Intracellular Cross Talk and Physical Interaction between Two Classes of Neurotransmitter-Gated Channels

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Fast chemical communications in the nervous system are mediated by several classes of receptor channels believed to be independent functionally and physically. We show here that concurrent activation of $P2X_2$ ATP-gated channels and 5-HT_3 serotonin-gated channels leads to functional interaction and nonadditive currents (47–73% of the predicted sum) in mammalian myenteric neurons as well as in *Xenopus* oocytes or transfected human embryonic kidney (HEK) 293 cell heterologous systems. We also show that these two cation channels coimmunoprecipitate constitutively and are associated in clusters. In heterologous systems, the inhibitory cross talk between $P2X_2$ and 5-HT_3 receptors is disrupted when the intracellular C-terminal domain of the $P2X_2$ receptor subunit is deleted and when minigenes coding for $P2X_2$ or 5-HT_3A receptor subunit cytoplasmic domains are overexpressed. Injection of fusion proteins containing the C-terminal domain of $P2X_2$ receptors in myenteric neurons also disrupts the functional interaction between native $P2X_2$ and 5-HT_3 receptors. Therefore, activity-dependent intracellular coupling of distinct receptor channels underlies ionotropic cross talks that may significantly contribute to the regulation of neuronal excitability and synaptic plasticity.

Key words: **P2X purinoceptor; ATP; 5-HT_3; serotonin; ionotropic; ligand-gated cation channel; myenteric neurons**

Introduction

Fast communication in the nervous system is critical for information processing and synaptic plasticity; it is achieved through the activation of neurotransmitter-gated channels or ionotropic receptors (Sakmann, 1992) believed to be independent functionally and physically. The four known, structurally distinct classes of neurotransmitter-gated channels are represented by P2X ATP-gated receptors (Khakh et al., 2001), Phe-Met-Arg-Phe-amide-gated channels (Lingueglia et al., 1995), nicotinic acetylcholine receptors (Ortells and Lunt, 1995), and ionotropic glutamate receptors (Hollmann and Heinemann, 1994). Previous studies have shown that, in peripheral neurons and in neuronal cell lines, coactivation of P2X and nicotinic receptors elicits nonadditivity of ATP- and acetylcholine-induced currents (Nakazawa et al., 1991, 1994; Barajas-López et al., 1998; Searl et al., 1998; Zhou and Galligan, 1998; Khakh et al., 2001). A cross inhibition between the $P2X_2$ and the $\alpha_3\beta_4$ nicotinic receptor subtypes coexpressed in *Xenopus* oocytes has been reported previously (Khakh et al., 2000), and nonadditivity of P2X- and $GABA_A$-mediated currents has been observed in rat dorsal root ganglion neurons (Sokolova et al., 2001). Although these data indicate nonindependence of activity between P2X and several members of the nicotinic receptor family, the mechanisms involved in this inhibitory cross talk remain to be elucidated.

5-Hydroxytryptamine (5-HT) receptor channels (5-HT_3) belong to the nicotinic acetylcholine receptor superfamily (Maricq et al., 1991; Davies et al., 1999) and mediate fast excitatory transmission in the nervous system (Derkach et al., 1989; Ugita et al., 1992; Barnes and Sharp, 1999). 5-HT_3 and $P2X_2$ ATP receptors are coexpressed in several populations of central, sensory, sympathetic, and myenteric neurons (Tecott et al., 1993; Barajas-López et al., 1996; Zhou and Galligan, 1996; Morales et al., 2001). Both neurotransmitter receptor subunits can assemble into functional homomeric channels, providing a unique molecular model to investigate whether specific interactions involving subunit domains may underlie functional coupling between excitatory receptor channels.

Materials and Methods

Receptor channels, minigenes, and glutathione S-transferase fusion proteins. The original wild-type rat $P2X_2$, 5-HT_3A, 5-HT_3B, and ρ1 clones were provided by D. Julius (University of California, San Francisco, CA), S. F. Heinemann (Salk Institute, La Jolla, CA), E. Kirkness (The Institute for Genomic Research, Rockville, MD), and M. Garret (University of Bordeaux, Bordeaux, France), respectively. The truncated $P2X_2$ ($P2X_2$TR) construct was available from previous work (Boué-Grabot et al., 2000). The cDNAs coding for 5-HT_3A–Flag, enhanced green fluorescent protein (EGFP)-tagged $P2X_2$, the main intracellular domain of 5-HT_3A (5-HT_3A–IL2), the C-terminal domain of $P2X_2$ ($P2X_2$–CT), and the N-terminal domain of $P2X_2$ ($P2X_2$–NT) were generated by PCR and

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subcloned into pcDNA3. $P2X_2$–CT was also subcloned into pGEX2T (Amersham Biosciences, Arlington Heights, IL) to produce glutathione *S*-transferase (GST)–$P2X_2$–CT fusion protein in bacteria. All constructs were verified by DNA sequencing.

Electrophysiology in myenteric neurons. Whole-cell voltage-clamp recordings from cultured myenteric neurons of guinea pig proximal jejunum were performed as described previously (Barajas-López et al., 1996). Briefly, the neurons were dissociated using sequential enzymatic treatments with papain solution (10 μl/ml; activated with 0.4 mg/ml L-cysteine) followed by collagenase (1 mg/ml) and dispase (4 mg/ml). After washout, neurons were plated on coverslips coated with sterile rat tail collagen, placed in a recording chamber, and continuously superfused (2 ml/min) with an external solution containing (in mM): 160 NaCl, 2 $CaCl_2$, 11 glucose, 5 HEPES, and 3 CsCl, pH 7.4. Whole-cell currents were made using glass pipettes filled with internal solution containing (in mM): 160 Cs-glutamate, 10 EGTA, 5 HEPES, 10 NaCl, 3 ATP-Mg, and 0.1 GTP, pH 7.3, and recorded via an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) at a holding potential (V_H) of −60 mV. For competition experiments, GST protein or GST–$P2X_2$–CT fusion protein (75 μM) was included in the intracellular recording solution. Fast applications of 5-HT and ATP (Sigma, St. Louis, MO) were made using an eight barreled device. Because solutions were applied by gravity, we verified that the flow between different lines did not change significantly from the beginning to the end of the recording session. Results are reported as means ± SEM; statistical differences were evaluated using Student's *t* test.

Heterologous expression systems. Oocytes were prepared as described previously (Boué-Grabot et al., 2000). Stage V and VI oocytes were manually defolliculated before the microinjection of cRNAs. After injection (0.2 ng of RNA coding for $P2X_2$ and 15–20 ng of RNA coding for $P2X_2$TR, 5-HT_3A, or nicotinic receptor subunits), oocytes were incubated with Barth's solution containing 1.8 mM $CaCl_2$ at 19°C for 24–72 hr before electrophysiological recordings. For competition experiments, RNAs corresponding to minigenes were injected (ranging between 20 and 60 ng for each) independently in a second round of microinjection. Two-electrode voltage-clamp recordings were performed using glass pipettes (1–3 MΩ) filled with 3 M KCl solution. Oocytes were placed in a recording chamber and were perfused at a flow rate of 10–12 ml/min with Ringer's solution containing (in mM): 115 NaCl, 5 NaOH, 2.5 KCl, 1.8 $CaCl_2$ or $BaCl_2$, and 10 HEPES, pH 7.4. Membrane currents (DC; 1 kHz) were recorded through an OC-725B amplifier (Warner Instruments, Hamden, CT) and digitized at 500 Hz. All drugs (purchased from Sigma) were dissolved in the perfusion solution and applied using a computer-driven valve system. Because of the difference in time-to-peak between 5-HT_3 and $P2X_2$ currents recorded in oocytes (see Fig. 2*A*), we compared the peak of actual responses with the peak of predicted additive responses and not with the sum of the peaks of individual responses. All recordings were made at room temperature. Statistical differences between means were assessed using Student's *t* test.

Whole-cell voltage clamps (V_H of −60 mV) from transfected human embryonic kidney (HEK) 293 cells were made using pipettes filled with internal solution containing (in mM): 120 K-gluconate, 1 $MgCl_2$, 10 HEPES, and 4 NaOH, pH 7.18. Cells were perfused (2 ml/min) with external solution (22–24°C) containing (in mM): 14 NaCl, 3 KCl, 1 $MgCl_2$, 1 $BaCl_2$, 10 HEPES, and 5 NaOH, pH 7.35. Currents (DC; 5 kHz) were recorded using an Axopatch-200B amplifier (Axon Instruments) and digitized at 500 Hz. All values are reported as means ± SEM, and differences were assessed using Student's *t* test.

Immunoprecipitations and confocal imaging. Membrane proteins from HEK293 cells, transiently transfected with $P2X_2$, $P2X_2$–GFP, or 5-HT_3A–Flag or cotransfected with $P2X_2$–GFP plus 5-HT_3A–Flag or $P2X_2$ plus 5-HT_3A–Flag using the calcium phosphate method, were homogenized in 10 mM HEPES and 0.3 M sucrose and solubilized in 1% Triton X-100 with protease inhibitors (Sigma) at 4°C before immunoaffinity purification on anti-Flag M2 resin (Sigma) as described previously (Boué-Grabot et al., 2000). In two experiments, HEK293 cells were incubated before homogenization in PBS buffer containing ATP plus 5-HT (100 μM each). Bound proteins were eluted and then loaded onto a 10% SDS-PAGE and transferred to a nitrocellulose membrane. Labeling of immunoprecipitated associated receptors was performed with anti-GFP antibodies (1:5000; Molecular Probes, Eugene, OR), affinity-purified anti-$P2X_2$ antibodies (1:1000; Chemicon, Temecula, CA). or anti-Flag M2 antibodies (1:1000; Sigma) followed by incubation with corresponding peroxidase-labeled secondary antibodies (1:2000; Jackson ImmunoResearch, West Grove, PA) for visualization by enhanced chemiluminescence. In the experiments of competition, HEK293 cells were cotransfected with wild-type $P2X_2$, 5-HT_3A–Flag subunits and $P2X_2$–CT or $P2X_2$–NT constructs at a cDNA ratio of 3:1 (minigene:subunits). Confocal fluorescence microscopy images were obtained from Chinese hamster ovary (CHO) cells stably expressing 5-HT_3A subunits after transient transfection with $P2X_2$–GFP and treatment with 5 μM latrunculin-A for 18 hr. 5-HT_3 receptors were immunolocalized using anti-5-HT_3A antibodies (Doucet et al., 2000) and cyanine dye 3-conjugated secondary antibody; tagged $P2X_2$ receptors were visualized with GFP.

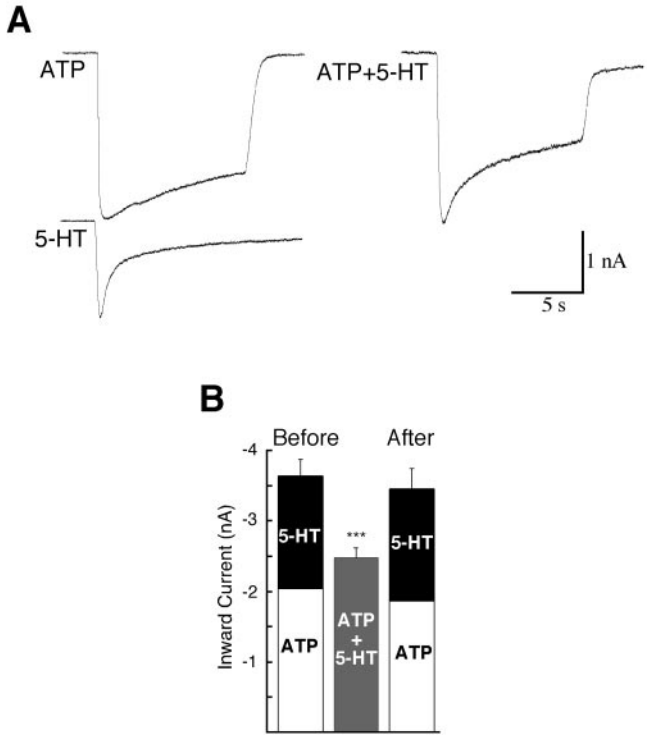

Figure 1. Inhibitory cross talk between ATP and 5-HT responses in guinea pig myenteric neurons. *A*, Representative whole-cell current responses induced by ATP, 5-HT, and ATP plus 5-HT (1 mM each). *B*, Mean values of ATP- and 5-HT-induced responses recorded 5 min before and 5 min after ATP plus 5-HT responses from nine experiments. Whole-cell currents were measured at a V_H of −60 mV. ***$p < 0.001$.

Results

Whole-cell voltage-clamp recordings were obtained from myenteric neurons acutely dissociated from guinea pig proximal jejunum. The application of saturating concentrations of ATP (1 mM) evoked a slowly desensitizing inward current (I_{ATP} = −2.0 ± 0.2 nA; n = 9), whereas an application of saturating concentrations of 5-HT (1 mM) induced a rapidly desensitizing current ($I_{5\text{-HT}}$ = −1.61 ± 0.1 nA) (Fig. 1*A*). Interestingly, the coapplication of ATP and 5-HT (1 mM each) to the same neurons induced a large reversible inward current (I_{ATP} + $I_{5\text{-HT}}$ = −2.48 ± 0.1 nA; n = 9) (Fig. 1) that was significantly smaller than the arithmetic sum of I_{ATP} and $I_{5\text{-HT}}$ currents (69 ± 4% of predicted; $p < 0.001$). If responses to ATP and 5-HT were attributable to the activation of functionally independent channels, the current induced by the simultaneous application of saturating concentrations of these transmitters, when the occupancy of both

receptors reaches 100%, should have been additive. The nonadditivity of currents induced by the concurrent activation of both native receptors indicates that ATP-gated and 5-HT-gated channels do not function independently in myenteric neurons. Nonadditivity was also recorded when subsaturating concentrations of ATP and 5-HT were used (data not shown).

Several neuronal P2X subtypes can generate slowly desensitizing currents; however, the distribution of $P2X_2$ in guinea pig myenteric plexus (Castelluci al., 2002) and the loss of somatic P2X currents in myenteric neurons of $P2X_2$ knock-out mice (Cockayne et al., 2002) suggest that these currents are mediated by homomeric $P2X_2$- or heteromeric $P2X_2$-containing ATP receptors. Therefore, the kinetic profiles of I_{ATP} and $I_{5\text{-}HT}$ recorded in myenteric neurons (Fig. 1) are consistent with the activation of ionotropic $P2X_2$ and 5-HT_3 receptor subtypes. Therefore, we expressed both receptor subunit cDNAs in *Xenopus* oocytes to test whether they also interact functionally in a heterologous system, to investigate the functional and molecular characteristics of the inhibitory cross talk between $P2X_2$ and 5-HT_3 channels. During two-electrode voltage-clamp recording in oocytes expressing both $P2X_2$ and 5-HT_3A subunits, the simultaneous application of ATP and 5-HT (100 μM each) evoked an inward current (-11.9 ± 1.5 μA; $n = 22$) that was significantly smaller ($p < 0.001$) than the sum of responses to separate applications of 5-HT ($I_{5\text{-}HT} = -2.7 \pm 0.5$ μA) and ATP ($I_{ATP} = -14.5 \pm 1.8$ μA) (Fig. 2*A*), demonstrating nonadditivity by occlusion. In agreement with our recordings in myenteric neurons, the amplitude of $I_{ATP+5\text{-}HT}$ represents $69 \pm 1\%$ ($n = 28$) of the predicted current corresponding to the arithmetic sum of I_{ATP} and $I_{5\text{-}HT}$. Moreover, when 5-HT was applied during the continuous application of ATP, no modification or a rapid reduction in the amplitude of the I_{ATP} was observed (Fig. 2*B*), revealing an instantaneous reciprocal current occlusion. The nonadditivity between $I_{5\text{-}HT}$ and I_{ATP} was also recorded at submaximal concentrations of either agonist (Fig. 2*C*), by measuring outward currents at positive potentials (Fig. 2*D*), or in the absence of extracellular Ca^{2+} (data not shown). Coactivation of $P2X_2$ and heteromeric 5-HT_3A+B receptors also produced current responses significantly smaller than the predicted current ($73 \pm 1\%$; $n = 17$) (Fig. 2*E*), suggesting that the 5-HT_3A subunit is essential in the interaction between 5-HT_3 and P2X receptors. Therefore, recombinant $P2X_2$ and 5-HT_3A receptors do not function independently in *Xenopus* oocytes.

In oocytes expressing only $P2X_2$ receptors, the application of saturating concentrations of 5-HT did not activate $P2X_2$ receptors, and the coapplication of ATP and 5-HT induced a current response identical in kinetics and in amplitude to I_{ATP} ($I_{ATP+5\text{-}HT} = 102 \pm 5\%$ of I_{ATP}; $n = 6$) (Fig. 3*A*). Similarly, ATP did not activate 5-HT_3A channels, nor did it modulate $I_{5\text{-}HT}$ ($I_{ATP+5\text{-}HT} = 102 \pm 2\%$ of $I_{5\text{-}HT}$; $n = 6$) (Fig. 3*B*) indicating that the cross-inhibition between 5-HT_3A and $P2X_2$ is not attributable to receptor cross-modulation.

In oocytes coexpressing 5-HT_3A receptors and homomeric ρ1 $GABA_C$ receptors, simultaneous applications of 5-HT (100 μM) and GABA (10 μM) evoked an inward current (-2.66 ± 0.58 μA; $n = 12$) that was not different from the sum of $I_{5\text{-}HT}$ (-1.54 ± 0.23 μA) and I_{GABA} (-1.18 ± 0.4 μA) (Fig. 3*C*). $I_{5\text{-}HT+GABA}$ represented $97 \pm 4\%$ of the predicted current (Fig. 3*E*). Additive currents were also recorded when 5-HT was applied during the prolonged application of GABA and vice versa (Fig. 3*D*). These results indicate that homomeric 5-HT_3A receptors and ρ1 $GABA_C$ receptors act as independent channels without cross talk in *Xenopus* oocytes.

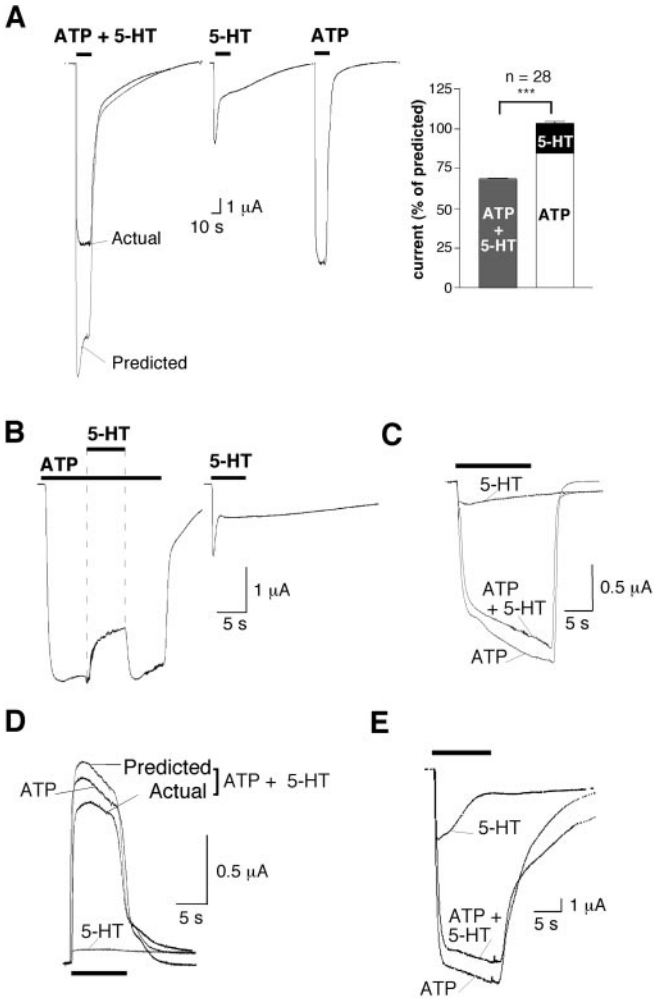

Figure 2. Cross-inhibition between recombinant $P2X_2$ and 5-HT_3 receptor channels in *Xenopus* oocytes. *A, B*, Coapplication of ATP and 5-HT evoked inward currents (actual) significantly smaller than the arithmetic sum (predicted) of the individual ATP and 5-HT responses. ***$p < 0.001$; $n = 28$. The amplitudes of the responses are normalized to the predicted response from each oocyte (bar graph). V_H is -60 mV. *B*, Current occlusion also occurs when 5-HT application starts during ATP application. *C, D*, Superimposed traces obtained from *Xenopus* oocytes expressing $P2X_2$ and 5-HT_3A receptors activated with 1 μM ATP, 100 μM 5-HT, and both ATP and 5-HT (*C*) and at $+40$ mV V_H with a saturating concentration of agonists (100 μM each) (*D*). *E*, Nonadditivity of ATP and 5-HT responses is also observed in oocytes coexpressing $P2X_2$ and heteromeric 5-HT_3A+B channels.

To determine whether intracellular domains could be involved in the functional interaction between $P2X_2$ and 5-HT_3A receptors, we first truncated most of the cytoplasmic C-terminal domain of the $P2X_2$ receptor subunit by adding a stop codon at amino acid 365 ($P2X_2$TR). Truncated $P2X_2$ subunits have been shown previously to assemble into functional receptors (Boué-Grabot et al., 2000). Contrary to the data obtained with wild-type $P2X_2$, coactivation of $P2X_2$TR and 5-HT_3A receptors evoked a current response (-3.4 ± 0.8 μA; $n = 14$) that was not significantly different ($p > 0.5$) from the sum of I_{ATP} and $I_{5\text{-}HT}$ (-0.72 ± 0.24 μA and -2.07 ± 0.42 μA, respectively; $n = 14$) (Fig. 4*A*). $I_{ATP+5\text{-}HT}$ represented $115 \pm 6\%$ of the predicted current (Fig. 4*B*). The additivity of ATP and 5-HT responses was also observed when 5-HT applications started during the continuous application of ATP (Fig. 4*C*). The application of 5-HT to oocytes expressing $P2X_2$TR receptors alone did not activate or modulate I_{ATP} ($I_{ATP+5\text{-}HT}$ was $100 \pm 11\%$ of I_{ATP}) (Fig. 4*D*). Therefore,

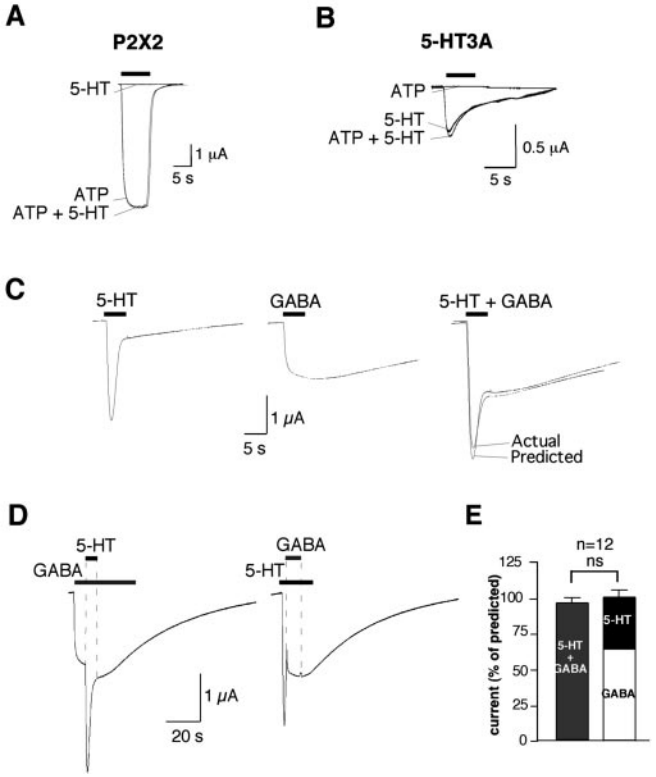

Figure 3. Cross talk between $P2X_2$ and 5-HT_3A receptors is not attributable to cross-modulation and additivity of 5-HT_3- and $GABA_C$-mediated responses. Superimposed control traces obtained from *Xenopus* oocytes expressing $P2X_2$ (*A*) or 5-HT_3A (*B*) receptors alone or with ATP, 5-HT, or a mixture of ATP and 5-HT (100 μM each) are shown. *C*, Coexpression of homomeric 5-HT_3A and ρ1 $GABA_C$ receptors in *Xenopus* oocytes. Currents evoked by the application of 100 μM 5-HT and 10 μM GABA and by the coapplication of 5-HT plus GABA are shown. *D*, Additivity was also observed when GABA application started during 5-HT application and reciprocally. *E*, Inward currents evoked by the coapplication of GABA and 5-HT (actual) were not significantly different from the arithmetic sum (predicted) of the individual GABA and 5-HT currents ($n = 12$). *ns*, Not significant.

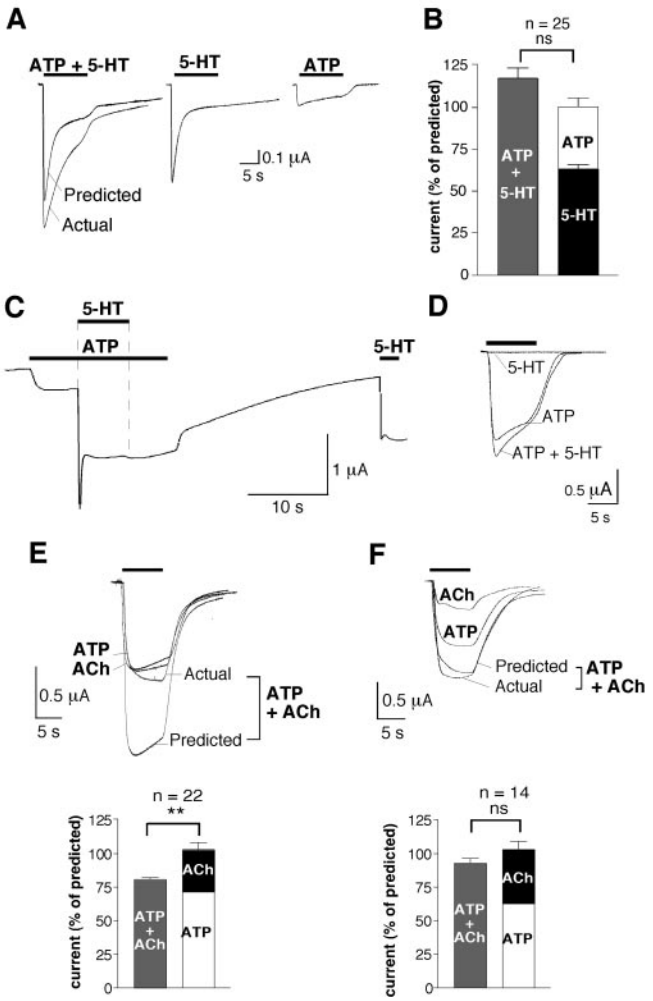

Figure 4. Additivity of agonist-induced responses in oocytes coexpressing $P2X_2TR$ and either 5-HT_3A or $\alpha_3\beta_4$ nicotinic receptors. *A, C*, Representative current traces showing coexpression of $P2X_2TR$ and 5-HT_3A receptors in *Xenopus* oocytes. *B*, Mean current amplitudes showing the additivity of responses mediated by $P2X_2TR$ and 5-HT_3A channels. *D*, Representative superimposed currents with individual and combined applications of ATP and 5-HT (100 μM) recorded from one oocyte expressing $P2X_2TR$ alone. *E*, Nonadditivity of $P2X_2$ and $\alpha_3\beta_4$ nicotinic currents in oocytes. $^{**}p < 0.005$; $n = 22$. *F*, In oocytes coexpressing $P2X_2TR$ and $\alpha_3\beta_4$ nicotinic channels, coapplications of ATP and ACh evoked inward currents (actual; $n = 14$) that were not different from the sum of individual ATP and ACh responses (predicted). *ns*, Not significant.

$P2X_2TR$ and 5-HT_3A receptors act as independent channels in *Xenopus* oocytes, suggesting an important role for the intracellular $P2X_2$ C-terminal domain in the reciprocal cross-inhibition between wild-type $P2X_2$ and 5-HT_3A activity. The functional interaction between $P2X_2$ and neuronal $\alpha_3\beta_4$ nicotinic channels ($I_{ATP+ACh} = 80 \pm 2\%$ of predicted) (Fig. 5*E*) was also abolished when wild-type $P2X_2$ receptors were replaced with $P2X_2TR$ receptors ($I_{ATP+ACh} = 93 \pm 4\%$ of predicted) (Fig. 4*F*), suggesting that the reciprocal inhibitory cross talk between P2X receptors and nicotinic or 5-HT_3 receptors is based on similar intracellular mechanisms.

To generate competitive inhibitors of the functional interaction between $P2X_2$ and 5-HT_3 receptors, we designed two minigenes encoding soluble cytoplasmic forms of the C-terminal domain of $P2X_2$ receptors ($P2X_2$–CT, corresponding to amino acids 365–469) or of the N-terminal domain of $P2X_2$ receptors ($P2X_2$–NT, corresponding to amino acids 1–29). As illustrated in Figure 5*A–C*, expression of $P2X_2$–CT disrupted the interaction between $P2X_2$ and either 5-HT_3A or $\alpha_3\beta_4$ nicotinic receptors, as demonstrated by the additive responses induced by the coapplication of ATP and either 5-HT or acetylcholine, without affecting the magnitude of the respective transmitter-evoked currents. This inhibition of the interaction by the $P2X_2$–CT minigene was concentration dependent (Fig. 5*E*). Nonadditive responses to coapplications of ATP and 5-HT were observed with the coexpression of $P2X_2$–NT, indicating that the N-terminal domain of the $P2X_2$ subunit is not involved in the cross-inhibition between $P2X_2$ and 5-HT_3A receptors (Fig. 5*B,E*). Thus, the intracellular C-terminal domain of $P2X_2$ receptors is determinant in their functional interaction with excitatory members of the nicotinic receptor gene superfamily.

To determine whether the cross talk between $P2X_2$ and 5-HT_3A is also dependent on a cytoplasmic domain of 5-HT_3A receptor subunit, we generated a minigene (5-HT_3A–IL2, corresponding to amino acids 316–418) encoding the large intracellular loop between the third and the fourth transmembrane domains. Coexpression of 5-HT_3A–IL2 with $P2X_2$ and 5-HT_3A channels also disrupted the functional interaction (Fig. 5*D*) in a concentration-dependent manner (Fig. 5*E*).

A physical association between $P2X_2$ and 5-HT_3A receptor channels could underlie their functional interaction. Therefore, we performed affinity purification of Triton X-100-solubilized membrane protein extracts from HEK293 cells cotransfected

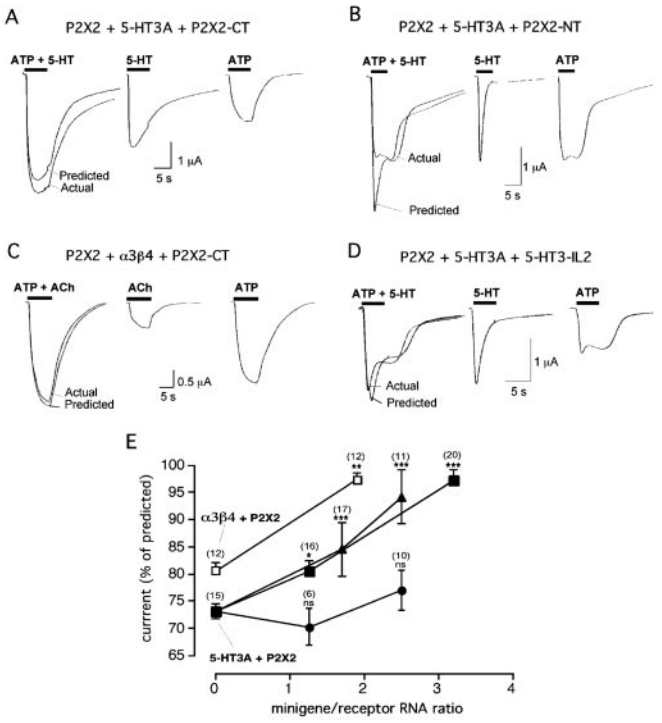

Figure 5. Cross talk between $P2X_2$ and 5-HT_3A channels or between $P2X_2$ and $\alpha_3\beta_4$ nicotinic acetylcholine receptors is dependent on specific intracellular subunit domains. *A*, *B*, *D*, Inward currents evoked with 100 μM ATP, with 100 μM 5-HT, or with both agonists (actual) in oocytes coexpressing $P2X_2$ and 5-HT_3A channels. *A*, In the presence of a minigene encoding $P2X_2$–CT. *B*, In the presence of a minigene encoding $P2X_2$–NT. *D*, In the presence of a minigene encoding 5-HT3–IL2. *C*, Currents induced with 100 μM ATP, 100 μM ACh, and both ATP and ACh (100 μM each) in oocytes coexpressing $P2X_2$, $\alpha_3\beta_4$ nicotinic channels, and $P2X_2$–CT. *E*, Concentration-dependent inhibitory effect of $P2X_2$–CT (■, □) or 5-HT3A–IL2 (▲) on the functional interaction between $P2X_2$ and 5-HT_3A and between $P2X_2$ and $\alpha_3\beta_4$ nicotinic channels. *Numbers* in *parentheses* indicate numbers of cells. *$p < 0.05$. $P2X_2$–NT (●) had no effect on the cross-inhibition between 5-HT_3 and $P2X_2$ receptors ($p > 0.5$). Mean values of 5-HT plus ATP responses normalized to the predicted response without minigene and with increasing amounts of minigenes are shown. **$p < 0.005$; ***$p < 0.0005$. *ns*, Not significant.

with functionally interacting GFP-tagged $P2X_2$ and Flag-tagged 5-HT_3A receptors to test this hypothesis. The coapplication of ATP and 5-HT (100 μM) evoked an inward current (-2.2 ± 0.5 nA; $n = 5$) that was significantly smaller than the sum of I_{ATP} and $I_{5\text{-HT}}$ (-4.7 ± 0.8 nA) in transfected HEK293 cells (actual $I_{5\text{-HT+ATP}} = 47 \pm 7\%$ of predicted response) (Fig. 6*A*,*E*). After immunopurification on anti-Flag resin, a band of 95 kDa relative molecular mass corresponding to the expected size of the $P2X_2$–GFP subunit was revealed with anti-GFP antibodies, demonstrating a physical association between $P2X_2$ and 5-HT_3 receptors (Fig. 6*B*). The specificity of the coimmunoprecipitation was verified by the absence of the signal detected with purified proteins from HEK293 cells transfected with $P2X_2$–GFP alone, with membrane proteins from nontransfected cells (Fig. 6*B*), or after mixing membrane proteins from two batches of HEK293 cells expressing either $P2X_2$ or 5-HT_3 receptors (data not shown). A physical association between the two receptors was observed with or without activation of the receptors by a 100 μM concentration of their respective agonists ATP and 5-HT (Fig. 6*B*). Coexpression of $P2X_2$ and 5-HT_3–Flag receptors with minigenes encoding $P2X_2$ N- or C-terminal domains did not inhibit their physical interaction, as shown by the detection of $P2X_2$ after immunopurification on anti-Flag resin (Fig. 6*D*). Overexpression of the $P2X_2$–CT minigene was checked by recording the loss of functional interaction between $P2X_2$ and 5-HT_3 receptors in patch clamp. Coapplication of ATP and 5-HT (100 μM) evoked an inward current (-3.2 ± 0.5 nA; $n = 3$) that was not significantly different from the sum of I_{ATP} and $I_{5\text{-HT}}$ (-2.7 ± 0.3 nA) in transfected HEK293 cells (actual $I_{5\text{-HT+ATP}} = 84 \pm 6\%$ of predicted response) (Fig. 6*C*). These results indicate the existence of constitutive $P2X_2$ plus 5-HT_3A complexes in the plasma membrane and suggest that if intracellular domains are necessary for the functional cross-inhibition, other domains of $P2X_2$ and 5-HT_3 receptors are possibly involved in the physical association. Indeed, multireceptor clusters containing GFP-tagged $P2X_2$ and 5-HT_3A receptors were localized at the surface of transfected cells using confocal fluorescence microscopy (Fig. 6*F*).

Because the cross talk between recombinant $P2X_2$ and 5-HT_3A receptors was disrupted by the expression of minigenes encoding specific intracellular receptor subunit domains, we then tested whether the cross talk between native $P2X_2$ and 5-HT_3 receptors could also be disrupted in neurons using a similar strategy of competition. Indeed, we observed that the intracellular infusion of GST–$P2X_2$–CT fusion protein through the recording pipette into the cytoplasm of myenteric neurons significantly reduced the functional interaction ($I_{5\text{-HT+ATP}} = 86 \pm 3\%$ of the predicted; $p < 0.005$; $n = 8$) (Fig. 7*A*–*C*) recorded in neurons infused with buffer alone ($I_{5\text{-HT+ATP}} = 72 \pm 2\%$ of predicted; $n = 8$) (Fig. 7*C*) or GST alone ($I_{5\text{-HT+ATP}} = 73 \pm 5\%$ of predicted; $n = 6$) (Fig. 7*B*,*C*). Responses to 5-HT and ATP were not additive at the time at which the whole-cell configuration was established but became additive 30 min later in the same neuron, after dialysis of the cytoplasm with the fusion protein GST–$P2X_2$–CT (Fig. 7*D*). Therefore, the inhibitory cross talk between native and recombinant 5-HT_3 and $P2X_2$ receptor channels is mediated by the activity-dependent coupling of specific intracellular subunit domains.

Discussion

Here we present the first evidence that two structurally unrelated ligand-gated channels, $P2X_2$ and 5-HT_3 receptors, are physically associated, and that specific intracellular domains are necessary for the expression of their cross-inhibition. Coactivation of both receptor channels expressed natively in myenteric neurons or in recombinant heterologous systems triggers an instantaneous reciprocal current occlusion in a situation similar to the cross-inhibition between $P2X_2$ and $\alpha_3\beta_4$ nicotinic receptors reported previously (Barajas-López et al., 1996; Zhou and Galligan, 1996; Khakh et al., 2000). This receptor-mediated cross talk between $P2X_2$ and 5-HT_3 responses is calcium, voltage, and agonist concentration independent. It is not attributable to cross-modulation, because ATP has no effect on 5-HT_3 receptors and 5-HT does not activate $P2X_2$ receptors, and the cross talk shows some receptor specificity, because 5-HT_3 receptors do not interact with $GABA_C$ receptors.

The functional independence observed between truncated $P2X_2$ and 5-HT_3A receptors as well as the suppression of the cross talk between wild-type $P2X_2$ and 5-HT_3 receptors in competition experiments with the intracellular loop of 5-HT_3A subunit and with the C-terminal (but not the N-terminal) domain of $P2X_2$ demonstrate the involvement of cytoplasmic sequences from both receptor subunits in the functional interaction in native neurons and in heterologous expression systems. These results, in line with the absence of cross-modulation, eliminate the possibility of a major role for second messengers generated by endogenous and electrophysiologically silent metabotropic P2Y or 5-HT receptors in this inhibitory cross talk. The involvement of the

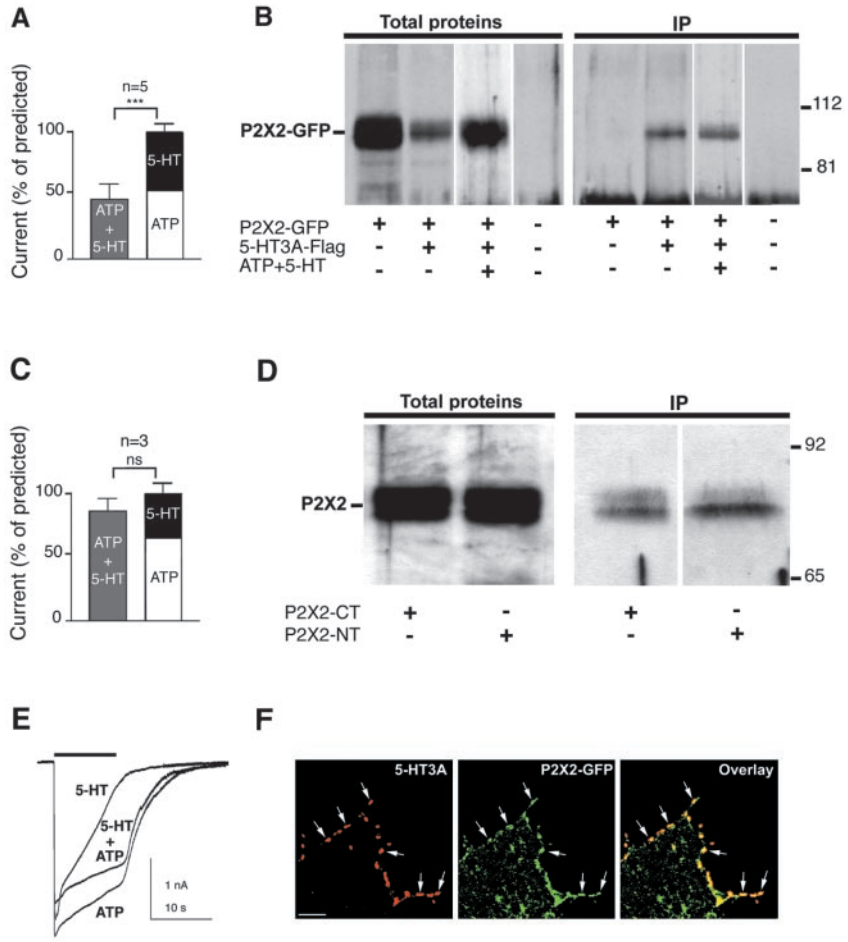

Figure 6. Constitutive physical association and coclustering of $P2X_2$ and $5\text{-}HT_3A$ receptor channels. *A*, *E*, Nonadditive current responses from HEK293 cells transfected with $P2X_2$–GFP and $5\text{-}HT_3A$–Flag to separate applications of 100 μM ATP, 100 μM 5-HT, and ATP plus 5-HT (100 μM each). ***$p < 0.001$. *B*, Copurification of $P2X_2$–GFP and $5\text{-}HT_3A$–Flag receptors from transfected HEK293 cells. Transfected HEK293 cells were incubated in the presence (+) or absence (−) of the agonists ATP and 5-HT (100 μM each) before homogenization. *IP*, Immunoprecipitation. *C*, Additivity of the $P2X_2$ and $5\text{-}HT_3$ responses in HEK293 cells cotransfected with wild-type $P2X_2$, $5\text{-}HT_3A$–Flag, and $P2X_2$–CT minigene. *ns*, Not significant. *D*, $P2X_2$ and $5\text{-}HT_3$ receptors were coimmunopurified when coexpressed with the $P2X_2$–CT or $P2X_2$–NT minigene. The Western blots are representative of 10 independent experiments; *numbers* indicate molecular mass markers in kilodaltons. *F*, Confocal microscopy images showing membrane colocalization of $5\text{-}HT_3A$ and $P2X_2$–GFP receptors in transfected CHO cells. *Arrows* in the *overlay panel* indicate coclusters of the two types of neurotransmitter-gated channels. Scale bar, 10 μm.

types (Khakh et al., 2000; Sokolova et al., 2001). The fact that the C-terminal sequences in the P2X family or the intracellular domains of the nicotinic receptor family members display no clear homology at the level of their primary sequence argues in favor of a coupling between channel motifs with conserved tertiary structures. Moreover, the lack of a modulatory effect of overexpressed cytoplasmic domains on the function of the other receptor partners (at least for current amplitudes and kinetics) in our competition experiments suggests an activity-dependent coupling. Interestingly, overexpression of a minigene encoding the C-terminal domain of $P2X_2$ in transfected cells has a clear competitive disrupting effect on the functional interaction between $P2X_2$ and $5\text{-}HT_3$ receptors but did not prevent their coimmunoprecipitation. Although we cannot exclude the allosteric participation of extracellular or transmembrane regions of $P2X_2$ and $5\text{-}HT_3$ subunits to the cross talk, our results strongly support the existence of two distinct types of interaction between the receptor channels: an activity-dependent intracellular coupling and a constitutive physical association involving other determinants that remain to be identified.

Functional cross talks between P2X ATP-gated channels and $5\text{-}HT_3A$ or nicotinic receptors were recorded in *Xenopus* oocytes and in mammalian cell lines as well as in several neuronal types. This widespread occurrence suggests that, if indirect, receptor–receptor functional couplings might depend on the expression of ubiquitous and conserved intracellular partners.

The large cytoplasmic domain of channel subunits belonging to the nicotinic receptor superfamily is necessary for the functional coupling with P2X receptors, but it is also required for targeting and/or postsynaptic clustering through specific interactions with receptor-associated proteins. For example, muscle nicotinic acetylcholine receptors associate with rapsyn (Maimone and Enigk, 1999) and glycine, $GABA_A$ receptors associate with gephyrin (Meyer et al., 1995; Essrich et al., 1998), and specific GABA receptor subunits associate with GABA receptor-associated protein or MAP-1B (Hanley et al., 1999; Wang et al., 1999). Although proteins associated with $5\text{-}HT_3$ receptors and neuronal P2X receptors are not yet known, direct or indirect constitutive interactions of P2X receptors with $5\text{-}HT_3$ receptors might also play a role in targeting both of them to specific synaptic or extrasynaptic localizations in coclusters at the neuronal surface (Rubio and Soto, 2001).

In vivo, several transmitters can be coreleased in the synaptic cleft (Docherty et al., 1987; Jonas et al., 1998), and ATP is known to be a cotransmitter in a variety of neuroneuronal and neuroeffector synapses (Burnstock, 1986; Jo and Schlichter, 1999; Poelchen et al., 2001). In the guinea pig myenteric nervous system, the high density of serotonergic varicose nerve fibers originates primarily from intrinsic neurons (Furness and Costa, 1982;

intracellular C-terminal domain of $P2X_2$ in the cross talk with $\alpha_3\beta_4$ nicotinic acetylcholine-gated channels demonstrated here strongly suggests that a generic molecular mechanism underlies the functional coupling observed between P2X ATP receptors and members of the nicotinic receptor superfamily. Although the inhibitory cross talk between $GABA_A$ and P2X receptors appears to be chloride and calcium dependent in dorsal root ganglion sensory neurons (Sokolova et al., 2001), the possibility of intracellular interactions between $GABA_A$ and P2X subunits should now be investigated.

Specific associations linking metabotropic G-protein-coupled receptors and ion channels have been shown to mediate, for example, the inhibition of neurotransmitter-gated channels by dopamine receptors (Liu et al., 2000; Lee et al., 2002) and the increase in L-type voltage-gated calcium channel activity by the stimulation of β_2 adrenergic receptors (Davare et al., 2001). It is clear now that P2X channels interact with several members of the nicotinic receptor superfamily, and conversely, nicotinic and GABA receptors interact with several ATP-gated channel sub-

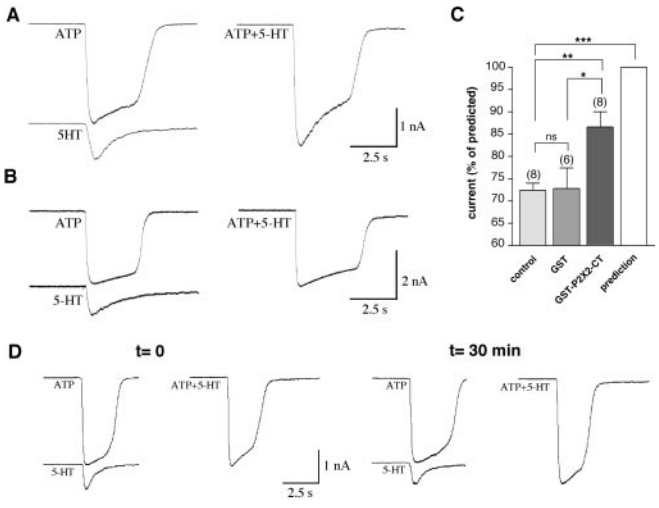

Figure 7. Disruption of cross talk between $P2X_2$ and $5\text{-}HT_3$ receptors in myenteric neurons by intracellular GST–$P2X_2$–CT fusion protein. *A*, Representative additive whole-cell current responses induced by ATP, 5-HT, and ATP plus 5-HT (1 mM each) recorded 30 min after the infusion of GST–$P2X_2$–CT fusion protein. *B*, Control inhibitory cross talk after infusion with GST alone in the recording pipette. *C*, Mean values of 5-HT plus ATP responses normalized to the predicted response with buffer only (*control*) and with fusion proteins (GST or GST–$P2X_2$–CT). $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$. *ns*, Not significant. *D*, Current traces obtained from one typical neuron before ($t = 0$) and after the infusion of GST–$P2X_2$–CT fusion protein ($t = 30$ min). Whole-cell currents were measured at a V_H of -60 mV.

Wardell et al., 1994). Enterochromaffin cells (Racké et al., 1996), platelets, and mast cells (Bueno and Fioramonti, 1999) provide non-neuronal sources of ATP and 5-HT. Both excitatory mediators have the ability to depolarize the mucosal nerve terminals (Bertrand et al., 2000, 2002). The interactions between P2X and $5\text{-}HT_3$ receptors coexpressed in the terminals of myenteric sensory neurons (Bertrand et al., 2002) could thus play a regulatory role by buffering the paracrine effects of ATP and 5-HT on reflex actions.

The functional cross-inhibition between ATP-gated channels and other excitatory transmitter-gated channels of the nicotinic receptor family may regulate neuronal excitability and synaptic plasticity by limiting both the level of depolarization and the flow of calcium ions through calcium-permeable P2X receptors (Koshimizu et al., 2000). Alternatively, in pathological conditions of neuronal hyperactivity, it may also play a protective role by preventing overexcitation and calcium-dependent excitotoxicity.

Thus, the existence of intracellular interactions in multireceptor complexes linking the activity of different types of receptor channels reveals a novel mode of fast signal processing and coincidence detection at the membrane level whose extent in the nervous system and other excitable tissues remains to be explored.

References

Barajas-López C, Huizinga JD, Collins SM, Gerzanich V, Espinosa-Luna R, Peres AL (1996) P2X-purinoceptors of myenteric neurones from the guinea-pig ileum and their unusual pharmacological properties. Br J Pharmacol 19:1541–1548.

Barajas-López C, Espinosa-Luna R, Zhu Y (1998) Functional interactions between nicotinic and P2X channels in short-term cultures of guinea-pig submucosal neurons. J Physiol (Lond) 513:671–683.

Barnes NM, Sharp T (1999) A review of central 5-HT receptors and their function. Neuropharmacology 38:1083–1152.

Bertrand PP, Bornstein JC (2002) ATP as a putative sensory mediator: activation of intrinsic sensory neurons of the myenteric plexus via P2X receptors. J Neurosci 22:4767–4775.

Bertrand PP, Kunze WAA, Furness JB, Bornstein JC (2000) The terminals of myenteric intrinsic primary afferent neurons of the guinea-pig ileum are excited by 5-hydroxytryptamine acting at 5-hydroxytryptamine-3 receptors. Neuroscience 101:459–469.

Boué-Grabot E, Archambault V, Séguéla P (2000) A protein kinase C site highly conserved in P2X subunits controls the desensitization kinetics of P2X2 ATP-gated channels. J Biol Chem 275:10190–10195.

Bueno L, Fioramonti J (1999) Effects of inflammatory mediators on gut sensitivity. Can J Gastroenterol 13:42A–46A.

Burnstock G (1986) Purines and cotransmitters in adrenergic and cholinergic neurones. Prog Brain Res 68:193–203.

Castelucci P, Robbins HL, Poole DP, Furness JB (2002) The distribution of purine $P2X_2$ receptors in the guinea-pig enteric nervous system. Histochem Cell Biol 117:415–422.

Cockayne DA, Dunn PM, Burnstock G, Ford APD (2002) Generation and electrophysiological characterization of $P2X_2$ and $P2X_{2/3}$ knockout mice. Soc Neurosci Abstr 28:12.

Davare MA, Avdonin V, Hall DD, Peden EM, Burette A, Weinberg RJ, Horne MC, Hoshi T, Hell JW (2001) A β2 adrenergic receptor signaling complex assembled with the Ca^{2+} channel Cav1.2. Science 293:98–101.

Davies PA, Pistis M, Hanna MC, Peters JA, Lambert JJ, Hales TG, Kirkness EF (1999) The 5-HT3B subunit is a major determinant of serotonin-receptor function. Nature 397:359–363.

Derkach V, Surprenant A, North RA (1989) 5-HT3 receptors are membrane ion channels. Nature 339:706–709.

Docherty M, Bradford HF, Wu JY (1987) Co-release of glutamate and aspartate from cholinergic and GABAergic synaptosomes. Nature 330:64–66.

Doucet E, Miquel MC, Nosjean A, Verge D, Hamon M, Emerit MB (2000) Immunolabeling of the rat central nervous system with antibodies partially selective of the short form of the 5-HT3 receptor. Neuroscience 95:881–892.

Essrich C, Lorez M, Benson JA, Fritschy JM, Luscher B (1998) Postsynaptic clustering of major $GABA_A$ receptor subtypes requires the γ2 subunit and gephyrin. Nat Neurosci 1:563–572.

Furness JB, Costa M (1982) Neurons with 5-hydroxytryptamine-like immunoreactivity in the enteric nervous system: their projections in the guinea-pig small intestine. Neuroscience 7:341–349.

Hanley JG, Koulen P, Bedford F, Gordon-Weeks PR, Moss SJ (1999) The protein MAP-1B links GABAc receptors to the cytoskeleton at retinal synapses. Nature 397:66–69.

Hollmann M, Heinemann S (1994) Cloned glutamate receptors. Annu Rev Neurosci 17:31–108.

Jo YH, Schlichter R (1999) Synaptic corelease of ATP and GABA in cultured spinal neurons. Nat Neurosci 2:241–245.

Jonas P, Bischofberger J, Sandkuhler J (1998) Corelease of two fast neurotransmitters at a central synapse. Science 281:419–424.

Khakh B, Zhou X, Sydes J, Galligan JJ, Lester HA (2000) State-dependent cross-inhibition between transmitter-gated cation channels. Nature 406:405–410.

Khakh B, Burnstock G, Kennedy C, King BF, North RA, Séguéla P, Voigt M, Humphrey PPA (2001) Current status of the nomenclature and properties of P2X receptors and their subunits. Pharmacol Rev 5:107–118.

Koshimizu TA, Van Goor F, Tomic M, Wong AOL, Tanoue A, Tsujimoto G, Stojilkovic S (2000) Characterization of calcium signaling by purinergic receptor-channels expressed in excitable cells. Mol Pharmacol 58:936–945.

Lee FJ, Xue S, Pei L, Vukusic B, Chery N, Wang Y, Wang YT, Niznik HB, Yu X, Liu F (2002) Dual regulation of NMDA receptor functions by direct protein-protein interactions with the dopamine D1 receptor. Cell 111:219–230.

Lingueglia E, Champigny G, Lazdunski M, Barbry P (1995) Cloning of the amiloride-sensitive FMRFamide peptide-gated sodium channel. Nature 378:730–733.

Liu F, Wan Q, Pristupa ZB, Yu XM, Niznik HB (2000) Direct protein-protein coupling enables cross-talk between dopamine D5 and γ-aminobutyric acid A receptors. Nature 403:274–280.

Maimone MM, Enigk RE (1999) The intracellular domain of the nicotinic acetylcholine receptor α subunit mediates its coclustering with rapsyn. Mol Cell Neurosci 14:340–354.

Maricq AV, Peterson AS, Brake AJ, Myers RM, Julius D (1991) Primary structure and functional expression of the 5HT3 receptor, a serotonin-gated ion channel. Science 254:432–437.

Meyer G, Kirsch J, Betz H, Langosch D (1995) Identification of a gephyrin binding motif on the glycine receptor β subunit. Neuron 15:563–572.

Morales M, McCollum N, Kirkness EF (2001) 5-HT3-receptor subunits A and B are coexpressed in neurons of the dorsal root ganglion. J Comp Neurol 438:163–172.

Nakazawa K (1994) ATP-activated current and its interaction with acetylcholine-activated current in rat sympathetic neurons. J Neurosci 14:740–750.

Nakazawa K, Fujimori K, Takanaka A, Inoue K (1991) Comparison of adenosine triphosphate- and nicotine-activated inward currents in rat phaeochromocytoma cells. J Physiol (Lond) 434:647–656.

Ortells MO, Lunt GG (1995) Evolutionary history of the ligand-gated ion-channel superfamily of receptors. Trends Neurosci 18:121–127.

Poelchen W, Sieler D, Wirkner K, Ille P (2001) Co-transmitter function of ATP in central catecholaminergic neurons of the rat. Neuroscience 102:593–602.

Rubio ME, Soto F (2001) Distinct localization of P2X receptors at excitatory postsynaptic specializations. J Neurosci 15:641–653.

Sakmann B (1992) Elementary steps in synaptic transmission revealed by currents through single ion channels. Neuron 8:613–629.

Searl TJ, Redman RS, Silinski EM (1998) Mutual occlusion of P2X ATP receptors and nicotinic receptors on sympathetic neurons of the guinea-pig. J Physiol (Lond) 510:783–791.

Sokolova E, Nistri A, Giniatullin R (2001) Negative cross talk between anionic $GABA_A$ and cationic P2X ionotropic receptors of rat dorsal root ganglion neurons. J Neurosci 21:4958–4968.

Tecott LH, Maricq AV, Julius D (1993) Nervous system distribution of the serotonin 5-HT3 receptor mRNA. Proc Natl Acad Sci USA 90:1430–1434.

Ugita S, Shen KZ, North RA (1992) 5-hydroxytryptamine is a fast excitatory transmitter at 5-HT3 receptors in rat amygdala. Neuron 8:199–203.

Wang H, Bedford FK, Brandon NJ, Moss SJ, Olsen RW (1999) $GABA_A$-receptor-associated protein links $GABA_A$ receptors and the cytoskeleton. Nature 397:69–72.

Wardell CF, Bornstein JC, Furness JB (1994) Projections of 5-hydroxytryptamine-immunoreactive neurons in guinea-pig distal colon. Cell Tissue Res 278:379–387.

Zhou X, Galligan JJ (1996) P2X purinoceptors in cultured myenteric neurons of guinea-pig small intestine. J Physiol (Lond) 496:719–729.

Zhou X, Galligan JJ (1998) Nonadditive interaction between nicotinic cholinergic and P2X purine receptors in guinea-pig enteric neurons in culture. J Physiol (Lond) 513:685–697.