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"Functional Interactions Between Nicotinic and P2X Receptors in Guinea-pig Celiac Ganglia Neurons"

Interacciones Funcionales Entre los Receptores Nicotínicos y P2X en Neuronas del Ganglio Celiaco de Cobayo

Tesis que presenta
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Para obtener el grado de
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Constancia de aprobación de la tesis

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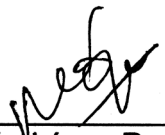

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Dedico este trabajo a mi hija, Carlis, la estrella que ha dado dirección a mi vida. A mi esposa, Almuchis, por su amor, apoyo, paciencia, y por siempre creer en mi. A mis papás, Jesusita y José, por darme principios sólidos y el valor para seguir mis sueños. A mis hermanos y hermanas por ser mis compañeros y amigos.

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Abbreviations

nACh	Acetylcholine
ATP	Adenosine 5' triphosphate
GABA	γ-Aminobutyric acid
5-HT	Serotonin
PPADS	Pyridoxalphosphate-6-azophenyl-2´,4´-disulfonic acid
NS	not significant
I_{ACh}	Current induced by ACh
I_{ATP}	Current induced by ATP
$I_{ACh+ATP}$	Current induced by ACh+ATP
$I_{5\text{-}HT}$	Current induced by 5-HT
I_{nic}	Current induced by nicotine
nACh	Channel activated by ACh
P2X	Channel activated by ATP
$GABA_A$	Channel activated by GABA
$5HT_3$	Channel activated by 5-HT
P2Y	Receptores activated by ATP and associated to G-proteins
LGCs	Ligand Gated Channels
HEK cells	Human Embrionic Kidney cells

Resumen

Interacciones Funcionales Entre los Receptores Nicotínicos y P2X en Neuronas del Ganglio Celiaco de Cobayo

Palabras clave: *Canalaes iónicos; Receptores P2X; ATP; Receptores nACh; Acetlcolina; Receptores ionotropicos; Canales activados por ligando; Neuronas celiacas; Neuronas simpaticas; Patch-Clamp; Electrofisiología.*

En el ganglio celiaco, la liberación de la acetilcolina (ACh) y del ATP activan canales nicotínicos y P2X, respectivamente, y son responsables de la generación de potenciales postsinápticos excitatorios rápidos. Recientemente, se ha reportado que los canales nicotínicos y los P2X mantienen interacciones inhibitorias alostéricas en neuronas de los plexos entéricos. Debido a su importancia funcional, en el presente estudio se investigó la existencia de una interacción similar en las neuronas del ganglio celiaco, utilizando la técnica de *Patch Clamp* en su configuración de célula completa. Las corrientes inducidas por ACh (I_{ACh}) o ATP (I_{ATP}) fueron inhibidas por hexametonio o PPADS, respectivamente, indicando que son mediadas por diferentes receptores. Las corrientes inducidas por ACh+ATP ($I_{ACh+ATP}$), solo fueron tan grandes como la corriente inducida por el trasmisor mas efectivo, revelando una oclusión de corriente. La aplicación secuencial de neurotransmisor y las propiedades farmacológicas de $I_{ACh+ATP}$ indican que esta corriente es acarreada por ambos canales, los nicotínicos y los P2X. ATP no afecta I_{ACh} en neuronas: i) en presencia de PPADS, ii) después de la desensibilización de los receptores P2X o iii) durante las corrientes salientes registradas a potenciales positivos. De igual manera ACh no afecta I_{ATP} en neuronas: i) en presencia de hexametonio, ii) después de la desensibilización de los receptores nicotínicos o iii) durante las corrientes salientes registradas a potenciales positivos. La oclusión de corriente se observa tan pronto como se activan los canales, en experimentos realizados a 10ºC, y cuando se agregó estaurosporina (un inhibidor de las proteína-cinasas) al interior de la pipeta, indicando que la oclusión es independiente del metabolismo neuronal. En conclusión, estos resultados son consistentes con un modelo de inhibición cruzada entre receptores nicotínicos y purinérgicos a través de cambios alostéricos. Por el momento, desconocemos la función de tales interacciones, sin embargo, es muy probable que tengan un papel importante en la integración sináptica rápida en el ganglio celiaco.

Abstract

Functional Interactions Between Nicotinic and P2X Receptors in Guinea-pig Celiac Ganglia Neurons

Key words: *Ion Channels; ATP; nACh receptors; Acetylcholine; Ionotropic Receptors; Ligand-gated Ion Channel; Celiac Neurons; Sympathetic Neurons; Patch-Clamp Techniques; Electrophysiology.*

Functional interactions between nicotinic (activated by acetylcholine) and P2X (activated by ATP) receptors of celiac neurons from the guinea pig were characterized using *whole-cell* recordings. Currents induced by ACh (I_{ACh}) and ATP (I_{ATP}), were inhibited by hexamethonium and PPADS, respectively. Currents induced by ACh+ATP ($I_{ACh+ATP}$), were only as large as the current induced by the most effective transmitter, revealing current occlusion. This occlusion requires maximal activation of both receptors. Sequential applications of neurotransmitters and pharmacological properties of $I_{ACh+ATP}$ indicate that this current is carried through both nicotinic (nACh) and P2X channels. ATP did not affect I_{ACh} in neurons: i) in the presence of PPADS, a P2X receptor blocker, ii) after P2X receptor desensitization, or iii) during outward currents registered at positive potentials. Similarly, ACh did not affect I_{ATP} in neurons: i) in the presence of hexamethonium, a nicotinic receptor blocker, ii) after nicotinic receptor desensitisation, or iii) during outward currents registered at positive potentials. Current occlusion occurred as fast as current activation, and remained present at 10ºC, after adding staurosporine (a protein kinase inhibitor) to the pipette solution. However, current occlusion was not observed at lower concentrations (30 μm). In conclusion, these results are consistent with a model of cross-inhibition between nACh and P2X receptors.

Introduction

General Background

Cells of the nervous system use substances, called neurotransmitters, to communicate with each other. Some of these substances act by directly regulating ionic channels on neuronal postsynaptic neurons. These channels are also receptors and therefore are known as ionotropic receptors. These receptors include those activated by nicotine and acetylcholine (ACh; nACh receptors), by γ-amino butyric acid (GABA; $GABA_A$ receptors), by serotonin (5-HT; $5HT_3$ receptors), and by adenosine trifosfate (ATP; P2X receptors).

Besides directly activating channels, neurotransmitters can bind to receptors associated to G-proteins. These can then directly regulate other ion channels (e.g. calcium) or enzymes (phospholipase C and adenyl cyclase). Because these receptors are associated to a cascade of biochemical events inside the membrane, they are known as metabotropic receptors. Examples of these receptors are the adrenergic, muscarinic (activated by ACh), and P2Y (activated by ATP).

ACh and ATP are known to be neurotransmitters in the peripheral nervous system

The role of ACh and nACh receptors in fast synaptic neurotransmission is well documented in the peripheral nervous system and has been classically recognized. The role of ATP as a fast neurotransmitter was shown in more recent

studies carried out in celiac ganglia neurons in culture (Evans, 1992; Silinsky, 1992; Silinsky & Gerzanich, 1993). These authors showed that excitatory postsynaptic events (potentials and currents) were mimicked by external application of ATP. Both, the events and the ATP effects were blocked by P2X antagonists (e.g. suramin) but were not sensitive to agonists acting on receptors of the family nicotinic. Furthermore, these synaptic events and the ATP effects have the same electrophysiological properties.

Neurotransmitters and Neuromodulators are coreleased and play a role as cotransmitters

The concept of co-transmission has been reviewed by Burnstock (Burnstock, 1990) and its implications in neural network modulation have recently been review by Nusbaum *et al.* (Nusbaum *et al.*, 2001). In mammals, one of the best studied examples of co-transmission was observed in sympathetic nerve terminals of some smooth muscle tissues. In these tissues, ATP and noradrenaline are released from the same nerves and synergistically determine smooth muscle contraction (Burnstock, 1990). These type of interactions might also be expected to occur when neuroactive substances are released from nonneuronal cells; for example, platelets and mast cells are known to release several neuromodulators including ATP, serotonin, and histamine (Fraser & Simpson, 1983; Arvier *et al.*, 1996). Simultaneous release of neuroactive substances from these and other cell types may also occur during complex responses such as inflammation (Collins *et al.*, 1992; Arvier *et al.*, 1996).

The synaptic or nonsynaptic effects of transmitters that are simultaneously released can be complex when their receptor/signalling systems cross-talk with each other. This makes the study of co-transmission both exciting and challenging. Relevant to this co-transmission concept in the enteric nervous system, we and others have made a number of interesting observations on the interaction between purinoceptors and other G-protein-linked receptors (Palmer *et al.*, 1987; Barajas-Lopez, 1994). In the myenteric plexus, Palmer *et al.* (Palmer *et al.*, 1987; Barajas-Lopez, 1994) showed that adenosine can prevent the slow depolarization induced by a number of substances (including bombesin, vasoactive intestinal peptide, and histamine), without affecting those induced by serotonin and substance P. In the submucosal plexus, we showed that activation of $\propto_2$-adrenoceptors and somatostatin receptors, coupled through a pertussis toxin-sensitive receptor, can prevent the slow depolarization induced by adenosine, but not that induced by substance P (Barajas-Lopez, 1994). Several observations indicate that these effects could be mediated by the inhibition of adenyl cyclase activity (Barajas-Lopez, 1994).

Even more relevant are our latest findings regarding the cross-inhibitory interactions between different populations of ligand-gated channels (LGCs) known to mediate fast neurotransmission. Specifically those between P2X and nACh receptors (Barajas-Lopez *et al.*, 1998; Boue-Grabot *et al.*, 2003), P2X and $5HT_3$ channels (Barajas-Lopez *et al.*, 2002; Boue-Grabot *et al.*, 2003), and between P2X and $GABA_A$ (Sokolova *et al.*, 2001; Karanjia *et al.*, 2006).

Nakazawa (1994) suggested that there is an overlap of P2X and nACh channels, and that ATP could open a subpopulation of nACh channels by binding to P2X receptors in rat sympathetic neurons (Fig 1). This "channel overlap" hypothesis was based on the finding that the current induced by co-application of ATP and ACh ($I_{ACh+ATP}$) is less than the addition ($I_{ACh}+I_{ATP} = I_{expected}$) of the individual currents (I_{ACh} and I_{ATP}) induced by either substance.

We proposed a different model based on our findings in submucosal neurons (Barajas-Lopez *et al.*, 1998). In these studies we found similar observations as Nakazawa, when recording at –60 mV. However, at +40 mV, currents were additive. At this potential the amplitude of $I_{ACh+ATP}$ was not different from $I_{expected}$, ruling out Nakazawa's hypothesis (Barajas-Lopez *et al.*, 1998). This finding shows that I_{ATP} and I_{ACh} are carried through two different populations of channels. In agreement with this idea is the fact that P2X and nACh receptors have completely different pharmacological and physiological properties. These include: 1) different current rundown in membrane patches (see below); 2) different sensitivity to open channel blockers, e.g. hexamethonium blocks only nACh channels; 3) small but significant difference in reversal potentials of I_{ACh} and I_{ATP}, indicating a different ionic permeability for these channels; and 4) different desensitisation kinetics, and independent desensitisation (Barajas-Lopez *et al.*, 1998). These observations are in agreement with the fact that nACh and P2X channels have very different molecular structures (Brake *et al.*, 1994; Lindstrom, 1996; Lindstrom *et al.*, 1996; Soto *et al.*, 1996; North & Barnard, 1997; Le *et al.*, 1998), and illustrates the fact that P2X and nACh receptors do not share the same channel.

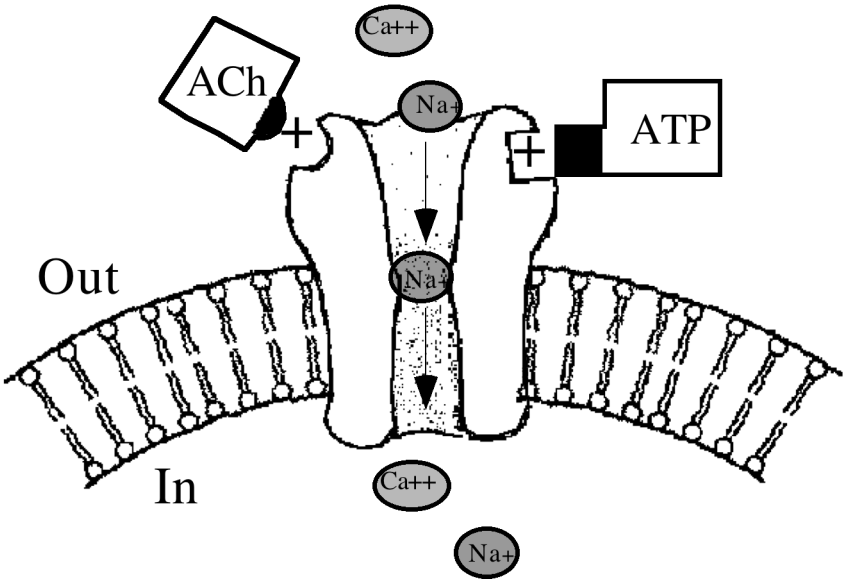


Figure 1. **Nakazawa's hypothesis to explain lack of current additivity**

Schematic representation of Nakazawa's (1994) hypothesis to explain the lack of additivity of the ion currents induced by ATP and ACh. This author proposed the presence of one channel activated by these two agonists.

This implies that other mechanisms are responsible for the occlusion observed between I_{ACh} and I_{ATP}.

Our observations also give some insight into the possible mechanisms involved in this occlusion (Barajas-Lopez *et al.*, 1998). We found that the current occlusion occurs as fast as channel activation (within a few ms), appears to be triggered by ion influx through the channels, and does not require Ca^{2+}, Na^{+}, Mg^{2+}, G-protein activation, or protein phosphorylation. Such observations suggest that this interaction is mediated by direct allosteric interactions between P2X and nACh channels or by allosteric interactions that involve a neighbouring structure (e.g. a protein) located beside these channels. Both explanations would imply that nACh and P2X channels are located very close to each other in the neuronal membrane, perhaps forming functional units possessing at least one channel of each type. A schematic representation of this hypothesis is shown and discussed in Figure 12 (see Disscusion).

Similar findings were recently claimed in sympathetic (Searl *et al.*, 1998) and demonstrated in myenteric neurons (Zhou & Galligan, 1998). In general, the conclusions of these studies are similar to our interpretations and were discussed in a short review (Searl & Silinsky, 1998). Various models that could explain the current occlusion were discussed in this review, including the one proposed by our laboratory (Barajas-Lopez *et al.*, 1998). According to the authors, our model would have been proven "*if selective nicotinic channel blockers, such as procaine (known not to bind to the agonist recognition site), should reverse the occlusion of*

the ATP-receptor." These experiments were actually done, as stated in the Discussion of the paper from our group (see Fig 2F and 2G of ref (Barajas-Lopez *et al.*, 1998)), but we used hexamethonium (Barajas-Lopez *et al.*, 1998); another channel blocker (Hille, 1992). Nevertheless, we consider that our model has to be further tested using single channel recordings.

Recently, our group has also demonstrated that the carboxy-terminal segment of the P2X receptors is essential for the cross-inhibition between LGCs (Boue-Grabot *et al.*, 2003). It is not known, however, if this interaction is prevented because the subunit terminal is required to anchor P2X receptors close to nACh or because it is directly involved in the cross-inhibition mechanisms.

The functional significance of the inhibitory interaction between cationic LGCs is not clear. A simple hypothetical explanation, however, is that it could serve to save cellular energy by limiting ion movements through the neuronal membrane. This could be important if we consider the already high metabolic rate of neurons, the relatively high density of these channels, and the fact that these channels are also permeable to Ca^{2+} (an intracellular messenger that is highly regulated) (Barajas-Lopez *et al.*, 2000).

Our group (Barajas-Lopez *et al.*, 1998) and others (Zhou & Galligan, 1998) investigated various alternative explanations for current occlusion. For instance, the reversal potential of I_{ACh}, I_{ATP} and $I_{ATP+ACh}$ is very similar, thus, indicating that the ionic driving forces did not change between these three conditions. Therefore, changes in the intracellular or extracellular concentrations of cations cannot be the cause of the current occlusion. A similar cross-inhibition between the $P2X_2$

and nicotinic $\propto_3\beta_4$ receptor subtypes was observed when these were co-expressed in *Xenopus* oocytes and they are present in functional enteric synapses where ATP and ACh appear to be co-released (Khakh *et al.*, 2000).

Cross-inhibitory interactions are present between P2X and $GABA_A$ channels (Karanjia *et al.*, 2006) or $5HT_3$ (Barajas-Lopez *et al.*, 2002), and other LGCs present in submucosal neurons. The rationale for this research was: 1) to investigate if other LGCs could also interact with P2X receptors; 2) to gain some insight on how these channels are organized in the membranes of submucosal neurons; and 3) to capitalize on the fact that these channels have different electrophysiological properties that can be useful to investigate specific questions regarding the mechanisms involved in channel interactions. For instance, $GABA_A$ channels are Cl^- permeable, and the currents induced by 5-HT (I_{5HT}) have a very slow deactivation process (current recovery during agonist removal).

Altogether, these observations support the working hypothesis that LGCs are organized into functional clusters formed by at least one channel of each type, and that there is a cross-inhibitory interaction between them when they are simultaneously activated.

This cross-inhibition might be indirect, involving other proteins and not only the LGCs. For instance, channel opening might lead to activation of an intracellular protein, which could then modulate (inhibit) the activity of the neighbouring channel. There is a lot of indirect evidence in favour of this hypothesis. Recent experimental evidence (reviewed by Sheng & Pak, 2000), indicates that cytoplasmic domains of ion channels, including LGCs, interact with

various intracellular proteins. These proteins link channels to the cytoskeleton and to appropriate signal transduction pathways in the cell. These protein-channel complexes are likely involved in the subcellular targeting, cytoskeletal anchoring and clustering of ion channels to specific submembrane regions (e.g. postsynaptic or presynaptic region) (Sheng & Pak, 2000). The fact that channel cross-interactions occur in *Xenopus* oocytes and in HEK cells (Boue-Grabot *et al.*, 2003) (see also below) suggest that they are independent of specific subsynaptic proteins or that if intracellular proteins are involved these might be present in a wide variety of cells.

Various experimental observations from our group favour an alternative hypothesis according to which channel cross-interactions occur by a direct molecular communication between channels, independent of the cytoskeleton. Thus, LGCs do co-immunoprecipitate (Boue-Grabot *et al.*, 2003), the interaction kinetics is very fast, the interaction occurs at low temperatures (8°C) (Barajas-Lopez *et al.*, 1998; Barajas-Lopez *et al.*, 2002), and it is present in membrane patches (when a lot of intracellular components have been disrupted or are totally absent). All these data would indicate that current occlusion is mediated by direct interactions between channels (Barajas-Lopez *et al.*, 1998; Barajas-Lopez *et al.*, 2002). 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). In a more recent study carried out in *Xenopus* oocytes (Boue-Grabot *et al.*, 2004a; 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.

Structure of the nACh and P2X channels

nACh receptors are part of the *Cys-loop* superfamily of ligand-gated ion channel and twelve different neural subunits have been cloned (Jensen *et al.*, 2005). Each of the nACh receptor subunits has four transmembrane domains, the amino- and carboxy-terminals are located extracellularly (Cockcroft *et al.*, 1990; Ortells & Lunt, 1995; McGehee, 1999). 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; Vial *et al.*, 2004). P2X subunits have only two transmembrane domains and the amino- and carboxy-terminals are citoplasmic (Dunn *et al.*, 2001; Khakh *et al.*, 2001; North, 2002). How these subunits are organized in the membrane to form ion channels is not completely understood. However, P2X and nAChR subunits appear to form both homomeric and heteromeric receptors. P2X channels are believed to be trimers and the nACh channels are likely pentamers.

Rationale, Hypothesis and Objectives

As stated before, pioneering studies demostrated that activation of nACh and P2X channels is not independent (Nakazawa, 1994) and that these receptors can inhibit each other when they are simultaneously activated in enteric neurons (Barajas-Lopez *et al.*, 1998; Zhou & Galligan, 1998). This inhibitory interaction is observed when channels are maximally activated (Nakazawa, 1994; Barajas-Lopez *et al.*, 1998). In apparent contradiction with these studies, a similar phenomenon was reported in celiac ganglia neurons (Searl *et al.*, 1998). This study shows that nicotine can inhibit ATP-induced currents (I_{ATP}), at very low concentrations. ATP or α,β-methylene ATP can also inhibit nicotine-induced currents (I_{nic}). I_{ATP} and I_{nic} were also induced by relatively low agonist concentrations and were clearly mediated by activation of nACh and P2X receptors. Importantly, ACh was not used as an agonist in such a study.

These discrepancies might indicate that a different type of interaction appears to exist in celiac neurons to that reported by our laboratory and others (Nakazawa, 1994; Barajas-Lopez *et al.*, 1998; Zhou & Galligan, 1998).

General Objective:

To investigate and characterize the putative inhibitory interactions between nACh and P2X native receptors of celiac neurons.

General Hypothesis:

Based on previous studies carried out in enteric neurons we propose that nACh and P2X native receptors of celiac neurons are engaged in allosteric inhibitory interactions that can limit the ion currents throught them, when both channels are simultaneously and maximally activated.

Specific Objectives:

1. To investigate the pharmacological properties of the nACh and P2X native receptors expressed in celiac neurons.

2. To characterize the kinetics of the whole-cell currents induced by the application of ACh and ATP in celiac neurons.

3. To characterize the inhibitory interaction between nACh and P2X receptors in celiac ganglia neurons.

Contrary to what has previously been reported (Searl *et al.*, 1998), we found that currents induced by ACh and ATP in celiac neurons are additive at lower concentrations. This effect 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 G-proteins or protein

phosphorylation, implying that it is mediated by allosteric interactions between the receptors.

Materials and Methods

Young guinea pigs (150-300 g), either male or female, were killed by decapitation and the celiac ganglia was removed, placed in modified Krebs solution (in mM: NaCl 126, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $CaCl_2$ 2.4, KCl 4.5, $NaHCO_3$ 25, glucose 11; gassed with 95% O_2 and 5% CO_2). The kidneys, suprarenal glands, and lipid tissue were dissected away from the ganglia, which was then sectioned in 4 to 5 small pieces. The celiac ganglia pieces were then dissociated using a sequential treatment with two enzymatic solutions, as described elsewhere (Barajas-Lopez *et al.*, 1996). The first contained papain (0.01 ml/ml; activated with 0.4 mg/ml of L-cysteine) and the second collagenase (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. Cells were cultured in Minimum Essential Medium 97.5%, containing 2.5% guinea-pig serum, penicillin 10 U/ml, streptomycin 10 µg/ml and glucose 16.7 mM.

In order to decrease the participation 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 ACh were recorded from celiac ganglia neurons using a GeneClamp 500B amplifier (Axon Instruments,

Inc.). Short-term (24-72 hours) primary cultures were used to prevent space-clamp problems due to neurite growth, which becomes a common problem on the fourth day after plating the neurons. Patch pipettes were made as previously described (Barajas-Lopez *et al.*, 1996) and had resistances between 1-4 MΩ. Except when otherwise mentioned 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, unless otherwise mentioned, had the following compositions (in mM). Inside the pipette: CsCl 140, EGTA 10, HEPES 5, NaCl 10, ATPMg 4.5, and GTP 0.1; external solution: NaCl 160, $CaCl_2$ 2, glucose 11, HEPES 4.8 and CsCl 3. The pH of all solutions was adjusted to 7.3-7.4 with either CsOH 1M (pipette solutions) or NaOH 1M (external solutions). Under these conditions, the usual input resistance of the neurons was >1 GΩ.

Whole-cell currents were recorded on a PC using Axoscope software (Axon instruments) 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 10°C were carried out using the eight-tubes device jacketed with a segment (~14 cm long) from a plastic pipette (10 ml). This plastic jacket was perfused with ice-cooled water so that the temperature in front of the delivering tube was at 10°C.

Hexamethonium and pyridoxalphosphate-6-azophenyl-2´,4´-disulfonic acid (PPADS) were purchased from Research Biomedical Inc. (Natick, MA, 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 sample of cells. Two-tailed P values of 0.05 or less were considered significant.

Results

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

Electrophysiological and pharmacological properties of the whole-cell currents activated by ACh (I_{ACh}) and ATP (I_{ATP}) in sympathetic neurons have been previously characterized. These currents are mediated by activation of channels with similar ion permeabilities, namely non-specific cationic channels (Zhong *et al.*, 2000) (Mandelzys *et al.*, 1995).

Using the standard internal solution, ACh and ATP induced an inward current in 198 (89%) of 222 and in 246 (95%) neurons out of 258 recorded neurons, respectively. Concentration-response curves were obtained for these transmitters and analysed (Fig 2). The EC_{50} values for ACh and ATP were 96 and 73 μM, respectively. The current amplitude induced by maximal concentrations of ACh and ATP (1 mM) was variable in different cells but a typical value was between -2 to -4 nA (range from -0.4 up to -6 nA). The mean peak-amplitude of these currents was of -3.34±0.26,and -3.08±0.28 nA, respectively. The amplitude of these currents was independent of each other and in fact some neurons only responded to ACh (12 out of 218) or ATP (24 out of 218 neurons), indicating that these channels are expressed independently in these neurons.

As shown in Figure 3A and 3B, inward currents induced by ACh and ATP (1 mM) were totally inhibited by hexamethonium (1 mM; n=7) and PPADS (30 μM; n=8), respectively. Hexamethonium did not affect I_{ATP} nor did PPADS alter I_{ACh} (see Fig 9).

Figure 2. ACh and ATP activated inward currents in a concentration-dependent manner.

Currents induced by three different concentrations of ACh (**A**) or ATP (**B**). Notice that the amplitude of the recorded currents increased by increasing the agonist concentration. **C**, Concentration-response curves for both agonists. Calculated EC_{50} are indicated. Symbols are means±S.E.M.

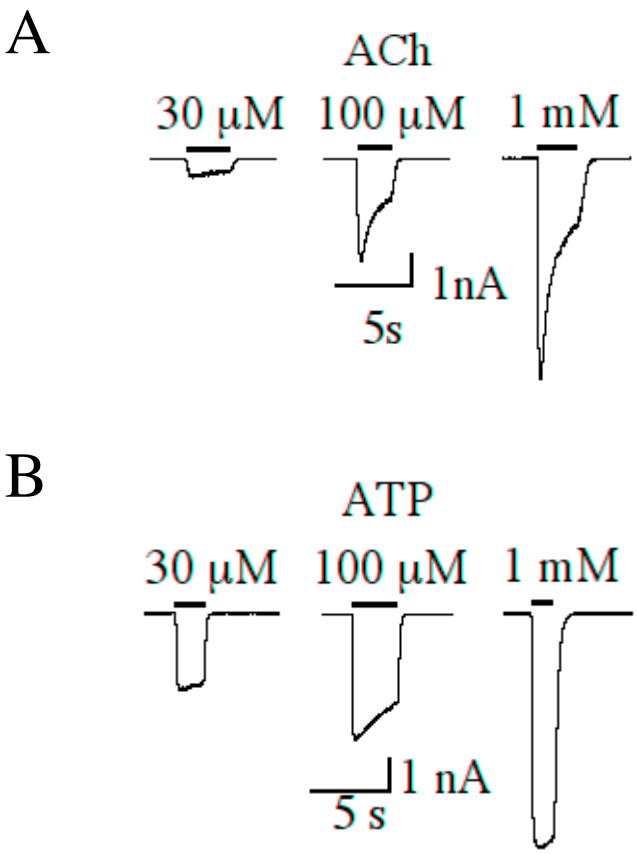
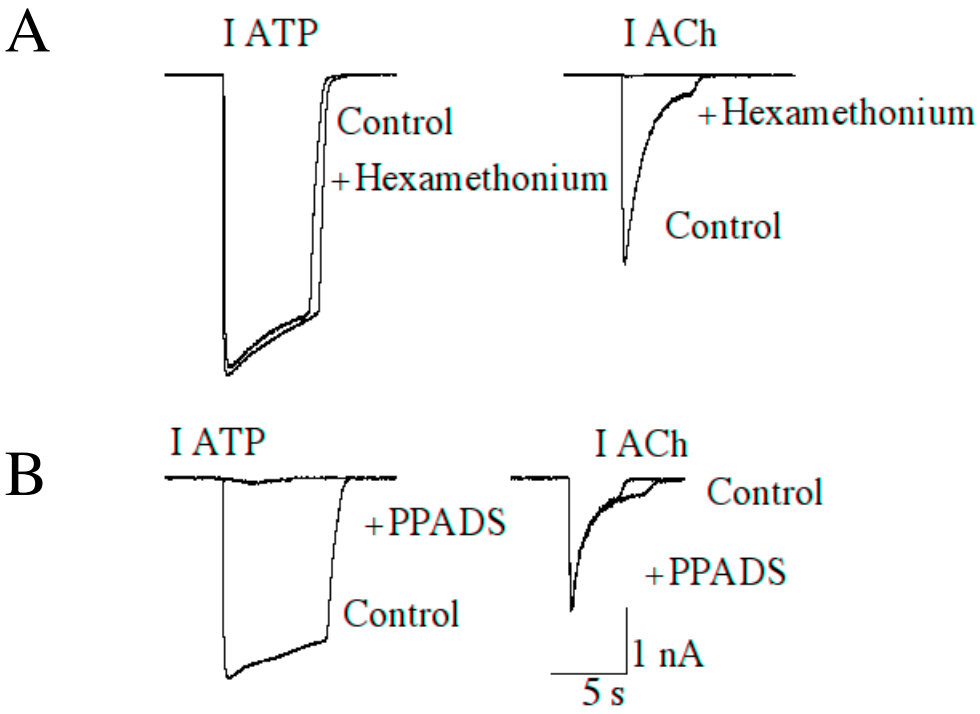

A
ACh
30 μM
100 μM
1 mM
1nA
5s
B
ATP
30 μM
100 μM
1 mM
1 nA
5 s
C
150
100
50
0
Relative Current (%)
ATP
ACh
EC50=73 μM
EC50=96 μM
1
10
100
1000
Agonist Concentration (μM)

Figure 3. Whole-cell inward currents induced by ATP (I_{ATP}) and ACh (I_{ACh}) are mediated by two distinct receptors

A, Hexamethonium (1 mM; a nACh receptor antagonist) blocks I_{ACh} without modifying I_{ATP}. **B**, PPADS (30 μM; a P2X receptor antagonist) blocks I_{ATP} without affecting I_{ACh}. **C**, Bars are averages of I_{ACh} and I_{ATP} in the presence of hexamethonium and PPADS. Lines on bars are S.E.M. values. Whole-cell inward currents shown in **A** and **B** were measured from two different celiac neurons. All these experiments were carried out at a holding potential of -60 mV, and were induced by ACh (1 mM) and ATP (1 mM). I_{ACh} and I_{ATP} were recorded 5 min before (Control) and fifteen seconds after of hexamethonium and PPADS. Notice that despite the relative high concentrations of the agonists and the inhibitors (PPADS, Hexamethonium) all their effects are, as expected, on only one of the channels.


A
I ATP
Control
+Hexamethonium
I ACh
+Hexamethonium
Control
B
I ATP
+PPADS
Control
I ACh
Control
+PPADS
1 nA
5 s
C
Current Amplitude
(% of Control Values)
120
100
80
60
40
20
0
NS
P < 0.001
P < 0.01
NS
ATP
ACh
Hexametonium
1 mM
PPADS 30 μM
n = 6
n = 7

These currents usually reached their peak within the initial second. After reaching its maximal amplitude, the currents decreased despite the continuous presence of the transmitters (current inactivation), indicating receptor tachyphylaxis. nACh receptors desensitisation was faster than the one observed for P2X. In all nine cells treated with long-term (approximately 2 min) applications of ACh and ACh+ATP, receptor desensitisation was better fitted by the sum of three exponential functions. However, the τ of the third exponential had a large value, which some times was longer than our recording time (>120 s). Likely in all nine cells treated with long-term (approximately 2 min) applications of ATP, current desensitisation was better fitted by the sum of two exponential functions. After 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 0.34±0.04 and 0.37±0.04 s for I_{ACh} and I_{ATP}, respectively (n=6). These values were not significantly different.

Currents induced by ACh and ATP are not additive

The experiments described above demonstrated that I_{ACh} 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 ACh and ATP (1 mM; when receptor occupancy would be close to 100%) should be additive.

To investigate if this is the case, we measured the peak of I_{ATP}, I_{ACh}, and the current induced by the simultaneous application of a maximal concentration of both agonists ($I_{ACh+ATP}$) in the same neuron.

We found that mathematical addition of individual currents yielded a current (I_{ATP}+ I_{ACh}=$I_{Expected}$; -6.43±0.48 nA) that was significantly larger ($P<0.001$; n=24) than $I_{ACh+ATP}$; (-4.27±0.27 nA), revealing an occlusion between I_{ACh} and I_{ATP} (Fig 4). For instance, if I_{ATP} was larger than I_{ACh}, then $I_{ACh+ATP}$ had different magnitude as I_{ATP} (not showed). Indeed, in five analyzed experiments in which I_{ATP} (-4.33±0.58 nA) was significantly ($P<0.05$) larger than I_{ACh} (-2.00±0.41 nA), $I_{ACh+ATP}$ (-4.72±0.51 nA) was significantly ($P<0.05$) larger than I_{Ach} and was different ($P<0.01$) than I_{ATP}. This indicates that the magnitude of the current that is being occluded is similar to the current induced by the less effective agonist (ACh). In seven analyzed neurons in which I_{ACh} (-3.84±0.56 nA) was significantly ($P<0.001$) larger than I_{ATP} (-1.88±0.49 nA), $I_{ACh+ATP}$ (-4.28±0.59 nA) was also significantly ($P<0.001$) larger than I_{ATP} in this case, it was also different ($P<0.01$) than I_{ACh}.

Current kinetics induced by single and simultaneous application of ATP and ACh

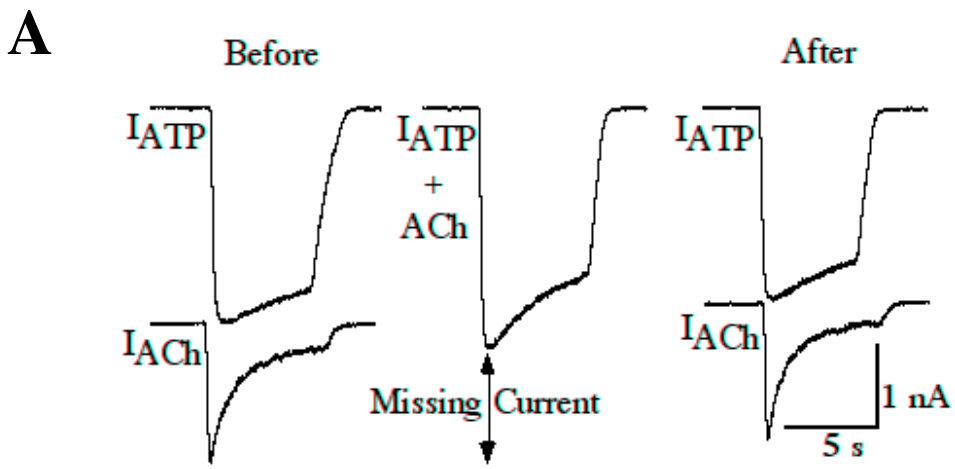
Figure 5A shows the average onset of I_{ACh}, I_{ATP}, $I_{ACh+ATP}$, and $I_{Expected}$ from six analyzed neurons and demonstrates the presence of current occlusion at the onset of these currents. This occlusion was significantly different ($P<0.05$), 100 ms after the beginning of these currents. At this point, the average $I_{ACh+ATP}$ was -

2.36±0.41 and $I_{Expected}$ -2.9±0.63 nA. In other words, current occlusion occurs rapidly.

After the removal of the agonists from the external solution, the decay of $I_{ACh+ATP}$, I_{ACh}, or I_{ATP} was best fitted by a single exponential function, as shown in Fig 5B. These observations would indicate that $I_{ACh+ATP}$ is mediated by the opening of both or either (P2X and nACh) channel and suggests other non-specific effects of the agonists do not mediate that current occlusion.

Figure 4. Whole-cell inward currents induced by ACh (I_{ACh}) and ATP (I_{ATP}) are not additive

A, Current recordings from a typical experiment in the same celiac neuron and **B**, average (bars) values of twenty-four experiments like the one shown in **A**. Currents were induced by application of either 1mM ACh (I_{ACh}) or ATP (I_{ATP}) and by the simultaneous application of both agonists ($I_{ACh+ATP}$). I_{ACh} and I_{ATP} were recorded five minutes before and five minutes after $I_{ACh+ATP}$. **B**, the first and last bars are the combined representation of I_{ACh} and I_{ATP} and the addition of these currents represents the expected current ($I_{Expected}$= I_{ACh}+I_{ATP}). S.E.M. are shown as lines in the top of the bars for $I_{Expected}$ and $I_{ACh+ATP}$. The later two currents are significantly different (P≤0.001).


A
Before
After
I_{ATP}
I_{ATP} + ACh
I_{ACh}
Missing Current
1 nA
5 s

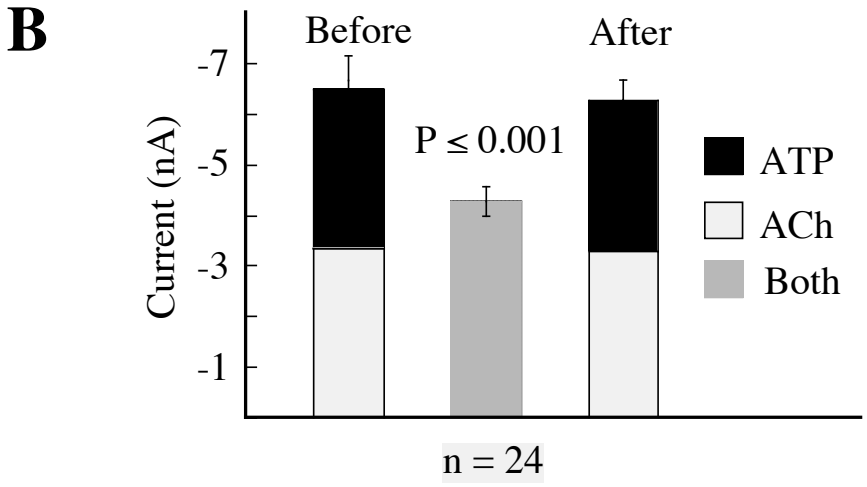
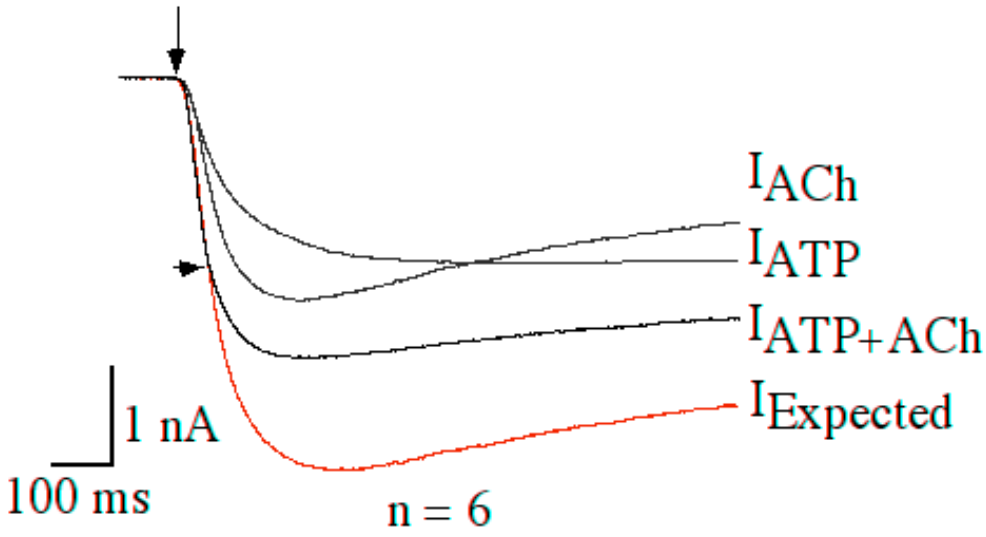

B
Before
After
-7
-5
-3
-1
Current (nA)
P ≤ 0.001
ATP
ACh
Both
n = 24

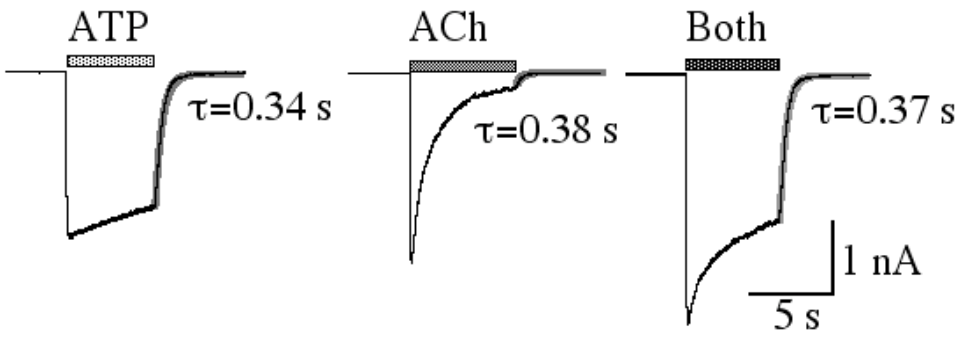
Figure 5. Onset kinetics of three currents indicates that the occlusion is quite fast whereas the decay kinetics is virtually the same for the current induced by both agonists and for the individual currents

A: Current occlusion is observed very rapidly, 100 ms after activation of the currents. All current tracings are mean currents from the same six celiac neurons. The current induced after 100 ms by simultaneous application of both agonists ($I_{ACh+ATP}$) is smaller (P<0.0.01) than the expected current ($I_{Expected}$). Whole-cell currents were measured at a holding potential of -60 mV. **B**, the decay kinetics of I_{ATP}, I_{ACh}, and $I_{ACh+ATP}$ is very similar and indeed, it is best fitted by a single exponential function with a very similar □ value, as it is indicated. This would indicate that $I_{ACh+ATP}$ could be mediated by either channel or by both channels. Best fitted exponential functions (thick grey lines) are shown. In these experiments the holding potential was -60 mV.

A


I_ACh
I_ATP
I_ATP+ACh
I_Expected
1 nA
100 ms
n = 6

B


ATP
ACh
Both
τ=0.34 s
τ=0.38 s
τ=0.37 s
1 nA
5 s

To obtained more information on this, we analysed the desensitisation kinetic by a long application (at least 90 s) of ACh, ATP or both. Within this time, $I_{ACh+ATP}$ kinetics appears to be different from that of either I_{ACh} or I_{ATP} (Fig 6A). Indeed, the kinetics of $I_{ACh+ATP}$ was well fitted by the sum of two exponential functions (Fig 6B; n=9). We tested the hypothesis that these two exponentials of $I_{ACh+ATP}$ are the mix of the two exponentials of the I_{ATP} and I_{ACh} kinetic. Thus, the average τ value of the first exponential (τ_1) of $I_{ACh+ATP}$ was statistically diferent to τ_1 of I_{ATP} ($P<0.01$) or τ_1 of I_{ACh} ($P<0.05$) kinetics and the average τ value of the second exponential (τ_2) of $I_{ACh+ATP}$ was diferent to τ_2 of I_{ATP} ($P<0.01$) or τ_2 of I_{ACh} ($P<0.05$) (Fig 6B). These observations indicate that $I_{ACh+ATP}$ are mediated by the opening of both P2X and nACh channels.

In order to further investigate this phenomenon, we tested the hypothesis that the simultaneous application of both agonists can desensitise both nACh and P2X receptors.

Simultaneous application of both agonists desensitise nACh and P2X receptors

During the following experiments, the amplitude of both I_{ACh} and I_{ATP} was measured before and immediately after (approximately 5 s) a long application of ACh+ATP. This long application lasted for at least 90 s or until the induced current had desensitised more than 80% of its initial amplitude (usually within this time). Some typical recordings and the average data from such experiments are shown in Figs 7A-B. We observed that application of ATP+ACh, decreased both

I_{ACh} to ~70 % and I_{ATP} to ~20 % of their control amplitude (Fig 7E). Such an inhibition was not due to cross-desensitisation since nACh receptor desensitisation alone did not affect I_{ATP} (Fig 7C) and P2X receptor desensitisation alone did not affect I_{ACh} (Fig 7D). These observations, coupled with the fact that $I_{ACh+ATP}$ kinetics is different from the kinetics of I_{ACh} or I_{ATP} alone (Fig 6), indicate that $I_{ACh+ATP}$ is carried through both nACh and P2X channels. This would also imply that current occlusion is mediated by partial inhibition of both channels.

Current occlusion requires channel activation

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

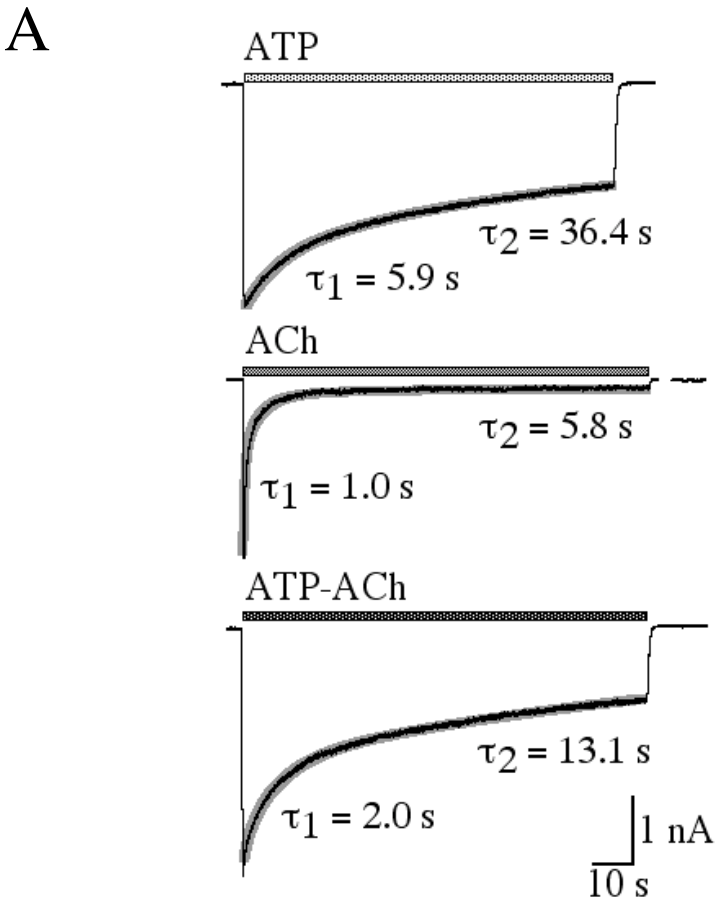
Inhibition of I_{ATP} prevented ATP actions on I_{ACh} (Fig 8B). Thus, when I_{ATP} was inhibited to a value of -0.07±0.01 nA by addition with 30 μM of PPADS (a P2X receptor antagonist) to the external solution, $I_{ACh+ATP}$ (-2.43±0.38 nA) had similar amplitude and similar kinetics to I_{ACh} (-2.38±0.40 nA; n=8).

In another seven cells in which I_{ACh} was totally blocked with 1 mM hexamethonium (a nACh channel blocker), $I_{ACh+ATP}$ (-3.39±0.52 nA) had the same amplitude and similar kinetics to I_{ATP} (-3.19±0.48 nA; Fig 8C).

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 nACh receptors or ACh molecules acting on P2X receptors.

Figure 6. Desensitization kinetics of these currents indicate that occlusion is bidirectional

A, Currents induced by ACh (I_{ACh}), ATP (I_{ATP}), and by application of both agonists ($I_{ACh+ATP}$) from one celiac neuron. Desensitization of $I_{ACh+ATP}$ and I_{ACh} was best fitted by the sum of three exponential functions (thick grey line). The □ value of the third exponential function was long-lasting (considering our recording time) and therefore, they were neither shown nor considered for this analysis. I_{ATP} desensitization was best fitted by two exponential functions, as it is shown. **B**, bars and lines on their top are the mean ± S.E.M. values of the □ for these exponential functions. The τ_1 and τ_2 of I_{ATP} and I_{ACh} were significantly ($P \leq 0.05$) different than τ_1 and τ_2 of $I_{ACh+ATP}$, respectively. In these experiments the holding potential was -60 mV.


A
ATP
τ2 = 36.4 s
τ1 = 5.9 s
ACh
τ2 = 5.8 s
τ1 = 1.0 s
ATP-ACh
τ2 = 13.1 s
τ1 = 2.0 s
1 nA
10 s

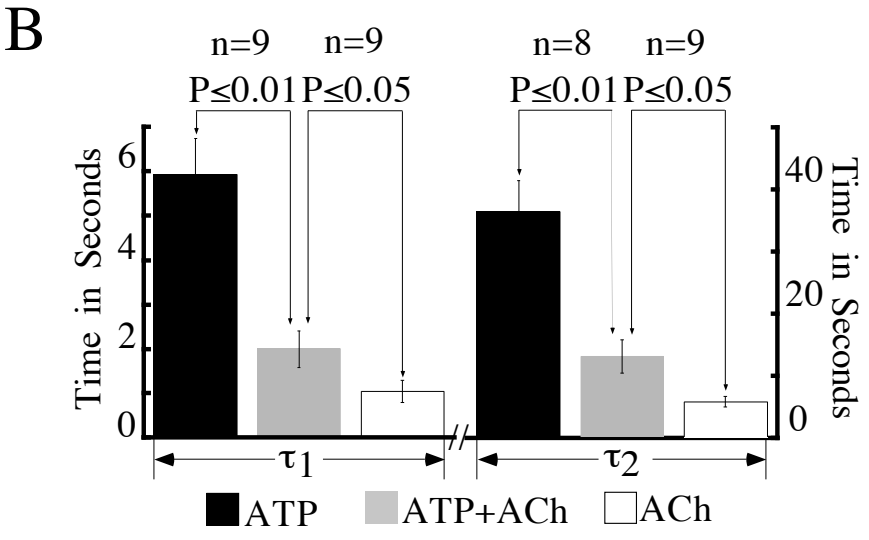
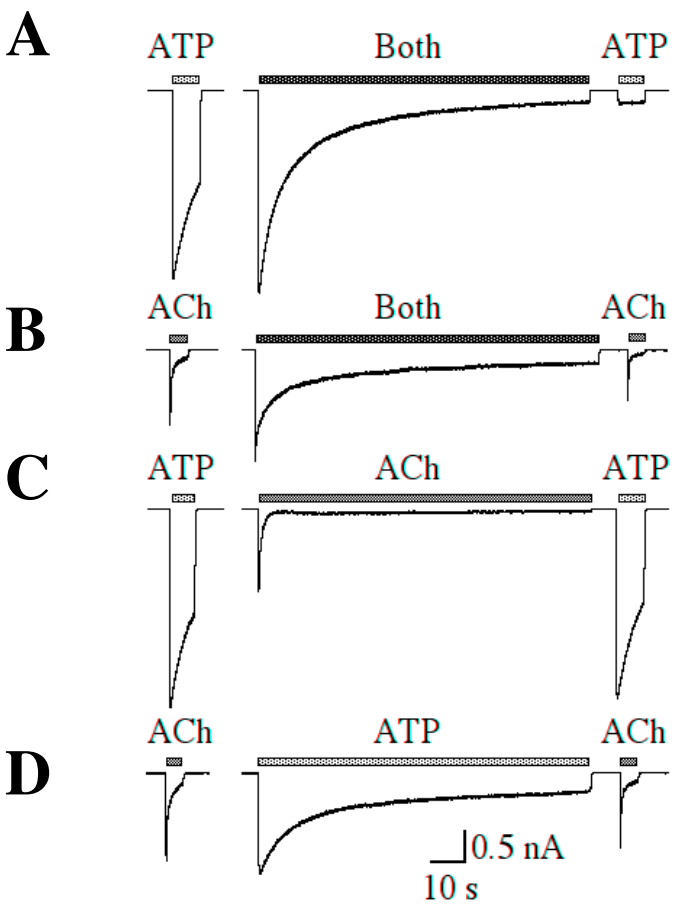

B
n=9 n=9 n=8 n=9
P≤0.01 P≤0.05 P≤0.01 P≤0.05
Time in Seconds
6
4
2
0
40
20
0
τ1
τ2
ATP
ATP+ACh
ACh

Figure 7. Simultaneous application of ACh and ATP induced desensitization of both nACh and P2X receptors, which was not due to cross-desensitization between these receptors

Control I_{ATP} (**A** and **C**) and I_{ACh} (**B** and **D**) was recorded five minutes before (left recordings) and immediately after (~5 s), of the prolonged application of both agonists (**A** and **B**), ACh (**C)** or ATP (**D**). **E**: average amplitude of I_{ACh} and I_{ATP} recorded after the prolonged application of ATP, ACh or ACh+ATP, as a percentage of control response (n=7 to ATP; n=5 to ACh). Line on top of the bars represents S.E.M. Recordings shown in A-D are from four different neurons taken at the holding potential of -60 mV.


A
ATP
Both
ATP
B
ACh
Both
ACh
C
ATP
ACh
ATP
D
ACh
ATP
ACh
0.5 nA
10 s

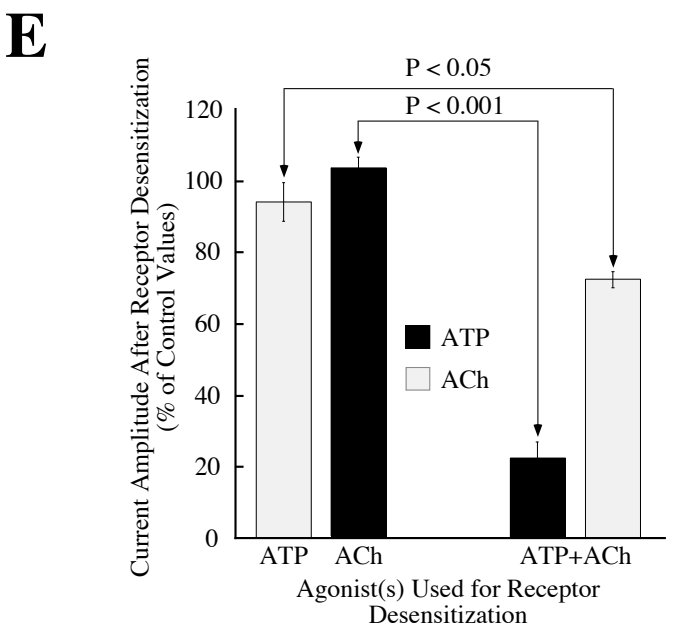
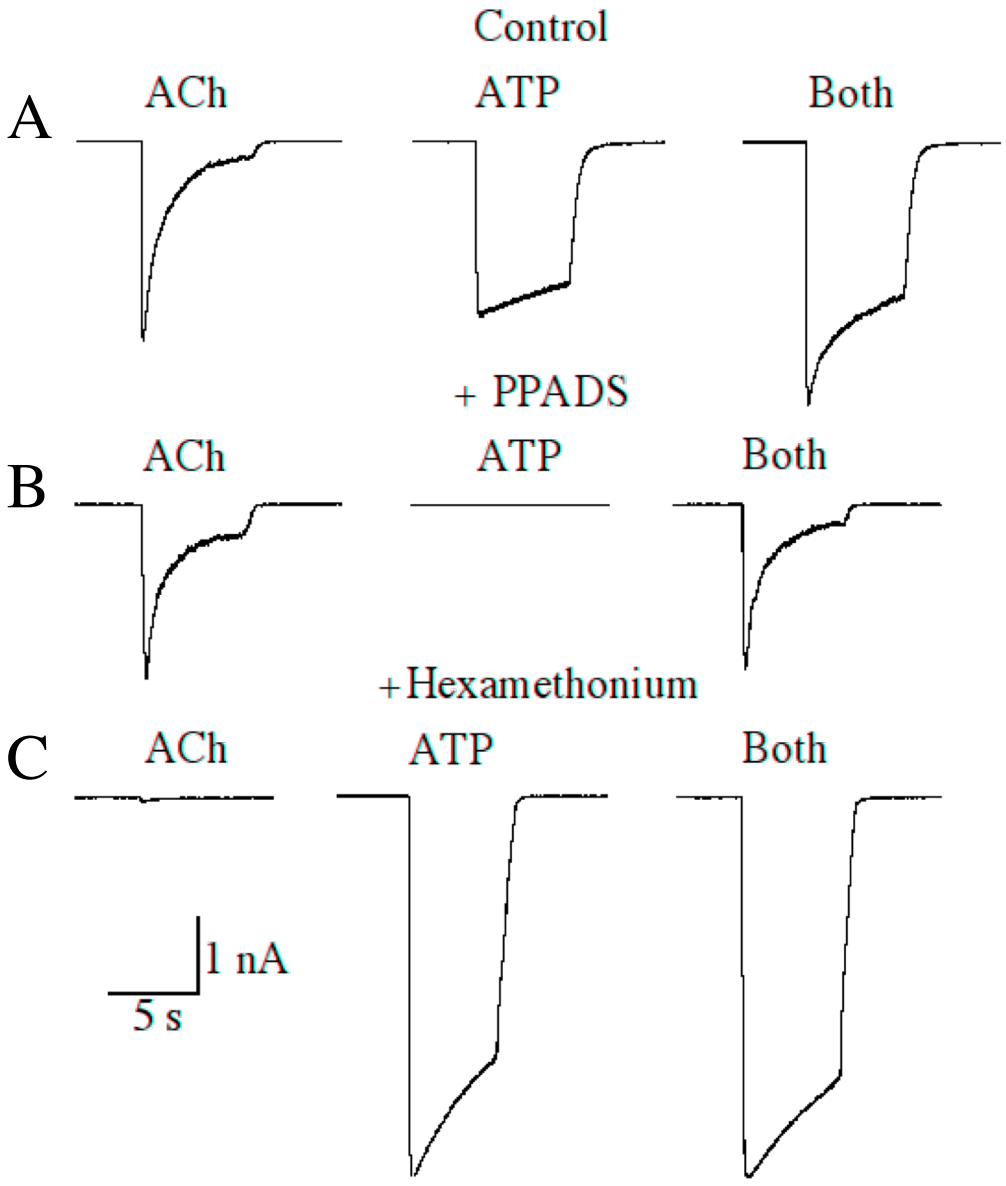

E
P < 0.05
P < 0.001
120
100
80
60
40
20
0
ATP
ACh
ATP
ACh
ATP+ACh
Current Amplitude After Receptor Desensitization
(% of Control Values)
Agonist(s) Used for Receptor
Desensitization

Figure 8. Current occlusion required something more that channel activation

Inward currents induced by application of ACh (I_{Ach}), ATP (I_{ATP}) or ACh+ATP ($I_{ACh+ATP}$) in the absence of any receptor antagonist (**A**), in the presence of PPADS (a P2X receptor antagonist, **B**) and hexamethonium (a nACh channel blocker, **C**). Notice that PPADS prevents the effects of ATP on I_{ACh} and hexamethonium prevents the effects of ACh on I_{ATP}.


Control
A
ACh
ATP
Both
+ PPADS
B
ACh
ATP
Both
+Hexamethonium
C
ACh
ATP
Both
1 nA
5 s

Role of protein phosphorylation in current occlusion

ATP and ACh are also known to activate metabotropic P2Y and mACh receptors in enteric and sympathetic neurons (Smith & Kessler, 1988; Caulfield & Birdsall, 1998; Barajas-Lopez *et al.*, 2000; Stemkowski *et al.*, 2002; Wan *et al.*, 2003; Spalding & Burstein, 2006). Activation of these receptors would lead to activation of G-proteins, changes in second messengers, and likely to 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 9) by either lowering the temperature to 10°C (n=8) or adding 5 μM staurosporine (n=6) a non-specific protein kinase inhibitor (Ruegg & Burgess, 1989) to the internal solution. Importantly staurosporine, at a concentration of 3 μM 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-Lopez *et al.*, 2000).

Channel permeability versus receptor activation as the origin for current occlusion

The fact that hexamethonium (nACh channel blocker) (Liu *et al.*, 2002) prevents ACh-mediated inhibition of I_{ATP}, suggests that channel cross-inhibition requires ion flow through the channels and is not enough to activate the receptors. In

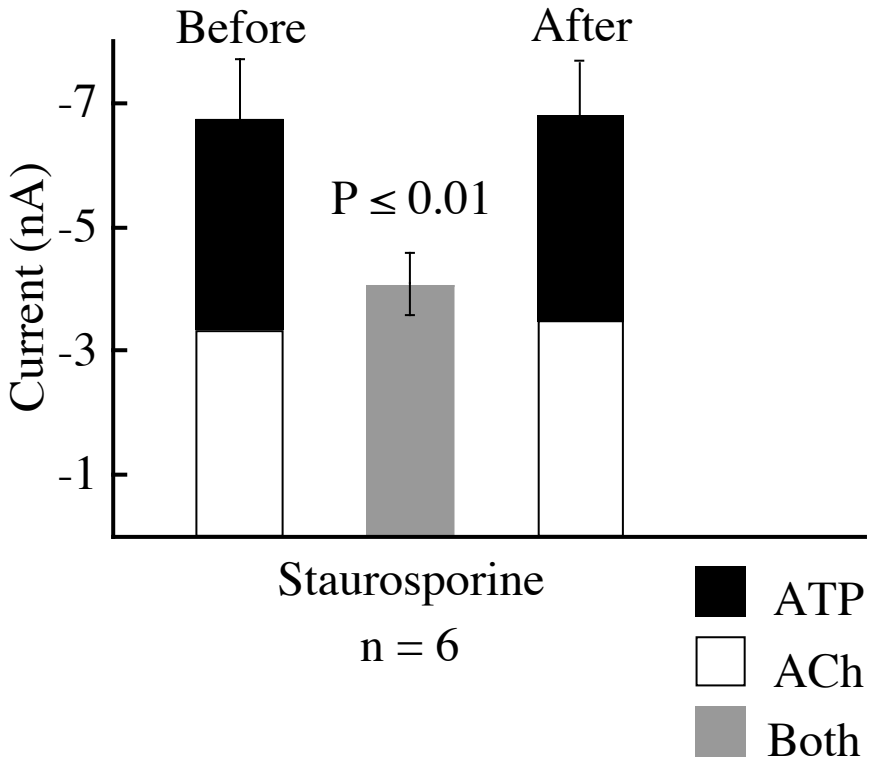
order to further study this hypothesis we investigated the effects of ACh on I_{ATP} and ATP on I_{ACh} at a positive potential (+5 mV). At this potential, these currents were outward and neither ATP alters I_{ACh}, nor ACh modifies I_{ATP} (Fig 10). These experiments support the hypothesis that cross-inhibition between nACh and P2X channels required an ion influx through them and that it is not enough to activate their receptors.

Current occlusion is concentration dependent

According to our observations, a model (Fig 12) that considers a cross inhibition between nACh and P2X channels, due to allosteric changes in these proteins, might explain the occlusion between I_{ATP} and I_{ACh}. In agreement with this model, we observed that occlusion was still observed when a saturating concentration of agonists was used (see Fig 4). Although, occlusion was absent when non-saturating concentrations (30 μM) were used for both agonists (Fig 11; n=9).

Figure 9. Cell metabolism and protein phosphorylation are not required for current occlusion

Average amplitude of inward currents induced by application of ACh (I_{ACh}), ATP (I_{ATP}) or ACh+ATP ($I_{ACh+ATP}$) in two different experimental groups of celiac neurons. Data for each group are represented in each plot bars. In both graphs, the first and last bars show the average I_{ATP} and I_{ACh} before and after application of ACh+ATP. This combined bar, therefore, represents the average expected current ($I_{Expected} = I_{ACh} + I_{ATP}$). The middle bar represents the mean $I_{ACh+ATP}$. Error lines on the top of the bars are S.E.M. for $I_{Expected}$ and $I_{ACh+ATP}$. Experiments at at 10°C were performed by cooling down the external solution. Staurosporine (5 μM), a protein kinase inhibitor, was added to the standard intracellular solution. All these experiments were carried out at a holding potential of -60 mV.


Before
After
-7
-5
-3
-1
Current (nA)
P ≤ 0.01
Staurosporine
n = 6
ATP
ACh
Both

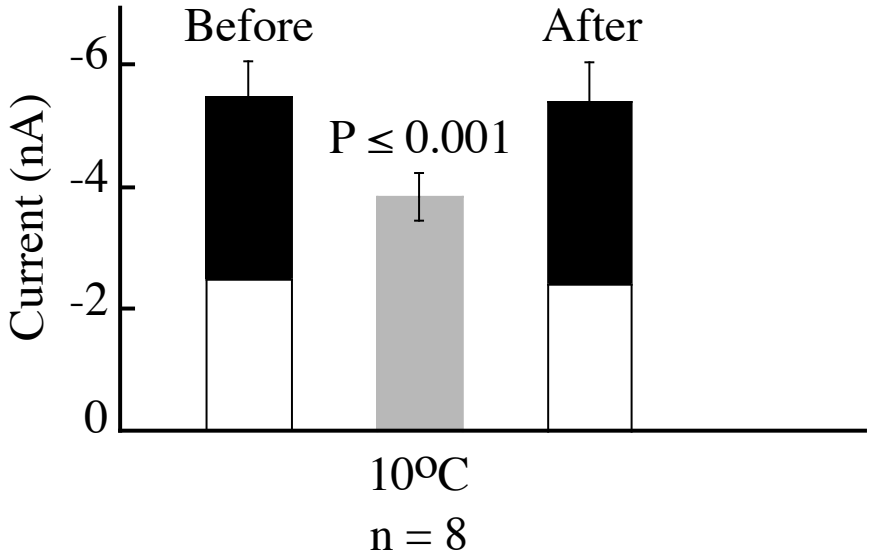
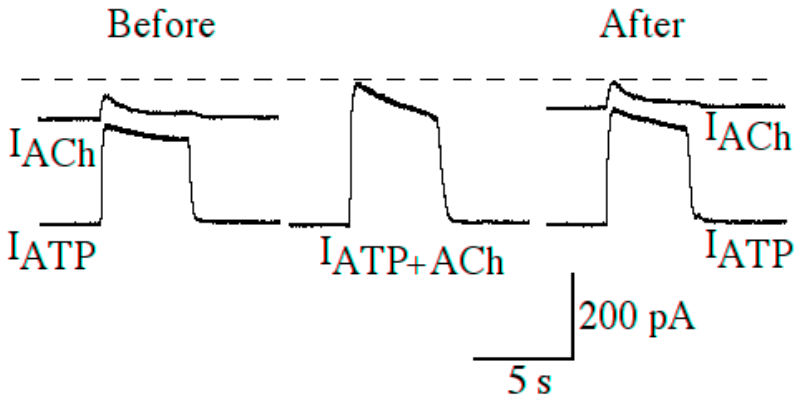

Before
After
-6
-4
-2
0
Current (nA)
P ≤ 0.001
10ºC
n = 8

Figure 10. Current occlusion is not observed for outward currents

Bars are mean amplitude of outward currents induced by application of ACh (I_{ACh}), ATP (I_{ATP}) or ACh+ATP ($I_{ACh+ATP}$). These current were measured at positive holding potentials. At these potentials it is observed that the sum of I_{ATP} and I_{ACh}, then $I_{ACh+ATP}$ was equal to $I_{expected}$. Line on top of the bars represents S.E.M (n=3).

A


Before
After
I ACh
I ATP
I ATP+ACh
I ACh
I ATP
200 pA
5 s

B

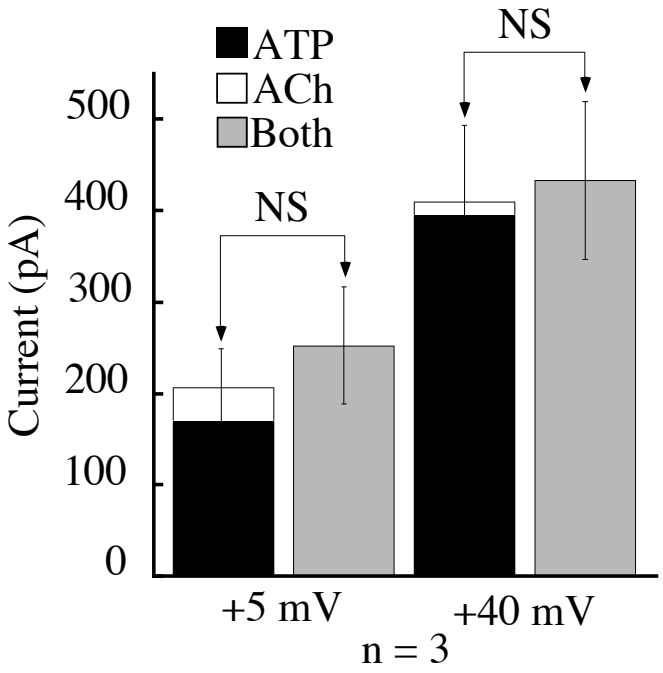
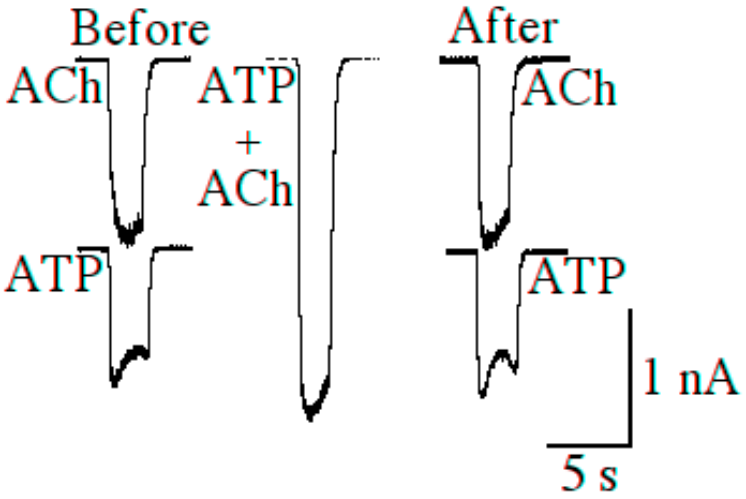

ATP
ACh
Both
NS
NS
Current (pA)
500
400
300
200
100
0
+5 mV
+40 mV
n = 3

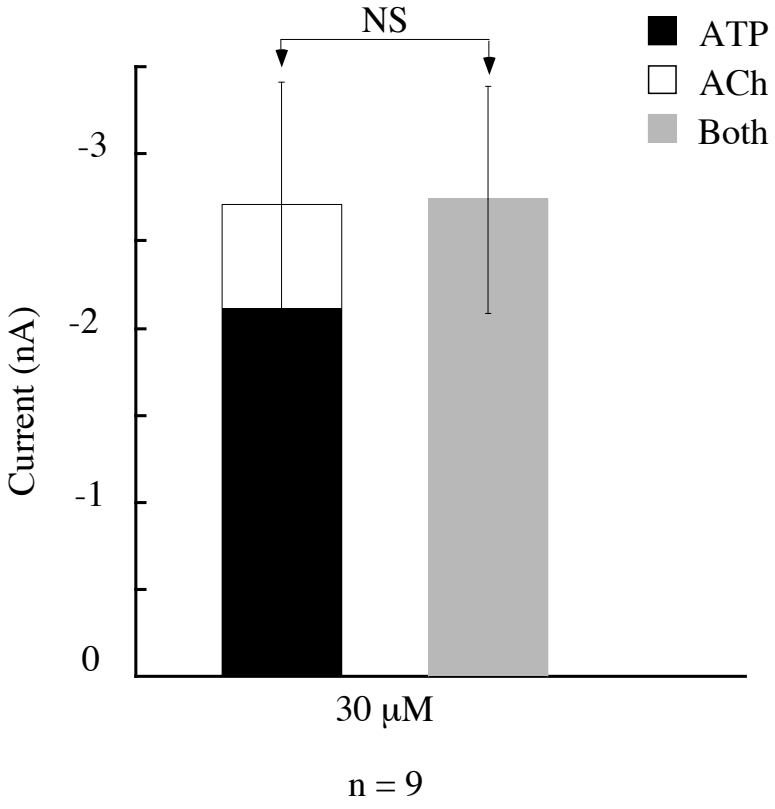
Figure 11. Current occlusion is not observed at low agonist concentration

A, Typical recordings of I_{ATP}, I_{ACh} and $I_{ATP+ACh}$ induced by 30 μM agonists concentration. **B**, Bars represent the averages of nine experiments. Notice that $I_{ATP+ACh}$ was not significantly (NS) different than the sum of the individual currents. S.E.M. are plotted as lines on the top of bars.

A


Before
ACh
ATP
+
ACh
ATP
After
ACh
ATP
1 nA
5 s

B


NS
ATP
ACh
Both
-3
-2
-1
0
Current (nA)
30 μM
n = 9

Discussion

This study shows, for the first time, the presence of inhibitory interactions between nACh and P2X native receptors in celiac neurons, which are only present when receptors are activated by maximal concentrations of ACh and ATP. Our observations indicate that ATP and ACh activate two independent channels, whose activation is, however, not independent and ionic currents carried through them are occluded when these receptors are simultaneously activated. Furthermore, we ruled out the possibility than either ATP or ACh molecules have any direct effect on nACh or P2X receptors. This current occlusion occurs very fast since it is observed 100 ms after that the currents are activated, appears to involve partial inhibition of both channels and to be independent of the cell metabolism and protein phosphorylation. Taken together, these observations suggest that current occlusion is mediated by cross-inhibition throught direct interaction between nACh and P2X channels.

Contrary to what has been reported previously (Searl *et al.*, 1998), we found here that currents induced by ACh and ATP in celiac neurons are additive at low concentrations. Searl et al. (1998) reported that nicotine, at very low concentrations, can to inhibit I_{ATP}. ATP or α,β-methylene ATP, also at low concentrations, can also inhibit nicotine-induced currents (I_{nic}). These authors also indicated that I_{ATP} and I_{nic} induced by relatively low agonist concentrations and were clearly mediated by activation of nACh and P2X receptors. This discrepancy might be explained by the fact that we used ACh and they used

nicotine. Thus, it is possible that nicotine might be acting on a pathway different than the nACh channels. This explanation, however, is unlikely because blocking nACh channels with hexamethonium prevented the inhibitory effects of nicotine on P2X channels (Searl *et al.*, 1998).

Consistent with the findings reported here, our laboratory and others (Nakazawa, 1994; Barajas-Lopez *et al.*, 1998; Zhou & Galligan, 1998) have found lack of occlusion at low concentrations of agonists in enteric and sympathetic neurons.

It is clear that what we observed here is different than what Searl et al. (1998) reported. Thus, they found that the inhibitory effect of nicotine on P2X-mediated currents do not required of nACh activation. Similarly the inhibitory effects of ATP on nicotinic-mediated currents also do not required of the opening of P2X channels. The type of current occlusion reported here requires the opening of channels (Nakazawa, 1994; Barajas-Lopez *et al.*, 1998; Zhou & Galligan, 1998) and channels conducting inwardly.

We have previously proposed a cross-inhibition model to explain current occlusion between I_{ATP} and I_{ACh} in submucous neurons, whose properties are very similar to those described here (Barajas-Lopez *et al.*, 1998). Therefore, a similar model (Fig 12) could explain current occlusion between I_{ATP} and I_{ACh} in celiac neurons. According to which, P2X and nACh channels would be located very close to each other forming complexes, each of them formed by one or more channels of each type. Direct support for our cross-inhibition model comes from recent studies (Boue-Grabot *et al.*, 2004a; Khakh *et al.*, 2005). The later

group 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, with their channels ~80 Å appart. According to the cross-inhibition model, when channels of a given complex are simultaneously activated, the net ion inflow through the first channel (e.g. P2X) would induce allosteric inhibition of the second channel of the same complex (e.g. nAChR). Inhibition of the second channel would stop once the ion flow through the first channel ceased. According to this model only one (either) channel is open in a given complex. The use of non-saturating agonist concentrations would also decrease channel cross-inhibition because the probability that two different channels are simultaneously activated in a given complex will be lower with non-saturating than with saturating concentrations.

Functional implications for these channel interactions

Our current experimental information suggests that inhibitory interactions between ligand-gated channels may be a mechanism widely used to limit the ionic currents through the cellular membrane. Channel interactions similar to those reported here, exist between $GABA_A$ and P2X channels (Sokolova *et al.*, 2001; Boue-Grabot *et al.*, 2004a); nACh and P2X receptors (Barajas-Lopez *et al.*, 1998; Zhou & Galligan, 1998; Khakh *et al.*, 2000); 5-HT_3 and P2X receptors (Barajas-Lopez *et al.*, 2002; Boue-Grabot *et al.*, 2003); and between glycine and $GABA_A$ channels (Li *et al.*, 2003). A recent study (Boue-Grabot *et al.*, 2004a)

reports that $P2X_2$ helps to re-target and recruit mainly intracellular ρ1/GABA receptors, to surface clusters formed by these two receptors.

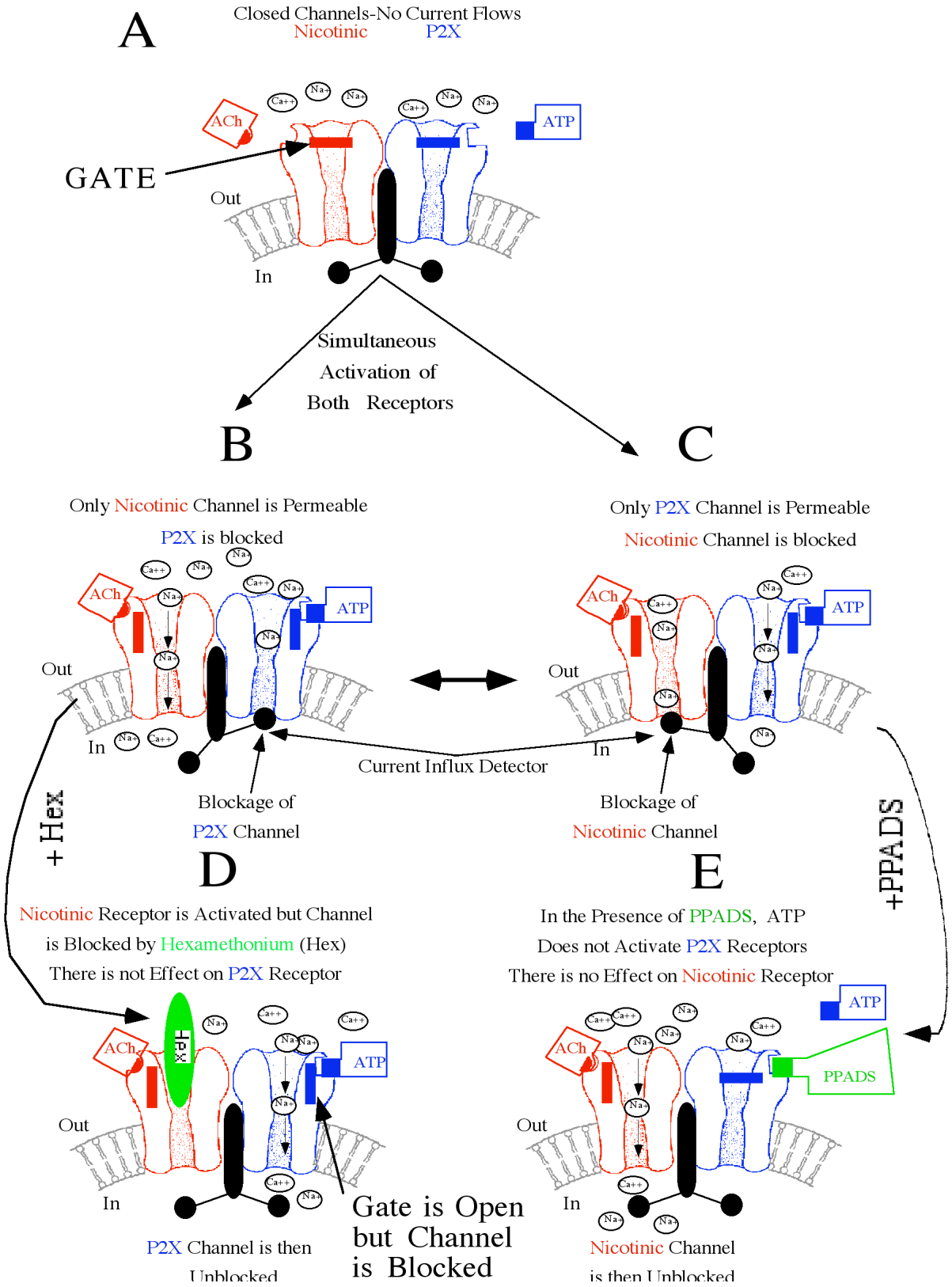
The fact that ATP, the endogenous agonist for most P2X receptors, has been shown to be co-released with various neurotransmitters, including GABA (Jo & Schlichter, 1999), noradrenaline (Burnstock & Kennedy, 1986; Starke *et al.*, 1991) and ACh (Unsworth & Johnson, 1990; Redman, 1994); the latter has been shown to be co-released with Glu (Li *et al.*, 2004; Nishimaru *et al.*, 2005), leading to the hypothesis that the inhibitory interactions between neurotransmitter receptors might be an important modulator of synaptic transmission. In celiac neurons, fast synaptic potentials mediated by both ACh and ATP have been reported (Evans, 1992). Therefore, at this point it is still to be discovered what is the role of the cross-inhibition between P2X and nACh receptors for synaptic integration.

Figure 12. Diagram showing the model of channel interactions between P2X and nACh receptors in celiac neurons

A: The model proposes that channels exist in functional clusters each of them containing at least one channel of each type. If channels are closed, no current flows through them. When channels are simultaneously activated by the agonists, current flows through them. However, only one channel would be open for every given cluster because the other would be allosterically inhibited (**B** or **C**).

B and C: For a given cluster, when the P2X channel is permeable, the nACh channel is blocked (C). When the nACh channel is permeable, the P2X channel is blocked (B). When maximal agonist concentrations are used to activate all channels on the neuronal membrane, interactions such as those seen in B and C occur. As a result, current occlusion occurs because current is not flowing through every channel present in each cluster; one type of channel is inhibited in each cluster. This also indicates that the current induced by simultaneous agonist application is mediated by both channels.

D and E: The model also considers that for current occlusion to occur, both channels must be functional. If one channel is activated but blocked, it does not affect the current, which can then flow through the other channel and further, no interaction between the channels is observed. **D,** In the presence of hexamethonium (Hex), ACh can activate its receptor but hexamethonium blocks the channel and thus, the combined current observed is similar to the ATP current alone (not shown). **D,** In the presence of PPADS, ATP cannot activate P2X receptors and the combined current observed is similar to the ACh current alone (not shown).


A
Closed Channels-No Current Flows
Nicotinic P2X
GATE
ACh
ATP
Out
In
Simultaneous
Activation of
Both Receptors
B
C
Only Nicotinic Channel is Permeable
P2X is blocked
Only P2X Channel is Permeable
Nicotinic Channel is blocked
Current Influx Detector
Blockage of
P2X Channel
Blockage of
Nicotinic Channel
+Hex
+PPADS
D
E
Nicotinic Receptor is Activated but Channel
is Blocked by Hexamethonium (Hex)
There is not Effect on P2X Receptor
In the Presence of PPADS, ATP
Does not Activate P2X Receptors
There is no Effect on Nicotinic Receptor
HEX
PPADS
P2X Channel is then
Unblocked
Gate is Open
but Channel
is Blocked
Nicotinic Channel
is then Unblocked

Conclusion

Our results indicate that there is a fast cross-inhibitory interaction between nACh and P2X channels in celiac neurons. The properties described for these interactions 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.

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