+} in current occlusion

ATP and GABA are also known to activate metabotropic P2Y and $GABA_B$ receptors in enteric neurons (Christofi *et al.*, 1997; Barajas-López *et al.*, 2000; Krantis, 2000). Activation of these receptors would lead to activation of G-proteins, changes in second messengers, and likely protein phosphorylation. This would suggest that at least part of the current occlusion observed here could be

mediated by metabotropic receptors. The following observations, however, do not support this hypothesis.

Current occlusion was still observed after inhibiting protein phosphorylation (Fig 2.7) by either lowering the temperature to 11°C or adding 50 μM genistein (a tyrosine kinase inhibitor; (O'Dell *et al.*, 1991)) together with two non-specific protein kinase inhibitors to the internal solution; 5 μM K-252a plus 5 μM staurosporine ((Ruegg & Burgess, 1989). Importantly, a concentration of 3 μM of staurosporine, applied extracellularly, is enough to inhibit the slow membrane depolarization induced by forskolin, phorbol esters, adenosine, and ATP in enteric neurons (Barajas-Lopez, 1993; Barajas-López *et al.*, 2000).

Under the same experimental conditions used in the current study, we previously found that adenosine inhibits voltage activated calcium currents by a G-protein mediated mechanism (Barajas-López *et al.*, 1996b). This effect was prevented by the addition of *N*-ethylmaleimide (NEM; 30 μM; known to uncouple receptors from G-proteins; (Allende *et al.*, 1991; Shapiro *et al.*, 1994)). In the present study, the same concentration of NEM (30 μM) did not alter the occlusion between I_{GABA} and I_{ATP} (Fig 2.7). Similarly, replacing GTP with GDP-β-S in the internal solution (Fig 2.7) did not prevent this current occlusion. This latter experimental manoeuvre would be expected to also inhibit G-protein mediated events (Anwyl, 1991; Ikeuchi & Nishizaki, 1996).

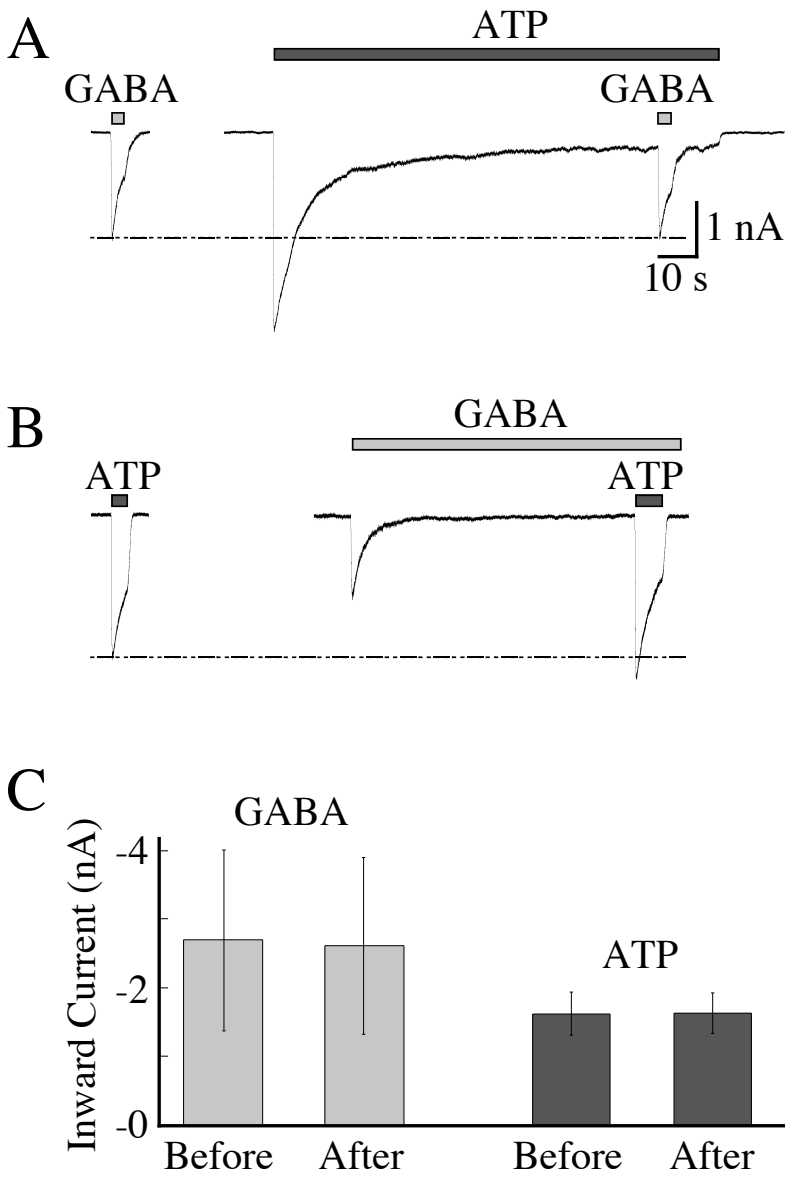


Figure 2.6 Inhibitory interactions between $GABA_A$ and P2X receptors disappear after receptor desensitisation

I_{GABA} (**A**) and I_{ATP} (**B**) were recorded five minutes before (control recordings, left) and during continuous application of the other agonist. Note that currents induced by this second application have similar kinetics and amplitude to control currents. These two sets of currents were recorded in two different myenteric neurons at a holding potential of -60 mV. **C**: Means ± S.E.M. values of similar experiments are indicated by the bars and lines, respectively.

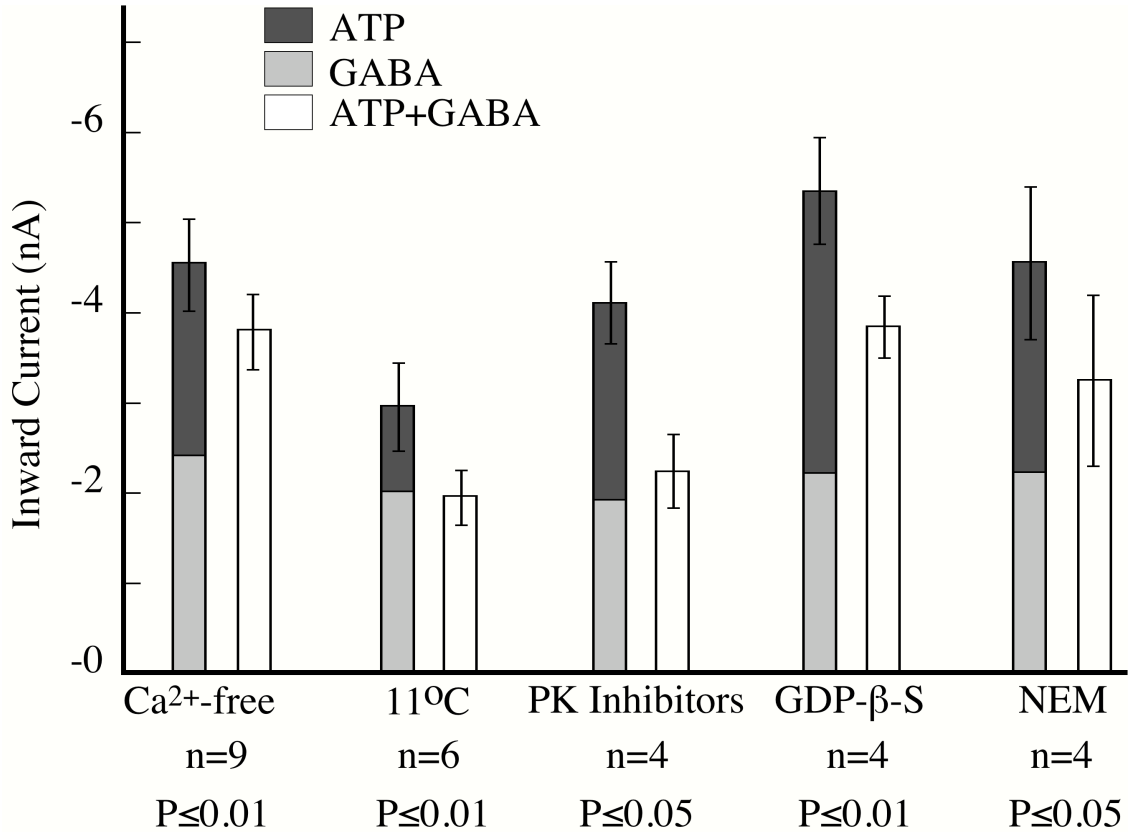


Figure 2.7 Calcium ions, G-proteins, and protein phosphorylation are not required for current occlusion

Average amplitude of inward currents induced by application of GABA (I_{GABA}), ATP (I_{ATP}) or GABA+ATP ($I_{GABA+ATP}$) in five different experimental groups of myenteric neurons. Data for each group are represented by a pair of bars. The first bar of each pair is a combined bar and shows the average I_{ATP} and I_{GABA} before application of GABA+ATP. This combined bar, therefore, represents the average expected current ($I_{Expected} = I_{GABA} + I_{ATP}$). The second bar represents the mean $I_{GABA+ATP}$. Error lines on the top of the bars are S.E.M. for $I_{Expected}$ and $I_{GABA+ATP}$. Ca^{2+}-free experiments were carried out in 0-Ca^{2+} plus 50 μM EGTA extracellular media and with the standard intracellular solution. Six experiments were performed by cooling down the external solution to 11°C. The protein kinase (PK) inhibitors experiments were carried out using standard intracellular solution plus K-252a (5 μM), Staurosporine (5 μM) and Genistein (50 μM). In the GDP-β-S experiments, the pipette solution contained these substances instead of GTP, respectively. During the N-ethylmaleimide (NEM) experiments, recorded neurons were pre-treated for 5 to 10 min with standard extracellular solution plus 30 μM NEM. During these experiments, only one cell from each c overslip was recorded and coverslips were discarded after a neuron had been exposed to NEM. All these experiments were carried out at a holding potential of -60 mV.

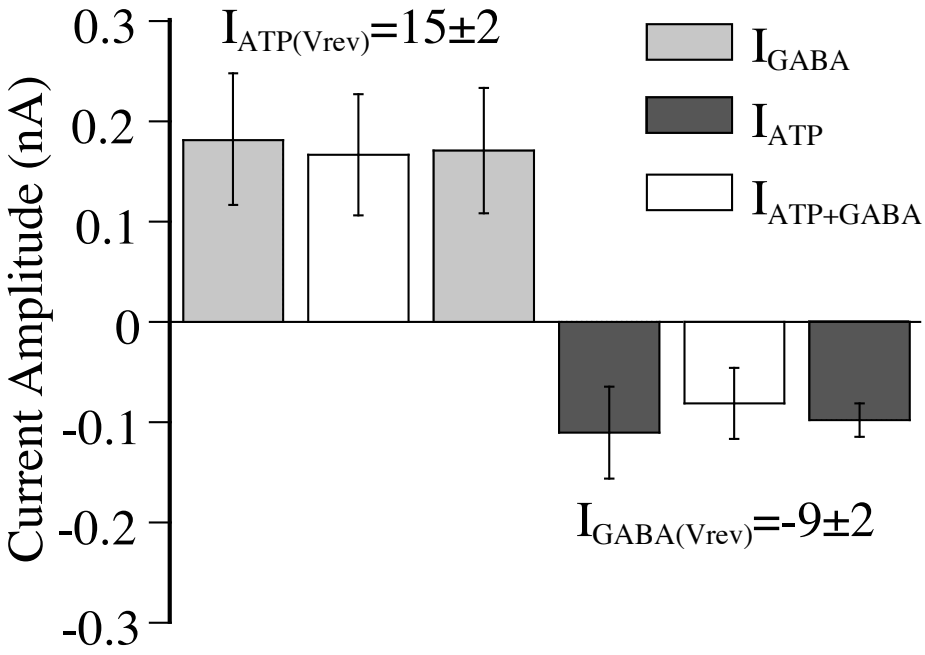


Figure 2.8 Channels required to be conducting for cross-inhibition to occur

Bars are mean amplitude of inward currents induced by application of GABA (I_{GABA}), ATP (I_{ATP}) or GABA+ATP ($I_{GABA+ATP}$). These current were measured at the reversal potential for I_{ATP} (15±2 mV) and for I_{GABA} (-9±2 mV). Reversal potentials were measured experimentally in every neuron. Line on top of the bars represents S.E.M (n=6).

2.4.8 Role of Ca^{2+} influx in the current occlusion

Activation of P2X receptors can elevate the intracellular [Ca^{2+}] (Barajas-López *et al.*, 2000) so current occlusion might be mediated by an increase in the intracellular concentration of this ion. However, the current occlusion was still observed in total absence of Ca^{2+} in the extracellular and intracellular medium (Fig 2.7). These experiments were carried out with the standard intracellular and extracellular solutions but the latter containing no Ca^{2+} plus 50 μM EGTA.

2.4.9 Channel permeability versus receptor activation as the origin for current occlusion

The fact that picrotoxin (a $GABA_A$ channel blocker) prevents GABA effect on I_{ATP} and Ca^{2+} (a P2X channel blocker) also avoids ATP action on I_{GABA}, suggests the hypothesis that current occlusion requires ion channel. In order to further investigate this hypothesis, some recordings (n=6) were carried out using a low Cl^- intracellular solution, which have 65 mM of Cs-gluconate plus 140 mM of sacarose instead of 160 mM CsCl. In these experiments, the reversal potential for I_{GABA} was -9±2 mV and at this potential GABA did not alter I_{ATP} (Fig 2.8). Similarly, at the reversal potential for I_{ATP} (15±2 mV) ATP failed to modify I_{GABA}. These experiments support the hypothesis that cross-inhibition between $GABA_A$ and P2X channels required ion flow through them and that is not enough to activate their receptors.

2.4.10 Current occlusion is concentration dependent

According to our observations, a model that considers a cross inhibition between $GABA_A$ and P2X channels, due to allosteric changes in these proteins, might

explain the occlusion between I_{ATP} and I_{GABA}. This model was proposed before to explain a similar occlusion between I_{ATP} and I_{ACh} (Barajas-López *et al.*, 1998). In agreement with this model, we observed that occlusion was still observed when a saturating concentration of only one agonist was used (n=7). Thus, currents induced by a concentration of 1 mM ATP and 60 μM GABA, or 1 mM GABA and 60 μM ATP, were not additive (Figs 2.9A and 2.9D). In addition, occlusion was absent when not saturating concentrations (30 or 100 μM) were used for both agonists (Fig 2.9B-D; n=7).

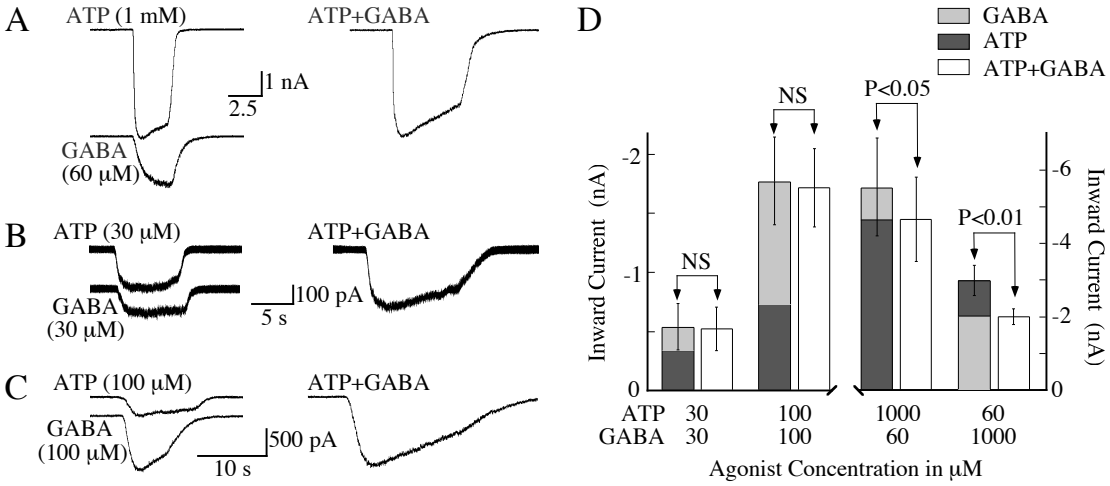


Figure 2.9 Current occlusion requires maximal activation of at least one of the channels

A-C: Typical recordings of I_{ATP}, I_{GABA} and $I_{ATP+GABA}$ induced by indicated agonist concentrations and recorded from three different neurons. **A**: Notice that it was enough to maximally activate one of the channels to observe current occlusion. **B** and **C**: In these experiments $I_{ATP+GABA}$ was no different (no significantly, NS) from the sum of the individual currents **D**: Average (bars) membrane currents of similar experiments are shown. S.E.M. are plotted as lines on the top of bars.

2.5 Discussion

This study shows the presence of inhibitory interactions between $GABA_A$ and P2X native receptors present in myenteric neurons, in a disagreement with the conclusions of a previous report (Zhou & Galligan, 1998). Our observations demonstrate that activation of $GABA_A$ and P2X channels is not independent and ionic currents carried through them are occluded when these receptors are simultaneously activated. This current occlusion is observed as soon as currents are activated and does not require Ca^{2+}, G-protein activation, or protein phosphorylation and is mediated by inhibition of both channels. Altogether, these observations suggest that current occlusion is mediated by cross-inhibition and a direct interaction between $GABA_A$ and P2X receptors/channels.

We have previously proposed a cross-inhibition model to explain the current occlusion between I_{ATP} and I_{ACh}, whose properties are very similar to those of the current occlusion described here. Therefore, a similar model could explain current occlusion between I_{ATP} and I_{GABA}. According to this model, P2X and $GABA_A$ channels would be located very close to each other forming complexes, each of these formed by one channel of each type. Direct support for our cross-inhibition model comes from Khakh's group (Khakh *et al.*, 2005). These authors used fluorescence resonance energy transfer and total internal reflection fluorescence microscopy and found that $P2X_2$ and $\alpha_4\beta_2$ channels (nicotinic channels) appear to form complexes, where each channel lies ~80 Å apart from each other. According to the cross-inhibition model, when channels of a given complex are simultaneously activated, the ion flow through the first channel (p. e. P2X) would

induce allosteric inhibition of the second channel of the same complex (p. e. $GABA_A$). Inhibition of the second channel would stop once the ion flow through the first channel ceases. According to this model only one (either) channel is open in a given complex. Furthermore, it is likely that some complexes could be formed by the same type of channels decreasing the amount of current occlusion observed in whole-cell recordings. The use of non-saturating agonist concentrations would also decreased channel cross-inhibition because the likelihood that two different channels are simultaneously activated in a given complex will be lower than with saturating concentrations. In agreement with the cross-inhibition model we found that saturation of either channel is enough to observe current occlusion.

2.5.1 Functional implications for these channel interactions

Our 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. Channel interactions similar to those reported here, exist between: $GABA_A$ and P2X channels in neurons of the dorsal root ganglia (Sokolova *et al.*, 2001); nACh and P2X receptors (Barajas-López *et al.*, 1998; Zhou & Galligan, 1998; Khakh *et al.*, 2000); 5-HT_3 and P2X receptors (Barajas-López *et al.*, 2002; Boue-Grabot *et al.*, 2003); and between glycine and $GABA_A$ channels (Li *et al.*, 2003). Furthermore, interactions have also been reported between metabotropic receptors, and metabotropic and ionotropic receptors. For instance, between: dopamine (D2) and somatostatin (SSTR5) receptors (Rocheville *et al.*, 2000), and dopamine (D5) and $GABA_A$ receptors (Liu

et al., 2000). All these studies suggest that interactions between receptor-proteins play an important role in neuronal signalling.

The fact that ATP, the endogenous agonist for most P2X receptors, has been shown to be co-released with various neurotransmitters, including GABA (Jo & Schlichter, 1999), ACh, and noradrenaline (Burnstock, 1986), leads the hypothesis that the inhibitory interactions between neurotransmitter receptors might be an important modulator of synaptic transmission. In the myenteric plexus, fast synaptic potentials mediated by ATP have been reported by Galligan's group, but GABAergic synaptic potentials have not (Galligan *et al.*, 2000). GABAergic neurons innervate the circular and longitudinal muscle layers but do not appear to innervate other myenteric neurons (Pompolo & Furness, 1990). Therefore, it is unclear, at this point, what is the role of the cross-inhibition between P2X and $GABA_A$ channels for synaptic integration in the myenteric plexus. However, $GABA_A$ channels are present in intrinsic afferent neurons (IPANs) of this plexus and some of their processes arrive from the muscularis externa (Galligan *et al.*, 2000). It is possible that putative $GABA_A$ receptors in these processes are activated when GABA is released from motor neurons. ATP has also been considered as a major inhibitory neurotransmitter in the muscularis externa (Crist *et al.*, 1992; Smits & Lefebvre, 1996; Fernandez *et al.*, 1998) and importantly, GABA immunoreactivity is found in a large portion of inhibitory motor neurons (nitric oxide immunoreactive) (Williamson *et al.*, 1996), opening the possibility that it might be co-released also with ATP. Despite the uncertainty regarding its physiological role at this time, the cross-inhibition between P2X and

$GABA_A$ channels in myenteric neurons can be used as a model for future studies aimed to understanding the mechanisms for this interaction or as a pharmacological target once we learn how to control it.

In conclusion, our results indicate that there is a very fast cross-inhibitory interaction between $GABA_A$ and P2X channels. The properties described for this interaction support 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.

Capítulo 3

Cross-Inhibitory Interactions Between $GABA_A$ and $5\text{-}HT_3$ Channels in Myenteric Neurons

3.1 Abstract

Inhibitory interactions between $GABA_A$ [activated by γ-aminobutyric acid (GABA)] and $5\text{-}HT_3$ [activated by serotonin (5-HT)] receptors of myenteric neurons from the guinea pig small intestine were characterized using whole-cell recordings. Currents induced by GABA (I_{GABA}) or by 5-HT ($I_{5\text{-}HT}$) were inhibited by bicuculline or ondansetron, respectively. Currents induced by GABA+5-HT ($I_{GABA+5\text{-}HT}$) were significantly lower than the sum of I_{GABA} and $I_{5\text{-}HT}$, revealing a current occlusion. This occlusion requires maximal activation of these receptors. Kinetic and pharmacological properties of $I_{GABA+5\text{-}HT}$ indicate that they are carried through both $GABA_A$ and $5\text{-}HT_3$ channels. 5-HT did not affect I_{GABA} in neurons in which $5\text{-}HT_3$ channels were not present or after inhibiting $5\text{-}HT_3$ channels with ondansetron; a $5\text{-}HT_3$ receptor antagonist. Similarly, GABA did not affect 5-HT mediated currents in neurons in which $GABA_A$ channels were not present or in the presence of bicuculline, a $GABA_A$ receptor antagonist. Current occlusion requires maximal activation of $GABA_A$ and $5\text{-}HT_3$ receptors, occurred as fast as current activation, was present in the absence of Ca^{2+}, at low temperature (11ºC), and after adding to the pipette solution a protein kinase inhibitor (staurosporine). These results are consistent with a model of cross-inhibition between $GABA_A$ and $5\text{-}HT_3$ channels.

3.2 Introduction

γ-Aminobutyric acid (GABA) and serotonin (5-HT) are known to play a role as neurotransmitters (DeFeudis, 1990; Sugita *et al.*, 1992) by directly activating $GABA_A$ (chloride) and 5-HT_3 (cationic) channels, respectively. Both are part of the *Cys-loop* ligand-gated ion channels superfamily. Twenty different $GABA_A$ subunits have been cloned whereas for 5-HT_3 subunits only three have been cloned (Reeves & Lummis, 2002). Each of the ligand-gated subunits has four transmembrane domains (Cockcroft *et al.*, 1990b; Ortells & Lunt, 1995). 5-HT_3 subunits appear to form both homomeric and heteromeric channels while the $GABA_A$ channels are predominantly heteropentamers.

Various studies carried out in peripheral neurons have demonstrated that P2X native channels can establish an inhibitory interaction with members of the *Cys-loop* ligand-gated channels superfamily. In particular, with nicotinic channels (nACh) (Nakazawa, 1994; Barajas-López *et al.*, 1998; Zhou & Galligan, 1998), with 5-HT_3 channels (Barajas-López *et al.*, 2002; Boue-Grabot *et al.*, 2003), and with $GABA_A$ channels (Sokolova *et al.*, 2001; Karanjia *et al.*, 2006). This inhibitory interaction is very fast and might be mediated by an allosteric interaction between P2X and the mentioned *Cys-loop* ligand-gated channels. In favour of this hypothesis, $P2X_2$ and the $\alpha_4\beta_2$ nACh channels appear to form complexes, with channels lying ~80 Å apart (Khakh *et al.*, 2005). In a recent study carried out in *Xenopus* oocytes (Boue-Grabot *et al.*, 2004b), it was found that the C-terminal domain of $P2X_2$ and the intracellular loop of $GABA_A$ subunits are required for the functional interaction between ATP- and GABA-gated channels.

So far, only Li et al., (Li *et al.*, 2003) have reported the existence of similar interactions between two members of the same *Cys-loop* channel superfamily. These authors described the presence of an asymmetric cross-inhibition between glycine and $GABA_A$ channels, which are both permeable to Cl^-. Therefore, our aim, in the present study, was to investigate and to characterize the putative inhibitory interactions between $GABA_A$ and 5-HT_3 native receptors of myenteric neurons. Our findings indicate that activation of 5-HT_3 and $GABA_A$ receptors opens two different channel populations. These two channels, however negatively modulate each other when they are simultaneously and maximally activated. This inhibitory interaction occurs simultaneously with current activation and does not require Ca^{2+} or protein phosphorylation. Altogether, these results imply that it is mediated by allosteric interactions between these receptors.

3.3 Materials and methods

Young guinea pigs (150-200 g), either male or female, were killed by decapitation and a segment of five cm of proximal jejunum 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 and submucosal layers of this intestinal segment were dissected, before removing most of the circular muscle layer leaving behind the longitudinal layer with the myenteric plexus embedded with it. All procedures involving animals had the prior approval of the Queen's University Animal Care Committee.

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

5-HT and GABA are known to modulate various membrane ion channels of enteric neurons via G-protein linked receptors (Cherubini & North, 1984, 1985; Galligan *et al.*, 1988; Wang *et al.*, 1996; Gershon, 1999; Krantis, 2000). In order to decrease the involvement of membrane currents other than those mediated by activation of ligand-gated channels, the experiments were carried out in the

presence of Cs^+ (a potassium channel blocker). Furthermore, 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 from myenteric neurons using an Axopatch 1D amplifier (Molecular Devices, Sunnyvale, CA, USA). Short-term (2-80 hours) primary cultures were used to prevent space-clamp problems due to neurite growth, which become a common problem on the fourth day after plating the neurons. Patch pipettes were made as previously described (Barajas-López *et al.*, 1996b) and had resistances between 2-4 MΩ. These low resistance electrodes and a slight suction inside the pipette help to maintain a low access resistance (usually 6 MΩ). Sixty to ninety-five percent of the series resistance was compensated in 9 neurons of the experiments reported here. This compensation, however, did not affect the lack of additivity of $I_{5\text{-}HT}$ and I_{GABA} (see Results). The holding potential was -60 mV. To ensure the best voltage clamp, we rejected the data of neurons showing fast Na-mediated inward currents, during neurotransmitter stimulation. The standard solutions used had the following compositions, inside the pipette (in mM): 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 calculated junction potential (using the PCLAMP software, Molecular Devices) for the pipette was -5 mV however, holding potential described here were not adjusted. Under these conditions, the

usual input resistance of the neurons was ≥1 GΩ. Whole-cell currents were recorded on a PC using AXOTAPE software (Molecular Devices) and analyzed on a Macintosh computer using AXOGRAPH software (Molecular devices). 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-tubes device, with the tubes connected to syringes containing the control and experimental solutions. The tube containing the control solution was placed in front of the cell being recorded and the external application of experimental substances was achieved by abruptly changing this tube for a tube delivering the same solution plus the drug(s). Experimental substances were removed by returning back to the control solution. External solutions were delivered by gravity and the level of the syringes was continuously adjusted to minimize changes in the flow rate. Experiments, unless otherwise stated, were performed at room temperature (~23°C). Experiments at 11°C were carried out using the eight-tubes device jacketed with a segment (~6 cm long) from a plastic pipette (10 ml). This plastic jacket was perfused with ice-chilled water so that the temperature in front of the delivering tube was at 11°C.

Bicuculline was purchased from Research Biomedical Inc. (Natick, MA, USA) and staurosporine from Kamiya (Thousand Oaks, CA, USA). Ondansetron was purchased from Glaxo Smithkline (Parma, Italy) and all other substances from Sigma (St. Louis, MO., USA).

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 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.

3.4 Results

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

Electrophysiological and pharmacological properties of the membrane conductances and whole-cell currents activated by GABA (I_{GABA}) and 5-HT ($I_{5\text{-}HT}$) in enteric neurons have been previously characterized (Cherubini & North, 1984, 1985; Derkach *et al.*, 1989; Zhou & Galligan, 2000). These whole-cell currents are mediated by activation of channels permeable to chloride ($GABA_A$) and cations (5-HT_3), respectively.

Using the standard chloride internal solution, individual applications of GABA and 5-HT induced inward currents at a holding potential of -60 mV. Concentration-response curves (not shown) were obtained for these transmitters and analyzed as previously reported (Barajas-López *et al.*, 2002). The EC_{50} values for GABA and 5-HT were 104 and 55 μM, whereas the Hill coefficient values were 1.3±0.1 and 1.0±0.1 (not significantly different than unity), respectively. The current amplitude induced by maximal concentrations of GABA (0.3 mM) and 5-HT (1 mM) was variable in different cells but a typical value was between 1-3 nA (range from only few pA up to -6.9 nA). However, currents were included in the analysis when their amplitude was at least 200 pA. In these cells, the mean peak-amplitude of I_{GABA} and $I_{5\text{-}HT}$ -2.19±0.18 (n=106 out of 139) and -1.48±0.09 nA (n=124 out of 136), respectively. A larger variability was noticed in the amplitude of I_{GABA} than in $I_{5\text{-}HT}$. The amplitude of these currents was independent of each other and in fact some cells only displayed I_{GABA} (12 out of 136) or $I_{5\text{-}HT}$ (33 out of

139 neurons), indicating that these channels are expressed independently in myenteric neurons.

As shown in Figures 3.1A-C, inward currents induced by GABA (0.3 mM) and 5-HT (1 mM) were totally inhibited by bicuculline (30 μM; n=6) and ondansetron (3 μM; n=5), respectively. Ondansetron was especific on $I_{5\text{-}HT}$ and did not affect I_{GABA}, however, bicuculline (30 μM) partially blocked 25% of $I_{5\text{-}HT}$. This later effect of bicuculline on $I_{5\text{-}HT}$ has previously been reported (Takenouchi & Munekata, 1998; Sun & Machu, 2000).

$I_{5\text{-}HT}$ and I_{GABA} onsets were virtually the same. Thus the time required to reach the half-maximal current was not significantly different in six analyzed cells. This time had mean values of 191±33 and 174±35 ms for I_{GABA} and $I_{5\text{-}HT}$, respectively. These currents usually reached their peak within the following second. After reaching their maximal amplitude the currents decreased despite the continuous presence of the transmitters (current inactivation), indicating receptor tachyphylaxis. Desensitization of $GABA_A$ receptors was clearly slower than the one observed for $5\text{-}HT_3$ receptors. In seven analyzed cells treated with long-term (90 s) applications of GABA, receptor desensitization was better fitted by the sum of two exponential functions (τ values of these functions are given below). In the same seven neurons treated with long-term (90 s) applications of 5-HT, current desensitization was better fitted by the sum of three exponential functions (τ values of these functions are given below). $\tau 1$ and $\tau 2$ values of I_{GABA} were significantly different ($P<0.05$) than $\tau 1$ and $\tau 2$ values of $I_{5\text{-}HT}$. After removing the agonists from the external solution, currents decreased until they disappeared.

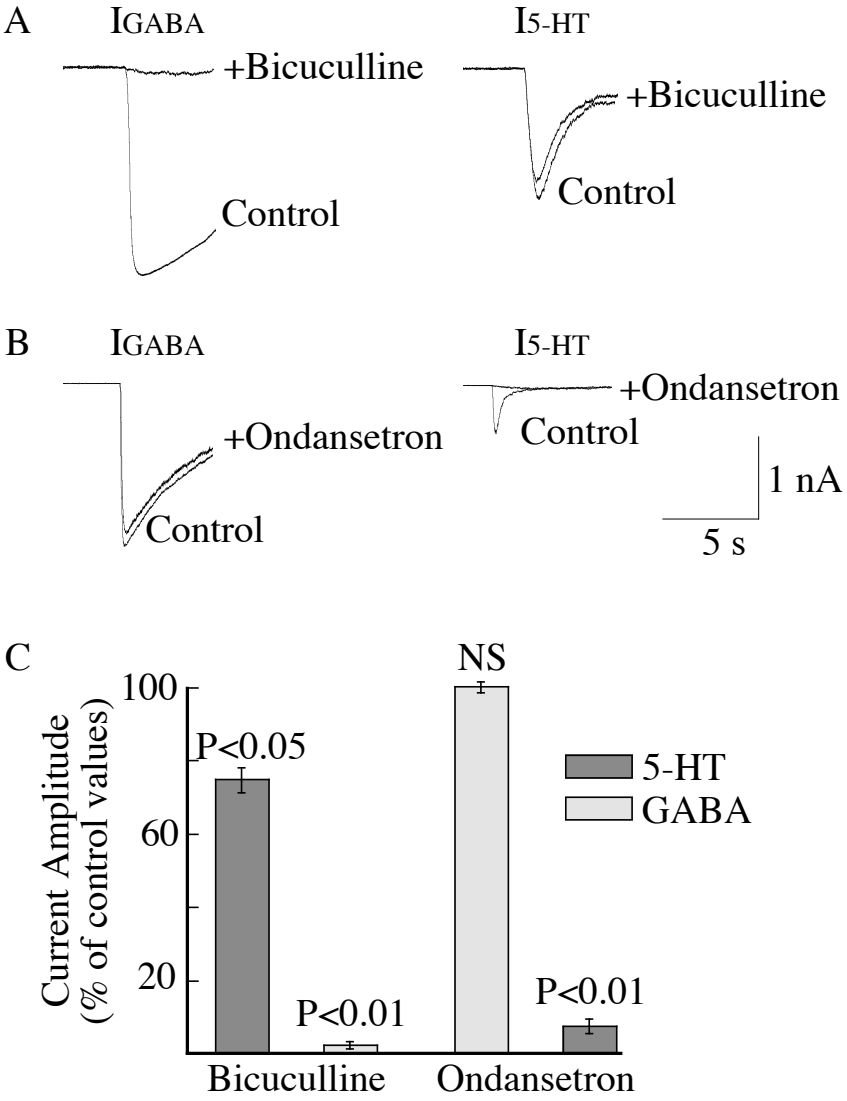


Figure 3.1 Whole-cell currents induced by 5-HT ($I_{5\text{-}HT}$) and GABA (I_{GABA}) are mediated by two distinct receptors

A: Bicuculline (30 μM; a $GABA_A$ receptor antagonist) blocks I_{GABA}. **B**: Ondansetron (3 μM; a 5-HT_3 receptor antagonist) blocks $I_{5\text{-}HT}$ without affecting I_{GABA}. **C**: mean ± S.E.M. values (bars and lines on their top) obtained from six bicuculline experiments. **A** and **B** were measured from two different myenteric neurons, at a holding potential of –60 mV, and were induced by GABA (0.3 mM) and 5-HT (1 mM). Only the initial portion of the currents (~5 s) is shown, which were recorded before (Control) and five min after starting the receptor antagonist application. Notice that despite the relative high concentrations of the receptor agonists (GABA and 5-HT) and ondansetron their effects are, as expected, on only one of the channels. Bicuculline effect was not specific, thus, it totally inhibited I_{GABA} but only blocked ~25% of $I_{5\text{-}HT}$.

This decay was well fitted by a single exponential function with mean τ values of 0.8±0.1 and 10.4±1.6 s for I_{GABA} and $I_{5\text{-}HT}$, respectively (n=10). These values were significantly different (P<0.001).

3.4.2 Currents induced by GABA and 5-HT were not additive

The experiments described above demonstrated that I_{GABA} and $I_{5\text{-}HT}$ have different kinetics and that they are mediated by activation of pharmacologically distinct receptors. If the two currents are mediated by functionally independent ion channels then the currents induced by concentrations of GABA and 5-HT of 0.3 mM and 1 mM, respectively (when receptor occupancy would be expected to be 70% to 100%) should be additive. To investigate if this is the case, we measured the peak of $I_{5\text{-}HT}$, I_{GABA}, and the current induced by the simultaneous application of the same concentrations of both agonists ($I_{GABA+5\text{-}HT}$) in the same neuron.

It was found mathematical addition of individual currents yielded a current ($I_{5\text{-}HT}$+ I_{GABA}=$I_{Expected}$; -3.05±0.25 nA), which was significantly larger (P<0.001; n=33) than $I_{GABA+5\text{-}HT}$; (-2.51±0.24 nA), revealing an occlusion between I_{GABA} and $I_{5\text{-}HT}$ (Fig. 3.2A and 3.2B). In order to rule out the possibility that current occlusion was due to the whole-cell access resistance, sixty to ninety-five percent of this (ussually 6 MΩ) was compensated in nine out of the previously described 33 neurons. In these experiments, $I_{Expected}$ (-4.21±0.64 nA) was still significantly larger than $I_{GABA+5\text{-}HT}$ (-3.27±0.48 nA; P<0.01).

3.4.3 Current kinetics of responses induced by simultaneous application of both agonists

Figure 3.3 shows the average onset of $I_{GABA,}$ $I_{5\text{-}HT}$, $I_{GABA+5\text{-}HT}$, and $I_{Expected}$ from nine analyzed neurons and demonstrates the presence of current occlusion since the beginning of their activation. This occlusion was significant ($P<0.01$) 15 ms after (time indicated by arrow) the beginning of these currents. At this time, the average $I_{Expected}$ was -100±16 pA and $I_{GABA+5\text{-}HT}$ -60±15 pA. In other words, current occlusion occurs rapidly and concomitantly with channel activation.

Figure 3.4A shows the average I_{GABA}, $I_{5\text{-}HT}$, $I_{GABA+5\text{-}HT}$, and $I_{Expected}$ from seven neurons exposed to long application (90 s) of the neurotransmitters. Visual inspection of these recordings revealed that $I_{GABA+5\text{-}HT}$ desensitized faster than I_{GABA} but slower than $I_{5\text{-}HT}$. To quantify this, exponential fits were performed using the data of the desensitization phase (inactivation). Desensitization of $I_{GABA+5\text{-}HT}$ was better fitted by the sum of three exponential functions, as with $I_{5\text{-}HT}$. τ values of the third exponentials ($\tau 3$) were larger than the analyzed recording period and were rejected for further analysis. τ values of the first ($\tau 1$) and second ($\tau 2$) exponentials for $I_{GABA+5\text{-}HT}$ were significantly different ($P<0.05$) than the correnpondent τ values of either $I_{5\text{-}HT}$ or I_{GABA} (Fig 3.4B).

After washing out the agonists from the external solution, $I_{GABA+5\text{-}HT}$ decay appears to be different from that of either I_{GABA} or $I_{5\text{-}HT}$ (Fig 3.5A and 3.5B). Indeed, the decay of $I_{GABA+5\text{-}HT}$ was well fitted by the sum of two exponential functions (n=10). We tested the hypothesis that these two exponential functions of $I_{GABA+5\text{-}HT}$ are the single exponentials of the $I_{5\text{-}HT}$ and I_{GABA} decay. Thus, average τ

value of the first exponential (τ_1) was virtually the same to τ_1 of I_{GABA} decay and the average τ value of the second exponential (τ_2) of $I_{GABA+5\text{-}HT}$ did not differ from τ_1 of $I_{5\text{-}HT}$ decay (Fig 3.5B). These observations indicate that $I_{GABA+5\text{-}HT}$ is mediated by the opening of both 5-HT_3 and $GABA_A$ channels. In order to further investigate this interpretation, we test the hypothesis that the simultaneous application of both agonists desensitised both $GABA_A$ and 5-HT_3 receptors.

3.4.4 Simultaneous application of both agonists desensitized $GABA_A$ and 5-HT_3 receptors

In the following experiments, we measured the amplitude of both I_{GABA} and $I_{5\text{-}HT}$ before and immediately after (~5 s) a long application of GABA+5-HT. This long application lasted until the peak current had decreased more than 80% of its initial amplitude (usually within 90 seconds). Some typical recordings and the average data from such experiments are shown in Figs 3.6A-E. We observed that application of GABA+5-HT, decreased both I_{GABA} (Fig 3.6A) and $I_{5\text{-}HT}$ (Fig 3.6B) to less than 20% of their control amplitude (Fig 3.6E). Such an inhibition was not due to cross-desensitization because 5-HT_3 receptor desensitization did not affect I_{GABA} (Fig 3.6C) and $GABA_A$ receptor desensitization alone did not affect $I_{5\text{-}HT}$ (Fig 3.6D). These observations, coupled with the fact that $I_{GABA+5\text{-}HT}$ kinetics is different from the kinetics of I_{GABA} or $I_{5\text{-}HT}$ alone (Fig 3.3-5), indicate that $I_{GABA+5\text{-}HT}$ is carried through both $GABA_A$ and 5-HT_3 channels. This would also imply that current occlusion is mediated by partial inhibition of both channels.

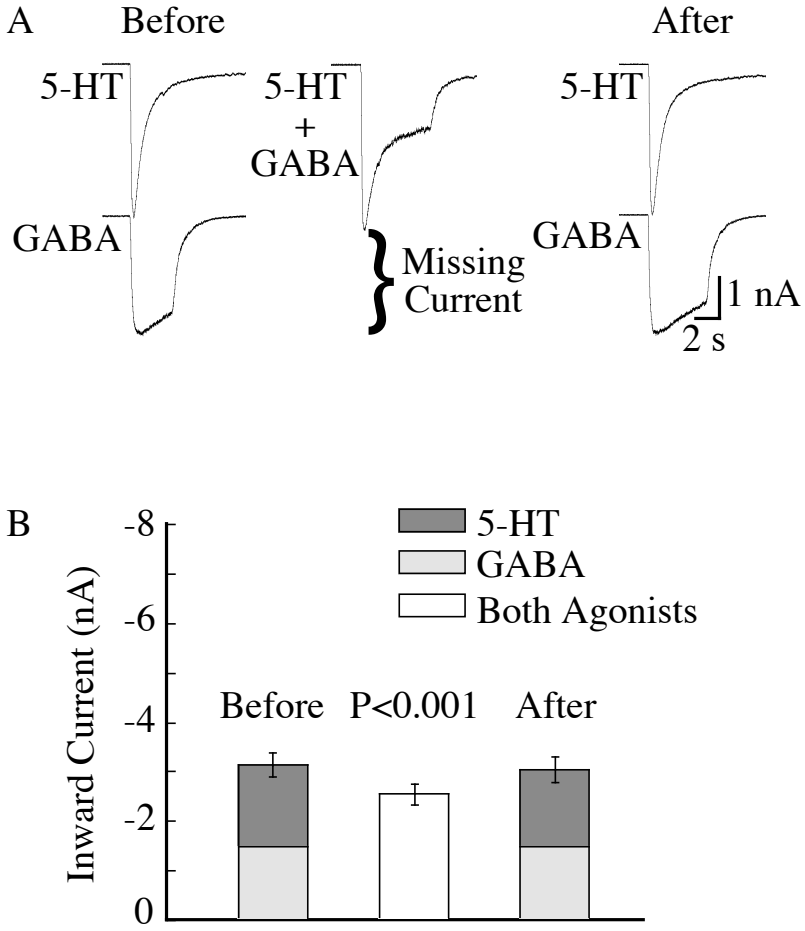


Figure 3.2 Whole-cell inward currents induced by GABA (I_{GABA}) and 5-HT ($I_{5\text{-}HT}$) are not additive, revealing a current occlusion

A: shows recordings from one neuron of a typical experiment and **B**: the average (bars) values of thirty-three experiments. Currents were induced by application of either GABA (0.3 mM) or 5-HT (1 mM) and by the simultaneous application of both agonists ($I_{GABA+5\text{-}HT}$). I_{GABA} and $I_{5\text{-}HT}$ were recorded five min before and five min after $I_{GABA+5\text{-}HT}$. **B**: the first and third bars show the mean values of I_{GABA} and $I_{5\text{-}HT}$, the addition of these currents represents expected current ($I_{Expected}$= I_{GABA}+$I_{5\text{-}HT}$). S.E.M. are shown as lines in the top of the bars for $I_{Expected}$ and $I_{GABA+5\text{-}HT}$.

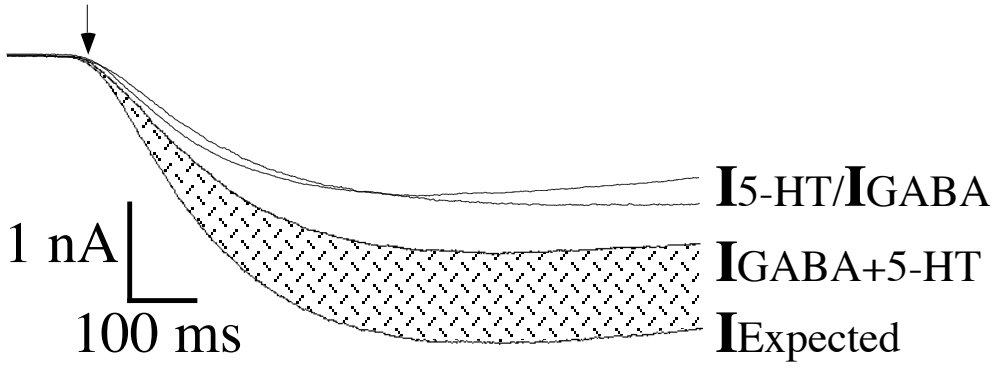


Figure 3.3 Onset of membrane currents indicate that occlusion is fast and bidirectional
Tracings are mean currents from a total of nine analyzed myenteric neurons. The current induced by simultaneous application of both agonists ($I_{GABA+5\text{-}HT}$) is always smaller than the expected current ($I_{Expected}$= I_{GABA}+$I_{5\text{-}HT}$). The difference between these two currents is highlighted by shadow area. $I_{GABA+5\text{-}HT}$ and $I_{Expected}$ are already significantly different ($P<0.05$) fifteen ms (instant indicated by arrow) after starting their activation. Whole-cell currents were measured at a holding potential of -60 mV and only the initial portion of currents (~1 s) is depicted.

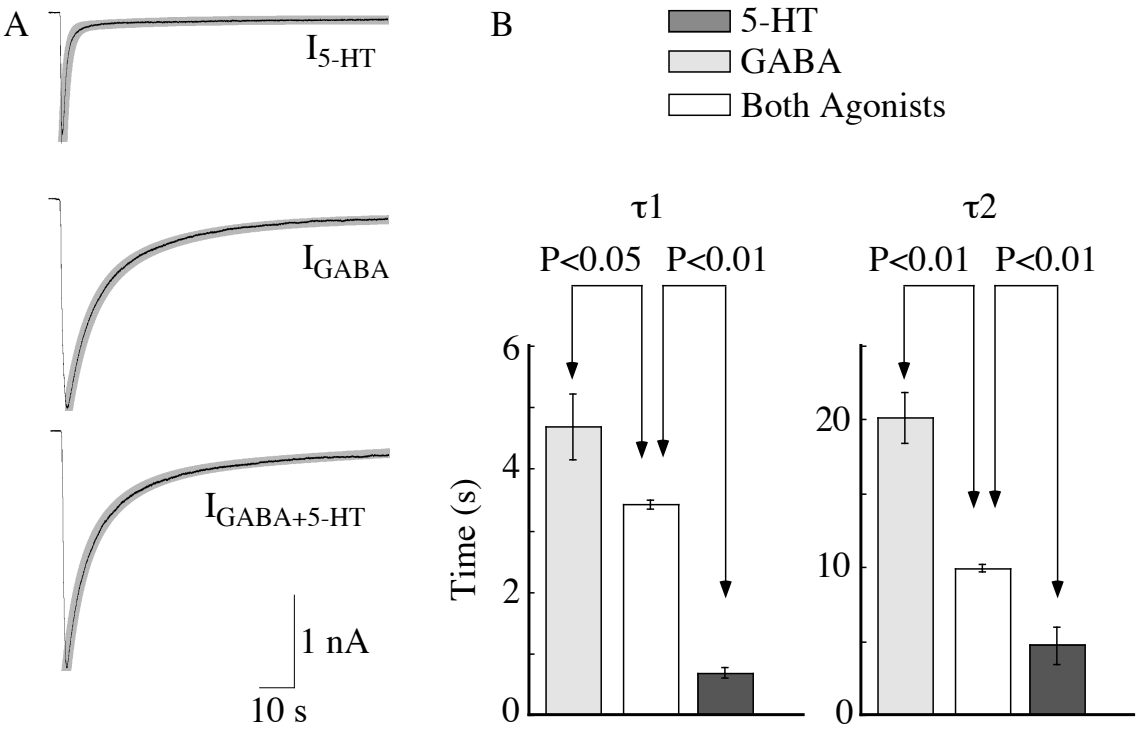


Figure 3.4 Desensitization kinetics of $I_{GABA+5\text{-}HT}$ is not explained by the desensitization kinetics of I_{GABA} or $I_{5\text{-}HT}$ alone

A: tracings are mean currents from a total of seven analized myenteric neurons. Desensitization segment of $I_{GABA+5\text{-}HT}$ and $I_{5\text{-}HT}$ was best fitted by the sum of three exponential functions (thick grey lines), whereas I_{GABA} was best fit by the sum of two. τ values of the third exponentials (τ3) were larger than the analyzed recording period and were rejected for further analysis. Note that $I_{GABA+5\text{-}HT}$ desensitized faster than I_{GABA} but slower than $I_{5\text{-}HT}$. B: bars represent mean ± S.E.M. values of the t of these exponential functions. t1 and t2 of I_{GABA} were larger (P<0.05) than t1 and t2 of $I_{GABA+5\text{-}HT}$, respectively. t1 and t2 of $I_{5\text{-}HT}$ were smaller (P<0.01) than t1 and t2 of $I_{GABA+5\text{-}HT}$, respectively. In these experiments agonists were applied for ~90 s and the holding potential was -60 mV.

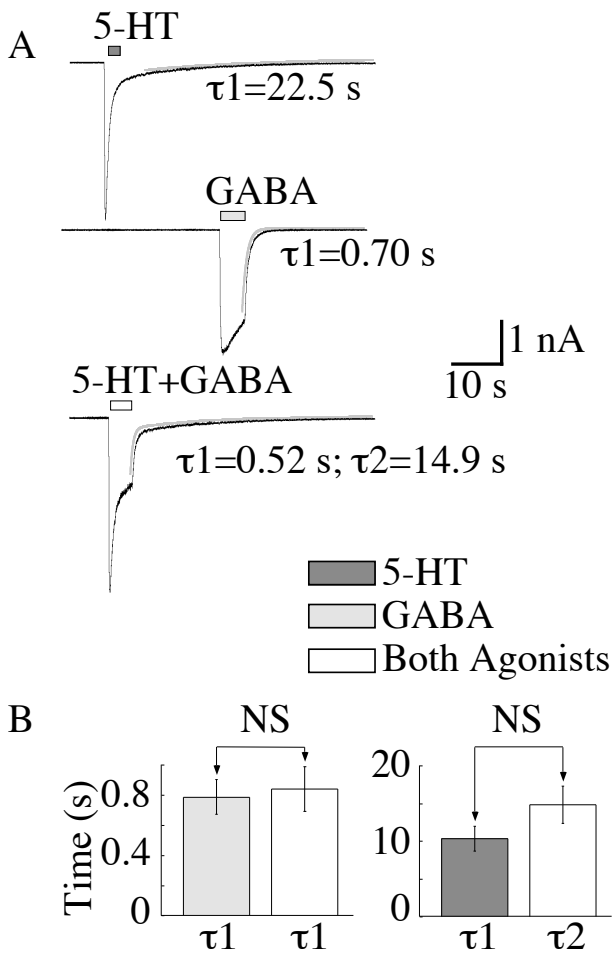


Figure 3.5 Decay kinetics of $I_{GABA+5\text{-}HT}$ can be explained by the combined decay kinetics of I_{GABA} or $I_{5\text{-}HT}$.

A: representative currents induced by GABA (I_{GABA}), 5-HT ($I_{5\text{-}HT}$), and GABA+5-HT ($I_{GABA+5\text{-}HT}$) are from the same myenteric neuron. Decay of $I_{GABA+5\text{-}HT}$ was best fitted by the sum of two exponential functions (thick grey line), whereas I_{GABA} and $I_{5\text{-}HT}$ desensitization were best fitted by a single exponential function. τ of these exponential functions are indicated in seconds. B: bars represent the mean ± S.E.M. values (n = 10) of τ values of these exponential functions. τ_1 of I_{GABA} was not significantly (NS) different from τ_1 of $I_{GABA+5\text{-}HT}$. τ_1 of $I_{5\text{-}HT}$ did also not differ from τ_2 of $I_{GABA+5\text{-}HT}$. Exponential fits were performed using the data from a couple hundred milliseconds after removing the agonists to the "steady-state" component. Experiments were performed at the holding potential of -60 mV.

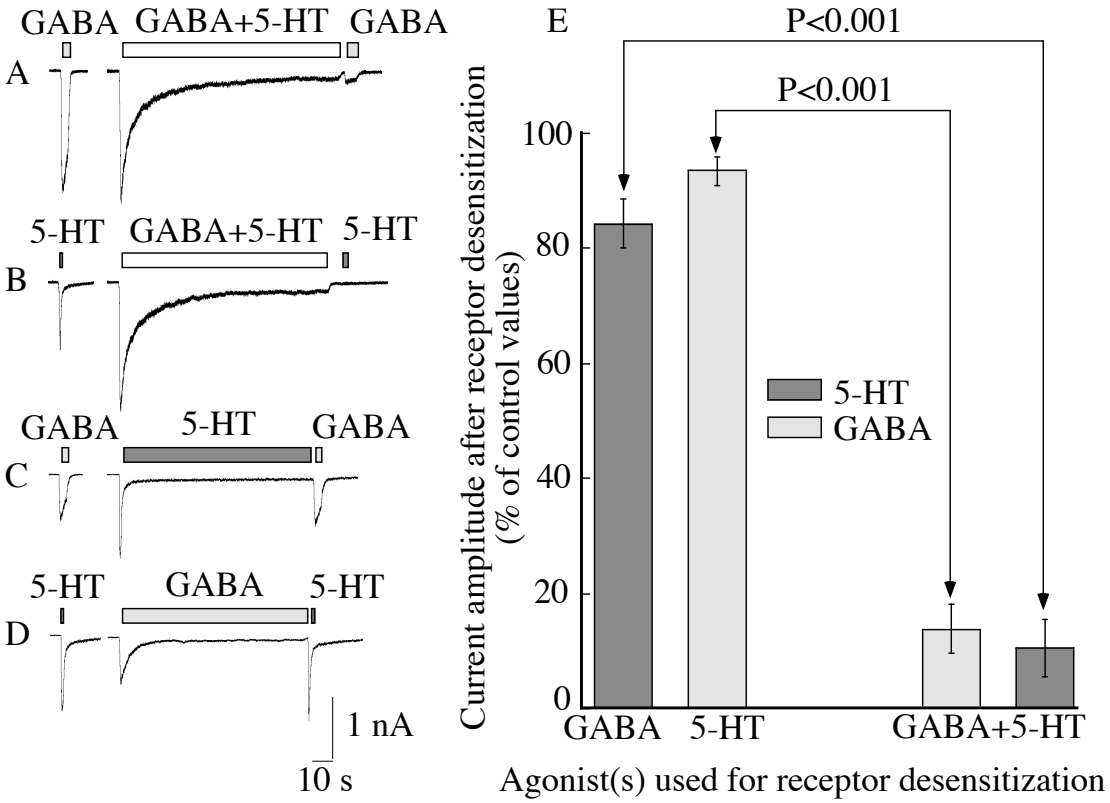


Figure 3.6 Application of GABA+5-HT induced desensitization of both $GABA_A$ and $5\text{-}HT_3$ receptors, which was not due to cross-desensitization between these receptors

Control I_{GABA} (A and C) and $I_{5\text{-}HT}$ (B and D) was recorded five minutes before (left recordings) and immediately after (~5 s), the prolonged application of both agonists (A and B), 5-HT (C), or GABA (D). E: average amplitude of I_{GABA} and $I_{5\text{-}HT}$ recorded after the prolonged application of GABA, 5-HT or GABA+5-HT, as a percentage of control response (n=13). Lines on top of the bars represent S.E.M. Recordings are from two neurons taken at the holding potential of -60 mV.

3.4.6 Current occlusion requires channel activation

In a previous study, it was demonstrated that serotonin molecules directly block nACh channels of enteric neurons (Barajas-López *et al.*, 2001), suggesting that current occlusion might be mediated by a similar mechanism. This hypothesis and other pre-receptor mechanisms are ruled out by the following observations.

In eight cells with a marginal or no response to GABA, $I_{GABA+5\text{-}HT}$ had similar amplitude (-1.20±0.38 nA) and kinetics to $I_{5\text{-}HT}$ (-1.10±0.16 nA; Fig 3.7A). In another six cells in which I_{GABA} was totally blocked with bicuculline (30 μM), $I_{GABA+5\text{-}HT}$ had similar amplitude (-1.72±0.25 nA) and kinetics to $I_{5\text{-}HT}$ (-1.72±0.24 nA; Fig 3.7B).

In seven cells with a marginal or no initial response to 5-HT, $I_{GABA+5\text{-}HT}$ had a similar amplitude (-2.54±0.73 nA) and kinetics to I_{GABA} alone (-2.38±0.77 nA; Fig 3.7C). Similarly, in another six neurons, in which $I_{5\text{-}HT}$ (-72±20 pA) had previously been inhibited with ondansetron, $I_{GABA+5\text{-}HT}$ had similar amplitude (-2.36±0.81 nA) and kinetics to I_{GABA} (-2.21±0.67 nA; Fig 3.7D).

These observations together with the ones described in the previous paragraph indicate that current occlusion is mediated by a post-receptor mechanism(s). These data also rule out the possibility that occlusion is mediated by 5-HT molecules acting on $GABA_A$ channels (an effect demonstrated before for 5-HT on native nACh channels (Barajas-López *et al.*, 2001)), or GABA molecules acting on $5\text{-}HT_3$ channels.

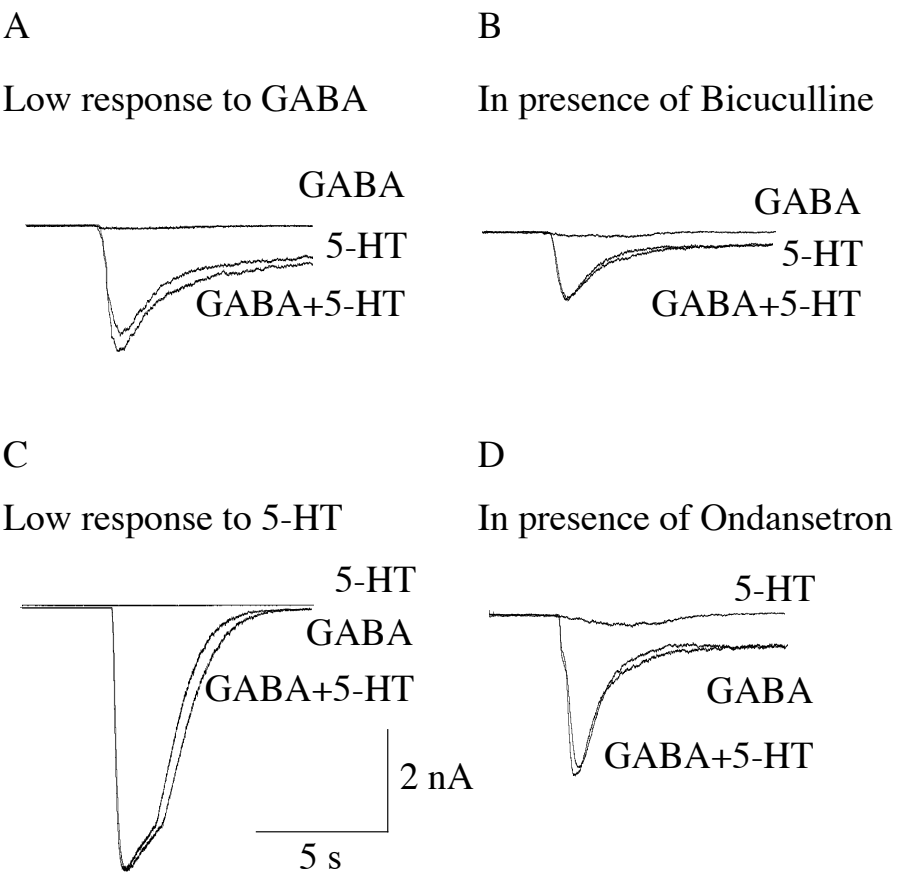


Figure 3.7 Inhibitory interactions between 5-HT_3 and $GABA_A$ receptors required the presence of functional channels.
A: recordings from a neuron in which GABA did not induce any current (I_{GABA}) but 5-HT induced a prominent inward current ($I_{5\text{-}HT}$). Note that GABA did not alter either the amplitude or kinetics of $I_{5\text{-}HT}$. B: similar results were obtained when the I_{GABA} was blocked by 30 μM bicuculline. C: recordings from a neuron with no response to 5-HT but with a prominent I_{GABA}. Note that 5-HT did not modify either the amplitude or kinetics of I_{GABA}. D: similar results were obtained when the $I_{5\text{-}HT}$ was blocked by 3 μM ondansetron.

3.4.6 Role of protein phosphorylation and intracellular Ca^{2+} in current occlusion

5-HT and GABA are also known to activate metabotropic receptors (5-HT and $GABA_B$) in enteric neurons (Cherubini & North, 1984, 1985; Gershon, 1999; Krantis, 2000). Activation of these receptors would lead to activation of G-proteins, changes in second messengers, activation of protein kinases, and protein phosphorylation. Therefore, one possibility is that at least part of the current occlusion observed here could be mediated by activation of metabotropic receptors. The fact that bicuculline and ondansetron prevents the effects of GABA on $I_{5\text{-}HT}$ and 5-HT actions on I_{GABA}, suggests that activation of metabotropic receptors are not required for current occlusion. The following observations are in agreement with such an interpretation.

Current occlusion was still observed after inhibiting protein phosphorylation (Fig 3.8) by either lowering the temperature to 11°C or adding a non-specific protein kinase inhibitor (5 μM staurosporine) to the internal solution (Ruegg & Burgess, 1989).

$5\text{-}HT_3$ receptors are permeable to Ca^{2+}, which suggests that current occlusion is mediated by a increase in the intracellular concentration of this ion. Against this hypothesis, however, the current occlusion was still observed in total absence of Ca^{2+} in the extracellular and intracellular medium (Fig 3.8). These experiments were carried out with the standard intracellular and extracellular solutions but the latter containing no Ca^{2+} plus 50 μM EGTA.

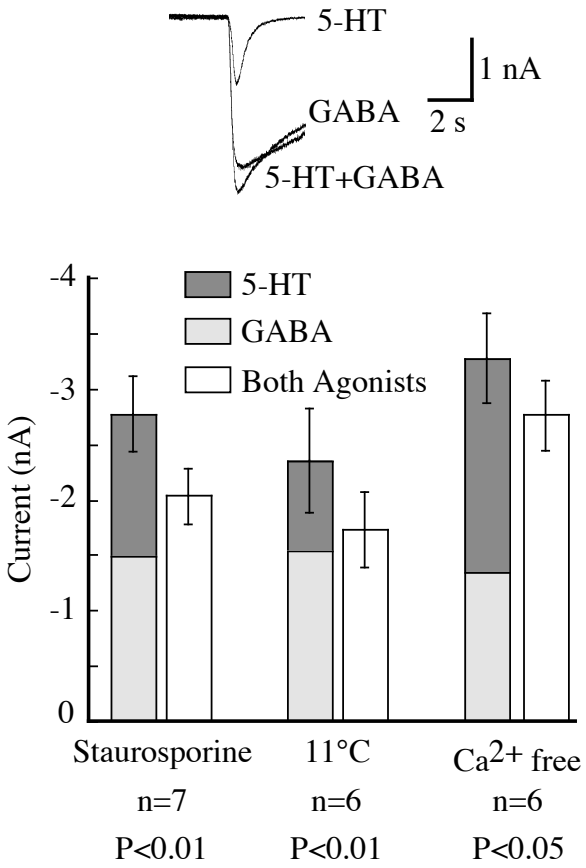


Figure 3.8 Calcium ions and protein phosphorylation are not required for current occlusion

Average amplitude of inward currents induced by application of GABA (I_{GABA}), 5-HT ($I_{5\text{-}HT}$) or GABA+5-HT ($I_{GABA+5\text{-}HT}$) in three different experimental groups of myenteric neurons. Average values of each group, are represented by a pair of bars. The first bar of each pair is a combined bar and shows $I_{5\text{-}HT}$ and I_{GABA} before application of GABA+5-HT. This combined bar represents the average expected current ($I_{Expected} = I_{GABA} + I_{5\text{-}HT}$). The second bar represents the mean $I_{GABA+5\text{-}HT}$. Error lines on the top of the bars are mean ± S.E.M. for $I_{Expected}$ and $I_{GABA+5\text{-}HT}$. Ca^{2+}-free experiments were carried out in 0-Ca^{2+}, plus 50 μM EGTA in the extracellular media, and using the standard intracellular solution in the pipette. Some experiments (n=6) were performed at 11°C and a set of recordings from a typical experiment are shown in the inset. Staurosporine (5 μM) experiments were carried out adding this protein kinase inhibitor to the pipette solution. All these experiments were carried out at a holding potential of -60 mV.

3.4.7 Current occlusion is concentration dependent

According to our observations, a model that considers a cross inhibition between $GABA_A$ and $5\text{-}HT_3$ channels, due to allosteric changes in these proteins, might explain the occlusion between $I_{5\text{-}HT}$ and I_{GABA}. In agreement with this model, we observed that currents induced by a concentration of 30 μM 5-HT and 30 μM GABA, were additive (n=6; Fig 3.9).

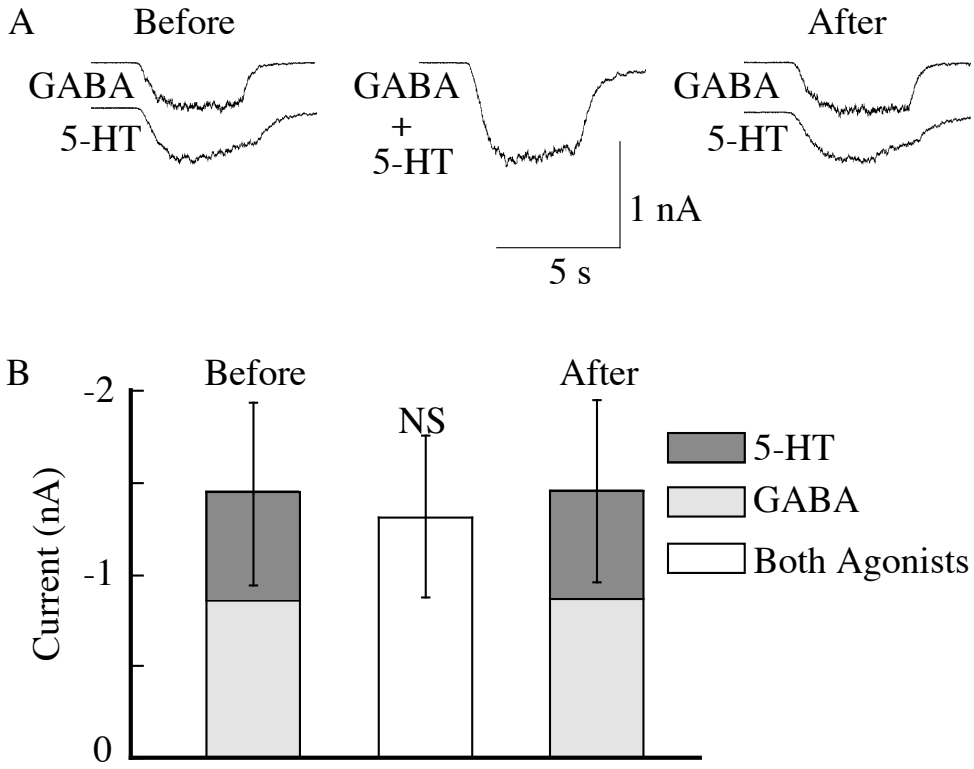


Figure 3.9 Current occlusion requires maximal activation of the channels

A: Currents induced by application of non-saturating concentrations of GABA (30 μM), 5-HT (30μM), or by GABA+5-HT recorded from the same myenteric neuron. B: Average (bars) membrane currents and mean ± S.E.M. of six similar experiments. In these experiments $I_{GABA+5\text{-}HT}$ was no different (no significantly, NS) from the sum of the individual currents. Experiments were carried out at a holding potential of -60 mV.

3.5 Discussion

This is the first demonstration that simultaneous activation of $GABA_A$ and 5-HT_3 receptors is not independent and ionic currents carried through them are occluded when they are simultaneously activated. This current occlusion is observed as soon as currents are activated, and does not require Ca^{2+} or protein phosphorylation. Altogether, these observations suggest that this current occlusion is mediated by cross-inhibition and by a direct interaction between $GABA_A$ and 5-HT_3 channels. Similar interactions to the ones proposed here have been shown between nACh and P2X receptors (Barajas-López *et al.*, 1998; Zhou & Galligan, 1998; Khakh *et al.*, 2000), between $GABA_A$ and P2X receptors (Sokolova *et al.*, 2001; Karanjia *et al.*, 2006). Interactions of metabotropic receptors have also been reported between dopamine (D2) and somatostatin (SSTR5) receptors (Rocheville *et al.*, 2000), and between dopamine (D5) and $GABA_A$ channels (Liu *et al.*, 2000). This is the second study that demonstrates interactions between two members of the *Cys-loop* family, which would suggest the existence of inhibitory interactions between the same populations of ligand-gated channels (p. e. $GABA_A$). This would imply that interactions between receptor proteins play a major role in neuronal signalling in many if not all synapses. This hypothesis, however, requires further experimental analysis and it is clearly out of the scope of the present study.

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

The whole cell currents induced by either GABA (300 μM) or 5-HT (1 mM), despite these relatively high concentrations, are mediated by pharmacologically

distinct receptors. This is demonstrated by the specific inhibitory effect of ondansetron (a 5-HT_3 receptor antagonist (Hoyer *et al.*, 1994)) on $I_{5\text{-}HT}$, and by the more potent effect of bicuculline (a $GABA_A$ antagonist (Li *et al.*, 2003)) on I_{GABA} than on $I_{5\text{-}HT}$. Activation of these channels is, however, not independent as shown by the fact that inward currents carried through $GABA_A$ and 5-HT_3 receptors were not additive when maximally activated.

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

Enteric neurons also express metabotropic GABA and 5-HT receptors (Cherubini & North, 1984, 1985; Derkach *et al.*, 1989; Zhou & Galligan, 2000), which are known to be linked to second messengers and protein phosphorylation cascades (Gershon, 1999; Krantis, 2000). Several of our observations, however, indicate that activation of these receptors is not required for the current occlusion observed here. First, current occlusion occurs, as soon as $GABA_A$ and 5-HT_3 channels are activated, indicating that this occlusion is as fast as the activation of these ligand-gated channels. Second, bicuculline prevents effects of GABA on the 5-HT_3 channels (present study) and ondansetron prevented the effect of 5-HT on the $GABA_A$ channels. Third, inhibition of protein phosphorylation (with staurosporine or by lowering experimental temperature to 11ºC) did not prevent the occlusion observed between I_{GABA} and $I_{5\text{-}HT}$.

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

At least three different observations indicate that $I_{GABA+5\text{-}HT}$ is carried through both $GABA_A$ and 5-HT_3 channels. 1) $I_{GABA+5\text{-}HT}$ desensitizes faster than I_{GABA} but slower than $I_{5\text{-}HT}$. 2) The decay of $I_{GABA+5\text{-}HT}$ resembles the decay of both I_{GABA} and $I_{5\text{-}HT}$. 3) When GABA and 5-HT are applied simultaneously, 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 desensitized individually. Altogether, these observations also imply that inhibition between these channels is reciprocal.

3.5.4 Functional implications for these channel interactions

The present and previous 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. Thus functional interaction such as one demonstrated here has been shown to exist between P2X and nicotinic channels in enteric neurons (Barajas-López *et al.*, 1998; Zhou & Galligan, 1998) and between P2X and other ligand-gated channels ($GABA_A$) in enteric (Karanjia *et al*., 2006) and dorsal root ganglion neurons (Sokolova *et al.*, 2001). Finally, the present study and that of Li *et al.* (Li *et al.*, 2003), show that two structurally related members (Ortells & Lunt, 1995) can interact indicating that the same type of channel (p. e. $GABA_A$) might also maintain cross-interactions between themselves and implying that this type of interactions might play an essential role in synaptic integration.

In conclusion, our results indicate that there is a very fast inhibitory interaction between $GABA_A$ and $5\text{-}HT_3$ channels. These interactions occur as fast as the activation of $GABA_A$ and $5\text{-}HT_3$, 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. Since these channels are structurally similar, it might be possible that these interactions are present between the same channels of the same type (p. e. $5\text{-}HT_3$) implying an important role in neuronal signalling.

Capítulo 4

4 Direcciones futuras

Varios estudios muestran la importancia del dominio carboxilo terminal de los receptores P2X y el asa intracelular (entre el dominio M3 y M4) de los receptores *Cys-loop* en el mecanismo de la interacción inhibitoria (Boue-Grabot *et al.*, 2003; Boue-Grabot *et al.*, 2004a; Boue-Grabot *et al.*, 2004b). Sin embargo, también existen interacciones entre miembros de la superfamilia *Cys-loop*, como fue mostrado por Li *et al.* (2003) y por nosotros (capítulo 3), sugiriendo que el asa intracelular podría ser la responsable de la interacción de estos canales. Esta es una de las hipótesis que están siendo investigadas actualmente en nuestro laboratorio.

Los canales de la superfamilia *Cys-loop* están estructuralmente relacionados (Ortells & Lunt, 1995) y mantienen una secuencia de aminoácidos muy similar, típicamente tienen del 30 al 40% de identidad (Absalom *et al.*, 2004). El hecho de que canales *Cys-loop* mantengan interacciones inhibitorias sugiere que los canales del mismo tipo (p.ej., $GABA_A$) puedan inhibirse de manera similar. De ser éste el caso, las interacciones entre canales jugarían un papel central en la integración sináptica. En apoyo a esta hipótesis los LGIC se localizan en la membrana postsináptica en densidades altas (Kandel & Siegelbaum, 2000). Además, las concentraciones de neurotransmisor en la hendidura sináptica parecen ser lo suficientemente altas como para saturar los receptores (Clements *et al.*, 1992). El análisis experimental de esta hipótesis requiere de la creación de un canal artificial que puedan ser activado con un agonista distinto y que sólo forme canales homoméricos.

En conclusión, nuestros resultados sugieren que las interaciones inhibitorias juegan un papel fundamental en la comunicación neuronal.

Capítulo 5

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Capítulo 6
(Anexos)

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Cross-inhibitory interactions between $GABA_A$ and P2X channels in myenteric neurones

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Keywords: enteric nervous system, $GABA_A$ channels, guinea pig, ion channels, ligand-gated channels, P2X channels

Abstract

Inhibitory interactions between $GABA_A$ [induced by γ-aminobutyric acid (GABA)] and P2X [activated by adenosine 5′-triphosphate (ATP)] receptors of myenteric neurones from the guinea pig small intestine were characterized using whole-cell recordings. Currents induced by GABA (I_{GABA}) or ATP (I_{ATP}) were inhibited by picrotoxin or pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid, respectively. Currents induced by GABA + ATP ($I_{GABA+ATP}$) were only as large as the current induced by the most effective transmitter, revealing current occlusion. This occlusion requires maximal activation of at least one of these receptors. Sequential applications of neurotransmitters, and kinetic and pharmacological properties of $I_{GABA+ATP}$ indicate that they are carried through both $GABA_A$ and P2X channels. ATP did not affect I_{GABA} in neurones: (i) in which P2X channels were not present; (ii) after inhibiting P2X channels with Ca^{2+} (iii) in the presence of pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid, a P2X receptor antagonist; (iv) after P2X receptor desensitization or (v) at I_{ATP} reversal potential. Similarly, GABA did not affect P2X-mediated currents in neurones: (i) in which $GABA_A$ channels were not present; (ii) in the presence of picrotoxin, a $GABA_A$ channel blocker; (iii) after $GABA_A$ receptor desensitization or (iv) at the I_{GABA} reversal potential. Current occlusion occurred as fast as current activation and it was still present in the absence of Ca^{2+}, at 11 °C, after adding to the pipette solution a cocktail of protein kinase inhibitors (staurosporine + genistein + K-252a), after substituting the GTP in the pipette with GDP-β-S and after treating the cells with *N*-ethylmaleimide. Taken together, all of these results are consistent with a model of cross-inhibition between $GABA_A$ and P2X.

Introduction

γ-Aminobutyric acid (GABA) and adenosine 5′-triphosphate (ATP) are known to play a role as neurotransmitters (DeFeudis, 1990; Evans *et al.*, 1992; Silinsky & Gerzanich, 1993; Zhou & Galligan, 1996) by directly activating $GABA_A$ chloride and P2X cationic channels on the postsynaptic membrane. $GABA_A$ receptors are part of the Cys-loop superfamily of ligand-gated ion channels and 20 different subunits have been cloned. P2X receptors belong to a different ligand-gated channel superfamily and seven different P2X subunits have been cloned (Dunn *et al.*, 2001; Khakh *et al.*, 2001; North, 2002). Each of the $GABA_A$ subunits has four transmembrane domains (Cockcroft *et al.*, 1990; Ortells & Lunt, 1995), whereas P2X subunits appear to cross the membrane only twice (Dunn *et al.*, 2001; Khakh *et al.*, 2001; North, 2002). P2X subunits appear to form both homomeric and heteromeric channels whereas the $GABA_A$ channels are predominantly heteromeric pentomers.

Pioneering studies have shown that nicotinic acetylcholine (nACh) and P2X channels are not independent (Nakazawa, 1994) and that they can inhibit each other when they are simultaneously activated (Barajas-López *et al.*, 1998; Zhou & Galligan, 1998). This inhibitory interaction is very fast and might be mediated by an allosteric interaction between nACh and P2X channels. In favour of this hypothesis, $P2X_2$ and $\alpha_4\beta_2$ channels appear to form complexes with channels lying ~80 Å apart (Khakh *et al.*, 2005). Analogous interactions have been shown between P2X and $5\text{-}HT_3$ receptors in enteric neurones (Barajas-López *et al.*, 2002; Boue-Grabot *et al.*, 2003), and P2X and the $GABA_A$ receptors in dorsal root ganglia (Sokolova *et al.*, 2001). In a more recent study carried out in *Xenopus* oocytes (Boue-Grabot *et al.*, 2004b), it was found that the C-terminal domain of $P2X_2$ and the intracellular loop of $GABA_A$ subunits are required for the functional interaction between ATP- and GABA-gated channels. In apparent contradiction with these studies, in myenteric neurones, P2X channels were reported to interact specifically with nACh and not with other members of the Cys-loop superfamily present in these neurones (e.g. $GABA_A$ and $5\text{-}HT_3$ receptors; Zhou & Galligan, 1998). This discrepancy might indicate that these interactions are tissue specific, probably arising from receptor heterogeneity (see Boue-Grabot *et al.*, 2004b) and hence requiring further experimental analysis. Adding to this complexity, other types of pharmacological interactions appear to exist between the serotoninergic and cholinergic systems. Thus, it has been reported that serotonin molecules can themselves block nACh channels in various cell types including submucosal neurones and this occurs at concentrations

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Apéndice 2

Cross-Inhibitory Interactions Between $GABA_A$ and $5\text{-}HT_3$ Channels in Myenteric Neurons

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KEY WORDS γ-Aminobutyric acid (GABA); serotonin (5-HT); Neurotransmitters; Protein interactions; ligand-gated ion channels; *Cys-loop* receptor superfamily

ABSTRACT Inhibitory interactions between $GABA_A$ [activated by γ-aminobutyric acid (GABA)] and $5\text{-}HT_3$ [activated by serotonin (5-HT)] receptors of myenteric neurons from the guinea pig small intestine were characterized using whole-cell recordings. Currents induced by GABA (I_{GABA}) or by 5-HT ($I_{5\text{-}HT}$) were inhibited by bicuculline or ondansetron, respectively. Currents induced by GABA+5-HT ($I_{GABA+5\text{-}HT}$) were significantly lower than the sum of I_{GABA} and $I_{5\text{-}HT}$, revealing a current occlusion. This occlusion requires maximal activation of these receptors. Kinetic and pharmacological properties of $I_{GABA+5\text{-}HT}$ indicate that they are carried through both $GABA_A$ and $5\text{-}HT_3$ channels. 5-HT did not affect I_{GABA} in neurons in which $5\text{-}HT_3$ channels were not present or after inhibiting $5\text{-}HT_3$ channels with ondansetron; a $5\text{-}HT_3$ receptor antagonist. Similarly, GABA did not affect 5-HT mediated currents in neurons in which $GABA_A$ channels were not present or in the presence of bicuculline, a $GABA_A$ receptor antagonist. Current occlusion requires maximal activation of $GABA_A$ and $5\text{-}HT_3$ receptors, occurred as fast as current activation, was present in the absence of Ca^{2+}, at low temperature (11ºC), and after adding to the pipette solution a protein kinase inhibitor (staurosporine). These results are consistent with a model of cross-inhibition between $GABA_A$ and $5\text{-}HT_3$ channels. ENVIADO.

INTRODUCTION

γ-Aminobutyric acid (GABA) and serotonin (5-HT) are known to play a role as neurotransmitters (DeFeudis, 1990; Sugita *et al.*, 1992) by directly activating $GABA_A$ (chloride) and $5\text{-}HT_3$ (cationic) channels, respectively. Both are part of the *Cys-loop* ligand-gated ion channels superfamily. Twenty different $GABA_A$ subunits have been cloned whereas for $5\text{-}HT_3$ subunits only three have been cloned (Reeves & Lummis, 2002). Each of the ligand-gated subunits has four transmembrane domains (Cockcroft *et al.*, 1990b; Ortells & Lunt, 1995). $5\text{-}HT_3$ subunits appear to form both homomeric and heteromeric channels while the $GABA_A$ channels are predominantly heteropentamers.

Various studies carried out in peripheral neurons have demonstrated that P2X native channels can establish an inhibitory interaction with members of the *Cys-loop* ligand-gated channels superfamily. In particular, with nicotinic channels (nACh) (Nakazawa, 1994; Barajas-López *et al.*, 1998; Zhou & Galligan, 1998), with $5\text{-}HT_3$ channels (Barajas-López *et al.*, 2002; Boue-Grabot *et al.*, 2003), and with $GABA_A$ channels (Sokolova *et al.*, 2001; Karanjia *et al.*, 2006). This inhibitory interaction is very fast and might be mediated by an allosteric interaction between P2X and the mentioned *Cys-loop* ligand-gated channels. In favour of this hypothesis, $P2X_2$ and the $\alpha_4\beta_2$ nACh channels appear to form complexes, with channels lying ~80 Å apart (Khakh *et al.*, 2005). In a

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recent study carried out in *Xenopus* oocytes (Boue-Grabot *et al.*, 2004b), it was found that the C-terminal domain of $P2X_2$ and the intracellular loop of $GABA_A$ subunits are required for the functional interaction between ATP- and GABA-gated channels.

So far, only Li et al., (Li *et al.*, 2003) have reported the existence of similar interactions between two members of the same *Cys-loop* channel superfamily. These authors described the presence of an asymmetric cross-inhibition between glycine and $GABA_A$ channels, which are both permeable to Cl^-. Therefore, our aim, in the present study, was to investigate and to characterize the putative inhibitory interactions between $GABA_A$ and 5-HT_3 native receptors of myenteric neurons. Our findings indicate that activation of 5-HT_3 and $GABA_A$ receptors opens two different channel populations. These two channels, however negatively modulate each other when they are simultaneously and maximally activated. This inhibitory interaction occurs simultaneously with current activation and does not require Ca^{2+} or protein phosphorylation. Altogether, these results imply that it is mediated by allosteric interactions between these receptors.

MATERIALS AND METHODS

Young guinea pigs (150-200 g), either male or female, were killed by decapitation and a segment of five cm of proximal jejunum 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 and submucosal layers of this intestinal segment were dissected, before removing most of the circular muscle layer leaving behind the longitudinal layer with the myenteric plexus embedded with it. All procedures involving animals had the prior approval of the Queen's University Animal Care Committee.

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

5-HT and GABA are known to modulate various membrane ion channels of enteric neurons via G-protein linked receptors (Cherubini & North, 1984, 1985; Galligan *et al.*, 1988; Wang *et al.*, 1996; Gershon, 1999; Krantis, 2000). In order to decrease the involvement of membrane currents other than those mediated by activation of ligand-gated channels, the experiments were carried out in the presence of Cs^+ (a potassium channel blocker). Furthermore, 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 from myenteric neurons using an Axopatch 1D amplifier (Molecular Devices, Sunnyvale, CA, USA). Short-term (2-80 hours) primary cultures were used to prevent space-clamp problems due to neurite growth,

which become a common problem on the fourth day after plating the neurons. Patch pipettes were made as previously described (Barajas-López *et al.*, 1996b) and had resistances between 2-4 MΩ. These low resistance electrodes and a slight suction inside the pipette help to maintain a low access resistance (usually 6 MΩ). Sixty to ninety-five percent of the series resistance was compensated in 9 neurons of the experiments reported here. This compensation, however, did not affect the lack of additivity of $I_{5\text{-}HT}$ and I_{GABA} (see Results). The holding potential was -60 mV. To ensure the best voltage clamp, we rejected the data of neurons showing fast Na-mediated inward currents, during neurotransmitter stimulation. The standard solutions used had the following compositions, inside the pipette (in mM): 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 calculated junction potential (using the PCLAMP software, Molecular Devices) for the pipette was -5 mV however, holding potential described here were not adjusted. Under these conditions, the usual input resistance of the neurons was ≥1 GΩ. Whole-cell currents were recorded on a PC using AXOTAPE software (Molecular Devices) and analyzed on a Macintosh computer using AXOGRAPH software (Molecular devices). 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-tubes device, with the tubes connected to syringes containing the control and experimental solutions. The tube containing the control solution was placed in front of the cell being recorded and the external application of experimental substances was achieved by abruptly changing this tube for a tube delivering the same solution plus the drug(s). Experimental substances were removed by returning back to the control solution. External solutions were delivered by gravity and the level of the syringes was continuously adjusted to minimize changes in the flow rate. Experiments, unless otherwise stated, were performed at room temperature (~23°C). Experiments at 11°C were carried out using the eight-tubes device jacketed with a segment (~6 cm long) from a plastic pipette (10 ml). This plastic jacket was perfused with ice-chilled water so that the temperature in front of the delivering tube was at 11°C.

Bicuculline was purchased from Research Biomedical Inc. (Natick, MA, USA) and staurosporine from Kamiya (Thousand Oaks, CA, USA). Ondansetron was purchased from Glaxo Smithkline (Parma, Italy) and all other substances from Sigma (St. Louis, MO., USA).

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 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.

Apéndice 2

RESULTS

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

Electrophysiological and pharmacological properties of the membrane conductances and whole-cell currents activated by GABA (I_{GABA}) and 5-HT ($I_{5\text{-}HT}$) in enteric neurons have been previously characterized (Cherubini & North, 1984, 1985; Derkach *et al.*, 1989; Zhou & Galligan, 2000). These whole-cell currents are mediated by activation of channels permeable to chloride ($GABA_A$) and cations ($5\text{-}HT_3$), respectively.

Using the standard chloride internal solution, individual applications of GABA and 5-HT induced inward currents at a holding potential of -60 mV. Concentration-response curves (not shown) were obtained for these transmitters and analyzed as previously reported (Barajas-López *et al.*, 2002). The EC_{50} values for GABA and 5-HT were 104 and 55 μM, whereas the Hill coefficient values were 1.3±0.1 and 1.0±0.1 (not significantly different than unity), respectively. The current amplitude induced by maximal concentrations of GABA (0.3 mM) and 5-HT (1 mM) was variable in different cells but a typical value was between 1-3 nA (range from only few pA up to -6.9 nA). However, currents were included in the analysis when their amplitude was at least 200 pA. In these cells, the mean peak-amplitude of I_{GABA} and $I_{5\text{-}HT}$ -2.19±0.18 (n=106 out of 139) and -1.48±0.09 nA (n=124 out of 136), respectively. A larger variability was noticed in the amplitude of I_{GABA} than in $I_{5\text{-}HT}$. The amplitude of these currents was independent of each other and in fact some cells only display I_{GABA} (12 out of 136) or $I_{5\text{-}HT}$ (33 out of 139 neurons), indicating that these channels are expressed independently in myenteric neurons.

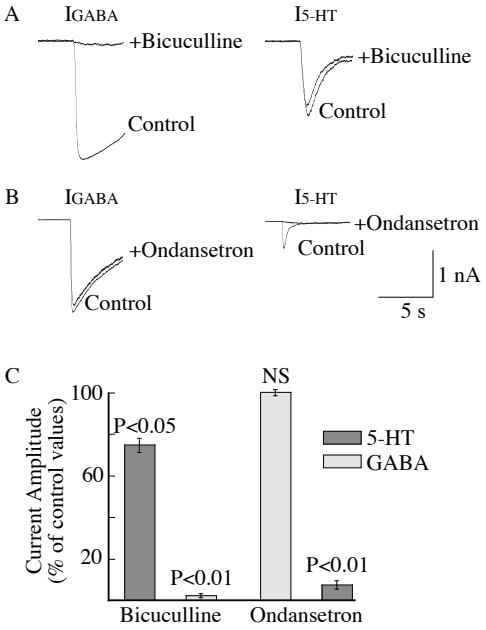

Figure 1 Whole-cell currents induced by 5-HT ($I_{5\text{-}HT}$) and GABA (I_{GABA}) are mediated by two distinct receptors

A: Bicuculline (30 μM; a $GABA_A$ receptor antagonist) blocks I_{GABA}. **B**: Ondansetron (3 μM; a $5\text{-}HT_3$ receptor antagonist) blocks $I_{5\text{-}HT}$ without affecting I_{GABA}. **C**: mean ± S.E.M. values (bars and lines on their top) obtained from six bicuculline experiments. **A** and **B** were measured from two different myenteric neurons, at a holding potential of –60 mV, and were induced by GABA (0.3 mM) and 5-HT (1 mM). Only the initial portion of the currents (~5 s) is shown, which were recorded before (Control) and five min after starting the receptor antagonist application. Notice that despite the relative high concentrations of the receptor agonists (GABA and 5-HT) and ondansetron their effects are, as expected, on only one of the channels. Bicuculline effect was not specific, thus, it totally inhibited I_{GABA} but only blocked ~25% of $I_{5\text{-}HT}$.

As shown in Figures 1A-C, inward currents induced by GABA (0.3 mM) and 5-HT (1 mM) were totally inhibited by bicuculline (30 μM; n=6) and ondansetron (0.3 μM; n=5), respectively. Ondansetron was especific on $I_{5\text{-}HT}$ and did not affect I_{GABA}, however, bicuculline (30 μM) partially blocked 25% of $I_{5\text{-}HT}$. This later effect of bicuculline on $I_{5\text{-}HT}$ has previously been reported (Takenouchi & Munekata, 1998; Sun & Machu, 2000).

$I_{5\text{-}HT}$ and I_{GABA} onsets were virtually the same. Thus the time required to reach the half-maximal current was not significantly different in six analyzed cells. This time had mean values of 191±33 and 174±35 ms for I_{GABA} and $I_{5\text{-}HT}$, respectively. These currents usually reached their peak within the following second. After reaching their maximal amplitude the currents decreased despite the continuous presence of the transmitters (current inactivation), indicating receptor tachyphylaxis. Desensitization of $GABA_A$ receptors was clearly slower than the one observed for 5-HT_3 receptors. In seven analyzed cells treated with long-term (90 s) applications of GABA, receptor desensitization was better fitted by the sum of two exponential functions (τ values of these functions are given below). In the same seven neurons treated with long-term (90 s) applications of 5-HT, current desensitization was better fitted by the sum of three exponential functions (τ values of these functions are given below). $\tau 1$ and $\tau 2$ values of I_{GABA} were significantly different ($P<0.05$) than $\tau 1$ and $\tau 2$ values of $I_{5\text{-}HT}$. After removing the agonists from the external solution, currents decreased until they disappeared.

This decay was well fitted by a single exponential function with mean τ values of 0.8±0.1 and 10.4±1.6 s for I_{GABA} and $I_{5\text{-}HT}$, respectively (n=10). These values were significantly different ($P<0.001$).

Currents induced by GABA and 5-HT were not additive

The experiments described above demonstrated that I_{GABA} and $I_{5\text{-}HT}$ have different kinetics and that they are mediated by activation of pharmacologically distinct receptors. If the two currents are mediated by functionally independent ion channels then the currents induced by concentrations of GABA and 5-HT of 0.3 mM and 1 mM, respectively (when receptor occupancy would be expected to be 70% to 100%) should be additive. To investigate if this is the case, we measured the peak of $I_{5\text{-}HT}$, I_{GABA}, and the current induced by the simultaneous application of the same concentrations of both agonists ($I_{GABA+5\text{-}HT}$) in the same neuron.

It was found mathematical addition of individual currents yielded a current ($I_{5\text{-}HT}+ I_{GABA}=I_{Expected}$; -3.05±0.25 nA), which was significantly larger ($P<0.001$; n=33) than $I_{GABA+5\text{-}HT}$; (-2.51±0.24 nA), revealing an occlusion between I_{GABA} and $I_{5\text{-}HT}$ (Fig. 2A and 3.2B). In order to rule out the possibility that current occlusion was due to the whole-cell access resistance, sixty to ninety-five percent of this (ussually 6 MΩ) was compensated in nine out of the previously described 33 neurons. In these experiments, $I_{Expected}$ (-4.21±0.64 nA) was still significantly larger than $I_{GABA+5\text{-}HT}$ (-3.27±0.48 nA; $P<0.01$).

Current kinetics of responses induced by simultaneous application of both agonists

Figure 3 shows the average onset of I_{GABA}, $I_{5\text{-}HT}$, $I_{GABA+5\text{-}HT}$, and $I_{Expected}$ from nine analyzed neurons and demonstrates the presence of current

Apéndice 2

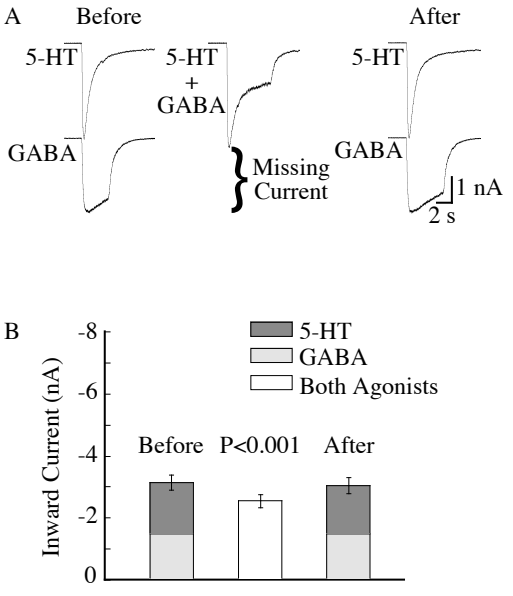

Figure 2 Whole-cell inward currents induced by GABA (I_{GABA}) and 5-HT ($I_{5\text{-}HT}$) are not additive, revealing a current occlusion

A: shows recordings from one neuron of a typical experiment and **B**: the average (bars) values of thirty-three experiments. Currents were induced by application of either GABA (0.3 mM) or 5-HT (1 mM) and by the simultaneous application of both agonists ($I_{GABA+5\text{-}HT}$). I_{GABA} and $I_{5\text{-}HT}$ were recorded five min before and five min after $I_{GABA+5\text{-}HT}$. **B**: the first and third bars show the mean values of I_{GABA} and $I_{5\text{-}HT}$, the addition of these currents represents expected current ($I_{Expected}$= I_{GABA}+$I_{5\text{-}HT}$). S.E.M. are shown as lines in the top of the bars for $I_{Expected}$ and $I_{GABA+5\text{-}HT}$.

occlusion since the beginning of their activation. This occlusion was significant (P<0.01) 15 ms after (time indicated by arrow) the beginning of these currents. At this time, the average $I_{Expected}$ was -100±16 pA and $I_{GABA+5\text{-}HT}$ -60±15 pA. In other words, current occlusion occurs rapidly and concomitantly with channel activation.

Figure 4A shows the average I_{GABA}, $I_{5\text{-}HT}$, $I_{GABA+5\text{-}HT}$, and $I_{Expected}$ from seven neurons exposed to long application (90 s) of the neurotransmitters. Visual inspection of these recordings revealed that $I_{GABA+5\text{-}HT}$ desensitized faster than I_{GABA} but slower than $I_{5\text{-}HT}$. To quantify this, exponential fits were performed using the data of the desensitization phase (inactivation). Desensitization of $I_{GABA+5\text{-}HT}$ was better fitted by the sum of three exponential functions, as with $I_{5\text{-}HT}$. τ values of the third exponentials ($\tau 3$) were larger than the analyzed recording period and were rejected for further analysis. τ values of the first ($\tau 1$) and second ($\tau 2$) exponentials for $I_{GABA+5\text{-}HT}$ were significantly different (P<0.05) than the correnpondent τ values of either $I_{5\text{-}HT}$ or I_{GABA} (Fig 4B).

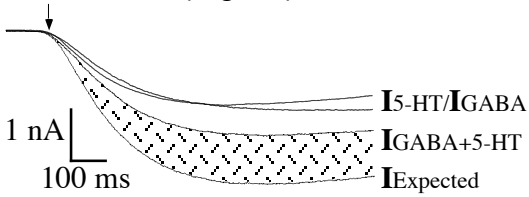

Figure 3 Onset of membrane currents indicate that occlusion is fast and bidirectional

Tracings are mean currents from a total of nine analyzed myenteric neurons. The current induced by simultaneous application of both agonists ($I_{GABA+5\text{-}HT}$) is always smaller than the expected current ($I_{Expected}$= I_{GABA}+$I_{5\text{-}HT}$). The difference between these two currents is highlighted by shadow area. $I_{GABA+5\text{-}HT}$ and $I_{Expected}$ are already significantly different (P<0.05) fifteen ms (instant indicated by arrow) after starting their activation. Whole-cell currents were measured at a holding potential of -60 mV and only the initial portion of currents (~1 s) is depicted.

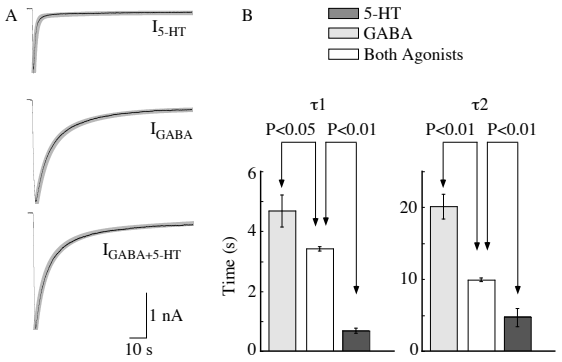

Figure 4 Desensitization kinetics of $I_{GABA+5\text{-}HT}$ is not explained by the desensitization kinetics of I_{GABA} or $I_{5\text{-}HT}$ alone

A: tracings are mean currents from a total of seven analized myenteric neurons. Desensitization segment of $I_{GABA+5\text{-}HT}$ and $I_{5\text{-}HT}$ was best fitted by the sum of three exponential functions (thick grey lines), whereas I_{GABA} was best fit by the sum of two. τ values of the third exponentials (τ3) were larger than the analyzed recording period and were rejected for further analysis. Note that $I_{GABA+5\text{-}HT}$ desensitized faster than I_{GABA} but slower than $I_{5\text{-}HT}$. B: bars represent mean ± S.E.M. values of the t of these exponential functions. t1 and t2 of I_{GABA} were larger (P<0.05) than t1 and t2 of $I_{GABA+5\text{-}HT}$, respectively. t1 and t2 of $I_{5\text{-}HT}$ were smaller (P<0.01) than t1 and t2 of $I_{GABA+5\text{-}HT}$, respectively. In these experiments agonists were applied for ~90 s and the holding potential was -60 mV.

After washing out the agonists from the external solution, $I_{GABA+5\text{-}HT}$ decay appears to be different from that of either I_{GABA} or $I_{5\text{-}HT}$ (Fig 5A and 5B). Indeed, the decay of $I_{GABA+5\text{-}HT}$ was well fitted by the sum of two exponential functions (n=10). We tested the hypothesis that these two exponential functions of $I_{GABA+5\text{-}HT}$ are the single exponentials of the $I_{5\text{-}HT}$ and I_{GABA} decay. Thus, average τ value of the first exponential (τ_1) was virtually the same to τ_1 of I_{GABA} decay and the average τ value of the second exponential (τ_2) of $I_{GABA+5\text{-}HT}$ did not differ from τ_1 of $I_{5\text{-}HT}$ decay (Fig 5B). These observations indicate that $I_{GABA+5\text{-}HT}$ is mediated by the opening of both 5-HT_3 and $GABA_A$ channels. In order to further investigate this interpretation, we test the hypothesis that the simultaneous application of both agonists desensitised both $GABA_A$ and 5-HT_3 receptors.

Simultaneous application of both agonists desensitized $GABA_A$ and 5-HT_3 receptors

In the following experiments, we measured the amplitude of both I_{GABA} and $I_{5\text{-}HT}$ before and immediately after (~5 s) a long application of GABA+5-HT. This long application lasted until the peak current had decreased more than 80% of its initial amplitude (usually within 90 seconds). Some typical recordings and the average data from such experiments are shown in Figs 6A-E. We observed that application of GABA+5-HT, decreased both I_{GABA} (Fig 6A) and $I_{5\text{-}HT}$ (Fig 6B) to less than 20% of their control amplitude (Fig 6E). Such an inhibition was not due to cross-desensitization because 5-HT_3 receptor desensitization did not affect I_{GABA} (Fig 6C) and $GABA_A$ receptor desensitization alone did not affect $I_{5\text{-}HT}$ (Fig 6D). These observations, coupled with the fact that $I_{GABA+5\text{-}HT}$ kinetics is different from the kinetics of I_{GABA} or $I_{5\text{-}HT}$ alone (Fig 3-5), indicate that $I_{GABA+5\text{-}HT}$ is carried through both $GABA_A$ and 5-HT_3 channels. This

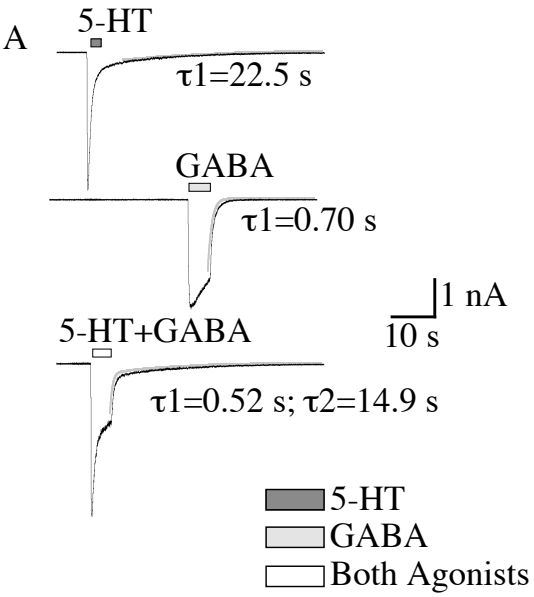

Figure 5 Decay kinetics of $I_{GABA+5\text{-}HT}$ can be explained by the combined decay kinetics of I_{GABA} or $I_{5\text{-}HT}$.

A: representative currents induced by GABA (I_{GABA}), 5-HT ($I_{5\text{-}HT}$), and GABA+5-HT ($I_{GABA+5\text{-}HT}$) are from the same myenteric neuron. Decay of $I_{GABA+5\text{-}HT}$ was best fitted by the sum of two exponential functions (thick grey line), whereas I_{GABA} and $I_{5\text{-}HT}$ desensitization were best fitted by a single exponential function. τ of these exponential functions are indicated in seconds. B: bars represent the mean ± S.E.M. values (n = 10) of τ values of these exponential functions. τ_1 of I_{GABA} was not significantly (NS) different from τ_1 of $I_{GABA+5\text{-}HT}$. τ_1 of $I_{5\text{-}HT}$ did also not differ from τ_2 of $I_{GABA+5\text{-}HT}$. Exponential fits were performed using the data from a couple hundred milliseconds after removing the agonists to the "steady-state" component. Experiments were performed at the holding potential of -60 mV.

would also imply that current occlusion is mediated by partial inhibition of both channels.

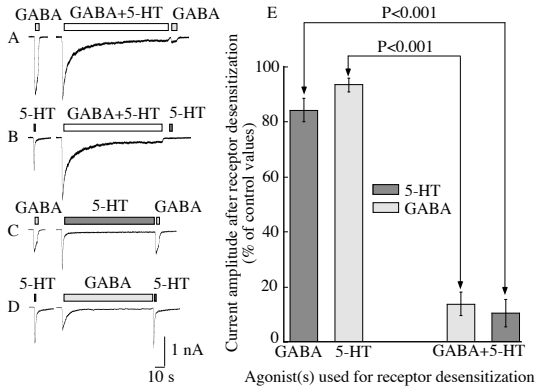

Figure 6 Application of GABA+5-HT induced desensitization of both $GABA_A$ and $5\text{-}HT_3$ receptors, which was not due to cross-desensitization between these receptors

Control I_{GABA} (A and C) and $I_{5\text{-}HT}$ (B and D) was recorded five minutes before (left recordings) and immediately after (~5 s), the prolonged application of both agonists (A and B), 5-HT (C), or GABA (D). E: average amplitude of I_{GABA} and $I_{5\text{-}HT}$ recorded after the prolonged application of GABA, 5-HT or GABA+5-HT, as a percentage of control response (n=13). Lines on top of the bars represent S.E.M. Recordings are from two neurons taken at the holding potential of -60 mV.

Current occlusion requires channel activation

In a previous study, it was demonstrated that serotonin molecules directly block nACh channels of enteric neurons (Barajas-López et al., 2001), suggesting that current occlusion might be mediated

by a similar mechanism. This hypothesis and other pre-receptor mechanisms are ruled out by the following observations.

In eight cells with a marginal or no response to GABA, $I_{GABA+5\text{-}HT}$ had similar amplitude (-1.20±0.38 nA) and kinetics to $I_{5\text{-}HT}$ (-1.10±0.16 nA; Fig 7A). In another six cells in which I_{GABA} was totally blocked with bicuculline (30 μM), $I_{GABA+5\text{-}HT}$ had similar amplitude (-1.72±0.25 nA) and kinetics to $I_{5\text{-}HT}$ (-1.72±0.24 nA; Fig 7B).

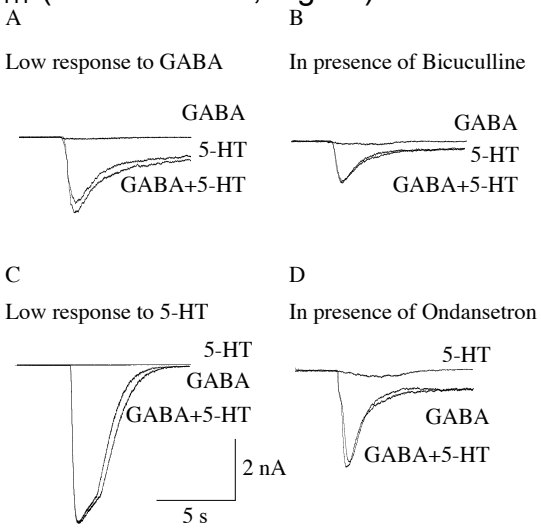

Figure 7 Inhibitory interactions between 5-HT_3 and $GABA_A$ receptors required the presence of functional channels

A: recordings from a neuron in which GABA did not induce any current (I_{GABA}) but 5-HT induced a prominent inward current ($I_{5\text{-}HT}$). Note that GABA did not alter either the amplitude or kinetics of $I_{5\text{-}HT}$. B: similar results were obtained when the I_{GABA} was blocked by 30 μM bicuculline. C: recordings from a neuron with no response to 5-HT but with a prominent I_{GABA}. Note that 5-HT did not modify either the amplitude or kinetics of I_{GABA}. D: similar results were obtained when the $I_{5\text{-}HT}$ was blocked by 3 μM ondansetron.

In seven cells with a marginal or no initial response to 5-HT, $I_{GABA+5\text{-}HT}$ had a similar amplitude (-2.54±0.73 nA) and kinetics to I_{GABA} alone (-2.38±0.77 nA; Fig 7C). Similarly, in another six neurons, in which $I_{5\text{-}HT}$ (-72±20 pA) had previously been inhibited with ondansetron, $I_{GABA+5\text{-}HT}$ had similar amplitude (-2.36±0.81 nA) and kinetics to I_{GABA} (-2.21±0.67 nA; Fig 7D).

These observations together with the ones described in the previous paragraph indicate that current occlusion is mediated by a post-receptor mechanism(s). These data also rule out the possibility that occlusion is mediated by 5-HT molecules acting on $GABA_A$ channels (an effect demonstrated before for 5-HT on native nACh channels (Barajas-López et al., 2001)), or GABA molecules acting on 5-HT_3 channels.

Role of protein phosphorylation and intracellular Ca^{2+} in current occlusion

5-HT and GABA are also known to activate metabotropic receptors (5-HT and $GABA_B$) in enteric neurons (Cherubini & North, 1984, 1985; Gershon, 1999; Krantis, 2000). Activation of these receptors would lead to activation of G-proteins, changes in second messengers, activation of protein kinases, and protein phosphorylation. Therefore, one possibility is that at least part of the current occlusion observed here could be mediated by activation of metabotropic receptors. The fact that bicuculline and ondansetron prevents the effects of GABA on $I_{5\text{-}HT}$ and 5-HT actions on I_{GABA}, suggests that

activation of metabotropic receptors are not required for current occlusion. The following observations are in agreement with such an interpretation.

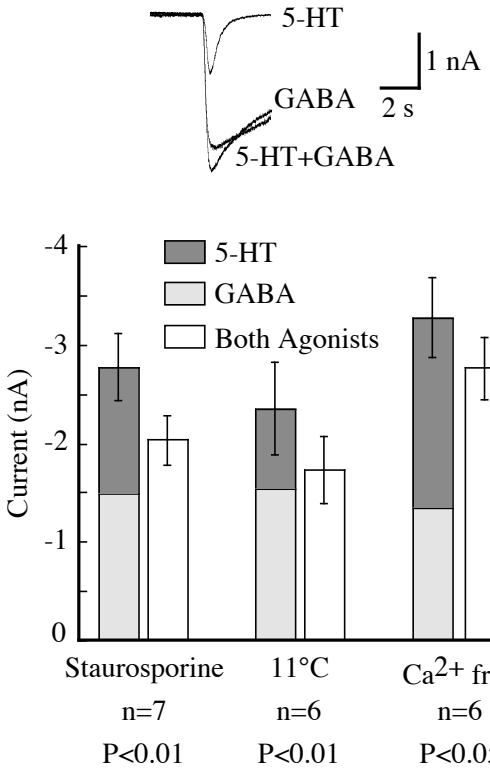

Figure 8 Calcium ions and protein phosphorylation are not required for current occlusion

Average amplitude of inward currents induced by application of GABA (I_{GABA}), 5-HT ($I_{5\text{-}HT}$) or GABA+5-HT ($I_{GABA+5\text{-}HT}$) in three different experimental groups of myenteric neurons. Average values of each group, are represented by a pair of bars. The first bar of each pair is a combined bar and shows $I_{5\text{-}HT}$ and I_{GABA} before application of GABA+5-HT. This combined bar represents the average expected current ($I_{Expected} = I_{GABA} + I_{5\text{-}HT}$). The second bar represents the mean $I_{GABA+5\text{-}HT}$. Error lines on the top of the bars are mean ± S.E.M. for $I_{Expected}$ and $I_{GABA+5\text{-}HT}$. Ca^{2+}-free experiments were carried out in 0-Ca^{2+}, plus 50 µM EGTA in the extracellular media, and using the standard intracellular solution in the pipette. Some experiments (n=6) were performed at 11°C and a set of recordings from a typical experiment are shown in the inset. Staurosporine (5 µM) experiments were carried out adding this protein kinase inhibitor to the pipette solution. All these experiments were carried out at a holding potential of -60 mV.

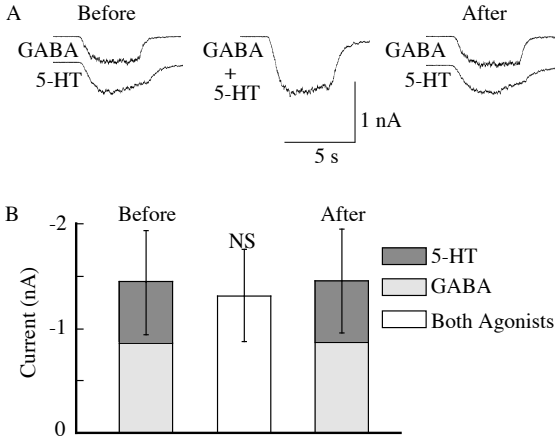

Figure 9 Current occlusion requires maximal activation of the channels

A: Currents induced by application of GABA (30 µM), 5-HT (30µM), or by GABA+5-HT recorded from the same myenteric neuron. B: Average (bars) membrane currents and mean ± S.E.M. of six similar experiments. In these experiments $I_{GABA+5\text{-}HT}$ was no different (no significantly, NS) from the sum of the individual currents. Experiments were carried out at a holding potential of -60 mV.

Current occlusion was still observed after inhibiting protein phosphorylation (Fig 8) by either lowering the temperature to 11°C or adding a non-specific protein kinase inhibitor (5 µM staurosporine) to the internal solution (Ruegg & Burgess, 1989).

$5\text{-}HT_3$ receptors are permeable to

Ca^{2+}, which suggests that current occlusion is mediated by an increase in the intracellular concentration of this ion. Against this hypothesis, however, the current occlusion was still observed in total absence of Ca^{2+} in the extracellular and intracellular medium (Fig 8). These experiments were carried out with the standard intracellular and extracellular solutions but the latter containing no Ca^{2+} plus 50 μM EGTA.

Current occlusion is concentration dependent

According to our observations, a model that considers a cross inhibition between $GABA_A$ and 5-HT_3 channels, due to allosteric changes in these proteins, might explain the occlusion between $I_{5\text{-}HT}$ and I_{GABA}. In agreement with this model, we observed that currents induced by a concentration of 30 μM 5-HT and 30 μM GABA, were additive (n=6; Fig 9).

DISCUSSION

This is the first demonstration that simultaneous activation of $GABA_A$ and 5-HT_3 receptors is not independent and ionic currents carried through them are occluded when they are simultaneously activated. This current occlusion is observed as soon as currents are activated, and does not require Ca^{2+} or protein phosphorylation. Altogether, these observations suggest that this current occlusion is mediated by cross-inhibition and by a direct interaction between $GABA_A$ and 5-HT_3 channels. Similar interactions to the ones proposed here have been shown between nACh and P2X receptors (Barajas-López *et al.*, 1998; Zhou & Galligan, 1998; Khakh *et al.*, 2000), between $GABA_A$ and P2X receptors (Sokolova *et al.*, 2001; Karanjia *et al.*, 2006). Interactions of metabotropic receptors have also been reported between dopamine (D2) and somatostatin (SSTR5) receptors (Rocheville *et al.*, 2000), and between dopamine (D5) and $GABA_A$ channels (Liu *et al.*, 2000). This is the second study that demonstrates interactions between two members of the *Cys-loop* family, which would suggest the existence of inhibitory interactions between the same populations of ligand-gated channels (p. e. $GABA_A$). This would imply that interactions between receptor proteins play a major role in neuronal signalling in many if not all synapses. This hypothesis, however, requires further experimental analysis and it is clearly out of the scope of the present study.

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

The whole cell currents induced by either GABA (300 μM) or 5-HT (1 mM), despite these relatively high concentrations, are mediated by pharmacologically distinct receptors. This is demonstrated by the specific inhibitory effect of ondansetron (a 5-HT_3 receptor antagonist (Hoyer *et al.*, 1994)) on $I_{5\text{-}HT}$, and by the more potent effect of bicuculline (a $GABA_A$ antagonist (Li *et al.*, 2003)) on I_{GABA} than on $I_{5\text{-}HT}$. Activation of these channels is, however, not independent as shown by the fact that inward currents carried through $GABA_A$ and 5-HT_3 receptors were not additive when maximally activated.

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

Enteric neurons also express metabotropic GABA and 5-HT receptors (Cherubini & North, 1984, 1985; Derkach *et al.*, 1989; Zhou & Galligan, 2000), which are known to be linked to second messengers and protein phosphorylation cascades (Gershon, 1999; Krantis, 2000).

Several of our observations, however, indicate that activation of these receptors is not required for the current occlusion observed here. First, current occlusion occurs, as soon as $GABA_A$ and 5-HT_3 channels are activated, indicating that this occlusion is as fast as the activation of these ligand-gated channels. Second, bicuculline prevents effects of GABA on the 5-HT_3 channels (present study) and ondansetron prevented the effect of 5-HT on the $GABA_A$ channels. Third, inhibition of protein phosphorylation (with staurosporine or by lowering experimental temperature to 11ºC) did not prevent the occlusion observed between I_{GABA} and $I_{5\text{-}HT}$.

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

At least three different observations indicate that $I_{GABA+5\text{-}HT}$ is carried through both $GABA_A$ and 5-HT_3 channels. 1) $I_{GABA+5\text{-}HT}$ desensitizes faster than I_{GABA} but slower than $I_{5\text{-}HT}$. 2) The decay of $I_{GABA+5\text{-}HT}$ resembles the decay of both I_{GABA} and $I_{5\text{-}HT}$. 3) When GABA and 5-HT are applied simultaneously, 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 desensitized individually. Altogether, these observations also imply that inhibition between these channels is reciprocal.

Functional implications for these channel interactions

The present and previous 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. Thus functional interaction such as one demonstrated here has been shown to exist between P2X and nicotinic channels in enteric neurons (Barajas-López *et al.*, 1998; Zhou & Galligan, 1998) and between P2X and other ligand-gated channels ($GABA_A$) in enteric (Karanjia *et al*., 2006) and dorsal root ganglion neurons (Sokolova *et al.*, 2001). Finally, the present study and that of Li *et al.* (Li *et al.*, 2003), show that two structurally related members (Ortells & Lunt, 1995) can interact indicating that the same type of channel (p. e. $GABA_A$) might also maintain cross-interactions between themselves and implying that this type of interactions might play an essential role in synaptic integration.

In conclusion, our results indicate that there is a very fast inhibitory interaction between $GABA_A$ and 5-HT_3 channels. These interactions occur as fast as the activation of $GABA_A$ and 5-HT_3, 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. Since these channels are structurally similar, it might be possible that these interactions are present between the same channels of the same type (p. e. 5-HT_3) implying an important role in neuronal signalling.

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