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 P2X₂ ATP-gated channels and 5-HT₃ 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 P2X₂ and 5-HT₃ receptors is disrupted when the intracellular C-terminal domain of the P2X₂ receptor subunit is deleted and when minigenes coding for P2X₂ or 5-HT_{3A} receptor subunit cytoplasmic domains are overexpressed. Injection of fusion proteins containing the C-terminal domain of P2X₂ receptors in myenteric neurons also disrupts the functional interaction between native P2X₂ and 5-HT₃ 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-HT₃; 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 P2X₂ 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-HT₃) 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-HT₃ and P2X₂ 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 P2X₂, 5-HT_{3A}, 5-HT_{3B}, and $\rho 1$ 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 P2X₂ (P2X₂TR) 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 P2X₂, the main intracellular domain of 5-HT_{3A} (5-HT_{3A}-IL2), the C-terminal domain of P2X₂ (P2X₂-CT), and the N-terminal domain of P2X₂ (P2X₂-NT) were generated by PCR and

Received Oct. 16, 2002; revised Nov. 25, 2002; accepted Nov. 26, 2002.

This work was supported by grants from the Canadian Institutes of Health Research, by the Heart and Stroke Foundation of Canada (P.S.), by Institut National de la Santé et de la Recherche Médicale (INSERM) (M.B.E.), by INSERM-Fonds de la Recherche en Santé du Québec (E.B.-G., P.S.), and by postdoctoral awards from the Savoy Foundation for Epilepsy (E.B.-G., Y.C.). C.B.-L. is a Scholar of the Ontario Ministry of Health, and P.S. is a Scholar of the Fonds de la Recherche en Santé du Québec. We thank Audrey Speelman for expert technical assistance as well as John MacDonald (University of Toronto, Toronto, Canada) and Brian Mac Vicar (University of Calgary, Calgary, Canada) for their helpful comments during the preparation of this manuscript.

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subcloned into pcDNA3. P2X₂-CT was also subcloned into pGEX2T (Amersham Biosciences, Arlington Heights, IL) to produce glutathione S-transferase (GST)-P2X₂-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 μ M/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 CaCl₂, 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-P2X₂-CT fusion protein (75 μ M) 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 $\pm$ 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 P2X₂ and 15–20 ng of RNA coding for P2X₂TR, 5-HT₃A, or nicotinic receptor subunits), oocytes were incubated with Barth's solution containing 1.8 mM CaCl₂ 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 M Ω) 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 CaCl₂ or BaCl₂, 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-HT₃ and P2X₂ currents recorded in oocytes (see Fig. 2A), 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 MgCl₂, 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 MgCl₂, 1 BaCl₂, 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 $\pm$ SEM, and differences were assessed using Student's *t* test.

Immunoprecipitations and confocal imaging. Membrane proteins from HEK293 cells, transiently transfected with P2X₂, P2X₂-GFP, or 5-HT₃A-Flag or cotransfected with P2X₂-GFP plus 5-HT₃A-Flag or P2X₂ plus 5-HT₃A-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 μ M each). Bound proteins were eluted and then loaded onto a 10% SDS-PAGE and transferred to a nitrocellulose membrane. Labeling of immunoprecip-

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. *** $p < 0.001$.

tated associated receptors was performed with anti-GFP antibodies (1:5000; Molecular Probes, Eugene, OR), affinity-purified anti-P2X₂ 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 P2X₂, 5-HT₃A-Flag subunits and P2X₂-CT or P2X₂-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-HT₃A subunits after transient transfection with P2X₂-GFP and treatment with 5 μ M latrunculin-A for 18 hr. 5-HT₃ receptors were immunolocalized using anti-5-HT₃A antibodies (Doucet et al., 2000) and cyanine dye 3-conjugated secondary antibody; tagged P2X₂ receptors were visualized with GFP.

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 ($I_{ATP} = -2.0 \pm 0.2$ nA; $n = 9$), whereas an application of saturating concentrations of 5-HT (1 mM) induced a rapidly desensitizing current ($I_{5-HT} = -1.61 \pm 0.1$ nA) (Fig. 1A). Interestingly, the coapplication of ATP and 5-HT (1 mM each) to the same neurons induced a large reversible inward current ($I_{ATP + 5-HT} = -2.48 \pm 0.1$ nA; $n = 9$) (Fig. 1) that was significantly smaller than the arithmetic sum of I_{ATP} and I_{5-HT} currents ($69 \pm 4\%$ 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 P2X₂ in guinea pig myenteric plexus (Castellucci et al., 2002) and the loss of somatic P2X currents in myenteric neurons of P2X₂ knock-out mice (Cockayne et al., 2002) suggest that these currents are mediated by homomeric P2X₂- or heteromeric P2X₂-containing ATP receptors. Therefore, the kinetic profiles of I_{ATP} and I_{5-HT} recorded in myenteric neurons (Fig. 1) are consistent with the activation of ionotropic P2X₂ and 5-HT₃ 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 P2X₂ and 5-HT₃ channels. During two-electrode voltage-clamp recording in oocytes expressing both P2X₂ and 5-HT₃A subunits, the simultaneous application of ATP and 5-HT (100 μ M each) evoked an inward current (-11.9 ± 1.5 μ A; $n = 22$) that was significantly smaller ($p < 0.001$) than the sum of responses to separate applications of 5-HT ($I_{5-HT} = -2.7 \pm 0.5$ μ A) and ATP ($I_{ATP} = -14.5 \pm 1.8$ μ A) (Fig. 2A), demonstrating nonadditivity by occlusion. In agreement with our recordings in myenteric neurons, the amplitude of $I_{ATP+5-HT}$ represents $69 \pm 1\%$ ($n = 28$) of the predicted current corresponding to the arithmetic sum of I_{ATP} and I_{5-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. 2B), revealing an instantaneous reciprocal current occlusion. The nonadditivity between I_{5-HT} and I_{ATP} was also recorded at submaximal concentrations of either agonist (Fig. 2C), by measuring outward currents at positive potentials (Fig. 2D), or in the absence of extracellular Ca^{2+} (data not shown). Coactivation of P2X₂ and heteromeric 5-HT₃A+B receptors also produced current responses significantly smaller than the predicted current ($73 \pm 1\%$; $n = 17$) (Fig. 2E), suggesting that the 5-HT₃A subunit is essential in the interaction between 5-HT₃ and P2X receptors. Therefore, recombinant P2X₂ and 5-HT₃A receptors do not function independently in *Xenopus* oocytes.

In oocytes expressing only P2X₂ receptors, the application of saturating concentrations of 5-HT did not activate P2X₂ receptors, and the coapplication of ATP and 5-HT induced a current response identical in kinetics and in amplitude to I_{ATP} ($I_{ATP+5-HT} = 102 \pm 5\%$ of I_{ATP} ; $n = 6$) (Fig. 3A). Similarly, ATP did not activate 5-HT₃A channels, nor did it modulate I_{5-HT} ($I_{ATP+5-HT} = 102 \pm 2\%$ of I_{5-HT} ; $n = 6$) (Fig. 3B) indicating that the cross-inhibition between 5-HT₃A and P2X₂ is not attributable to receptor cross-modulation.

In oocytes coexpressing 5-HT₃A receptors and homomeric $\rho 1$ GABA_C receptors, simultaneous applications of 5-HT (100 μ M) and GABA (10 μ M) evoked an inward current (-2.66 ± 0.58 μ A; $n = 12$) that was not different from the sum of I_{5-HT} (-1.54 ± 0.23 μ A) and I_{GABA} (-1.18 ± 0.4 μ A) (Fig. 3C). $I_{5-HT+GABA}$ represented $97 \pm 4\%$ of the predicted current (Fig. 3E). Additive currents were also recorded when 5-HT was applied during the prolonged application of GABA and vice versa (Fig. 3D). These results indicate that homomeric 5-HT₃A receptors and $\rho 1$ GABA_C receptors act as independent channels without cross talk in *Xenopus* oocytes.

Figure 2. Cross-inhibition between recombinant P2X₂ and 5-HT₃ 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 P2X₂ and 5-HT₃A receptors activated with 1 μ M ATP, 100 μ M 5-HT, and both ATP and 5-HT (*C*) and at $+40$ mV V_h with a saturating concentration of agonists (100 μ M each) (*D*). *E*, Nonadditivity of ATP and 5-HT responses is also observed in oocytes coexpressing P2X₂ and heteromeric 5-HT₃A+B channels.

To determine whether intracellular domains could be involved in the functional interaction between P2X₂ and 5-HT₃A receptors, we first truncated most of the cytoplasmic C-terminal domain of the P2X₂ receptor subunit by adding a stop codon at amino acid 365 (P2X₂TR). Truncated P2X₂ subunits have been shown previously to assemble into functional receptors (Boué-Grabot et al., 2000). Contrary to the data obtained with wild-type P2X₂, coactivation of P2X₂TR and 5-HT₃A receptors evoked a current response (-3.4 ± 0.8 μ A; $n = 14$) that was not significantly different ($p > 0.5$) from the sum of I_{ATP} and I_{5-HT} (-0.72 ± 0.24 μ A and -2.07 ± 0.42 μ A, respectively; $n = 14$) (Fig. 4A). $I_{ATP+5-HT}$ represented $115 \pm 6\%$ of the predicted current (Fig. 4B). The additivity of ATP and 5-HT responses was also observed when 5-HT applications started during the continuous application of ATP (Fig. 4C). The application of 5-HT to oocytes expressing P2X₂TR receptors alone did not activate or modulate I_{ATP} ($I_{ATP+5-HT}$ was $100 \pm 11\%$ of I_{ATP}) (Fig. 4D). Therefore,

Figure 3. Cross talk between P2X₂ and 5-HT_{3A} receptors is not attributable to cross-modulation and additivity of 5-HT₃- and GABA_C-mediated responses. Superimposed control traces obtained from *Xenopus* oocytes expressing P2X₂ (A) or 5-HT_{3A} (B) receptors alone or with ATP, 5-HT, or a mixture of ATP and 5-HT (100 μ M each) are shown. C, Coexpression of homomeric 5-HT_{3A} and ρ 1 GABA_C receptors in *Xenopus* oocytes. Currents evoked by the application of 100 μ M 5-HT and 10 μ M 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.

P2X₂TR and 5-HT_{3A} receptors act as independent channels in *Xenopus* oocytes, suggesting an important role for the intracellular P2X₂ C-terminal domain in the reciprocal cross-inhibition between wild-type P2X₂ and 5-HT_{3A} activity. The functional interaction between P2X₂ and neuronal $\alpha_3\beta_4$ nicotinic channels ($I_{ATP+ACh} = 80 \pm 2\%$ of predicted) (Fig. 5E) was also abolished when wild-type P2X₂ receptors were replaced with P2X₂TR receptors ($I_{ATP+ACh} = 93 \pm 4\%$ of predicted) (Fig. 4F), suggesting that the reciprocal inhibitory cross talk between P2X receptors and nicotinic or 5-HT₃ receptors is based on similar intracellular mechanisms.

To generate competitive inhibitors of the functional interaction between P2X₂ and 5-HT₃ receptors, we designed two minigenes encoding soluble cytoplasmic forms of the C-terminal domain of P2X₂ receptors (P2X₂-CT, corresponding to amino acids 365–469) or of the N-terminal domain of P2X₂ receptors (P2X₂-NT, corresponding to amino acids 1–29). As illustrated in Figure 5A–C, expression of P2X₂-CT disrupted the interaction between P2X₂ 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 P2X₂-CT minigene was concentration dependent (Fig. 5E). Nonadditive responses to coapplications of ATP and 5-HT were observed with the coex-

Figure 4. Additivity of agonist-induced responses in oocytes coexpressing P2X₂TR and either 5-HT_{3A} or $\alpha_3\beta_4$ nicotinic receptors. A, C, Representative current traces showing coexpression of P2X₂TR and 5-HT_{3A} receptors in *Xenopus* oocytes. B, Mean current amplitudes showing the additivity of responses mediated by P2X₂TR and 5-HT_{3A} channels. D, Representative superimposed currents with individual and combined applications of ATP and 5-HT (100 μ M) recorded from one oocyte expressing P2X₂TR alone. E, Nonadditivity of P2X₂ and $\alpha_3\beta_4$ nicotinic currents in oocytes. $**p < 0.005$; $n = 22$. F, In oocytes coexpressing P2X₂TR 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.

pression of P2X₂-NT, indicating that the N-terminal domain of the P2X₂ subunit is not involved in the cross-inhibition between P2X₂ and 5-HT_{3A} receptors (Fig. 5B,E). Thus, the intracellular C-terminal domain of P2X₂ receptors is determinant in their functional interaction with excitatory members of the nicotinic receptor gene superfamily.

To determine whether the cross talk between P2X₂ 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 P2X₂ and 5-HT_{3A} channels also disrupted the functional interaction (Fig. 5D) in a concentration-dependent manner (Fig. 5E).

A physical association between P2X₂ 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 P2X₂ and 5-HT₃A channels or between P2X₂ and α₃β₄ 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 P2X₂ and 5-HT₃A channels. *A*, In the presence of a minigene encoding P2X₂-CT. *B*, In the presence of a minigene encoding P2X₂-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 P2X₂, α₃β₄ nicotinic channels, and P2X₂-CT. *E*, Concentration-dependent inhibitory effect of P2X₂-CT (■) or 5-HT₃A-IL2 (▲) on the functional interaction between P2X₂ and 5-HT₃A and between P2X₂ and α₃β₄ nicotinic channels. Numbers in parentheses indicate numbers of cells. **p* < 0.05. P2X₂-NT (●) had no effect on the cross-inhibition between 5-HT₃ and P2X₂ 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 P2X₂ and Flag-tagged 5-HT₃A 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-HT} (-4.7 ± 0.8 nA) in transfected HEK293 cells (actual *I*_{5-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 P2X₂-GFP subunit was revealed with anti-GFP antibodies, demonstrating a physical association between P2X₂ and 5-HT₃ 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 P2X₂-GFP alone, with membrane proteins from nontransfected cells (Fig. 6*B*), or after mixing membrane proteins from two batches of HEK293 cells expressing either P2X₂ or 5-HT₃ 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 P2X₂ and 5-HT₃-Flag receptors with minigenes encoding P2X₂ N- or C-terminal domains did not inhibit their physical interaction, as shown by the detection of P2X₂ after immunopurification on anti-Flag resin (Fig. 6*D*). Overexpression of the P2X₂-CT minigene was checked by recording the loss of func-

tional interaction between P2X₂ and 5-HT₃ 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-HT} (-2.7 ± 0.3 nA) in transfected HEK293 cells (actual *I*_{5-HT+ATP} = $84 \pm 6\%$ of predicted response) (Fig. 6*C*). These results indicate the existence of constitutive P2X₂ plus 5-HT₃A complexes in the plasma membrane and suggest that if intracellular domains are necessary for the functional cross-inhibition, other domains of P2X₂ and 5-HT₃ receptors are possibly involved in the physical association. Indeed, multireceptor clusters containing GFP-tagged P2X₂ and 5-HT₃A receptors were localized at the surface of transfected cells using confocal fluorescence microscopy (Fig. 6*F*).

Because the cross talk between recombinant P2X₂ and 5-HT₃A receptors was disrupted by the expression of minigenes encoding specific intracellular receptor subunit domains, we then tested whether the cross talk between native P2X₂ and 5-HT₃ receptors could also be disrupted in neurons using a similar strategy of competition. Indeed, we observed that the intracellular infusion of GST-P2X₂-CT fusion protein through the recording pipette into the cytoplasm of myenteric neurons significantly reduced the functional interaction (*I*_{5-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-HT+ATP} = $72 \pm 2\%$ of predicted; *n* = 8) (Fig. 7*C*) or GST alone (*I*_{5-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-P2X₂-CT (Fig. 7*D*). Therefore, the inhibitory cross talk between native and recombinant 5-HT₃ and P2X₂ 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, P2X₂ and 5-HT₃ 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 P2X₂ and α₃β₄ nicotinic receptors reported previously (Barajas-López et al., 1996; Zhou and Galligan, 1996; Khakh et al., 2000). This receptor-mediated cross talk between P2X₂ and 5-HT₃ responses is calcium, voltage, and agonist concentration independent. It is not attributable to cross-modulation, because ATP has no effect on 5-HT₃ receptors and 5-HT does not activate P2X₂ receptors, and the cross talk shows some receptor specificity, because 5-HT₃ receptors do not interact with GABA_C receptors.

The functional independence observed between truncated P2X₂ and 5-HT₃A receptors as well as the suppression of the cross talk between wild-type P2X₂ and 5-HT₃ receptors in competition experiments with the intracellular loop of 5-HT₃A subunit and with the C-terminal (but not the N-terminal) domain of P2X₂ 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 P2X₂ and 5-HT₃A receptor channels. *A, E*, Nonadditive current responses from HEK293 cells transfected with P2X₂-GFP and 5-HT₃A-Flag to separate applications of 100 μ M ATP, 100 μ M 5-HT, and ATP plus 5-HT (100 μ M each). *** p < 0.001. *B*, Copurification of P2X₂-GFP and 5-HT₃A-Flag receptors from transfected HEK293 cells. Transfected HEK293 cells were incubated in the presence (+) or absence (–) of the agonists ATP and 5-HT (100 μ M each) before homogenization. *IP*, Immunoprecipitation. *C*, Additivity of the P2X₂ and 5-HT₃ responses in HEK293 cells cotransfected with wild-type P2X₂, 5-HT₃A-Flag, and P2X₂-CT minigene. *ns*, Not significant. *D*, P2X₂ and 5-HT₃ receptors were coimmunopurified when coexpressed with the P2X₂-CT or P2X₂-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-HT₃A and P2X₂-GFP receptors in transfected CHO cells. *Arrows* in the overlay panel indicate coclusters of the two types of neurotransmitter-gated channels. Scale bar, 10 μ m.

intracellular C-terminal domain of P2X₂ 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-

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 P2X₂ in transfected cells has a clear competitive disrupting effect on the functional interaction between P2X₂ and 5-HT₃ receptors but did not prevent their coimmunoprecipitation. Although we cannot exclude the allosteric participation of extracellular or transmembrane regions of P2X₂ and 5-HT₃ 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-HT₃A 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-HT₃ receptors and neuronal P2X receptors are not yet known, direct or indirect constitutive interactions of P2X receptors with 5-HT₃ 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;

Figure 7. Disruption of cross talk between P2X₂ and 5-HT₃ receptors in myenteric neurons by intracellular GST-P2X₂-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-P2X₂-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-P2X₂-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-P2X₂-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-HT₃ 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.

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